MODULATION OF SOYBEAN AND MAIZE ANTIOXIDANT ACTIVITIES BY CAFFEIC ACID AND NITRIC OXIDE UNDER SALT STRESS

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophiae in the Department of Biotechnology, University of the Western Cape.

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November 2012

Modulation of soybean and maize antioxidant activities by caffeic acid and nitric oxide under salt stress

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KEYWORDS

Nitric oxide

Salinity

Reactive oxygen species

Cell death

Antioxidant enzymes

Caffeic acid

Lipid peroxidation

Superoxide

Antioxidant gene expression

Salt stress tolerance

ABSTRACT

Modulation of soybean and maize antioxidant activities by caffeic acid and nitric oxide under salt stress

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This study explores the roles of exogenously applied nitric oxide, exogenously applied caffeic acid and salt stress on the antioxidant system in cereal (exemplified by maize) and legume (using soybean as an example) plants together with their influence on membrane integrity and cell death.

This study investigates changes in H_2O_2 content, root lipid peroxidation, root cell death and antioxidant enzymatic activity in maize roots in response to exogenously applied nitric oxide (NO) and salt stress. This part of the study is based on the partially understood interaction between NO and reactive oxygen species (ROS) such as H_2O_2 and the role of antioxidant enzymes in plant salt stress responses. The results show that application of salt (NaCl) results in elevated levels of H_2O_2 and an increase in lipid peroxidation, consequently leading to increased cell death. The study also shows that by regulating the production and detoxification of ROS through modulation of antioxidant enzymatic activities, NO plays a pivotal role in maize responses to salt stress. The study argues for NO as a regulator of redox homeostasis that prevents excessive ROS accumulation during exposure of maize to salinity stress that would otherwise be deleterious to maize.

This study extends the role of exogenously applied NO to improve salt stress tolerance in cereals crops (maize) further to its role in enhancing salt stress

tolerance in legumes. The effect of long-term exposure of soybean to NO and salt stress on root nodule antioxidant activity was investigated to demonstrate the role of NO in salt stress tolerance. The results show that ROS scavenging antioxidative enzymes like SOD, GPX and GR are differentially regulated in response to exogenous application of NO and salt stress. It remains to be determined if the NO-induced changes in antioxidant enzyme activity under salt stress are sufficient to efficiently reduce ROS accumulation in soybean root nodules to levels close to those of unstressed soybean root nodules.

Furthermore, this study investigates the effect of long-term exposure of soybean to exogenous caffeic acid (CA) and salt stress, on the basis of the established role of CA as an antioxidant and the involvement of antioxidant enzymes in plant salt stress responses. The effect of CA on soybean nodule number, biomass (determined on the basis of nodule dry weight, root dry weight and shoot dry weight), nodule NO content, and nodule cyclic guanosine monophosphate (cGMP) content in response to salt stress was investigated. Additionally, CA-induced changes in nodule ROS content, cell viability, lipid peroxidation and antioxidant enzyme activity as well as some genes that encode antioxidant enzymes were investigated in the presence or absence of salt stress. The study shows that long-term exposure of soybean to salt stress results in reduced biomass associated with accumulation of ROS, elevated levels of lipid peroxidation and elevated levels of cell death. However, exogenously applied CA reversed the negative effects of salt stress on soybean biomass, lipid peroxidation and cell death. CA reduced the salt stress-induced accumulation of ROS by mediating changes in root nodule antioxidant enzyme activity and gene expression. These CA-responsive antioxidant enzymes were found to be superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and

glutathione reductase (GR), which contributed to the scavenging of ROS in soybean nodules under salt stress.

The work reported in Chapter 2 has been published in a peer-reviewed journal [Keyster M, Klein A, Ludidi N (2012) Caspase-like enzymatic activity and the ascorbate-glutathione cycle participate in salt stress tolerance of maize conferred by exogenously applied nitric oxide. Plant Signaling and Behavior 7: 349-360]. My contribution to the published paper was all the work that is presented in Chapter 2, whereas the rest of the work in the paper (which is not included in Chapter 2) was contributed by Dr Marshall Keyster.

November 2012

AIMS OF THIS STUDY

The study aimed at the following:

i. Investigating the role of exogenously applied NO in antioxidant enzyme-mediated ROS scavenging in maize under salt stress.
ii. Detection of ROS scavenging maize antioxidant enzyme isoforms whose activity is mediated by exogenous NO in response to salt stress.
iii. Determining the effect of NO on antioxidant enzymes in soybean root nodules and how this influences soybean responses to salt stress.
iv. Investigating the role of exogenously applied CA in modulating the antioxidant system in soybean root nodules during long-term salt stress.

DECLARATION

I declare that "*Modulation of soybean and maize antioxidant activities by caffeic acid and nitric oxide under salt stress"* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Ashwil Johan Klein **November 2012**

Signed ……

ACKNOWLEDGEMENTS

- Firstly, I would like to thank my **Heavenly Father** for giving me the strength, guidance and wisdom to pursue my goals in life.
- Secondly, I would like to extend a special thanks to my supervisors Prof. Ndiko Ludidi and Dr. Marshall Keyster for providing me with the opportunity and necessary guidance throughout this study.
- I would like to acknowledge my parents (**Charmaine and Deon Klein**) and in-laws (**Salomo and Sandra Moses**), for their endless love, support and patience through all my years of study until now. All the phone calls, text messages and always being there with a word of encouragement in the difficult times. Words cannot express my gratitude.

- "To my fiancée (**Maretina Moses**) and daughter (**Chloé Avril Klein**), thank you for your steadfast patience and support throughout this study. The 2 of you were my true inspiration and motivation for completing this study. I love you to bits".

- To my siblings (**Ivoidia, Gwendolene, Jacques, Leandra**), the Van Wyk family (**John, Yolanda, Candice, Jason**) and the rest of the Moses family (**Cabral, Leoni**) thank you for always believing in me and your extra word of encouragement in difficult times.
- Thanks to my friends (**Eben B, Kevin, Jarries, Catz, DJ Linas, Randal**) and colleagues especially Dr. Marshall Keyster, Kyle Phillips, Dr. Morne Du Plessis, Dr. Oom Fani Egbichi, for keeping me sane throughout the writing process with all the silly talk. If I did not mention you, believe me you are not forgotten.
- The rest of the Plant Biotech Research Group (PBRG) past and present thank you very much for humor throughout this study.
- Last but not least, this work would not have been possible without financial support from **The University of the Western Cape** (UWC) and the **National Research Foundation (NRF**, South Africa). Your contribution is highly appreciated.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AO	amine oxidase
APX	ascorbate peroxidase
AsA	ascorbic acid
AtNOA1	*Arabidopsis thaliana* Nitric Oxide Associated 1
AtNOS1	*Arabidopsis thaliana* Nitric Oxide Synthase 1
BSA	bovine serum albumin
CA	caffeic acid
CAT	catalase
cGMP	cyclic guanosine monophosphate
CYP	cysteine protease/ cysteine endopeptidase
CYPs	cysteine proteases/ cysteine endopeptidases
DETA	Diethylenetriamine
DETA/NO	2,2′-(hydroxynitrosohydrazono)bisethanimine
DHAsA	dehydroascorbate
DNA	deoxyribonucleic acid
DHAR	dehydroascorbate reductase
EDTA	ethylenediaminetetraacetic acid
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
Lb	leghaemoglobin
MDA	malondialdehyde
MES	2-(N-Morpholino)ethanesulfonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitrotetrazolium blue chloride

NO	nitric oxide
NOS	nitric oxide synthase
NOX	NADPH oxidase
NR	Nitrate reductase
OXO	oxalate oxidase
PAGE	Polyacrylamide gel electrophoresis
PCD	programmed cell death
PVP	Polyvinylpyrrolidone
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SNP	sodium nitroprusside
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
XO	Xanthine oxidase
XTT	3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Salt stress is a major environmental constraint to crop productivity in the arid and semiarid regions of the world (Tunçtürk et al. 2011). It is estimated that over 30% of irrigated land used for crop production is adversely affected by high soil salinity, which causes salt stress in plants (Tunçtürk et al. 2011). In this way, salinity has a major impact on world food production, lowering yields of important crop plants such as wheat, soybean and maize. High concentrations of salt cause ion imbalance and osmotic stress in plants, disrupting ion and water homeostasis in plant cells. Drastic changes in ion and water homeostasis in plant cells leads to macromolecular damage, plant cell death and growth arrest (Zhu 2001). In addition to the decrease in water uptake and accumulation of ions to toxic levels, soil salinity affects plant growth by reducing plant nutrient uptake from the soil (Zhu 2001). Furthermore, salt stress also causes oxidative stress, resulting from the formation of ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\cdot$), peroxyl radicals, organic hydroperoxide (ROOH) and peroxynitrite ($ONOO^-$) (Turkan and Demiral 2008). ROS production caused by abiotic stress leads to disruption in metabolic processes such as photosynthesis, respiration and N_2-fixation (Becana et al. 2000). The downstream effects of ROS in plant cells include lipid peroxidation, protein oxidation and DNA/RNA damage (Mittler 2002).

In order to manage the effect of ROS damage, plants has evolved various strategies to regulate the levels of ROS in plant cells by controlling the activity of

ROS-producing enzymes such as xanthine oxidase (XO), oxalate oxidase (OXO), NADPH oxidase (NOX) and amine oxidase (AO) (Grant and Loake 2000; Mittler 2002). If that fails, another strategy for plants in distress is to activate ROS-scavenging enzymes which include superoxide dismutase (SOD) for O_2^- removal plus peroxidases and catalase (CAT) for H_2O_2 scavenging (Larson 1988; Mittler 2002). Some of the important peroxidases present in plants include ascorbate peroxidases (APX), glutathione peroxidases (GPX) and thioredoxin peroxidases (TPX) that primarily detoxify hydrogen peroxide to produce water. In addition to the ROS-scavenging enzymes, plants also utilize antioxidant metabolites such as ascorbic acid for O_2^- and H_2O_2 removal and glutathione for H_2O_2 obliteration (Apel and Hirt 2004; Mittler 2002).

Nitric oxide (NO) is an active molecule involved in mediation of various biotic and abiotic stresses that induce physiological responses in plants (Tan et al. 2008). NO modulates plant physiological processes during abiotic stress and this is thought to be via direct antioxidant activity of NO or via modification of the redox state of antioxidants and through regulating antioxidant enzyme activities (Hung and Kao 2004; Shi et al. 2005). According to work performed by Shi et al. (2005), antioxidant enzymes like SOD, CAT and APX were up-regulated by both endogenous and exogenous NO, thus lowering levels of ROS present in plant cells. NO has also been shown to regulate cell death in plants under abiotic stress by controlling cysteine protease (CYP) activity and lipid peroxidation (estimated from measurement of malondialdehyde levels) (Belenghi et al. 2003).

Caffeic acid [3-(3,4-dihydroxyphenyl)-2-propenoic acid, CA] on the other hand is a well-known phenolic compound that is widely present in plant kingdom (Jayanthi and Subash 2010). Recent experimental evidence by Gulcin (2006) suggests that

caffeic acid is a potent antioxidant and might have a beneficial impact on human health (Kroon and Williamson 1999; Fukumoto and Mazza 2000). Furthermore Jayanthi and Subash (2010) have shown that CA has several biological and pharmacological properties, such as antiviral, antioxidant, anti-inflammatory, anticarcinogenic, and immunomodulatory activites. It has also been documented that CA inhibits lipoxygenase activity and suppresses lipid peroxidation in plants cells (Gutteridge 1995). About two decades ago, CA was shown to completely block the production of ROS and inhibit the xanthine oxidase system (Sud'ina et al. 1993). It is likely that phenolic compounds trap free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes including superoxide dismutase (SOD), peroxidases (ascorbate peroxidase; APX, glutathione peroxidase; GPX) and reductases (glutathione reductase; GR).

This review will focus on the production and detoxification of ROS in plants as a consequence of salt stress. It will also explore the recent developments in the field of NO and simple phenolic compounds such as caffeic acid in cereal and legume plants as well as their respective roles in abiotic stress, with emphasis on salt stress tolerance.

1.2 ROS production in plants under salt stress

ROS are important signals in the biosynthesis of organic molecules, polymerization of cell wall constituents, and defense against abiotic and biotic stresses. They are produced in unstressed plant cells at the basal levels required for normal metabolic processes but are produced at elevated levels in stressed plant cells. Exposure of plants to salt stress enhances the production of ROS such as H_2O_2, O_2^-, $^1O_2$ and OH^-. Several lines of research have shown that excess ROS cause phytotoxic reactions such as lipid peroxidation, protein degradation and DNA

damage (McCord 2000; Vinocur and Altman 2005; Pitzschke et al. 2006). In plant cells, ROS, mainly H_2O_2, O_2^-, and OH^- are generated in the cytosol, chloroplasts, mitochondria, peroxisomes and the apoplastic space (Bowler and Fluhr 2000; Mittler 2002).

In the last decade studies have shown that ROS play a key role in plants as signal transduction molecules involved in mediating responses to pathogen infection, environmental stresses, programmed cell death (PCD) and developmental stimuli (Mittler et al. 2004; Torres and Dangl 2005). Membrane injury induced by salt stress is related to enhanced production of ROS (Shalata et al. 2001). Therefore a rise in ROS production may result in stomatal closure, causing a decrease in CO_2 concentration inside the chloroplasts. This in turn causes a decrease in $NADP^+$ concentration with the concomitant generation of ROS (Foyer and Noctor 2003). The increased concentration of ROS damages the D1 protein of photosystem II, leading to inhibition of photosynthesis. Stress-enhanced photorespiration and NADPH activity also contributes to increase in H_2O_2 accumulation, which may inactivate enzymes by oxidizing their thiol groups. This toxicity caused by H_2O_2 is not due to its reactivity alone, but requires the presence of a metal reductant to form the highly reactive hydroxyl radical, which has the ability to react with all biological molecules (Halliwell and Gutteridge, 1989). In order to counter the effect of ROS enhanced by salt-induced stress plants have to the resort to activating ROS scavenging enzymes such SOD, APX, GPX and GR to scavenge the respective toxic molecules.

1.3 Detoxification of ROS in plant cells by antioxidant enzymes under salt stress

ROS have the potential to interact with many cellular components, causing significant damage to membrane and other cellular structures, and consequently

growth inhibition (Verma and Mishra 2005; Agarwal and Shaheen 2007; Gao et al. 2008). Some of the ROS are highly toxic and must be detoxified by cellular responses in order for the plant to survive and grow (Gratäo et al. 2005). ROS scavenging depends on the detoxification mechanism, which may occur as a result of sequential or simultaneous action of a number of antioxidant enzymes, including CAT, GPX, SOD and APX. According to Parida and Das (2005), plants with high constitutive and induced antioxidant levels have a better chance to resist damage caused by overproduction of ROS and can thus withstand oxidative stress. The scavenging of ROS is one of the defense responses used by plants against abiotic stresses (Vranová et al. 2002). The degree of damage by ROS depends on the balance between the production of ROS and its removal by these antioxidant systems (Demiral and Turkan 2005; Khan and Panda 2008). A correlation between the antioxidant enzyme activity and salinity tolerance was demonstrated by comparison of a tolerant cultivar with a sensitive cultivar of maize (Azooz et al. 2009). The activities of antioxidant enzymes were reported to increase under salinity stress and closely related to salt tolerance of many plants (De Azevedo Neto et al. 2006; Koca et al. 2007; Athar et al. 2008). Superoxide dismutase (SOD; EC 1.15.1.1) is located in various cell compartments and is a major scavenger of superoxide (O_2^-). This enzyme converts O_2^- to H_2O_2, which is eliminated by ascorbate peroxidase (APX; EC 1.11.1.7) at the expense of oxidizing ascorbate to monohydroascorbate (Lee et al. 2001). Hydrogen peroxide is also scavenged by catalase (CAT; EC 1.11.1.6) and peroxidase (POD) to form water and oxygen (Mittler 2002; Chaparzadeh et al. 2004).

1.4 Nitric oxide (NO)

1.4.1 Enzymatic production of NO in plant cells

During the past two decades, NO has been established as a unique type of biological messenger in animal cells. According to Wendehenne et al. (2001) the interest of biologists was first captured when this highly reactive radical was identified as the endothelium-derived relaxing factor, a potent endogenous vasodilator. Further investigations led to the finding that NO is a multifunctional effector involved in numerous mammalian physiological processes, including the relaxation of smooth muscle, inhibition of platelet aggregation, neural communication and immune regulation (Wendehenne et al. 2001). Many insights into the understanding of NO functions came from the identification of nitric oxide synthase (NOS; EC 1.14.13.39), the enzyme responsible for NO production in mammalian cells.

According to studies conducted by Guo et al. (2003) and Zhao et al. (2007) NO is endogenously synthesized in plant cells in a similar manner as illustrated for animal models. There are two suggested enzymatic sources of NO synthesis in plants; the arginine-dependent and nitrite-dependent pathways.

The arginine pathway is mediated by NOS, which utilizes the substrates L-arginine, O_2, and NADPH to produce L-citrulline and NO (Figure 1.1). According to various authors (Cueto et al. 1996; Ninneman and Maier 1996) the existence of NOS activity was detected in higher plants and peroxisomes (Barrosso et al. 1999). This type of NO synthesis has also been detected in peas (Corpas et al. 2006) although NO production by plants was first observed by Klepper in soybean plants treated with photosynthetic inhibitor herbicides (Klepper 1979) or other chemicals (Klepper 1991) or under dark anaerobic conditions. However, to date no gene homologous to

the mammalian NOS has been successfully identified and isolated from higher plants. Guo et al. (2003) identified the *Arabidopsis thaliana* gene Nitric Oxide Associated 1 (AtNOS1) that they reported to be a "novel plant NOS" because of its sequence homology to the snail (*Helix pomata*) NOS, but AtNOS1 was later shown to have no NOS activity (Crawford et al. 2005) and instead was later proved to be a GTPase (Yamasaki et al. 2006). However recent work by Foresi et al. (2010) characterized a novel NOS gene from the single-celled alga *Osterococcus tauri*. *O. tauri* is the smallest-known free-living photosynthetic eukaryote which belongs to the group Chlorophyta in the plant kingdom, making the discovery of a NOS enzyme (in this class of the plant kingdom) extremely important to science because it provides a platform in the quest to discover a NOS enzyme in higher plants.

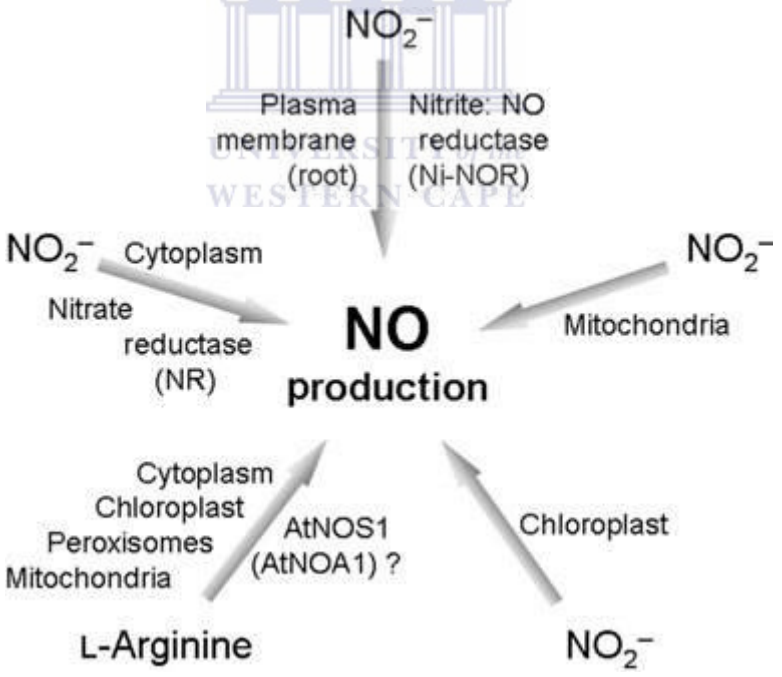

Figure 1.1 Routes of NO production in plants cells. NO can be synthesized enzymatically from nitrite (NO_2^-) by nitrate reductase (NR). There is also considerable evidence for L-arginine-dependent NOS activity in plant cells, although the protein *AtNOS1* is no longer considered to be a NOS and no other higher plant candidate for the role has been identified. (Figure was adapted from Wilson et al. 2008)

On the other-hand the nitrite-dependent NO production pathway catalyzed by plant nitrate reductase (NR; EC1.6.6.1) is a central enzyme in nitrogen assimilation in plants. To date, NR is the only isolated and characterized plant enzyme known to generate NO in plants (Dean and Harper 1988). It has two main forms, depending of its cellular location; plasma membrane (PM-NR) and cytosolic (cNR) NR (Figure 1.1). According to Rockel et al. (2002) the reaction catalyzed by NR to produce NO from nitrites uses NADPH as an electron donor. Various other studies have also reported the simultaneous generation of nitrous oxide, a pre-cursor of peroxynitrite and NO. In agreement with these findings is the fact that NR activity using nitrites as a substrate under aerobic conditions yields NO and peroxynitrite (Yamasaki and Sakihama 2000). This NR-dependent NO generation is nitrite-concentration-dependent. NO is generated from nitrites only if the nitrite levels exceed those of nitrates under dark and anoxic conditions. However, the fact that higher plants produce NO from NR only in the mitochondria of roots and not in the leaves is a major drawback in plant NO biology because it means that other sources of NO remain to be discovered to account for the production of NO by leaves (Gupta et al. 2005).

1.4.2 Non–enzymatic production of NO in plant cells

There are several known non-enzymatic reactions for NO production in plants. According to Stohr and Ullrich (2002), the non-enzymatic reduction of nitrite can lead to the formation of NO and this reaction is favoured at acidic pH where nitrite can be converted to NO and nitrate. Henry et al. (1997) also showed that nitrite can be chemically reduced by ascorbic acid at pH 3 to 6 to yield NO and dehydroascorbic acid. Furthermore it has been shown by Beligni et al. (2002) that NO can also be synthesized by reducing nitrite using ascorbate at acidic pH in barley aleurone cells.

1.4.3 NO signaling in plants

Recent research has established evidence of NO function in various plant systems (Crawford and Guo 2005; Lamotte et al. 2005). NO has emerged to be a key signaling molecule in plant signal transduction pathways, where cGMP may be its downstream mediator, and NO may directly or indirectly interact with other signaling molecules such as H_2O_2, salicylic acid, and cytosolic Ca_2^+ (Lamotte et al. 2004; Wendehenne et al. 2004). For example, NO alleviates the harmful effect from ROS in establishing stress resistance responses. NO also reacts with O_2^- to form peroxynitrite, heme-containing proteins, and thiol groups to form S-nitrosothiols (Neill et al. 2008). However, downstream signaling targets of NO during signaling are not well elucidated. Nevertheless, pathways for NO signaling may involve direct activation of ion channel proteins and/or proteins that regulate their gene expression or indirect regulation of proteins involved in various signal cascades (Neill et al. 2003). Salmi et al. (2007) argue that even though various lines of evidence indicate a role of NO in plant physiology; concrete information of how this molecule connects with upstream receptors and downstream response elements is still in its infancy. However, NO signaling research in plants has progressed tremendously and although the identification of a plant NOS enzyme remains uncertain (Travis 2004); there still is substantial evidence for a key role of NO in plant signaling systems.

1.4.4 The role of NO in abiotic stress responses

Abiotic stresses disrupt the cellular redox homeostasis in plants, leading to the onset of oxidative stress resulting from generation of ROS (Asada 2006). It has been reported that different types of chemical and mechanical stresses in various plant species seem to induce an increase in the level of NO and this altered NO content in plant cells is proposed to regulate plant responses to abiotic stresses (Table 1.1).

Many abiotic stresses generate free radicals and other oxidants, particularly from the chloroplasts, mitochondria and peroxisomes (Mano 2002), resulting in oxidative stress caused by an increased level of ROS in plant cells (Mittler 2002). ROS not only cause oxidative damage, but also exert some signaling responses that influence plant growth and physiology; therefore it is vital for plants to control the concentration of ROS in the cell (Vranova et al. 2002).

Table 1.1 NO-mediated responses to abiotic stresses. (Table was adapted and modified from Qiao and Fan 2008)

Abiotic stress	NO mediated effect	Species of induced NO	References
Drought/ osmotic stress	Involved in ABA signaling, stomatal closure Induction of ABA synthesis, LEA expression	*Nicotiana tabacum* *Pisum sativum*	Gould et al. (2003); Leshem and Haramaty (1996)
Salinity	Increased osmotic tolerance; Induced expression of Na^+/H^+ antiporter gene	*Nicotiana tabacum* Maize	Gould et al. (2003) Zhang et al. (2006)
Herbicide	Promoted the activity of antioxidant enzymes	*Scenedesmus obliquus* *Chlamydomonas reinhardtii*	Mallick et al. (2000); Sakihama et al. (2002)
Mechanical injury	NO burst result in cell death	*Arabidopsis thaliana* *Taxus brevifolia*	Pedroso et al. (2000)

It has been shown that NO eliminates the superoxide anion (O_2^-) and lipid radical (ROOH) and activates antioxidant enzyme activity. The scavenging of O_2^- by NO leads to the formation of peroxyntrite ($ONOO^-$), which is very toxic in animal cells but not as toxic for plant, cells (Tada et al. 2004; Kopyra and Gwozdz 2003).

The role of NO as an antioxidant appears to be based on its ability to sustain cellular redox homeostasis and regulate the toxicity of ROS. On the other-hand, NO was also proposed to eliminate excess nitrite from plant cells, as high concentrations of nitrite are toxic to plant cells (Shingles et al. 1996).

According to research conducted by Wendehenne et al. (2004) and Orozco-Cardenas and Ryan (2002), NO is involved in the signaling pathway downstream of jasmonic acid synthesis and upstream of H_2O_2 synthesis, thus regulating the expression of some genes involved in abiotic stress tolerance. Furthermore, NO influences Ca^{2+} levels in response to either salinity or osmotic stress caused by sorbitol (Zhao et al. 2004; Gould et al. 2003).

1.4.5 Salt stress tolerance mediated by NO

Salt stress is a world-wide agricultural problem and is one of the major abiotic stresses faced by plants, which adversely affect their productivity (Parida and Das 2005). Salt stress causes reduction of yield and alteration of plant metabolism, including a reduced water potential, ion imbalance and ion toxicity (Joseph and Jini 2011).

Salt tolerance on the other-hand is the ability of plants to grow and complete their life cycle on a substrate that contains high concentrations of soluble salt without drastic loss of yield (Cha-um et al. 2009). The involvement of NO in salt tolerance has drawn much attention in the past few years and has been demonstrated in many plant species. Some of these examples include the exogenous application of sodium nitroprusside (SNP) (NO donor) under salt stress, which significantly alleviated the oxidative damage to seedlings of rice (Uchida et al. 2002), lupin (Kopyra and Gwóźdź 2003), and cucumber (Fan et al. 2007). Song et al. (2009) also showed that NO enhanced growth and increased dry weight in *Suaeda salsa* seedlings. Zhang et al. (2006) reported that NO enhanced salt tolerance in maize seedlings through increasing K^+ accumulation in roots, leaves and shoots, while decreasing Na^+ accumulation. They further showed that both NO and salt treatment stimulated vacuolar H^+-ATPase and H^+-PPase activities, resulting in increased H^+ translocation

and Na^+/H^+ exchange. NO produced under salt stress could serve as a second messenger for the induction of plasma membrane H^+-ATPase expression (Zhao et al. 2004). Guo et al. (2009) suggested that NO might confer salt tolerance to plants by preventing both oxidative damage to membranes and translocation of Na^+ from root to shoots. We have recently shown that application of another NO donor (DETA/NO) to maize plants also enhanced salt stress tolerance by reversing the detrimental effects caused by salt stress via enhancement of maize root and shoot biomass (Keyster et al. 2012).

1.4.6 The role of NO in plant growth and development

NO is a free radical involved in numerous and diverse cellular pathways in mammals (He et al. 2007). In recent years, there has been much research on the occurrence of NO and its physiological role in the plant kingdom. It has been proposed that NO play a key role in various plant developmental processes (Anderson and Mansfield 1979). These processes include root growth and leaf expansion, photomorphogenesis and senescence; as comprehensively reviewed by Beligni and Lamattina (2001) and Neill et al. (2003). NO also functions in mediating rapid physiological reactions such as stomatal closure (Bright et al. 2006) and the cytokinin signalling pathway (Tun et al. 2006). NO stimulates morphological processes related to plant growth and development such as seed germination, de-etiolation in lettuce and wheat (Beligni et al. 2000), inhibition of hypocotyl elongation in lettuce and *Arabidopsis thaliana* (Pagnussat et al. 2002) and root formation in cucumber (Kopyra and Gwózdz 2003). Some of these processes indicating the biological activities of NO in plant growth and development are tabulated in Table 1.2.

Table 1.2 The role of NO in plant growth and development in various plant organs. Table was adapted and modified from Beligni and Lamattina 2001)

Tissue organs	Physiological action	Species	Optimum concentrations
Seeds	Induction of germination	Lettuce	10^{-6}
Hypocotyls	Inhibition of elongation under low light fluences	Lettuce, *Arabidopsis thaliana*	10^{-6}
Leaves	Induction of de-etiolation	Lettuce, wheat	10^{-6}
Roots	Root elongation, Induction of adventitious lateral root formation	Cucumber	10^{-10}

1.5 Phenolic acids

1.5.1 Structure of two classes of phenolic acids (hydroxybenzoic and hydroxycinnamic acids)

Hydroxybenzoic acids have the general structure of C6-C1 derived directly from benzoic acid (Figure 1.2). According to Macheix et al. (1990), variations in the structures of individual hydroxybenzoic acids lie in the hydroxylation and methylation of the aromatic ring. The four most commonly occurring hydroxybenzoic acids are phydroxybenzoic, vanillic, syringic, and protocatechuic acid. They may be present in soluble form conjugated with sugars or organic acids or bound to cell wall fractions such as lignin (Schuster and Herrmann, 1985). Gallic acid (Figure 1.2) is a trihydroxyl derivative which participates in the formation of hydrolysable gallotannins (Haslam, 1982; Haddock et al. 1982). Its dimeric condensation product (hexahydroxydiphenic acid) and related dilactone, ellagic acid (Figure 1.2) are common plant metabolites. Ellagic acid is usually present in ellagitannins as esters

of diphenic acid (Haddock et al. 1982; Castonguay et al. 1998; Komorsky-Lovrić and Novak 2011).

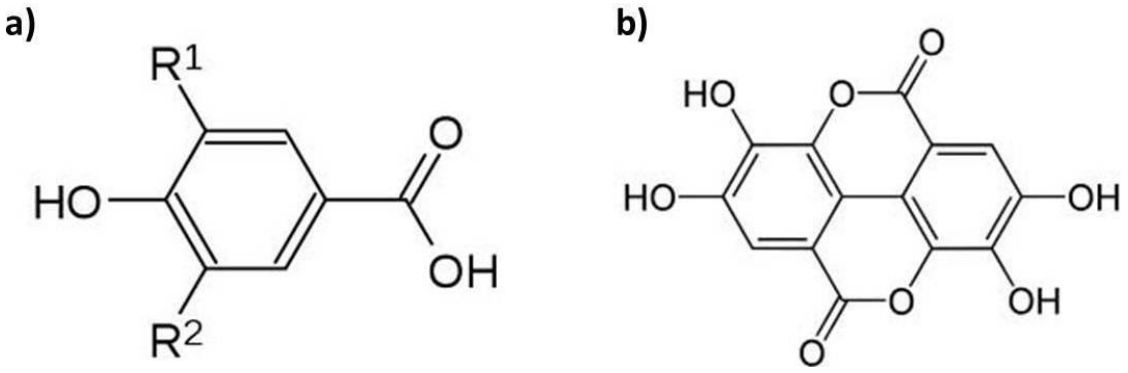

Figure 1.2 Chemical structures of (a) gallic acid and (b) ellagic acid.

On the other-hand, the four most widely distributed hydroxycinnamic acids in fruits are p-coumaric, caffeic, ferulic and sinapic acids (Figure 1.3) (Rice-Evans et al. 1997). Hydroxycinnamic acids usually occur in various conjugated forms, the free forms being artifacts from chemical or enzymatic hydrolysis during tissue extraction. The conjugated forms are esters of hydroxyacids such as quinic, shikimic and tartaric acid, as well as their sugar derivatives (Herrmann and Nagel, 1989; Macheix et al. 1990; Shahidi and Naczk 1995).

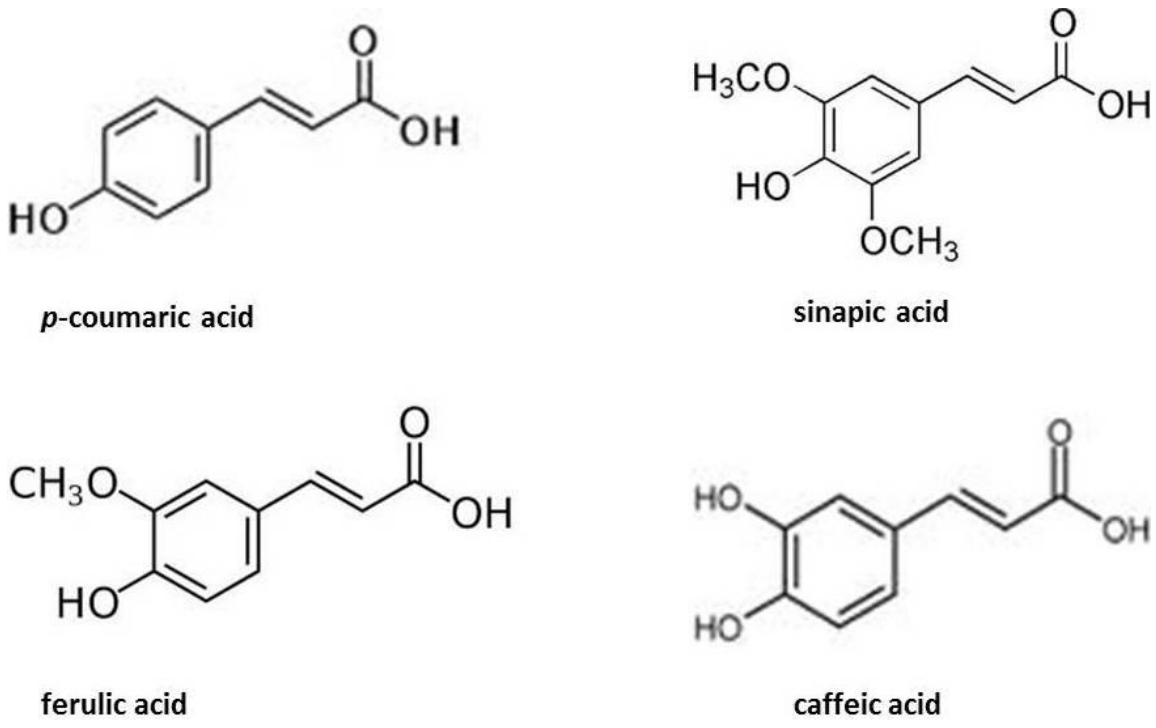

Figure 1.3 Chemical structures of four common hydroxycinnamic acids.

1.5.2 Phenolic acids in plants

1.5.2.1 Biosynthesis of phenolic compounds in plants

Phenolics display a wide variety of structures, ranging from simple moieties containing a single hydroxylated aromatic ring to highly complex polymeric substances (Dai and Mumper 2010). According to various authors the biosynthetic pathways of phenolic compounds in plants are well known (Haddock et al. 1982; Macheix et al. 1990; Dixon and Paiva 1995; Sakihama et al. 2002) and these pathways are illustrated in Figure 1.4. The biosynthesis and accumulation of secondary compounds can be an endogenously controlled process during development (Macheix et al. 1990) or regulated by exogenous factors such as light, temperature and wounding (Bennet and Wallsgrove 1994; Dixon and Paiva 1995). The diagram in Figure 1.4 illustrates that phenylalanine produced in plants via the shikimate pathway is a common precursor for most phenolic compounds in higher

plants (Macheix et al. 1990). Similarly, hydroxycinnamic acids, and particularly their coenzyme A esters, are common structural elements of phenolic compounds such as cinnamate esters and amides, lignin, flavonoids and condensed tannins (Macheix et al. 1990) (Figure 1.4). The phenylalanine/hydroxycinnamate pathway is defined as the 'general phenylpropanoid metabolism' pathway. It includes reactions leading from L-phenylalanine to hydroxycinnamates and their activated forms. The enzymes catalyzing the individual steps in general phenylpropanoid metabolism are phenylalanine ammonialyase (PAL), cinnamic acid 4-hydroxylase (C4H), and hydroxycinnamate: coenzyme A ligase (4CL) (Figure 1.5). These three steps are necessary for the biosynthesis of phenolic compounds (Whetten and Sederoff 1995; Douglas 1996).

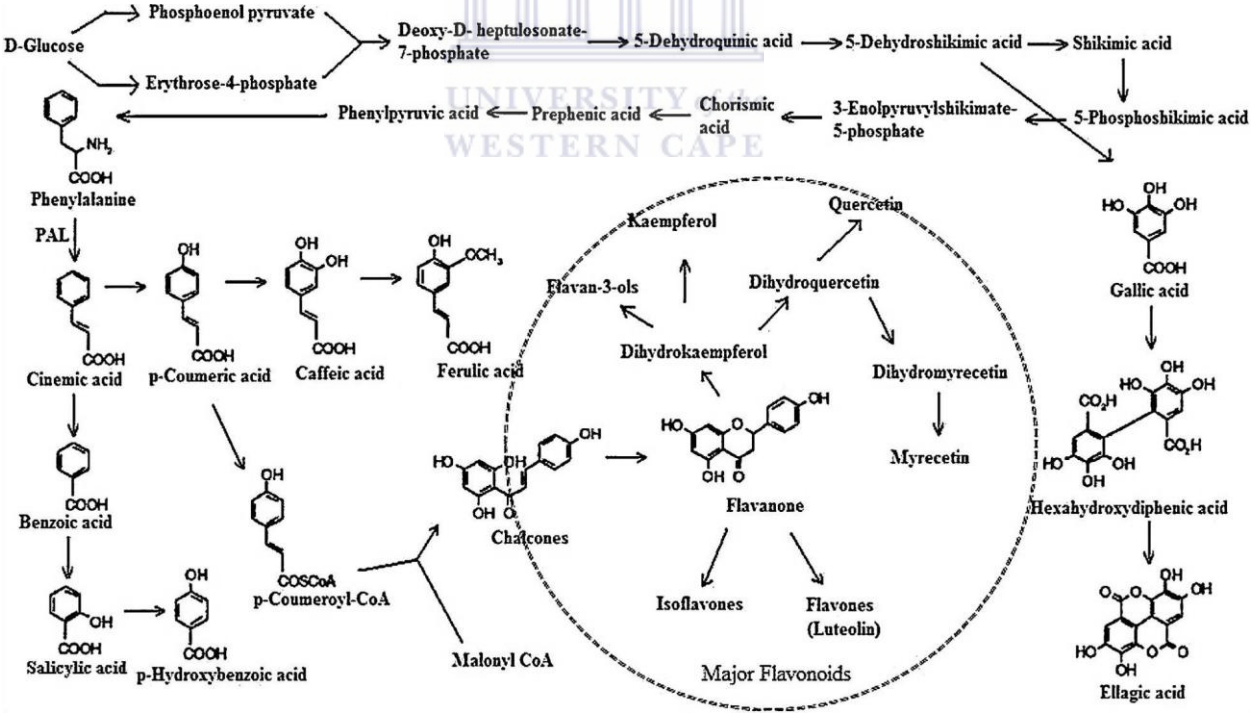

Figure 1.4 Biosynthesis of hydroxycinnamic acids, hydroxybenzoic acid and flavonoids.

1.5.2.2 Biosynthesis of hydroxycinnamic acids

The formation of hydroxycinnamic acids (caffeic, ferulic, 5-hydroxyferulic and sinapic acids) from *p*-coumaric acid requires two types of reactions, namely hydroxylation and methylation. The hydroxylation of *p*-coumaric acid to produce caffeic acid (Figure 1.4) is catalyzed by monophenol monooxygenases (a well-known group of plant enzymes) (Macheix et al. 1990). The methylation of caffeic acid leads to the formation of ferulic acid which, together with *p*-coumaric acid and sinapic acid, is the precursor of lignin (Figure 1.5). The methylation reaction is catalyzed by an *O*-methyltransferase (Humphreys and Chapple 2002). Caffeic acid is the substrate for 5-hydroxyferulic acid, which yields sinapic acid as a result of an O-methylation reaction (Figure 1.5).

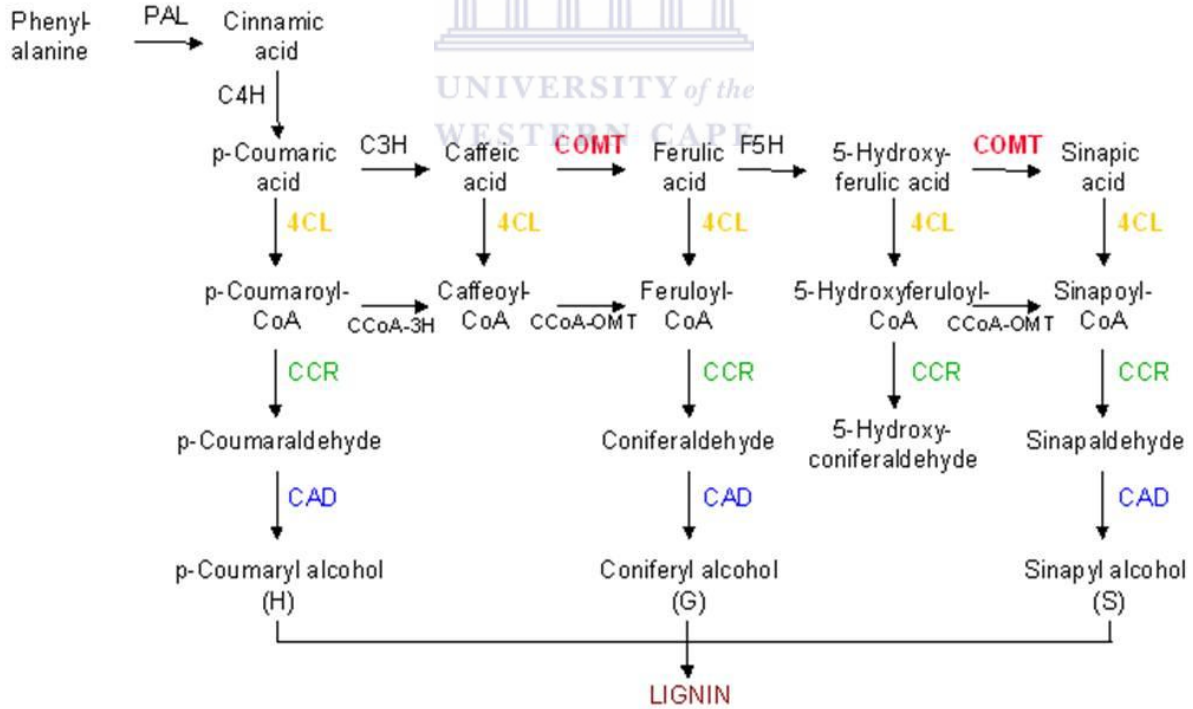

Figure 1.5 Biosynthesis of hydroxycinnamic acids and their derivatives involved in lignin production in plants. The biosynthetic enzymes resulting in the biosynthesis of lignin precursors (monolignols) and lignins are:phenylalanine ammonia-lyase (PAL); tyrosine ammonia-lyase (TAL); cinnamate 4-hydroxylase (C4H); 4-hydroxycinnamate 3-hydroxylase (C3H); caffeic acid 3-O-methyltransferase (COMT); ferulate 5-hydroxylase (F5H); 4-coumarate (4CL): CoA ligase; coumaroyl-coenzyme A 3-hydroxylase (CCoA 3H); caffeoyl-coenzyme A O-methyltransferase (CCoA OMT); cinnamoyl-CoA reductase (CCR); and cinnamyl alcohol dehydrogenase (CAD) (Figure was adapted from Spangenberg 2001).

The formation of hydroxycinnamic acid derivatives requires the formation of hydroxycinnamate- CoAs (e.g. *p*-coumaroyl-CoA) catalyzed by hydroxycinnamoyl-CoA ligases or by the action of O-glycosyl transferases. Hydroxycinnamate-CoA enter various specific phenylpropanoid reactions (Figure 1.5) such as condensations with malonyl-CoA, leading to flavonoids, or NADPH-dependent reductions that lead to lignins. Moreover, hydroxycinnamate-CoA can also conjugate with organic acids. In the biosynthesis of sugar derivatives of hydroxycinnamic acids, the transfer of glucose from uridine diphosphoglucose to hydroxycinnamic acid is catalyzed by glucosyl transferase (Macheix et al. 1990).

1.5.3 Function of phenolic acids in plants

Phenolics are of great importance as cell-wall support materials (Wallace and Fry 1994). They form an integral part of the cell-wall structure, mainly in the form of polymeric materials such as lignins, serving as mechanical support and barrier against microbial invasion. After cellulose, lignins are the second most abundant organic structures on earth (Wallace and Fry 1994). Besides the well-known volatile terpenoids and toxic water-soluble phenolics, such as simple phenols, hydroxybenzoic acids and hydroxycinnamic acids may serve as allelopathic compounds (Strack 1997). Phenolics, especially flavonoids, can act as signal molecules (host-recognition substances) in the interaction between the plant and nitrogen-fixing bacteria in certain leguminuos plants (Strack 1997). An important function of flavonoids and phenolic acids is their action in plant defence mechanisms (Bennet and Wallsgrove 1994; Dixon and Paiva 1995). Stress conditions such as excessive UV light, wounding, salinity or infection induces the biosynthesis of phenolic compounds. Thus, environmental factors may have a significant contribution to the content of phenolic acids and flavonoids in plants. Phenolic

compounds also contribute to the disease resistance mechanisms in plants. Two modes of action appear to operate in plant defence mechanisms, namely direct toxic effects (e.g. free radicals formed from lignin precursors) and the active and rapid deposition of barriers such as lignin (Bennet and Wallsgrove 1994; Strack 1997). Phenolics may accumulate as inducible low molecular weight compounds, called 'phytoalexins', as a result of microbial attack. Phytoalexins are post-infectional, i.e. although they might already be present at low concentrations in the plant; they rapidly accumulate upon pathogen attack (Strack 1997). In contrast, the pre-infectional toxins are constitutive compounds. They are present in healthy tissues at concentrations high enough for defence, either as free toxins or in conjugated forms (Strack 1997). Among the phenolic phytoalexins and toxins, hydroxycoumarins and hydroxycinnamic acids are of major importance (Strack 1997) but flavonols also play a role in plant defence.

1.5.4 Caffeic acid (CA) as a phenolic compound

CA is a well-known phenolic phytochemical present in many foods (Kang et al. 2009). Recent studies suggested that CA exerts anti-carcinogenic effects, but little is known about the underlying molecular mechanisms and specific target proteins. It is found in several grains, fruits, and vegetables (Table 1.3). Some of the best sources of CA are white grapes, white wine, olives, olive oil, spinach, cabbage, and asparagus. CA is one of the hydroxycinnamic acids, which are the most widely dispersed class of phenylpropanoids in plants. Like other polyphenolic acids such as ferulic, ellagic, and tannic acid, CA is thought to have considerable anti-carcinogenic potential, and is known to confer antioxidant activity (Chen and Ho 1997; Gülçin 2006).

Table 1.3 Detection of CA in different plant species. The table was adapted and modified from Stohr and Herrmann (1975).

Plant species	Organ	[] as caffeic acid (mg/kg), fresh weight	Hydrolysis
Vegetables			
Bean	Hulls, Unripe fruit	12-14; <0.5-9	Enzymatic
Cabbage	Outer leaves	11-44	Enzymatic
Carrot	Whole vegetables	18-96	Enzymatic
Pea	Unripe seeds	<0.5-1	Enzymatic
Potato	peel	63-280	Enzymatic
Fruits			
Grape fruit	Fruit/peel	11-40/ 14-51	Enzymatic
Lemon	Fruit/peel	13-27/ 16-35	Enzymatic
Orange	Fruit/peel	19-50/ 12-36	Enzymatic
Strawberry	Fruit	<0.5-14	Enzymatic
Watermelon	Fruit/peel	<0.5	Enzymatic

1.5.5 Antioxidant properties of CA

Antioxidants are organic molecules which can prevent or delay the progress of oxidation of other molecules. Hydroxycinammic acid compounds are widely distributed in the plant kingdom have been described as chain-breaking antioxidants, probably acting through radical-scavenging, which is related to their hydrogen-donating capacity and their ability to stabilize the resulting phenoxyl radical (Siquet et al. 2006). These compounds usually exist as glycosides or organic acids like CA. According to Leung et al. (1981), CA is the most predominant phenolic acid in sunflower seeds although its presence has been detected in various other plant species. As one of the most predominant phenolic compounds present in plants, CA can trap ROS directly or scavenge them through a series of coupled reactions with antioxidant enzymes. This has been shown in a study conducted by Jung et al. (2005). The role of CA as an antioxidant has been attributed to its ability to scavenge

alkoxyl radicals as a result of metal-ion breakdown of hydroperoxide-enriched methyl linoleate in sunflower oil at concentrations between 0.5-2 mM (Milic et al. 1998). Recently, it has been showed by Iglesias et al. (2009) that CA inhibited lipid oxidation in fish muscle while Cheng et al. (2007) showed that CA is an α-tocopherol protectant in human low density lipoprotein (LDL) as well as a radical and singlet oxygen quencher (Gülçin 2006). Furthermore, it was suggested by Sroka and Cisowski (2003) that the binding of quinic acid to CA resulted in an increase in antioxidant activity with decreased hydrogen peroxide and DPPH radical-scavenging activities.

1.5.6 Role of CA in plant growth

CA is one of the most common cinnamic acids isolated from a variety of crops, weed residues, as well as other plants (Rice 1995). It has been reported that CA inhibits plant growth in a concentration dependent manner. This is evident in studies conducted by various authors (Batish et al. 2008; Singh et al. 2009; Bubna et al. 2011). According to Batish et al. (2008), CA (at concentrations between 0 and 1000 mM) significantly suppressed root growth of mung bean, and impaired adventitious root formation (ARF) and root length in the mung bean hypocotyl cuttings. This inhibition was supported by Singh et al. (2009) who showed that at similar concentrations CA inhibits ARF in mung bean hypocotyls by inducing ROS-generated oxidative stress. The allelopathic effect of CA on soybean root growth was also tested by Bubna et al. (2011) who showed that the exogenous supplementation of CA to soybean seedlings seems to impair the growth of soybean roots. Based on these observations, CA appears to have a negative impact on plant growth.

CHAPTER 2

EXOGENOUSLY APPLIED NITRIC OXIDE ENHANCES SALT STRESS TOLERANCE IN MAIZE BY MEDIATING THE SCAVENGING OF ROS

2.1 Abstract

Salinity stress causes ionic stress (mainly from high Na^+ and Cl^- levels) and osmotic stress, resulting in cell death and inhibition of growth and ultimately adversely reducing crop productivity. In this study, changes in root H_2O_2 content, root lipid peroxidation, root cell death and root antioxidant enzymatic activity were investigated in maize seedlings (Zea mays L. cv. Silverking) after long-term (21 days) salt stress (150 mM NaCl) in the presence/absence of exogenously applied nitric oxide generated from the nitric oxide donor 2,2´-(Hydroxynitrosohydrazano)bis-ethane. Salt stress increased H_2O_2 content in the maize roots and resulted in elevated lipid peroxidation and cell death. Altered antioxidant enzymatic activities were observed in the roots in response to salt stress. Treatment of salt-stressed plants with exogenous nitric oxide reversed the detrimental effects caused by salt stress in the roots of maize. These results demonstrate that exogenously applied nitric oxide confers salt stress tolerance in maize by reducing salt stress-induced oxidative stress through a process that limits accumulation of ROS via enhanced antioxidant enzymatic activity.

2.2 Introduction

Salinity is the presence of the excessive concentrations of soluble salts in the soil, with the consequence of suppressed plant growth. The major cations contributing to salinity are Na^+, Ca^{2+}, Mg^{2+}, K^+ and anions are Cl^-, SO_4^{2-}, HCO_3^-, CO_3^{2-} and NO_3^{2-}.

Accumulation of salt in plant tissues to levels that limit water uptake by cells and interfere with normal metabolic functions in plant tissue can severely reduce plant growth and ultimately result in plant demise (Abogadallah, 2010; Mahajan and Tuteja, 2005; Munns and Tester, 2008). As a consequence of salt accumulation, ROS such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\cdot$) are produced in excess which leads to oxidative damage to cellular macromolecules (DNA, proteins and lipids) (Hasegawa et al. 2000; Abogadallah, 2010; Gill and Tuteja 2010). On the other hand, ROS can serve as signals that trigger antioxidant enzymatic activity so that excessive ROS can be efficiently scavenged to prevent their accumulation to toxic levels (Miller et al. 2008). According to Gill and Tuteja (2010), abiotic stresses such as salt stress are thought to trigger excessive ROS generation, to which plants respond by enhancing their antioxidant defenses in order to maintain equilibrium between antioxidants and ROS. Failure to maintain redox homeostasis would result in excessive accumulation of ROS to toxic levels, which would impose oxidative stress on the plant, for which the extent of lipid peroxidation is generally regarded as indicative of the severity of oxidative stress (Gill and Tuteja, 2010). Plants capable of triggering high levels of antioxidant enzyme activity and efficiently preventing excessive accumulation of ROS can thus be regarded as having high antioxidant capacity whereas those that cannot would be regarded as having lower antioxidant capacity. The induction of lipid peroxidation by excessive ROS accumulation caused by salt stress would ultimately result in plant cell death if redox homeostasis cannot be maintained (Gill and Tuteja, 2010).

The role of nitric oxide (NO) in facilitating salt stress tolerance in plants was confirmed by the identification of NO as a vital component in salt stress tolerance in *Atriplex centralasiatica* seedlings (Xu et al. 2011). It was recently demonstrated that

salt stress induces elevation of NO content in plant tissue in a pathway that involves nitric oxide synthase-like activity (Zhao et al. 2004; Zhang et al. 2006; Xu et al. 2011). There is also evidence that excessive salt stress suppresses NO biosynthesis, which suggests a role for NO in plant salt stress responses (Zhao et al. 2007). Furthermore, there is contradiction with regards to the timing of the onset and maintenance of the elevated NO content in response to salt stress as some reports show that salt induces elevation of NO content in plant for tissue only a few hours after exposure to salt stress and NO content returns to basal levels within 8 hours (Zhang et al. 2006); whereas some reports demonstrate that elevation of NO content is sustained in response to long-term salt stress up to at least 4 days during salt exposure (Xu et al. 2011). Furthermore, recent investigations have established that exogenously applied NO enhances plant tolerance against salt stress (Zhao et al. 2004; Zhang et al. 2006; Zhao et al. 2007). Furthermore, it appears that the enhancement of plant tolerance against salt stress may be mediated in part by antioxidant enzymes that act to prevent oxidative stress (Shi et al. 2007; Tanou et al. 2009; Zheng et al. 2009; Molassiotis et al. 2010; Wu et al. 2010).

Although the role of exogenously applied NO in enhancing salt stress tolerance and salt stress-induced oxidative stress tolerance in plants is partially understood, the majority of the reports focus on a subset of the plant antioxidant enzymes that are involved in the processes leading to NO-mediated salt stress tolerance. Therefore, in order to expand the understanding of the molecular processes participating in NO-transduced salt stress tolerance, the influence of exogenously applied NO on ROS accumulation and the extent of lipid peroxidation was investigated along with its regulating effects on the enzymatic activities of various antioxidant enzymes in response to salt stress in maize seedlings.

2.3 Materials and methods

2.3.1 Plant Growth

Maize (*Zea mays* L.) seeds (donated by Capstone Seeds Pty Ltd, Howick, South Africa) were surface-sterilized in 0.35% sodium hypochlorite for 10 minutes and then rinsed four times with sterile distilled water. Seeds were imbibed in sterile distilled water for 30 min and sown in 2 litres of pre-soaked (distilled water) filtered silica sand (98% SiO_2, Rolfes® Silica, Brits, South Africa), in 20 cm diameter plastic pots. The sand was kept moist by irrigation with distilled water during germination. Germinated seedlings (one plant per pot) were grown on a 25/19 °C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m^{-2}.s^{-1} during the day phase. Plants were supplied with nutrient solution [1mM K_2SO_4, 2 mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM KNO_3, 10 mM NH_4NO_3, 1 mM K_2HPO_4 buffer at pH 6.4, 5 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM ZnSO4, 1 µM $CuSO_4$, 2 µM Na_2MoO_4, 1 µM $CoSO_4$, 100 µM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 6.4] at the V1 stage (two fully expanded leaves and one emerging leaf). Plants at the same stage (V1) of development and similar height were selected for all subsequent experiments.

2.3.2 Treatment of plants

After one week of growth (from the V1 stage), control plants were supplied with nutrient solution every third day. For treatments, 200 ml of nutrient solution containing either 150 mM NaCl, 10 µM DETA/NO (NO donor), 10 µM DETA (chemically similar to DETA/NO but contains no NO moiety and thus does not release NO), a combination of 150 mM NaCl and 10 µM DETA/NO or a combination 150 mM NaCl and 10 µM DETA (all as final concentrations of these chemicals in the

nutrient solution) were applied to each plant directly to the sand at the base of the stem of the plant in the pot, every three days.

2.3.3 Evaluation of cell viability

A modified method was followed for the cell viability assays (i.e. after 21 days from the first treatment) (Sanevas et al. 2007). Briefly, fresh root tissue (100 mg per treatment) from five different plants of each of the treatment was harvested and stained at room temperature with 0.25% (w/v) Evans Blue dye for 15 minutes. The roots were washed for 45 min in distilled water to remove surface-bound dye, followed by extraction of the Evans Blue stain (taken up by dead root cells) from root tissue using 1% (w/v) SDS, after incubation for 1 hour at 55°C. Absorbance of the extract was measured at 600 nm to determine the level of Evans Blue up-take by the root tissue.

2.3.4 Preparation of protein extracts

Extracts were obtained from pre-washed maize roots by grinding the root tissue into a fine powder in liquid nitrogen and homogenizing 400 mg of the tissue with either 1 ml of homogenizing buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40 000] for determination of NO content, antioxidant enzymatic activities and caspase-like activity, or 1 ml of 10% trichloroacetic acid (TCA) for H_2O_2 content, lipid peroxidation, total ascorbate, dehydroascorbate, total reduced glutathione and oxidized glutathione. The resulting homogenates were centrifuged at 12 000 X *g* for 15 min and the supernatants were used for biochemical assays. Protein concentrations for all assays were measured in the extracts (prepared with homogenizing buffer) as instructed for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories).

2.3.5 Measurement of H_2O_2 content in maize roots

H_2O_2 content was determined based on a previously described method (Velikova et al. 2000). The reaction mixture contained 75 µl of the TCA extract, 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI. Samples were incubated at 25°C for 20 minutes and absorbance readings of the samples were taken at 390 nm. H_2O_2 content was calculated based on a standard curve constructed from the absorbance (A_{390} nm) of H_2O_2 standards.

2.3.6 Measurement of lipid peroxidation in maize roots

Products of lipid peroxidation (reflective of MDA content) were estimated (Buege and Aust 1978). For these measurements, 1 ml of TCA extract was taken and 4 ml 0.5% TBA in 20% TCA was added. The mixture was heated for 30 min at 95°C and then cooled in ice for 10 min. The specific absorbance of products was read at 532 nm and nonspecific background-absorbance at 600 nm was subtracted from the readings. The concentration of MDA was calculated using a molar extinction coefficient 155 mM^{-1} cm^{-1}.

2.3.7 Measurement of antioxidant enzyme activities

For all antioxidant enzyme activity assays, proteins were prepared using the homogenizing buffer. APX (EC1.11.1.11) activities were measured using a modified method (Asada 1984). In summary, 50 µg of the root extracts (extracts supplemented with ascorbate to a final concentration of 2 mM) were added to the assay buffer (50 mM K_2HPO_4, pH 7.0, 0.1 mM EDTA, 50 mM ascorbate). The reaction was initiated with 1.2 mM H_2O_2 in a final reaction volume of 200 µl and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of 2.8 $mM^{-1}.cm^{-1}$.

For total glutathione peroxidase (GPX, EC 1.11.1.9) activity, a modified method was used (Drotar et al. 1985). The reaction mixture contained 50 µg of protein extract, 50 mM K_2HPO_4, pH 7.0, 1 mM EDTA, 2 mM glutathione (GSH), 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 2.5 Units of glutathione reductase and 90 µM H_2O_2. GPX activity was calculated based on the change in absorbance at 340 nm resulting from the oxidation of NADPH in the reaction, using the extinction coefficient of 6.2 $mM^{-1}.cm^{-1}$ for NADPH.

Dehydroascorbate reductase (DHAR, EC. 1.8.5.1) activity was measured according to a modified method (Arrigoni et al. 1992). The reaction mixture contained 100 mM K_2HPO_4 pH 6.3, 1 mM dehydroascorbate (DHAsc), 2 mM reduced GSH and 50 µg of enzyme extract. The DHAR activity was monitored by following the formation of ascorbate at 265 nm for 5 min and calculated using the absorbance coefficient of 14 $mM^{-1}.cm^{-1}$.

Glutathione reductase (GR, EC. 1.6.4.2) activity was determined by following the rate of NADPH oxidation at 340 nm (Esterbauer and Grill 1978). The assay mixture contained 0.2 mM NADPH, 0.5 mM glutathione disulfide (GSSG), 1 mM EDTA, 100 mM K_2HPO_4, pH 7.8, and 50 µg of enzyme extract in a 200 µl reaction. GR activity was calculated based on the oxidation of NADPH in the reaction, using the extinction coefficient of 6.2 $mM^{-1}.cm^{-1}$. Controls for the antioxidant enzyme assays were performed as described above except that the protein extract was replaced with homogenizing buffer and absorbances from these controls were subtracted from the readings obtained in the assays.

2.3.8 Statistical analysis

All experiments were performed three times independently, with each experiment done in duplicate. Samples from different replicates were stored separately at -80°C

until analysed. Results were given as mean ± standard deviation from these experiments. For statistical analysis, One-way analysis of variance (ANOVA) test was used for all data and means were compared according to the Tukey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

2.4 Results

2.4.1 Salt stress-induced cell death is restricted by exogenously applied NO

The effect of the various treatments on cell death was investigated because salt stress induces programmed cell death (PCD) and such PCD is often linked to enhanced cysteine protease activity. For evaluating cell viability, the extent of Evans Blue uptake (indicative of dead cells that take up the Evans Blue stain because of ruptured cell membranes, since living cells with intact cell membranes do not take up the stain) was measured in roots of the maize plants at the end of the 21 days of treatment.

Maize plants treated with DETA/NO restricted root cell death by ± 20% compared to the cell death measured for the untreated plants, whereas the cell death in roots from plants treated with DETA was similar to that of untreated plants (Figure 2.1). In response to salt stress, the induction of cell death was evident from the fact that Evans Blue uptake in these roots was approximately twice the level of the cell death observed in roots from untreated plants and this salt stress-induced increase in cell death was not altered by treating maize with a combination of NaCl and DETA (Figure 2.1). However, treatment with a combination of NaCl and DETA/NO reversed the level of cell death caused by salt stress, although not to levels of the untreated plants but significantly lower to what was observed for the salt stressed plants. This NO-induced reduction in root cell death in the salt-treated plants was ± 30% lower compared to the salt-stressed plants without exogenously applied NO (Figure 2.1).

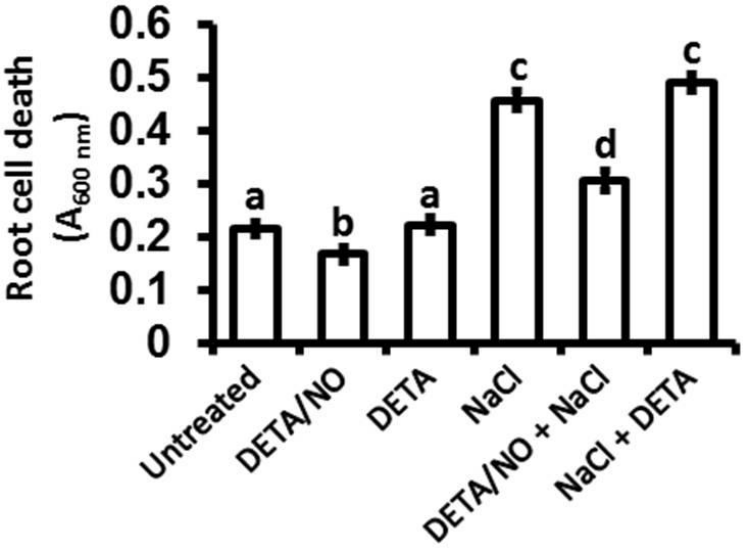

Figure 2.1 The effect of nitric oxide and salt stress on maize root cell death. Root cell death was measured in maize roots after 21 days of treatment that was initiated at the V1 stage of vegetative growth. Treatments were with nitrogen-free nutrient solution (UNTREATED), 10 µM DETA/NO, 10 µM DETA, 150 mM NaCl, 10 µM DETA/NO + 150 mM NaCl and 10 µM DETA + 150 mM NaCl. Error bars represent the means (±SE; n = 3) of three independent experiments.

2.4.2 Exogenously applied NO restricts salt stress-induced oxidative damage and the extent of lipid peroxidation

A consequence of detoxification of O_2^- by SOD is the production of H_2O_2 (Beyer and Fridovich 1987; Foyer and Noctor 2005). Several studies have established that salt stress leads to excessive accumulation of ROS, which cause oxidative stress in plant cells (Gill and Tuteja 2010). Therefore the levels of H_2O_2 and the effect of exogenously applied NO on the response of maize to salt-induced oxidative damage were investigated. The extent of malondialdehyde (MDA) accumulation, which is indicative of lipid peroxidation, in the various treatments was measured as an estimate of oxidative damage. Roots from plants exposed to salt stress accumulated H_2O_2 contents to levels that were ± 160% higher than the H_2O_2 content of roots from untreated plants, whereas treatment with DETA/NO caused a ± 30% reduction in root H_2O_2 levels compared to the H_2O_2 levels of roots from untreated plants.

Furthermore, there were no significant differences in H_2O_2 content between roots from DETA-treated and untreated plants (Figure 2.2A). Treatment of maize plants with a combination of NaCl and DETA/NO led to a reduction in salt stress-induced H_2O_2 accumulation to ± 40% compared to the NaCl-treated plants whereas a combination treatment with NaCl and DETA did not alter the salt stress-induced H_2O_2 accumulation (Figure 2.2A).

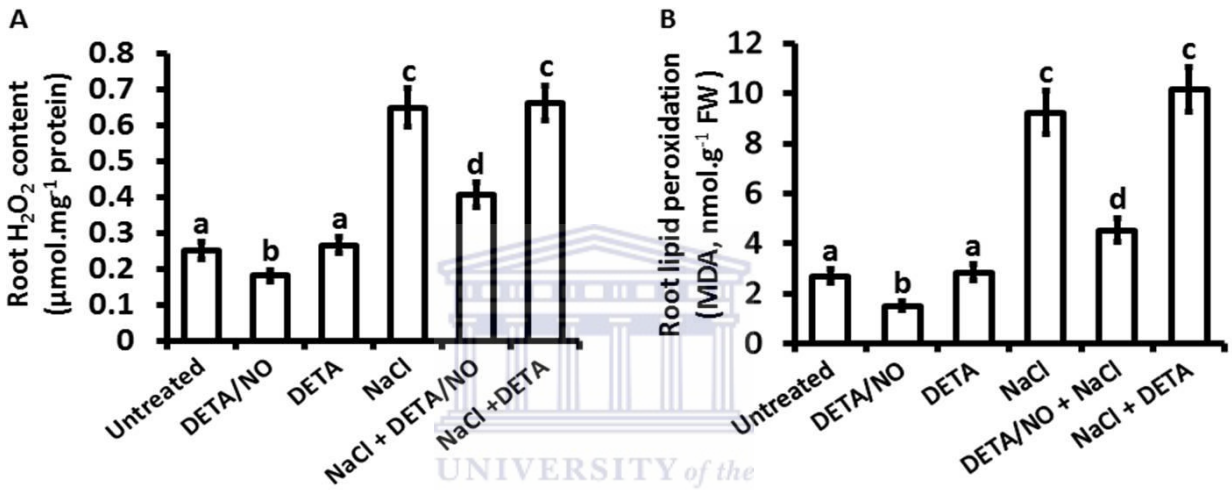

Figure 2.2 The effect of nitric oxide and salt stress on H_2O_2 content and lipid peroxidation. H_2O_2 content (A), lipid peroxidation (B) were measured in maize roots after 21 days of treatment that was initiated at the V1 stage of vegetative growth. Treatments were with nitrogen-free nutrient solution (UNTREATED), 10 µM DETA/NO, 10 µM DETA, 150 mM NaCl, 10 µM DETA/NO + 150 mM NaCl and 10 µM DETA + 150 mM NaCl. Error bars represent the means (±SE; n = 3) of three independent experiments.

No significant differences were observed between the MDA content of roots from untreated and DETA-treated plants but the MDA content of roots from plants treated with DETA/NO was reduced by ± 40% compared to the MDA content of roots from untreated plants (Figure 2.2B). Maize plants treated with NaCl accumulated root MDA content to levels that are ± 240% more than the root MDA content from untreated plants and this increase was similar to that of roots from plants treated with a combination of NaCl and DETA (Figure 2.2B). However, the salt stress-induced

increase in MDA content was ± 50% lower in roots from plants treated with a combination of NaCl and DETA/NO in comparison to the MDA content of roots from plants treated with NaCl alone (Figure 2.2B).

2.4.3 Exogenously applied NO modulates the activity of antioxidant enzymes in response to salt stress

Alteration of the activity of antioxidant enzymes in response to salt stress is well-documented in a variety of plant species (Abogadallah 2010; Gill and Tuteja 2010) and there is evidence that such responses are modulated by NO (Molassiotis et al. 2010; Shi et al. 2007; Tanou et al. 2009; Wu et al. 2010; Zheng et al. 2009). However, the reports on the modulation of these antioxidant responses by NO are limited to short-term salt stress. The effect of NO on the antioxidant enzyme responses to long-term salt stress were thus investigated here.

In response to treatment with DETA/NO, root APX activity was enhanced by ± 50%, whereas no change in APX activity was observed in response to DETA in comparison to roots from untreated plants (Figure 2.3A). On the other hand, the root APX activity in response to salt stress was induced to a slightly higher level than was observed in NO treated plants. This increase was ± 70% higher compared to the activity detected for the untreated plants (Figure 2.3A). However, the combination of NaCl and DETA/NO caused the highest induction of root APX activity since this activity was ± 120% higher than the activity observed from the untreated plants, whereas the combination of NaCl and DETA had a similar effect as NaCl alone (Figure 2.3A). According to the results, a similar trend of enzymatic activity was observed for root GPX as it was for root APX activity (Figure 2.3B).

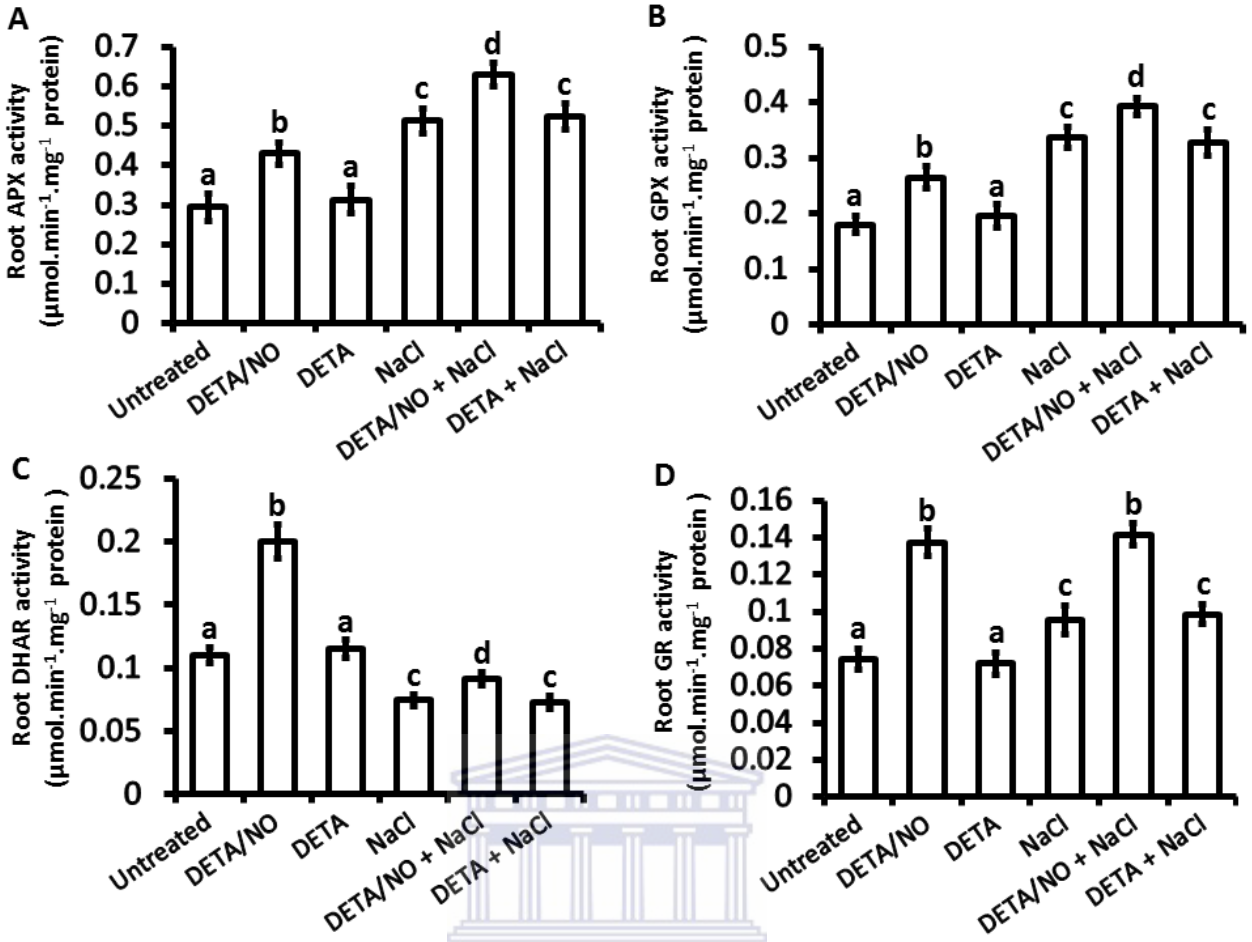

Figure 2.3 Changes in maize root antioxidant (APX, GPX, DHAR and GR) enzymatic activity is modulated by NO in response to salt stress. Total APX (A), GPX (B), DHAR (C) and GR (D) enzymatic activity were measured spectrophotometrically in roots of maize. Assays were done on maize plants that were treated at the V1 stage for a period of 21 days. Nitric oxide and salt-induced stress increase the enzymatic activity of APX, GPX and GR whereas a reduction for DHAR activity was observed in plants treated with salt. Error bars represent the means (±SE; n = 3) of three independent experiments.

In response to treatment with exogenously applied NO (DETA/NO) root dehydroascorbate reductase (DHAR) activity was enhanced by ± 100% compared to those of untreated plants whereas DETA did not alter the DHAR activity (Figure 2.3C). On the other hand, treatment of maize with salt stress inhibited root DHAR activity by ± 30% compared to the DHAR activity in roots from untreated plants (Figure 2.3C). The salt stress-induced inhibition of DHAR activity was alleviated by exogenously applied NO but not by DETA, as indicated by the observation that the

DHAR activity in roots from plants treated with a combination of NaCl and DETA/NO was ± 30% higher than the activity in roots from NaCl-treated plants. However root DHAR activity from plants treated with a combination of NaCl and DETA was similar to that of roots from NaCl-treated plants alone (Figure 2.4C).

Glutathione reductase (GR) activity in roots was enhanced by exogenously applied NO to levels that were ± 90% higher than those observed from the untreated plants, yet they were not altered by DETA in comparison to roots from untreated plants (Figure 2.3D). However, the induction of root GR activity in response to salt stress was limited to a level that is approximately 30% higher than the level observed for untreated plants (Figure 2.3D). The root GR activity observed in response to the combination of NaCl and DETA/NO was enhanced to a level similar to what was observed for exogenously applied NO, whereas the combination of NaCl and DETA had a similar effect as NaCl alone (Figure 2.3D).

2.5 Discussion

2.5.1 NO confers salt stress tolerance in maize by restricting cell death and lipid peroxidation induced by excessive ROS accumulation

The responses of maize to long-term (21 days) exposure to salt stress (150 mM) and exogenously applied nitric oxide (10 µM DETA/NO as the source of exogenously applied NO) was analyzed. In response to long term salt stress, there was an excessive accumulation of ROS. It has recently been shown by Keyster et al. (2012) that long-term salt stress resulted in the inhibition of maize biomass and that this poor growth performance of maize under salt stress can be attributed to excessive accumulation of ROS because leaf H_2O_2 levels increased drastically in response to salt stress and this corresponded to reduced dry weights in plants subjected to salt stress. This view is supported by the observation that extensive root cell death

(Figure 2.1) occurred in response to salt treatment and this trend observed for cell death corresponded to that seen for root lipid peroxidation (Figure 2.2B). It is thus reasonable to suggest that salt stress induces excessive accumulation of root H_2O_2 (as seen from results described in this chapter) and the resulting excessive ROS levels destabilize the cell membrane, leading to cell death and loss of biomass.

The fact that exogenously applied NO reduced root H_2O_2 content in plants treated with DETA/NO alone and in plants treated simultaneously with DETA/NO and NaCl; together with the finding in the results described in this chapter that such treatments reversed the extent of root lipid peroxidation, leaf cell death and root/shoot biomass loss (Keyster et al. 2012); implies that exogenously applied NO is involved in improving maize tolerance to salt stress by regulating H_2O_2 accumulation and cell death. Such effects are not seen with DETA, confirming that the effects on salt stress tolerance in the DETA/NO treatments are conferred by the exogenously applied NO.

2.5.2 Salt stress tolerance is mediated by NO-induced antioxidant activity

Involvement of ROS scavenging in the NO-mediated salt stress tolerance in maize is supported by the enhancement of antioxidant enzymatic activity in the treatments described here. APX, GPX and GR activities increased in response to both exogenously applied NO on its own and salt stress alone but the salt stress-induced increase in these enzymatic activities was significantly lower than the increase in the enzymatic activities seen for treatments where salt stress was applied in combination with exogenously applied NO. Given that H_2O_2 levels remained high in the salt-stressed maize and were lowered in the 'NaCl + DETA/NO' treatment when compared to the 'NaCl' treatment, together with a much more pronounced increase in APX, GPX and GR activities in the 'NaCl + DETA/NO' treatment, it is hypothesized that the increase in APX and GPX activity in response to NaCl is inadequate to

counteract the excessively high levels of H_2O_2 that accumulate in response to salt stress while the increase in GR activity under salt stress is insufficient to cater for efficient regeneration of GSH. The result of such inefficiency in the antioxidant system is accelerated cell death under salt stress. Salt-stressed plants supplemented with NO would thus be thought to induce sufficient APX and GPX activity to efficiently reduce H_2O_2 to levels that are less damaging to the plant, although not to the levels of untreated plants.

The inhibition of DHAR activity in response to long-term salt stress and the reversal in its activity (although not to the level of the untreated plants) when salt stressed plants are supplied with NO supports the involvement of exogenously applied NO in the enhancement of plant salt stress tolerance via regulation of antioxidant enzyme activity.

This further raises the possibility that ascorbate consumption is elevated, probably as a result of heightened oxidation of ascorbate because of augmented APX activity, under salt stress and the regeneration of reduced ascorbate under these conditions is restricted because of the inhibition of DHAR activity under salt stress. The fact that exogenously applied NO relieved the salt stress-induced inhibition of DHAR activity implies that efficient regeneration of reduced ascorbate ensures better availability of this reductant to sustain APX activity at levels sufficient to scavenge H_2O_2 and thus reduce cellular damage caused by this ROS.

CHAPTER 3

THE ENZYMATIC ACTIVITIES OF MAIZE ANTIOXIDANT ENZYME ISOFORMS ARE DIFFERENTIALLY MODULATED BY NITRIC OXIDE IN RESPONSE TO SALT STRESS

3.1 Abstract

NO is a bioactive, lipophilic free radical that acts as a signaling molecule with different kinds of physiological functions and its role in salt stress tolerance has been studied in various plant species. To counter the deleterious effects caused by salt stress, we have investigated the protective role of exogenously applied NO on maize plants under salt stress. Maize plants were subjected to various treatments (10 µM DETA/NO, 10 µM DETA, 150 mM NaCl, '10 µM DETA/NO + 150 mM NaCl' and '10 µM DETA + 150 mM NaCl') for a period of 21 days. The effect of these treatments on the activity of various isoforms of different antioxidant enzymes was evaluated in maize leaves. The results show that both NO and NaCl differentially regulate the enzymatic activity of isoforms belonging to four different antioxidant enzymes (SOD, APX, GPX and GR). It is also evident that NO increases the activity of some isoforms when plants are exposed to NaCl. Treatment with DETA, which serves as a control for DETA/NO, did not influence the enzymatic activity when supplied alone or in combination with NaCl. We therefore propose that isoforms for which enzymatic activities are augmented in salt-stressed plants supplemented with NO to levels higher than those observed for untreated or the various treatments (NO alone or NaCl alone) could play a significant role in NO-mediated salt stress tolerance in maize plants.

3.2 Introduction

Salinity is one of the most important environmental factors that cause reduction in plant growth, development and yield worldwide. Salt stress changes the morphological, physiological and biochemical responses of plants (Amirjani 2010; Siringam et al. 2011). There is evidence that high salt concentrations cause an imbalance in cellular ions, resulting in ion toxicity and osmotic stress, leading to the generation of ROS which cause damage to DNA, lipids and proteins (Yasar et al. 2006). Studies have revealed that ROS are accumulated in many plants exposed to high levels of salt. These plants include potato (Rahnama and Ebrahimzadeh 2005), wheat genotypes (Sairam et al. 2005) and tomato (Al-Aghabary et al. 2005), tomato and spinach (Gunes et al. 2007). The widespread nature of this problem underscores the importance of finding a way to protect plants from accumulation of ROS as a result of salinity stress and subsequent oxidative damage (Noctor and Foyer 1998) that would be caused by the ROS.

ROS have the potential to interact with many cellular components, causing significant damage to cell membranes and other cellular molecules, and consequently growth inhibition (Verma and Mishra 2005; Agarwal and Shaheen 2007; Gao et al. 2008). Some of the ROS are highly toxic and must be detoxified by cellular responses if the plant is to survive and grow (Gratäo et al. 2005). ROS scavenging depends on the detoxification mechanism, which may occur as a result of sequential and simultaneous action of a number of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX).

Nitric oxide (NO), a reactive nitrogen species, is believed to act as a signaling molecule mediating responses to both biotic and abiotic stresses in plants (Crawford

and Guo 2005; Delledonne 2005). The presence of NO has been shown to induce seed germination (Beligni and Lamattina 2000), to affect growth and development of plant tissue (Durner and Klessig 1999), to maintain iron homeostasis (Murgia et al. 2002) and to regulate plant maturation and senescence. Recently, a few studies suggested that NO can play a role in protecting plants from oxidative stresses (Garcıa-Mata and Lamattina 2001; Shi et al. 2005) by increasing the activity of antioxidative enzymes (Kopyra and Gwozdz 2003). This work describes how exogenously applied NO and NaCl regulates changes in the activity of isoforms of various antioxidant enzymes in the leaves of maize. The aim was to determine whether DETA/NO, as an NO donor, mediates salt stress tolerance in maize by enhancing the activity of specific isoforms of antioxidant enzymes. This was motivated by the observation that nitric oxide influences the activity of antioxidant enzymes in roots (Chapter 2) and leaves (Keyster 2011) of maize under salt stress but the influence of these treatments on the specific isoforms of these enzymes had not been characterized.

3.3 Materials and Methods

3.3.1 Plant Growth

Maize (*Zea mays* L.) seeds (donated by Capstone Seeds Pty Ltd, Howick, South Africa) were surface-sterilized in 0.35% sodium hypochlorite for 10 minutes and then rinsed four times with sterile distilled water. Seeds were imbibed in sterile distilled water for 30 min and sown in 2 litres of filtered silica sand (98% SiO_2, Rolfes® Silica, Brits, South Africa) that had been pre-soaked in distilled water in 20 cm diameter plastic pots. The sand was kept moist by irrigation with distilled water during germination. Germinated seedlings (one plant per pot) were grown on a 25/19 °C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic

photon flux density of 300 µmol photons.m^{-2}.s^{-1} during the day phase. Plants were supplied with nutrient solution [1 mM K_2SO_4, 2 mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM KNO_3, 10 mM NH_4NO_3, 1 mM K_2HPO_4 buffer at pH 6.4, 5 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM ZnSO4, 1 µM $CuSO_4$, 2 µM Na_2MoO_4, 1 µM $CoSO_4$, 100 µM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 6.4] at the V1 stage (two fully expanded leaves and one emerging leaf). At this stage (V1) plants of similar height were selected for all subsequent experiments.

3.3.2 Treatment of plants

After one week of growth (from the V1 stage), control plants were supplied with nutrient solution every third day. For treatments, 150 mM NaCl, 10 µM DETA/NO (NO donor), 10 µM DETA (contains no NO moiety), a combination of 150 mM NaCl and 10 µM DETA/NO and a combination 150 mM NaCl and 10 µM DETA were supplemented in nutrient solution and applied to each plant directly to the sand at the base of the stem of the plant in the pot every three days.

3.3.3 Preparation of protein extracts

Extracts were obtained from maize leaves by grinding the leaf tissue into a fine powder in liquid nitrogen and homogenizing 400 mg of the tissue with 1 ml of homogenizing buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetra acetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40 000]. The resulting homogenates were centrifuged at 12 000 X *g* for 15 min and the supernatants were used for the detection of antioxidant enzymes. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

3.3.4 Detection of antioxidant enzyme activity in maize leaves

3.3.4.1 Superoxide Dismutase (SOD)

For the detection of SOD isoforms in maize leaves, native PAGE was performed at 4°C in 10% polyacrylamide mini gels using 120 µg of protein per sample. SOD activity was detected by staining with 0.5 mM riboflavin and 2.5 mM nitroblue tetrazolium, as described by Beauchamp and Fridovich (1971). SOD isozyme patterns were determined by incubating the gels with 5 mM H_2O_2 to inhibit both Cu/ZnSOD and FeSOD, or with 5 mM KCN to inhibit only Cu/ZnSOD (Archibald and Fridovich 1982). MnSOD activity is resistant to both treatments.

3.3.4.2 Ascorbate peroxidase (APX)

APX activity staining was performed according to Lee and Lee (2000). Non-denaturing electrophoresis was performed in a buffer containing 2 mM ascorbate at 4°C in 10% polyacrylamide mini gels containing 10% glycerol. Subsequent to electrophoresis the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM ascorbate for a total of 20 min with the equilibration buffer changed every 10 min. This was followed by addition of 2 mM H_2O_2 to the gel in 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate, immediately after which the gel was incubated for 20 min. The gel was subsequently washed with sodium phosphate buffer (pH 7.8), 28 mM TEMED and 2.5 mM NBT with gentle agitation for approximately 10 min in the presence of light, after which the reaction was stopped by brief wash with distilled water.

3.3.4.3 Glutathione peroxidase (GPX)

GPX in-gel activity staining was carried out based on the procedure of Seckin et al. (2010), for detecting APX isoforms. Reduced glutathione (GSH) instead of

ascorbate was used in the staining solutions. Native PAGE was performed at 4°C in 10% polyacrylamide mini gels using 80 µg of protein. Prior to electrophoresis, gels were equilibrated in running buffer containing 2 mM GSH for 20 min at 4°C. Following electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM GSH for 20 min and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM GSH and 0.01% cumene hydroperoxide for 20 min. The gels were washed with distilled H_2O and transferred to a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.5 mM NBT for 10-20 min with gentle agitation in the presence of light.

3.3.4.4 Glutathione reductase (GR)

GR activity staining was performed in a 10% native polyacrylamide gel containing 10% glycerol at 4°C. GR activity was detected according to the procedure of Lee and Lee (2000) with slight modifications. GR was visualized in the native gel by incubation in 50 ml of 0.25 M Tris–HCl buffer (pH 7.9) containing 4.0 mM glutathione disulfide (GSSG), 1.5 mM NADPH, and 2 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for 20 min. The GR activity was negatively stained in the dark with a solution containing 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1.6 mM phenazine methosulfate (PMS) for 5-10 min at 30°C.

3.3.4.5 Quantitative evaluation of antioxidant enzymatic activities

Densitometry analysis was done on all the PAGE gels following image acquisition, using the Spot Denso tool (AlphaEase FC imaging software, Alpha Innotech Corporation). Individual gels were scored as arbitrary values (relative enzymatic activity) of three independent gels. The enzymatic activity (for the

respective antioxidants) of each isoform in the treatments was scored as an average of the relative pixel intensities from three independent gels and expressed in arbitrary units (by assigning a value of 1 for the isoform with the lowest pixel intensity in that type of isoform and expressing the rest of the pixel intensities for that type of isoform in the other treatments relative to this isoform, for example: if MnSOD1 has the lowest pixel intensity in the NaCl treatment, then this pixel intensity will be assigned a value of 1 and all the pixel intensities for MnSOD1 in the rest of the treatments will be expressed relative to the pixel intensity of MnSOD1 in the NaCl treatment).

3.3.5 Statistical analysis

The data was analyzed using the one-way analysis of variance (ANOVA) and tested for significance by the Tukey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

3.4 Results

3.4.1 NO and salt stress alter maize leaf superoxide dismutase (SOD) enzymatic activity

Superoxide dismutases (SOD, EC 1.15.1.1) represent the first line of plant defense against ROS in the array of enzymes that function to protect the plant cells against oxidative stress. For this reason SOD are classified as a chain-breaking group of enzymes since they scavenge superoxide and they yield another form ofROS; hydrogen peroxide (H_2O_2). This study shows the effect of exogenous NO (as the NO donor DETA/NO) and salt stress on the enzymatic activity of various SOD isoforms in maize leaves. SOD activity was differentially regulated in the presence of NO and salt stress (NaCl) as shown in Figure 3.1 (A-C) and Table 3.1. When protein

extracts from maize leaves were subjected to native PAGE and monitored for SOD activity, ten different SOD isoforms were observed in the untreated control plants (Figure 3.1A). Incubation of gels in 5 mM H_2O_2 (Figure 3.1C) and/or 6 mM KCN (Figure 3.2B) before staining for SOD activity identified three MnSOD isoforms (based on their resistance to both H_2O_2 and KCN inhibition), and seven Cu/Zn-SOD isoforms (inhibited by both H_2O_2 and KCN).

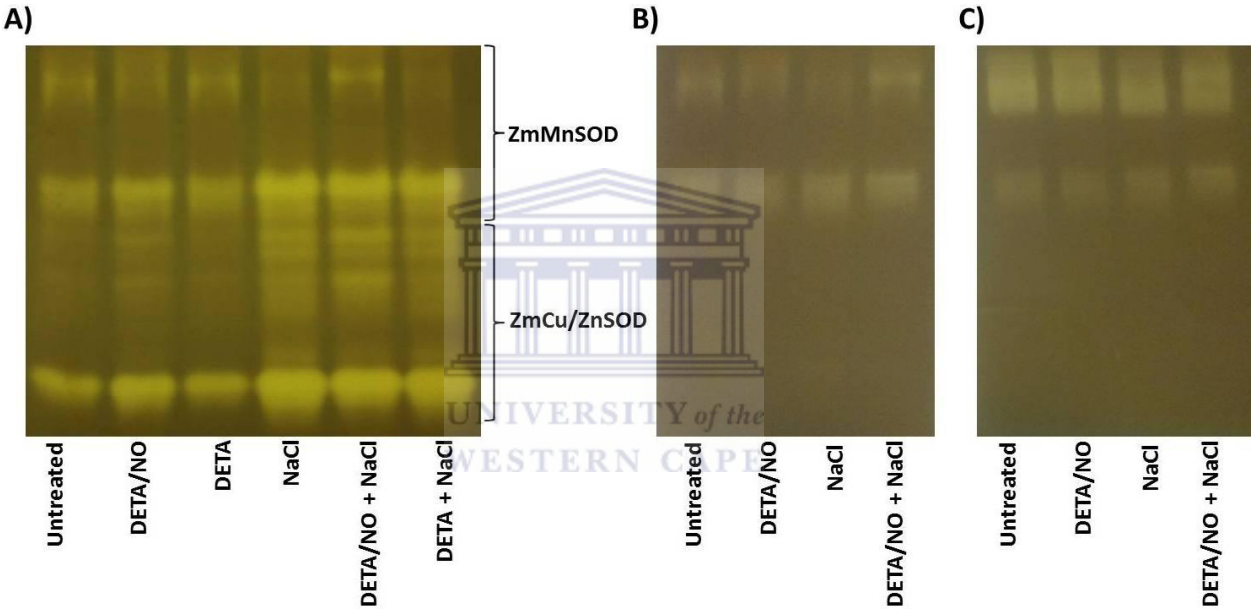

Figure 3.1 The effect of exogenous NO and salt stress on SOD enzymatic activity in maize. Assays were done on maize leaves taken from plants that were treated with the various treatments at the V1 stage for a period of 21 d (they were at the V3 stage at time of harvest). In-gel activities of various SOD isoforms in response to the following treatments are shown: 10 µM DETA/NO, 10 µM DETA, 150 mM NaCl, 10 µM DETA/NO + 150 mM NaCl, 10 µM DETA + 150 mM NaCl along with the untreated control. Individual SOD isoforms were identified by incubating gels in 6 mM KCN (B) and 5 mM H_2O_2 (C) respectively.

Under the influence of NO and/or salt stress, the intensities of some SOD isoforms (MnSOD2, MnSOD3, Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD3, Cu/ZnSOD4, Cu/ZnSOD5, Cu/ZnSOD6 and Cu/ZnSOD7) were preferentially enhanced, whereas

the intensity of MnSOD1 decreased when compared to the untreated plants (Figure 3.1A and Table 3.1). According to the densitometry analysis, MnSOD1 showed a ± 20% reduction in enzymatic activity in response to the treatment with the nitric oxide donor (DETA/NO) but no changes in response to DETA (used as a control for DETA/NO treatments) in comparison to the untreated plants (Figure 3.1A and Table 3.1). In fact, the DETA treatment had no effect on all the SOD isoforms detected when compared to the untreated controls. However, the activity of MnSOD1 in response to salt stress was reduced to a much lower level than it was in response to exogenously applied NO as indicated by the ± 37% reduction compared to the untreated plants (Table 3.1).

Table 3.1 Relative enzymatic activity of maize SOD isoforms.

Relative SOD Activity (Arbitrary Values)	Maize SOD isoforms	Treatments					
		Untreated	DETA/NO	DETA	NaCl	DETA/NO + NaCl	DETA + NaCl
	MnSOD1	1.58 ± 0.076^{a}	1.28 ± 0.065^{b}	1.57 ± 0.076^{a}	1.00 ± 0.052^{c}	1.85 ± 0.090^{d}	1.00 ± 0.050^{c}
	MnSOD2	1.01 ± 0.043^{a}	1.16 ± 0.055^{b}	1.00 ± 0.041^{a}	1.34 ± 0.063^{c}	1.32 ± 0.061^{c}	1.35 ± 0.065^{c}
	MnSOD3	1.00 ± 0.050^{a}	1.10 ± 0.065^{a}	1.00 ± 0.050^{a}	1.28 ± 0.064^{b}	1.06 ± 0.053^{a}	1.28 ± 0.064^{b}
	Cu/ZnSOD1	1.00 ± 0.050^{a}	1.57 ± 0.077^{b}	1.01 ± 0.050^{a}	1.74 ± 0.086^{c}	2.25 ± 0.112^{d}	1.73 ± 0.089^{c}
	Cu/ZnSOD2	1.00 ± 0.050^{a}	1.23 ± 0.061^{b}	1.00 ± 0.050^{a}	1.75 ± 0.088^{c}	1.44 ± 0.072^{d}	1.77 ± 0.089^{c}
	Cu/ZnSOD3	1.00 ± 0.050^{a}	1.37 ± 0.068^{b}	1.00 ± 0.050^{a}	1.14 ± 0.057^{a}	1.72 ± 0.086^{c}	1.14 ± 0.057^{a}
	Cu/ZnSOD4	NA	NA	NA	1.00 ± 0.050^{a}	1.19 ± 0.060^{b}	1.00 ± 0.050^{a}
	Cu/ZnSOD5	NA	NA	NA	1.00 ± 0.050^{a}	0.93 ± 0.047^{a}	1.00 ± 0.050^{a}
	Cu/ZnSOD6	NA	1.00 ± 0.050^{a}	NA	1.465 ± 0.074^{b}	1.175 ± 0.059^{a}	1.467 ± 0.073^{b}
	Cu/ZnSOD7	1.00 ± 0.050^{a}	1.35 ± 0.068^{b}	0.99 ± 0.050^{a}	1.87 ± 0.094^{c}	1.59 ± 0.08^{d}	1.87 ± 0.094^{c}

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate significant differences between treatments at 95% confidence according to the Tukey-Kramer test. The letters NA in the table indicate that very low or no activity was detected.

On the other hand, the combination of NaCl and DETA/NO caused an increase of ± 17% in MnSOD1 activity when compared to the untreated plants, whereas the

combination of NaCl and DETA had a similar effect as the NaCl treatment alone. MnSOD2 showed an increase in activity of ± 15% in response to DETA/NO and ± 33% in response to NaCl, whereas the combined treatment of DETA/NO and 150 mM NaCl resulted in an increase of ± 32% when compared to the control plants. For the MnSOD3 isoform there were no significant changes in the activity for (DETA/NO and 'DETA/NO + NaCl') treatments when compared to the untreated plants (Figure 3.1A and Table 3.1). However, there was a ± 27% increase in the intensities of the NaCl treated plants compared to the control plants. The results showed that all Cu/ZnSOD isoforms detected in this study were differentially regulated by both NO and salt stress. Cu/ZnSOD1 showed an increase of ± 56% in response to DETA/NO and a ± 74% increase in response to NaCl treatment compared to the untreated plants. However, the highest increase in SOD activity for Cu/ZnSOD1 was observed (a ± 125% increase) in the combined treatment of DETA/NO and NaCl. For Cu/ZnSOD2 an increase in enzymatic activity was observed for the various treatments. Apart from the slight increase in enzymatic activity observed in response to treatment with DETA/NO, both NaCl and the combination of DETA/NO and NaCl resulted in enhanced activity (by ± 75 and ± 44% respectively) for the Cu/ZnSOD2 isoform. For Cu/ZnSOD3, the enzymatic activity was slightly up-regulated in plants treated with salt stress, whereas plants treated with NO (including the NO-treated plants that were subjected to NaCl treatment) showed significant increase in enzymatic activity when compared to the untreated controls plants.

Furthermore, no or very low SOD activity was detected for three Cu/ZnSODs (Cu/ZnSOD4, Cu/ZnSOD5 and Cu/ZnSOD6) in the untreated as well as DETA/NO and DETA treated plants. However, SOD activity was detected in the salt stressed plants including the salt stressed plants supplemented with DETA/NO and DETA

respectively. For Cu/ZnSOD4, there was no significant change in the intensity in response to NaCl and DETA, whereas plants treated with the combination of DETA/NO and NaCl showed a slightly higher increase in SOD activity when compared to the salt stressed plants alone. No significant changes in activity were detected for Cu/ZnSOD5 in response to treatment with NaCl, DETA/NO + NaCl and DETA treatments respectively. For Cu/ZnSOD6, no activity was detected in the untreated and DETA treated plants whereas the activity in the salt stressed plants was significantly higher than those in the NO-treated plants. However there was no significant difference in activity in the plants treated with NO and the NO-treated plants supplemented with NaCl. For Cu/ZnSOD7, plants treated with DETA/NO resulted in an increase in SOD activity of ± 34% compared to the untreated and DETA treated plants. No significant change in activity was observed for the untreated and DETA treated plants. A similar trend was observed for the salt-stressed plants and those treated with a combination of DETA and NaCl. However, treatment with NaCl resulted in an increase of ± 86% in SOD activity whereas the combination of DETA/NO and NaCl increase the activity by ± 58% when compared to the untreated plants.

3.4.2 The effect of exogenous NO and salt stress on APX and GPX enzymatic activity in maize leaves

Given that NO and salt stress caused up-regulation of maize root APX and GPX enzymatic activity (Chapter 2), we analyzed the responses of individual APX and GPX isoforms (depicted as relative activity) to NO and salt stress, using in-gel enzymatic activity assays (Figures 3.2A and 3.2B) coupled with densitometry analyses (Table 3.2-APX activity).

For APX activity, the results show that in response to NO (DETA/NO as an NO donor) four APX isoforms were detected whereas in the salt-treated maize five APX isoforms were detected. The results further show that the activity (as band intensities) of the different isoforms was differentially regulated by the various treatments (Figure 3.2A). There was no distinct difference in the APX isoform activities from leaves of plants treated with DETA (control for DETA/NO) when compared to the untreated plants. Similarly, there were no differences between the APX isoform activities in leaves from plants treated with NaCl and those treated simultaneously with both NaCl and DETA. The densitometry analysis of all APX isoforms is tabulated in Table 3.2.

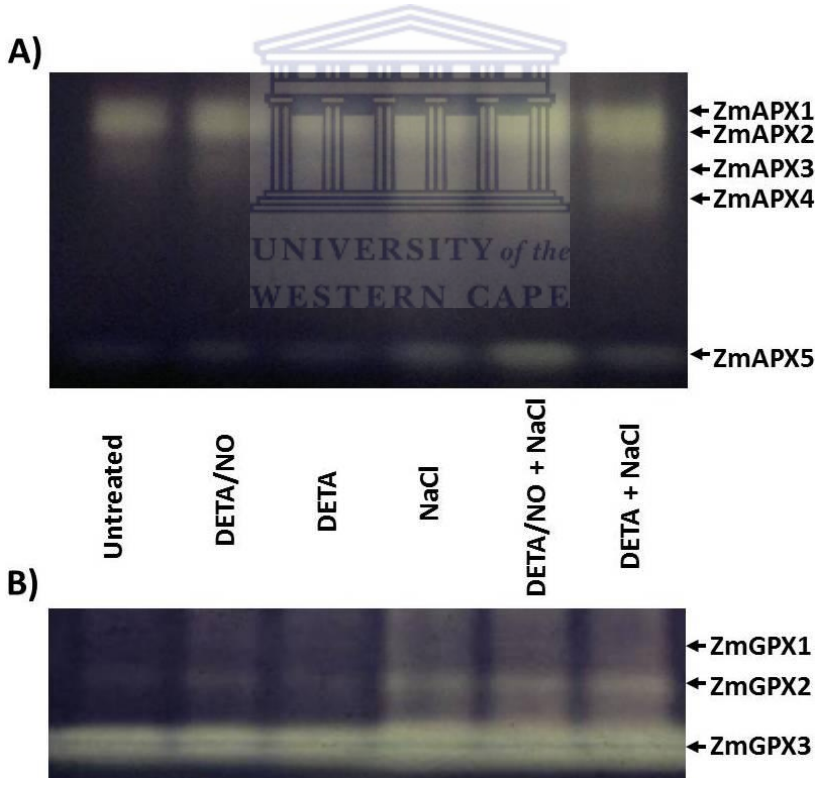

Figure 3.2 In-gel activity assays for APX and GPX in response to NO and salt stress. Assays were done on maize plants that were treated with the various treatments at the V1 stage for a period of 21 d (they were at the V3 stage at time of harvest). In-gel activities of various isoforms in response to treatment with 10 µM DETA/NO, 10 µM DETA, 150 mM NaCl, 10 µM DETA/NO + 150 mM NaCl or 10 µM DETA + 150 mM NaCl are shown for APX (A) and GPX (B) as detected in maize leaves.

The densitometry analysis show that both NO and salt stress enhanced APX1 activity compared to the untreated control. The most significant increase of ± 66% was observed for plants treated with both NO and salt stress (Table 3.2). The activity of APX2 was enhanced by ± 21% in response to treatment with DETA/NO. Plants treated with NaCl, including those NaCl-treated plants that were supplemented with DETA alone, had enhanced APX activity (increase by ± 26%) whereas the combined effect of DETA/NO and NaCl resulted in an increase of ± 86% in APX2 activity when compared to the untreated plants. The enzymatic activity of APX3 was slightly augmented in response to treatment with DETA/NO with an increase of ± 10% compared to the untreated plants (Table 3.2). However, plants treated with NaCl, including the salt stressed plants that were supplemented with DETA/NO, had significantly enhanced enzymatic activity for APX3; with the highest increase recorded for the plants treated with the combination of NaCl and DETA/NO (± 66%) (Table 3.1). According to the in-gel image (Figure 3.2A) and densitometry analysis (Table 3.2), very low or no activity was detected for APX4 in the leaves of plants treated with DETA, or DETA/NO and the untreated plants.

Table 3.2 Effect of NO and salt stress on APX activity in maize leaves.

Relative APX Activity (Arbitrary Values)	Maize APX isoforms	Treatments					
		Untreated	DETA/NO	DETA	NaCl	DETA/NO + NaCl	DETA + NaCl
	ZmAPX1	1.00 ± 0.050^{a}	1.10 ± 0.055^{b}	1.00 ± 0.050^{a}	1.33 ± 0.067^{b}	1.66 ± 0.083^{c}	1.34 ± 0.067^{b}
	ZmAPX2	1.00 ± 0.050^{a}	1.21 ± 0.061^{b}	1.01 ± 0.051^{a}	1.26 ± 0.063^{b}	1.85 ± 0.093^{c}	1.26 ± 0.063^{b}
	ZmAPX3	1.00 ± 0.050^{a}	1.09 ± 0.056^{a}	1.00 ± 0.050^{a}	1.32 ± 0.070^{b}	1.57 ± 0.086^{c}	1.33 ± 0.070^{b}
	ZmAPX4	NA	NA	NA	1.00 ± 0.050^{a}	1.45 ± 0.056^{b}	0.98 ± 0.050^{a}
	ZmAPX5	1.00 ± 0.050^{a}	1.47 ± 0.074^{b}	1.01 ± 0.051^{a}	1.89 ± 0.095^{c}	2.35 ± 0.118^{d}	1.88 ± 0.095^{c}

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate significant differences between treatments at 95% confidence according to Tukey-Kramer test. The letters NA in the table indicate that very low or no activity was detected.

However, there was activity detected for the salt stressed-plants (NaCl), salt-stressed plants supplemented with NO (DETA/NO + NaCl) as well as the salt-stressed plants supplemented with DETA (DETA + NaCl). No significant change in activity was observed between the NaCl-treated plants and the NaCl-treated plants supplemented with DETA. Plants treated with a combination of DETA/NO and NaCl showed an increase in activity to a level much higher than was observed for plants treated with NaCl alone or in combination with DETA. For APX5, application of DETA/NO enhanced enzymatic activity by ± 47% when compared to the untreated plants (Figure 3.2A). Furthermore, treatment with NaCl alone and the combination of DETA/NO + NaCl drastically enhanced the enzymatic activity by ± 88% and ± 135% respectively compared to untreated plants.

The results show that three GPX isoforms (Figure 3.2B and Figure 3.3) was detected in maize leaves in response to treatment with NO and salt stress. Apart from GPX3 (as shown by the green bar), plants treated with NO had elevated GPX activity to levels higher than the untreated plants. Salt stress on the other hand increased the enzymatic activity in all three GPX isoforms. In response to treatment with NO, the enzymatic activity for GPX1 (as shown by the blue bar) was slightly but significantly enhanced (by ± 17%) whereas NaCl and the combined treatment of NaCl and DETA/NO resulted in greater increases of between 43 and 45% respectively. For the DETA treatments, there were no significant differences in activity when compared to the untreated as well as the salt-treated plants supplemented with DETA. A similar pattern to GPX1 was observed for GPX2 (as shown by the maroon bar) where both NO and NaCl enhanced the enzymatic activity (Figure 3.2B and 3.3).

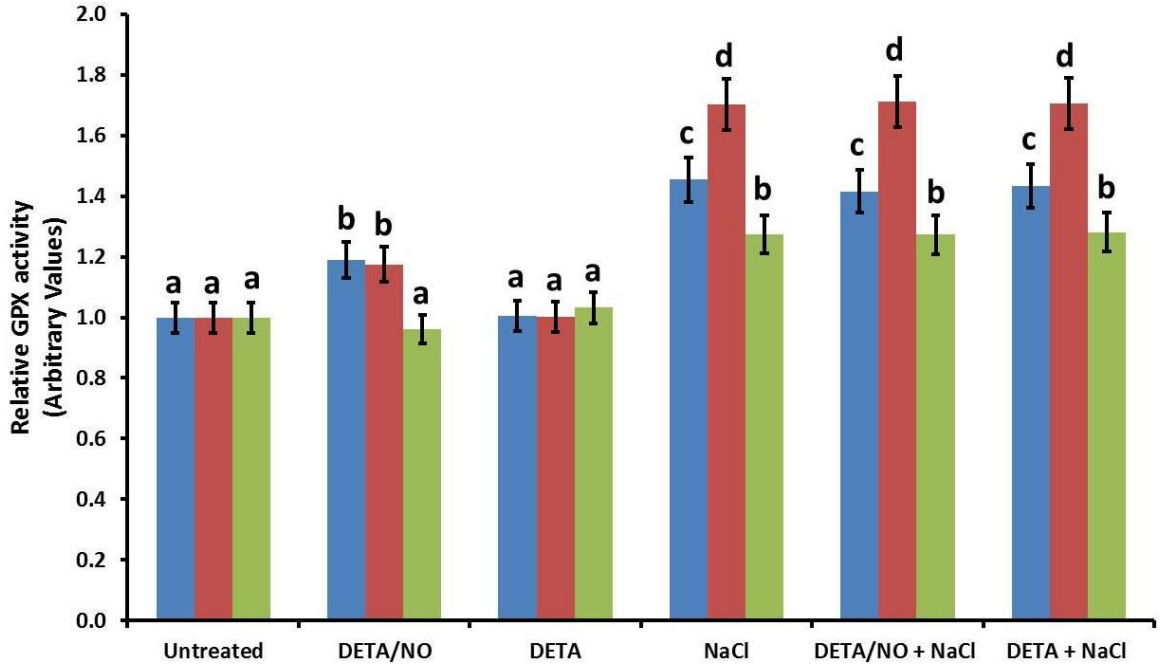

Figure 3.3 Effect of NO and salt stress on maize GPX activity. The level of enzymatic activity of maize GPX isoforms (GPX1-blue; GPX2-maroon; GPX3-green), derived from analysis of the intensity of the bands corresponding to each GPX isoform, are indicated. In-gel activities (as relative activity) in response to treatment with 10 µM DETA/NO, 10 µM DETA , 150 mM NaCl, 10 µM DETA/NO + 150 mM NaCl or 10 µM DETA + 150 mM NaCl are indicated for ZmGPX1, ZmGPX2 and ZmGPX3. Error bars represent the means (± SE; n = 4) of three independent experiments.

In response to treatment with NO, GPX2 activity was enhanced by ± 18% whereas plants treated with salt (including those supplemented with DETA and DETA/NO respectively) resulted in an increase of ± 71% compared to the untreated plants (Figure 3.2B and 3.3). For GPX3, there was a slight but insignificant reduction (± 4%) in activity when plants were treated with DETA/NO, but NO-treated plants simultaneously exposed to NaCl resulted in an increase of ± 27%. This percentage increase was also observed for NaCl-treated plants alone and NaCl-treated plants supplemented with DETA. It is therefore evident that the increase in activity observed for GPX3 was as a result of salt-induced stress.

3.4.3 The effect of exogenous NO and NaCl on GR activity in maize leaves

Although APX plays an important role in the H_2O_2 scavenging system, GR is also an essential scavenger in the removal of H_2O_2 in order to maintain the redox state of ascorbate and glutathione (Foyer et al. 1994). In order to analyze the enzymatic changes of GR isoforms in response to NO (DETA/NO) and salt stress (NaCl), leaf protein extracts from maize exposed to the various treatments were subjected to native polyacrylamide gel electrophoresis (PAGE) (Figure 3.4) and densitometry analysis (Table 3.3). In total, five GR isoforms were detected in maize leaves in response to the various treatments (Figure 3.4). According to Figure 3.4 and Table 3.3, there were no significant changes in GR1 and GR2 activity in response to the various treatments except for treatment with DETA/NO + NaCl. The addition of DETA/NO or NaCl on its own did not alter the enzymatic activity of both isoforms.

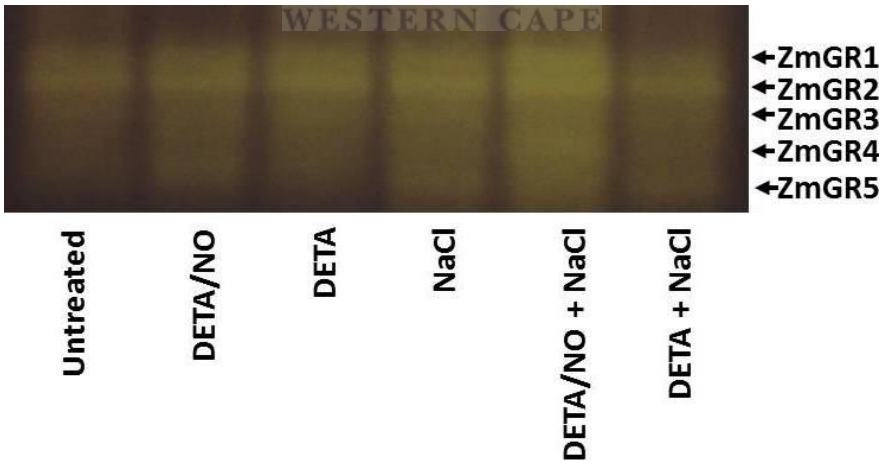

Figure 3.4 Detection of GR isoforms in maize leaves in response to nitric oxide and salt stress. Assays were done on maize plants that were treated with the various treatments at the V1 stage for a period of 21 d (they were at the V3 stage at time of harvest). In-gel activities (as GR isoforms 1-5) in response to treatment with 10 µM DETA/NO, 10 µM DETA, 150 mM NaCl, (10 µM DETA/NO + 150 mM NaCl) and (10 µM DETA + 150 mM NaCl) are shown.

Table 3.3 Relative GR enzymatic activity in response to NO and NaCl in maize.

Relative GR Activity (Arbitrary Values)	Maize GR isoforms	Treatments					
		Untreated	DETA/NO	DETA	NaCl	DETA/NO + NaCl	DETA + NaCl
	ZmGR1	1.00 ± 0.050[a]	1.02 ± 0.051[a]	1.00 ± 0.050[a]	1.01 ± 0.051[a]	1.20 ± 0.060[b]	1.01 ± 0.051[a]
	ZmGR2	1.00 ± 0.050[a]	1.00 ± 0.050[a]	1.00 ± 0.050[a]	1.01 ± 0.051[a]	1.16 ± 0.058[b]	1.01 ± 0.051[a]
	ZmGR3	1.00 ± 0.050[a]	1.01 ± 0.050[a]	1.00 ± 0.050[a]	1.01 ± 0.051[a]	0.99 ± 0.050[a]	1.00 ± 0.050[a]
	ZmGR4	1.00 ± 0.050[a]	1.13 ± 0.057[b]	1.03 ± 0.052[a]	1.04 ± 0.052[a]	1.51 ± 0.076[c]	1.03 ± 0.052[a]
	ZmGR5	1.00 ± 0.050[a]	1.26 ± 0.063[b]	0.99 ± 0.050[a]	1.19 ± 0.060[c]	1.50 ± 0.075[d]	1.19 ± 0.060[c]

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to the Tukey-Kramer test.

However, the combined treatments (DETA/NO + NaCl) enhanced both GR1 and GR2 activity by ± 20 and 16% respectively. For GR3, the densitometry analysis tabulated in Table 3.3 shows no significant changes in activity in response to all the treatments. Treatment of plants with DETA/NO enhanced leaf GR4 activity by ± 12% when compared to the untreated plants. The highest increase in GR4 activity was observed in plants treated with a combination of both DETA/NO and NaCl. This increase was ± 51% higher than was observed for the untreated plants.

On the other hand both NO and NaCl significantly enhanced enzymatic activity of GR5 when compared to the untreated plants. Plants treated with DETA/NO enhanced GR5 activity by ± 20% whereas NaCl alone or in combination with DETA increased the enzymatic activity by between 19 and 20% respectively. The highest increase in GR5 activity was observed in NO-treated plants supplemented with salt stress (as was the case of most of the GR isoforms). This increase was ± 50% higher than was observed for the untreated plants.

3.5 Discussion

In this study, we have analyzed the effect of NO (as 10 µM DETA/NO) and salt stress (150 mM NaCl) on the antioxidant enzyme activity of maize plants treated for

a period of 21 days. The degree to which the activities of antioxidant enzymes increase under salt stress generally varies among several plant species and even among different cultivars of the same species. The level of response depends on the species, the development and metabolic state of the plant, as well as the duration and intensity of the stress. Many stress situations cause an increase in the total antioxidant activity. The enzymatic activity (as total activity) for APX, GPX and GR in maize roots was previously described in Chapter 2. Here, we explore how long term exposure of maize plants to NO and salt stress influence the enzymatic activity of isoforms of four antioxidant enzymes (SOD, APX, GPX and GR).

3.5.1 SOD activity is differentially regulated by NO and NaCl in maize plants

Ten SOD isoforms were detected in maize leaves in response to treatment with NO and salt stress. In response to treatment with NO, the enzymatic activity detected for MnSOD2 was enhanced whereas the activity for MnSOD1 was slightly inhibited. However, no significant change was observed for MnSDO3 in response to treatment with NO when compared to the untreated plants. Treatment with NO seems to enhance the activity of five Cu/ZnSOD isoforms (Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD3, Cu/ZnSOD6 and Cu/ZnSOD7). No or very low activity was detected for Cu/ZnSOD4, Cu/ZnSOD5 and Cu/ZnSOD7 in response to treatment with NO.

Apart from MnSOD1, all the other SOD isoforms detected in response to NaCl were enhanced or remained unchanged when compared to the untreated control. This is in agreement with various other studies on salt stress (Wang et al. 2004; Lee et al. 2001) in which it was shown that high levels of NaCl cause an increased production of ROS such as O_2^- and H_2O_2, which then trigger enhanced antioxidant enzyme activity as a defense mechanism to the ROS. The increase in SOD isoform

activity in response to the combined treatment of NO and NaCl suggests that NO may play a significant role in salt stress tolerance. The enzymatic activity of eight SOD isoforms (MnSOD1, MnSOD2, Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD3, Cu/ZnSOD4, Cu/ZnSOD5 and Cu/ZnSOD7) was up-regulated in response to the combined treatment of NO and NaCl. The remaining two isoforms (MnSOD3 and Cu/ZnSOD3) shared the same profile as the untreated control or the NO treated samples. However, isoforms that will be potential targets for establishing salt tolerance in maize plants via regulating NO levels are MnSOD1, Cu/ZnSOD1, Cu/ZnSOD3 and Cu/ZnSOD4 because the activity of these isoforms is markedly enhanced by supplementation of NO to NaCl-treated plants. Apart from MnSOD1, whose activity was inhibited in response to NO and NaCl respectively (to levels lower than the controls), the activity of the other isoforms were enhanced in the same treatments (NO and NaCl respectively) and had an even higher increase in activity in response to the combined treatment with NO and NaCl. Furthermore other possible candidates that could be considered for salt stress tolerance is Cu/ZnSOD2, Cu/ZnSOD6 and Cu/ZnSOD7 as these isoforms are enhanced in the presence of NO and NaCl respectively but reduced in the combined treatment of NO and NaCl (although not to levels below the NO treated samples).

3.5.2 NO-induced increase in APX activity contributes to H_2O_2 detoxification and plant survival under salt stress

APX catalyzes the reduction of H_2O_2 into H_2O (Zhang et al. 2008) and modulates the concentration of H_2O_2 to a level sufficient for second messenger activity. There was a slight but significant increase (± 10%) in activity for three APX isoforms (APX1, APX2 and APX5) in response to NO, with the highest increase detected for APX5. There was an increase in APX activity upon the imposition of salt stress, to

levels higher than the untreated plants. The salt stressed plants supplemented with NO show a significant increase in APX activity compared to plants treated with salt alone or an even higher increase when compared to the untreated plants. These results suggest that exogenous NO contributes to the detoxification of H_2O_2 in maize under salt stress by enhancing maize APX enzymatic activity.

3.5.3 NO and salt stress differentially regulates maize leaf GPX activity

It has been shown that while salt-tolerant species increase their antioxidant enzyme activities and antioxidant contents in response to salt stress, salt sensitive species fail to do this efficiently (Shalata and Tal 1998). GPX is widely distributed in higher plants where it is involved in various processes, including lignification, auxin metabolism, salt tolerance and heavy metal stress (Passardi et al. 2005; Gao et al. 2008). Therefore, GPX has often served as a parameter of metabolic activity during growth alterations and environmental stress conditions (Gao et al. 2008). It was suggested by Rout and Shaw (2001) that GPX is one of the most important hydrogen peroxide scavenging enzymes leading to salt stress tolerance in aquatic macrophytes. GPXs were also suggested to be instrumental in salt tolerance in mulberry (Sudhakar et al. 2001), cotton (Gosset et al. 1994; Meloni et al. 2003) and barley (Liang et al. 2003) cultivars. Our analyses have shown that the activity of GPX isoforms in maize leaves was differentially regulated by NO and salt stress. In this study we have identified three GPX isoforms that were differentially regulated by both NO and salt stress. Apart from GPX3, whose enzymatic activity remained unchanged in response to NO, the enzymatic activities for the other two isoforms (GPX1 and GPX2) was enhanced to levels higher than the untreated plants in response to NO. On the other hand the enzymatic activity of all isoforms was enhanced in salt-stressed plants as well as the salt stressed plants supplemented

with NO and DETA respectively. We therefore propose that although all three isoforms were up-regulated in response to salt stress, this increase in response to long term exposure to NaCl is still not sufficient to scavenge the H_2O_2 generated in response to the NaCl concentration used in our experimental system. This could be the reason for the observed accumulation of H_2O_2 under salt stress in maize leaves (Keyster 2011). This observation was supported by de Azevedo Neto et al. (2005) who showed that GPX activity is more up-regulated in roots than the leaves of two maize genotypes. The results obtained in this study is in agreement with the findings of different authors (Mittova et al. 2002; Keyster et al. 2012) who have shown that total GPX activity was up-regulated in response to treatment with NaCl.

3.5.4 GR-induced changes in maize leaves are modulated by NO under salt stress

Glutathione reductase (GR) is responsible for recycling GSSG to GSH and controls the redox status in plant cells. It has been observed that stress-tolerant plants tend to have high GR activities (Mittova et al. 2003; Sekmen et al. 2007). It has been shown (Chapter 2) that GR activity (as total activity) in maize roots was up-regulated in response to treatment with NO. However in this study we have shown that apart from two isoforms (GR3 and GR4; whose enzymatic activity was more pronounced than the untreated plants) there is no significant difference in enzymatic activity in plants treated with NO compared to the untreated and DETA treated plants. Increases in GR activity in NO-treated plants have been previously reported in various studies (Laspina et al. 2005; Sang et al. 2008; Xu et al. 2010). Our results agree with those of Hasanuzzaman et al. (2011) that showed that salt-stressed plants supplemented with NO increase GR activity to levels higher than that obtained for salt-treated plants (Figure 3.3 and Table 3.3). Salt stress on the other-hand had

little effect on the activation or deactivation of almost all GR activity (isoforms) in the leaves apart from isoforms,GR3 and GR5 whose activity were slightly enhanced by salt stress (Figure 3.3 and Table 3.3). A similar profile was observed in the leaves of transgenic tobacco plants (Gupta et al. 1993). The enzymatic activity for all GR isoforms was enhanced in response to the combined treatment with NO and NaCl. These findings indicate maize plants fail to induce certain GR isoforms under salt stress and suggest that enhanced levels of GR (as seen for some isoforms) are necessary for protection from salt stress and the inability of maize to induce some of these isoforms in response to salt stress underlies the inability of the plant to withstand elevated salt concentrations. However, it is evident that three GR isoforms (GR3, GR4 and GR5) identified in this study could be considered as potential candidates for NO-mediated salt stress tolerance as these isoforms were enhanced in the presence of NaCl and/or NO but exhibited even higher increases in the combined treatment with NO and NaCl. The increased GR activity by NO under salt stress would ultimately maintain a higher GSH/GSSG ratio and GSH level to help the plants cope better under severe salt stress conditions.

In summary, salt stress may induce severe oxidative stress in the leaves of maize and the addition of NO (as DETA/NO) triggers a protective mechanism via induction of antioxidant enzymes against salt stress in these organs by enhancing the activity of the various isoforms of the antioxidant enzymes in salt-stressed plants. In this study, we have identified several potential candidates from different antioxidant enzymes that could be used as targets in salt tolerance studies in maize. Further analysis is required to fully understand both the regulatory mechanism of these antioxidant genes against environmental stresses such as salinity stress and the regulation of genes encoding these enzymes.

CHAPTER 4

NITRIC OXIDE AND SALT STRESS ALTER THE ACTIVITY OF ROS-SCAVENGING ENZYME ISOFORMS IN SOYBEAN ROOT NODULES

4.1 Abstract

The effects of exogenous NO and salt stress on the activity of isoforms of various antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GR), were investigated in root nodules of soybean plants. Soybean plants (at V5 of vegetative growth) were treated with DETA/NO (as an NO donor at 10 µM), DETA (serves as a control for NO; at 10 µM), NaCl (80 mM), DETA/NO + NaCl or DETA + NaCl for 12 days. The study show that the activities of the antioxidant enzymes are differentially regulated in response to NO and NaCl. NO enhanced SOD isoform activity whereas NaCl had the opposite effect. Varied responses occurred in salt-stressed plants supplemented with NO, where some isoform activities were enhanced and others were inhibited. GPX activity appears unaltered in response to NO, with the exception of four isoforms (GmGPX1, GmGPX2, GmGPX4, GmGPX5) that were slightly up-regulated. A similar trend was observed in response to NaCl and the combined treatment of NO + NaCl for the same isoforms. It appears that NO enhances GR activity, whereas the contrary can be said for NaCl and the combination of NO + NaCl. These results suggest that soybean protects itself against ROS by increasing the activity of certain antioxidant enzymes under salt stress and this protection is mediated by NO.

4.2 Introduction

Antioxidants encompass those molecules that quench ROS and/or inhibit their formation (Sies 1997), thus providing essential information on cellular redox homeostasis (Foyer and Noctor 2005). The ROS are generated as part of the normal cell functioning during the electron transport chain in plant cell mitochondria (Davies 1995), but accumulate more under salinity stress (Møller 2001), causing damage to proteins (Juszczuk et al. 2008), affecting membrane functioning (Ahmad et al. 2008) and thus leading to oxidative stress. Oxidative stress leads to destruction of plant cells and occur when the antioxidant system is inefficient or inadequate (Foyer and Noctor 2003) to counter the deleterious effects of overproduction of ROS.

Nitric oxide (NO), on the other hand is a diatomic and signaling molecule involved in various cellular processes and in particular the abiotic stress responses of plants (Neill et al. 2008; Lamotte et al. 2005). NO interacts with antioxidants to scavenge ROS, thus protecting plants under stress (Neill et al. 2008). Most of the studies that focus primarily on other plant tissues rather than legume nodules revealed that NO delays ROS-induced cell death (Beligni et al. 2002). The legume root nodule is symbiotic tissue which is both structurally and physiologically different from the other legume plant organs (Crespi and Gálvez 2000). Therefore, it cannot be assumed that there is an absolute similarity in antioxidant responses to stresses within the root nodule system compared to other plant tissues/organs. The unique nature of nodule physiology presents an opportunity to study legumes for improved nitrogen use efficiency and improved productivity under growth-limiting environmental conditions such as salt-induced stress. Therefore this study will explore the effect of exogenously applied NO on soybean root nodule antioxidant capacity under salt stress. The aim of this study was to determine if exogenous supplementation of NO

alters antioxidant enzyme activity in soybean root nodules from plants grown in the absence or presence of salt stress and whether this contributes to the protection of soybean plants from salt stress.

4.3 Materials and Methods

4.3.1 Plant Growth

Soybean (*Glycine max* L. merr. cv. PAN 626) seeds were surface-sterilized in 0.35% (v/v) sodium hypochlorite for 10 min, followed by five washes with sterile distilled water. The seeds were imbibed in sterile distilled water for 1 hour and sown in sand for plants used in treatments at the VC stage or inoculated with *Bradyrhizobium japonicum* supplied as the commercial peat-based HiStick2 Soybean Inoculant (Becker Underwood Ltd., West Sussex, United Kingdom) for plants used for treatments at the V5 stage. The *G. max* seeds were sown in 1 litre of filtered silica sand (98% SiO_2, Rolfes® Silica, Brits, South Africa) that had been pre-soaked in distilled water, in 15 cm diameter plastic pots (one plant per pot). The sand was kept moist by watering only with distilled water during germination. Germinated seedlings were grown on a 25/19 °C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m^{-2}.s^{-1} during the day phase, in a randomized design. Plants were supplied with nutrient solution [1 mM K_2SO_4, 2 mM $MgSO_4$, 3 mM $CaCl_2$, 1 mM K_2HPO_4 buffer at pH 7.3, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM ZnSO4, 2 µM $CuSO_4$, 2 µM Na_2MoO_4, 0.1 µM $CoSO_4$, 50 µM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3] at the VC stage (when the unifoliolate leaves are fully expanded and there is one node) until they reached the V5 stage (five trifoliolate leaves fully expanded). Plants of the same phenological stage and similar height were selected for analysis in each set of treatments.

4.3.2 Treatment of plants

Treatments were initiated at the V5 stage and done for 12 d. For these treatments plants were supplied with either nitrogen-free nutrient solution or nitrogen-free solution containing the nitric oxide donor DETA/NO at a final concentration of 10 µM and salt (NaCl) at a final concentration of 80 mM. As a negative control to the nitric oxide donor (DETA/NO), DETA was used at a final concentration of 10 µM instead of DETA/NO.

Plants were treated with either nitrogen-free nutrient solution, DETA/NO, NaCl or a combination treatment with the nitric oxide donor DETA/NO and NaCl. Diethylenetriamine (DETA), which is similar to DETA/NO except that it lacks the NO moiety that is otherwise attached to DETA in DETA/NO, was used as a negative control for all experiments that involve DETA/NO.

For treatment of plants at the V5 stage (plants for which inoculation with rhizobia was done to the seeds before sowing), a set of plants (denoted 'NaCl') was treated with 100 ml of the nitrogen-free nutrient solution containing 80 mM NaCl. To evaluate the effects of exogenously applied NO on plants exposed to NaCl, a set of plants (denoted 'DETA/NO + NaCl') was treated with a combination of 10 µM DETA/NO and 80 mM NaCl. An additional set of plants was treated with 100 ml (per plant) of the nitrogen-free nutrient solution supplemented with 10 µM DETA/NO in order to investigate the effect of NO on soybean. Another set of plants was treated with 100 ml (per plant) of the nitrogen-free nutrient solution supplemented with 10 µM DETA as a negative control for DETA/NO. The last set of plants (denoted 'DETA + NaCl') was treated as the 'DETA/NO + NaCl' set of plants, except that 10 µM DETA was used in the place of DETA/NO.

4.3.3 Preparation of protein extracts

Extracts were obtained from soybean root nodules by grinding the tissue into a fine powder in liquid nitrogen and homogenizing 400 mg of the tissue with 1 ml of homogenizing buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetra acetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40 000]. The resulting homogenates were centrifuged at 12 000 X *g* for 15 min and the supernatants were used for the detection of various ROS scavenging enzymes (as isoforms on native polyacrylamide gels). Protein concentrations were determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

4.3.4 Detection of antioxidant enzyme activity in soybean root nodules

4.3.4.1 Superoxide Dismutase (SOD)

For the detection of SOD isozymes in soybean root nodules, native PAGE was performed at 4°C in 10 % polyacrylamide mini gels using 120 µg of protein. SOD activity was detected by staining with 0.5 mM riboflavin and 2.5 mM nitroblue tetrazolium, as described by Beauchamp and Fridovich (1971). SOD isozyme classes were determined by incubating the gels with 5 mM H_2O_2 to inhibit both Cu/ZnSOD and FeSOD, or with 5 mM KCN to inhibit only Cu/ZnSOD (Fridovich 1982). MnSOD activity is resistant to both treatments.

4.3.4.2 Glutathione peroxidase (GPX)

GPX in-gel activity staining was carried out as a modified method based on the procedure of Seckin et al. (2010) for detecting APX isoforms. Reduced glutathione (GSH) instead of ascorbate was used in the staining solutions. Native PAGE was performed at 4°C in 10% polyacrylamide mini gels using 25 µg of protein. Prior to electrophoresis, gels were equilibrated in running buffer containing 2 mM GSH for 20

min at 4°C. Following electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM GSH for 20 min and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM GSH and 0.01% cumene hydroperoxide for 20 min. The gels were washed with distilled H_2O and transferred to a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.5 mM NBT for 10-20 min with gentle agitation in the presence of light.

4.3.4.3 Glutathione reductase (GR)

GR activity staining was performed in a 10% native polyacrylamide gel containing 10% glycerol at 4°C using 70 µg of protein per sample. GR activity was detected according to the procedure of Lee and Lee (2000) with slight modifications. GR was visualized in the native gel by incubation in 50 ml of 0.25 M Tris–HCl buffer (pH 7.9) containing 4.0 mM oxidized glutathione (GSSG), 1.5 mM NADPH, and 2 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for 20 min. The GR activity was negatively stained in the dark with a solution containing 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1.6 mM phenazine methosulfate (PMS) for 5-10 min at 30°C.

4.3.4.4 Quantitative evaluation of antioxidant enzymatic activities

Densitometry analysis was done on all the PAGE gels following image acquisition, using the Spot Denso tool (AlphaEase FC imaging software, Alpha Innotech Corporation). Individual gels were scored as arbitrary values (relative enzymatic activity) of three independent gels. The enzymatic activity (for the respective antioxidant enzymes) of each isoform in the treatments was scored as an average of the relative pixel intensities (by assigning a value of 1 for the isoform with

the lowest pixel intensity in that type of isoform and expressing the rest of the pixel intensities for that type of isoform in the other treatments relative to this isoform, for example: if MnSOD1 has the lowest pixel intensity in the NaCl treatment, then this pixel intensity will be assigned a value of 1 and all the pixel intensities for MnSOD1 in the rest of the treatments will be expressed relative to the pixel intensity of MnSOD1 in the NaCl treatment) from three independent gels and expressed in arbitrary units.

4.3.5 Statistical analysis

Data were reported as the mean of at least three analysis replicates (n=3). The data was analyzed using the one-way analysis of variance (ANOVA) and tested for significance by the Tukey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

4.4 Results

4.4.1 The effect of exogenous NO and salt stress on SOD activity in soybean root nodules

Superoxide dismutases (SODs) play an important role in stress tolerance of higher plants (Wang et al. 2010). This study shows the effect of exogenous NO (as the NO donor DETA/NO) and salt stress on SOD activity in soybean root nodules. SOD activity was differentially regulated in the presence of nitric oxide and salt stress as shown in Figure 4.1 and Table 4.1. A total of eleven different SOD isoforms were detected in soybean root nodules under the various treatments (Figure 4.1 and Table 4.1). SOD isoforms were determined by incubating the gels with 5 mM H_2O_2 to inhibit both Cu/ZnSOD and FeSOD, or with 5 mM KCN to inhibit only Cu/ZnSOD (images not shown). This analysis showed that the first two isoforms detected on the

gels were MnSOD1 and MnSOD2. The following three isoforms were identified as FeSOD 1-3 (after staining with KCN that only inhibit Cu/ZnSOD isoforms) whereas the last six bands were identified as Cu/ZnSOD1-6 (after staining with H_2O_2 that inhibit both FeSOD and Cu/ZnSOD isoforms respectively).

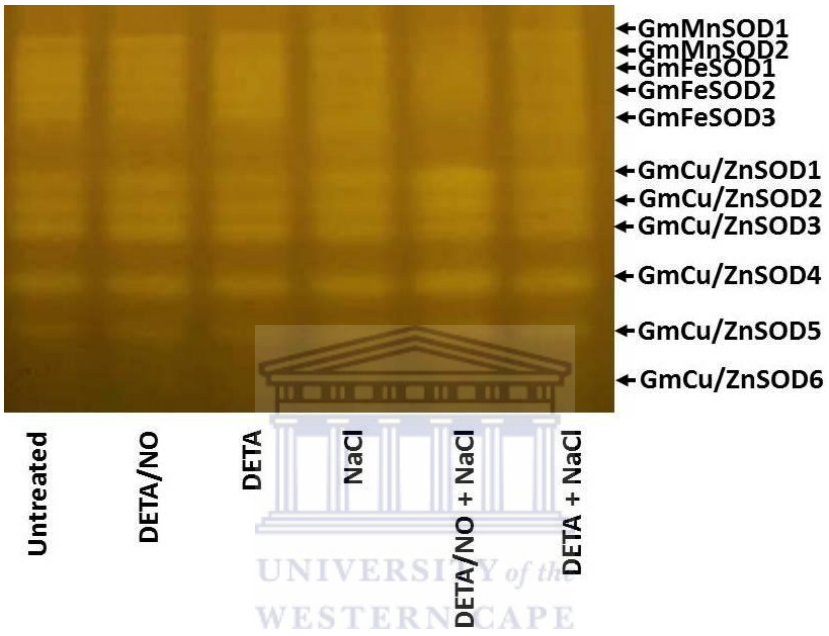

Figure 4.1 NO and salt stress differentially regulate SOD enzymatic activity in soybean root nodules. Assays were done on soybean nodules that were treated with the various treatments at the V5 stage for a period of 12 d (they were at the R1 stage at time of harvest). In-gel activities in response to treatment with 10 µM DETA/NO, 10 µM DETA, 80 mM NaCl, (10 µM DETA/NO + 80 mM NaCl) and (10 µM DETA + 80 mM NaCl) are shown for the SOD isoforms detected.

The results show that under the influence of NO (denoted 10 µM DETA/NO), the intensity (signifying enzymatic activity) of MnSOD1 was elevated by ± 10% whereas the intensity in response to the combined treatment of DETA/NO and NaCl decreased by ± 11% when compared to the control (untreated) plants (Figure 4.1 and Table 4.1). However, no changes in SOD activity occurred in response to treatment with DETA in comparison to the untreated plants (Figure 4.1 and Table 4.1). DETA resulted in nodule SOD activity that had a similar profile as the nodules

from untreated. Nodules from plants treated with a combination of DETA and NaCl showed results similar to those treated with NaCl alone. Treatment with DETA/NO did not alter the activity of MnSOD2 whereas NaCl and the combined treatment (DETA/NO + NaCl) reduced the enzymatic activity by ± 11% and ± 21% respectively when compared to the controls (Untreated and DETA).

Table 4.1 Relative enzymatic activity of GR in soybean root nodules.

Relative SOD Activity (Arbitrary Values)	Soybean nodule SOD isoforms	Treatments					
		Untreated	DETA/NO	DETA	NaCl	DETA/NO + NaCl	DETA + NaCl
	MnSOD1	1.00 ± 0.050^{a}	1.11 ± 0.056^{b}	1.00 ± 0.050^{a}	1.00 ± 0.050^{a}	0.90 ± 0.045^{a}	1.00 ± 0.050^{a}
	MnSOD2	1.00 ± 0.050^{a}	0.97 ± 0.049^{a}	1.00 ± 0.050^{a}	0.88 ± 0.044^{b}	0.79 ± 0.040^{b}	0.88 ± 0.044^{b}
	FeSOD1	1.16 ± 0.058^{a}	1.15 ± 0.058^{a}	1.17 ± 0.059^{a}	1.03 ± 0.051^{b}	1.00 ± 0.050^{a}	1.03 ± 0.051^{b}
	FeSOD2	1.20 ± 0.060^{a}	1.16 ± 0.058^{a}	1.20 ± 0.060^{a}	1.04 ± 0.052^{b}	1.00 ± 0.050^{a}	1.04 ± 0.052^{b}
	FeSOD3	1.22 ± 0.061^{a}	1.16 ± 0.058^{a}	1.22 ± 0.061^{a}	1.62 ± 0.081^{b}	1.00 ± 0.050^{a}	1.62 ± 0.081^{b}
	Cu/ZnSOD1	1.00 ± 0.050^{a}	1.29 ± 0.065^{b}	1.01 ± 0.050^{a}	1.09 ± 0.055^{a}	1.50 ± 0.075^{c}	1.08 ± 0.054^{a}
	Cu/ZnSOD2	1.00 ± 0.050^{a}	1.18 ± 0.059^{b}	1.00 ± 0.050^{a}	1.04 ± 0.052^{a}	1.27 ± 0.064^{c}	1.04 ± 0.052^{a}
	Cu/ZnSOD3	1.00 ± 0.050^{a}	1.13 ± 0.057^{b}	1.00 ± 0.050^{a}	1.00 ± 0.050^{a}	1.00 ± 0.050^{a}	1.00 ± 0.050^{a}
	Cu/ZnSOD4	1.00 ± 0.050^{a}	1.05 ± 0.052^{a}	1.00 ± 0.050^{a}	1.05 ± 0.053^{a}	1.21 ± 0.061^{b}	1.05 ± 0.053^{a}
	Cu/ZnSOD5	1.00 ± 0.050^{a}	1.14 ± 0.057^{b}	1.00 ± 0.050^{a}	1.13 ± 0.057^{b}	1.30 ± 0.065^{c}	1.14 ± 0.057^{b}
	Cu/ZnSOD6	1.00 ± 0.050^{a}	1.04 ± 0.052^{a}	1.02 ± 0.050^{a}	1.40 ± 0.070^{b}	1.37 ± 0.069^{b}	1.40 ± 0.070^{b}

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

The activity of FeSOD1 did not change in response to DETA/NO when compared to the untreated controls. Treatment with NaCl (alone), the combination of (DETA/NO + NaCl) and (DETA + NaCl) reduced the enzymatic activity by ± 11%. A similar pattern was observed for FeSOD2 in response to NaCl (alone) and the combined effects of (DETA/NO + NaCl) and (DETA + NaCl) respectively (Table 4.1). For FeSOD3, treatment with DETA/NO alone showed no changes in enzymatic activity when compared to the untreated controls. However treatments with NaCl and the combination of NaCl and DETA increased the activity of FeSOD3 by ± 33%

respectively. On the other hand FeSOD3 activity was reduced by ± 18% in response to treatment with the combination of 10 µM DETA/NO and 80 mM NaCl (Table 4.1). Treatment with DETA/NO alone resulted in a ± 29% increase in Cu/ZnSOD1 activity whereas NaCl alone did not significantly alter the activity of the isoform. The combination of DETA/NO and NaCl resulted in an significant increase of ± 50% whereas the combination of DETA and NaCl showed a similar response to the NaCl treatment alone. A similar response was observed for Cu/ZnSOD2 where only treatment with DETA/NO and the combination of DETA/NO and NaCl resulted in an increase of ± 18% and ± 27% respectively compared to the untreated controls. The NaCl treatments did not alter the activity of the Cu/ZnSOD2 isoform (as shown in Table 4.1).

Cu/ZnSOD3 showed a ± 12% increase in activity in response to treatment with DETA/NO whereas the other treatments did not result in any significant changes in the enzymatic activity of the isoform. For Cu/ZnSOD4, the only change in enzymatic activity was observed in response to the combined treatment of DETA/NO and NaCl with a ± 21% increase in enzymatic activity. The other treatments had no effect on the activity of this isoform when compared to the untreated controls (Table 4.1). For Cu/ZnSOD5, the combined treatment of plants with DETA/NO and NaCl resulted in a significant increase in enzymatic activity. Treatment with DETA/NO or NaCl enhanced enzymatic activity by ± 14% respectively when compared to the untreated controls. The combined treatment of DETA/NO and NaCl increased the enzymatic activity of the isoform by ± 30% compared to the untreated controls (Table 4.1). Treatment with DETA/NO alone did not change the enzymatic activity of Cu/ZnSOD6 instead it showed a similar response as the controls (Untreated and DETA). On the other hand, treatments with NaCl or the combination of DETA and NaCl showed an

increase of ± 40% respectively whereas the combination of DETA/NO and NaCl increased the enzymatic activity of the isoform by ± 37% (Table 4.1).

4.4.2 DETA/NO and salt-induced stress differentially regulate GPX enzymatic activity in soybean root nodules

Glutathione peroxidase (GPX) is perceived as one of the enzymes functioning in abiotic stress acclimatization during salt and heavy metal toxicity stress (Mittova et al. 2004). It also supports long-term survival of yeast during growth under abiotic stress (Lee et al. 2007). Furthermore, abiotic stress-tolerant plant genotypes have up-regulated gene expression for GPX that counteracts the effect of oxidative stress and this expression is observed after prolonged exposure to abiotic stress. GPX has often been used as a maker for oxidative stress tolerance because of its role in oxidative stress tolerance (Caregnato et al. 2008; Dazy et al. 2009).

Densitometry analysis showed that seven isoforms occur in soybean root nodules and that the enzymatic activity of each isoforms is differentially regulated by NO and NaCl (as shown in Figures 4.2 and Table 4.2). Significant increases in GPX activity was observed in response to treatment with both DETA/NO and NaCl when compared to the untreated plants. Although some isoforms remained unchanged in response to NO or NaCl; others did show a distinct difference in enzymatic activity as shown in the Figure 4.2 and after densitometry analyses (Table 4.2). For GmGPX1 and 2 there was no significant difference in isoform intensity in response to the various treatments, although there appears to be a slight increase in the NaCl-treated plants (level is not significantly higher than the other treatments). Plants treated with NO or NaCl or the combination of NO + NaCl had significantly enhanced intensities for the GmGPX3 isoform when compared to the control plants (Untreated and DETA).

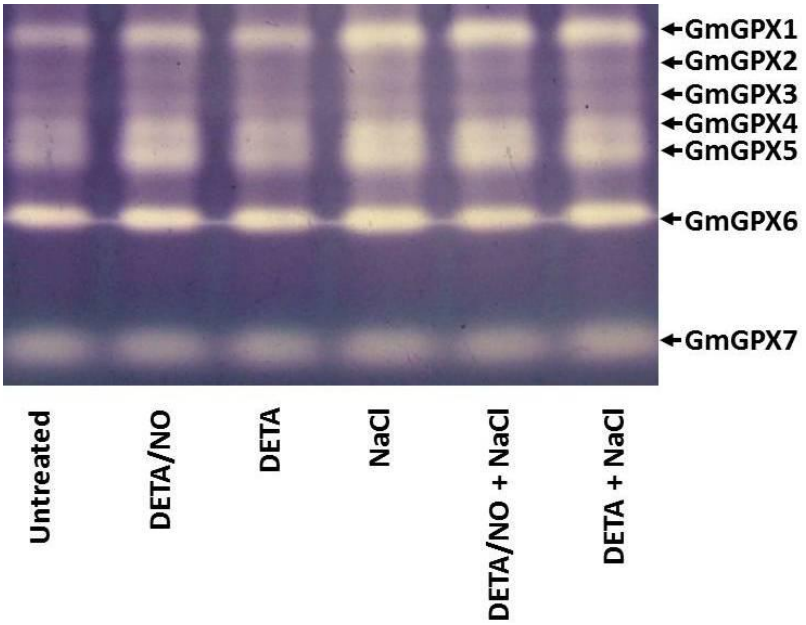

Figure 4.2 Exogenous NO and NaCl alter GPX enzymatic activity in soybean root nodules. In-gel assay for nodule GPX activity in response to treatment with 10 µM DETA/NO, 10 µM DETA, 80 mM NaCl, 10 µM DETA/NO + 80 mM NaCl or 10 µM DETA + 80 mM NaCl. Assays were done on soybean plants that were treated at the V5 stage for a period of 12 d (they were at the R1 stage at the time of harvest).

According to the in-gel assay (Figure 4.2) and densitometry analysis (as relative enzymatic activity (Table 4.2) some GPX isoforms (GmGPX3, GmGPX6 and GmGPX7) did not show any significant changes in enzymatic activity in response to the various treatments whereas GmGPX1, GmGPX4 and GmGPX5 were clearly up-regulated in response to various treatments (DETA/NO, NaCl, combination of DETA/NO and NaCl). Plants treated with DETA/NO alone had enhanced enzymatic activity of GmGPX1 (increase by ± 22%) compared to the untreated plants (Table 4.2). Treatment with NaCl or NaCl in combination with DETA/NO enhanced the GmGPX1 enzymatic activity by ± 39 and ± 37% respectively (Table 4.2). Plants treated with DETA or DETA + NaCl had similar activity profiles as the untreated and NaCl treated plants respectively.

Table 4.2 Relative GPX activity in soybean root nodules in response to NO and NaCl.

Relative GPX Activity (Arbitrary Values)	Soybean nodule SOD isoforms	Treatments					
		Untreated	DETA/NO	DETA	NaCl	DETA/NO + NaCl	DETA + NaCl
	GmGPX1	1.00 ± 0.050^{a}	1.22 ± 0.061^{b}	1.03± 0.051^{a}	1.39 ± 0.070^{c}	1.37 ± 0.070^{c}	1.40 ± 0.070^{c}
	GmGPX2	1.00 ± 0.050^{a}	1.16 ± 0.058^{b}	1.00 ± 0.050^{a}	1.17 ± 0.059^{b}	1.16 ± 0.058^{b}	1.17 ± 0.059^{b}
	GmGPX3	1.00 ± 0.050^{a}	1.00 ± 0.050^{a}	1.00 ± 0.050^{a}	1.05 ± 0.053^{a}	1.04 ± 0.052^{a}	1.05 ± 0.053^{a}
	GmGPX4	1.00 ± 0.050^{a}	1.30 ± 0.065^{b}	1.00 ± 0.050^{a}	1.37 ± 0.069^{b}	1.29 ± 0.065^{b}	1.37 ± 0.069^{b}
	GmGPX5	1.00 ± 0.050^{a}	1.16 ± 0.058^{b}	1.00 ± 0.050^{a}	1.15 ± 0.058^{b}	1.17 ± 0.059^{b}	1.15 ± 0.058^{b}
	GmGPX6	1.01 ± 0.050^{a}	1.02 ± 0.051^{a}	1.00 ± 0.050^{a}	1.01 ± 0.051^{a}	1.02 ± 0.051^{a}	1.01 ± 0.051^{a}
	GmGPX7	1.00 ± 0.050^{a}	1.03 ± 0.052^{a}	1.00 ± 0.050^{a}	1.03 ± 0.052^{a}	1.03 ± 0.052^{a}	1.03 ± 0.052^{a}

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at the 5% level of significance according to Tukey-Kramer test.

The densitometry analysis for GmGPX2 (Figure 4.2 and Table 4.2) showed that treatment with DETA/NO, NaCl or the combination of DETA/NO and NaCl enhanced enzymatic activity by ± 17% compared to the untreated plants.

No significant change in enzymatic activity for GmGPX3 was observed in response to the various treatments (Table 4.2). GmGPX4 showed a ± 30% increase in activity in response to treatment with DETA/NO whereas NaCl-treated plants and NO-treated plants supplemented with NaCl had enhanced enzymatic activity (increase of ± 37 and ± 29% respectively) compared to the control plants (Table 4.2). A similar activity profile to GmGPX4 was observed for GmGPX5 in response to the various treatments. Treatment with DETA/NO increased the activity of GmGPX5 by ± 16% whereas NaCl resulted in an increase of ± 15%, with the highest increase observed in plants treated with a combination of DETA/NO and NaCl (Table 4.2). For GmGPX6 and GmGPX7, no significant change in enzymatic activity was observed in response to the various treatments when compared to the untreated plants (Figure 4.2 and Table 4.2).

4.4.3 Glutathione reductase enzymatic activity is differentially regulated by exogenous nitric oxide and salt stress

Although APX plays an important role for the conversion of H_2O_2 to water, GR is also an essential catalyzer in the conversion of H_2O_2 in order to maintain the redox state of ascorbate and glutathione since it converts glutathione disulfide to the glutathione that is used by dehydroascorbate reductase to regenerate ascorbate (Hernandez et al. 1999). The potential of APX to metabolize H_2O_2 depends on the redox state of such compounds. Therefore, we studied the changes of the activities of GR isoforms in the root nodules of soybean plants in response to NO (DETA/NO) and salt stress (NaCl) (Figure 4.3). As shown in Figure 4.3, four GR isoforms were detected after separation of equal amounts of total root nodule protein on 10% polyacrylamide gels.

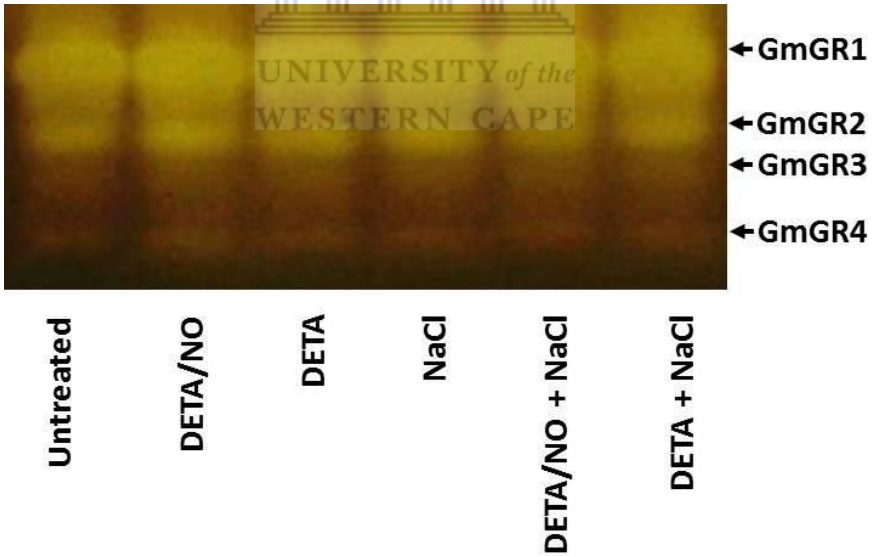

Figure 4.3 GR activities in response to NO (DETA/NO) and salt stress (NaCl) in soybean root nodules. In-gel assay for nodule GR activity in response to treatment with 10 µM DETA/NO, 10 µM DETA, 80 mM NaCl, (10 µM DETA/NO + 80 mM NaCl) and (10 µM DETA + 80 mM NaCl) are shown. Assays were done on soybean plants that were treated at the V5 stage for a period of 12 d (they were at the R1 stage at the time of harvest).

These isoforms were quantified as relative GR enzymatic activity using densitometry analysis (Figure 4.4). Treatment of soybean plants with NO or NaCl differentially regulated enzymatic activity of GR isoforms. For GmGR1 and GmGR3, no significant

differences were observed in response to the various treatments (Figures 4.3 and 4.4). Plants treated with NO or NaCl had enhanced the enzymatic activity of GmGR2 (increase of ± 18%) when compared to the untreated.

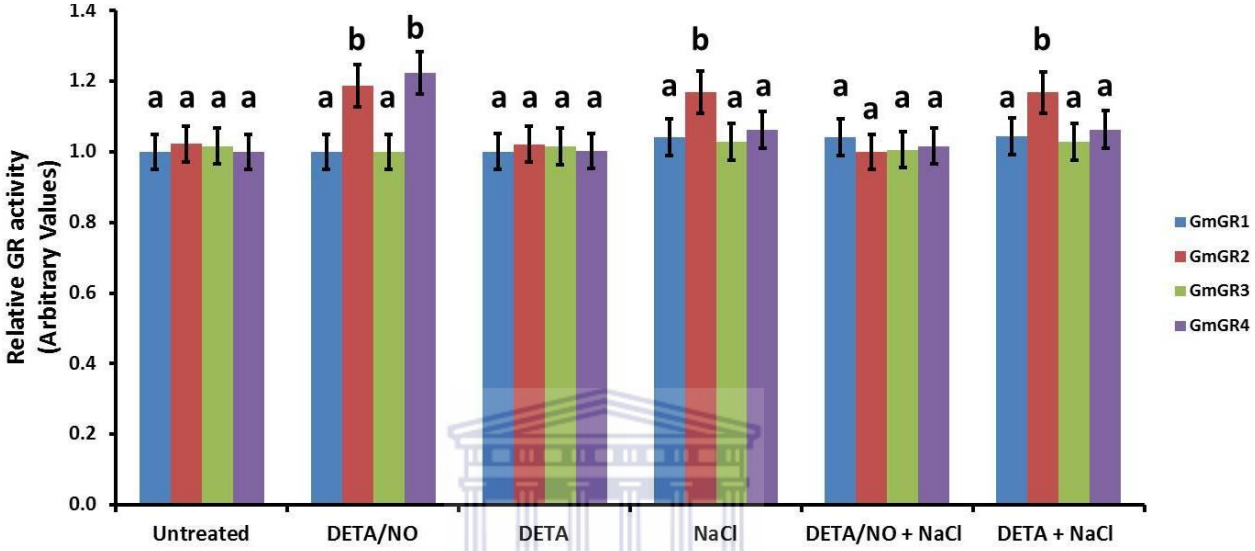

Figure 4.4 Effect of NO and salt stress on soybean root nodule GR activity. The graphs signifying the level of enzymatic activity of GR isoforms, derived from analysis of the activity of the bands corresponding to each GR isoform, are indicated. In-gel activities in response to treatment with 10 µM DETA/NO, 10 µM DETA , 80 mM NaCl, (10 µM DETA/NO + 80 mM NaCl) and (10 µM DETA + 80 mM NaCl) are indicated for GmGR1, GmGR2, GmGR3 and GmGR 4. Error bars represent the means (± SE; n = 3) of three independent experiments.

Although, individual treatments with NO or NaCl enhanced enzymatic activity of GmGR2 (to levels higher than the untreated plants); the combined treatment of NO and NaCl resulted in a slight but insignificant decrease in enzymatic activity compared to the untreated plants. According to the densitometry analysis obtained for GmGR4 (Figure 4.4), the enzymatic activity only increased in response to treatment DETA/NO by ± 22% compared to the control plants. The other treatments had no effect on the enzymatic activity of GmGR4 as they showed similar profiles to the control plants.

4.5 Discussion

4.5.1 NO-induced salt stress tolerance is mediated by regulation of SOD activity

In this study we have investigated the effects of exogenous application of NO and NaCl on the activity of various SOD isoforms in soybean root nodules. SODs have been identified in a wide range of organisms. These enzymes are classified into three eukaryotic classes according to their metal ion contents, namely the Cu/Zn, Mn, and Fe types (Bannister et al. 1987). The Cu/Zn type, with some exceptions (Puget and Michelson 1974), is restricted to eukaryotes and appears to be unrelated in amino acid sequence to the Mn and Fe types. The latter two classes have been identified in eukaryotes, prokaryotes, mitochondria, and chloroplasts and have a high degree of structural similarity and contain identical metal chelating amino acid groups at the active site. After separation on a native PAGE gels, eleven SOD isoforms were identified in soybean root nodules that comprise of two Mn (MnSOD1-2), three Fe (FeSOD1-3) and six Cu/ZnSOD (Cu/ZnSOD1-6) isoforms (Figure 4.1). These isoforms were identified by staining protein gels with two SOD inhibitors (H_2O_2; that inhibits both Fe and Cu/ZnSODs and KCN; that inhibits only Cu/ZnSODs) respectively (images not shown). It is evident that the relevant SOD isoforms in the NO-mediated scavenging of root nodule O_2^- are MnSOD1, Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD3 and Cu/ZnSOD5 as these isoforms are up-regulated in response to treatment with NO. The isoforms that remained unchanged in response to NO were MnSOD2, FeSOD1-3 and Cu/ZnSOD4. This result is contrary to what was observed by Niketic et al (1999) who suggested that NO did not significantly alter the activities of Cu/ZnSOD isoforms and caused deactivation of both MnSOD and FeSOD isoforms respectively.

In response to treatment with NaCl, the activity of MnSOD2 and FeSOD1-2 was reduced whereas the enzymatic activities of three Cu/ZnSOD isoforms (Cu/ZnSOD3, Cu/ZnSOD5 and Cu/ZnSOD6) were enhanced in the same treatment. Isoforms that remained unchanged in response to NaCl were MnSOD1 and three Cu/ZnSODs (Cu/ZnSOD1, Cu/ZnSOD2 and Cu/ZnSOD4). Out of the eleven SOD isoforms detected in response to NaCl, three (FeSOD3, Cu/ZnSOD5, Cu/ZnSOD6) were up-regulated whereas another three (MnSOD2, FeSOD1, FeSOD2) were down-regulated and the rest (MnSOD1, CuZnSOD1, Cu/ZnSOD2, Cu/ZnSOD3, Cu/ZnSOD4) remained unchanged. The observation that three SOD isoforms were inhibited and another three were enhanced by salt stress, together with the observation that the SOD activities of the rest (five) of the isoforms were not altered by NaCl implies that total SOD activity in response to long term salt stress in soybean root nodules would not be sufficient to scavenge ROS caused by NaCl stress. This hypothesis is in contradiction to what has been described in previous studies that show that long-term salt stress would result in an increase in O_2^- content thus trigger an increase in SOD activity to scavenge O_2^- to levels that is not toxic to the plants (Hernandez et al. 2001; Rubio et al. 2002). As it stands, it is inconclusive as to whether the amount of NaCl (over the stipulated period) used in this study was sufficient to increase the ROS to levels toxic to the plants, that would ultimately trigger the activation of downstream ROS scavenging enzymes such as APX, GPX and GR that detoxify H_2O_2 (by product of SOD detoxification) to non-toxic H_2O and O_2. It is important to note that the isoforms that are down-regulated in response to salt stress are potential targets for genetic improvement of salt tolerance in soybean because enhancement of their activities in response to long-term salt stress may

lead to efficient scavenging of O_2^-, which would contribute towards preventing O_2^--induced oxidative stress during long-term salt stress.

The results further show that salt-treated plants supplemented with NO over a period of 12 days differentially regulated the activity of the SOD isoforms. We have observed that two MnSOD isoforms (MnSOD1, MnSOD2) in addition to the three FeSOD isoforms (FeSOD1, FeSOD2, FeSOD3) were down-regulated whereas five Cu/ZnSOD isoforms (Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD4, Cu/ZnSDO5 and Cu/ZnSOD6) were up-regulated in response to combined treatment with NO and NaCl. Cu/ZnSOD3 was not altered in response to the combined treatment. It is worth noting that, in response to NO or NaCl, the activity of Cu/ZnSOD4 remained unchanged but the treatment with both NO and NaCl resulted in an increase in the activity of the various isoforms of SOD. As was the case for the other Cu/ZnSOD isoforms mentioned above, similar positive effects of NO on SOD activity under salt stress conditions have been reported (Kopyra and Gwozdz 2003; Shi et al. 2007).

4.5.2 The effect of exogenous NO and salt stress on soybean nodule GPX isoform activity

A family of seven genes encoding GPX has been identified in genome of *Arabidopsis thaliana*, named as AtGPX1- AtGPX8, respectively, which are located at the cytosol, chloroplasts, mitochondria, peroxisomes, and apoplast (Milla et al. 2003). These genes are expressed ubiquitously and are regulated by abiotic stresses through diverse signaling pathways (Milla et al. 2003). GPX is considered to be an important ROS scavenger because of its broader substrate specificities and stronger affinity for H_2O_2 compared to CAT (Brigelius-Flohe´ and Flohe´ 2003). The role of exogenous NO or NaCl stress on the activities of soybean root nodule GPX isoforms have been investigated (in this study) and it is clear that there is a distinct

pattern in enzymatic activity in response to the various treatments (as shown in Table 4.2). The results have shown that four GPX isoforms (GmGPX1, GmGPX2, GmGPX4, GmGPX5) were augmented whereas the rest of the isoforms (GmGPX3, GmGPX6, GmGPX7) remained unchanged in response to treatment with NO. Therefore, none of the isoforms was inhibited in response to treatment with NO. A similar profile depicting an increase in GPX activity was observed in response to treatment with NaCl or the combined treatment of NO and NaCl. Taking into account that four GPX isoforms were up-regulated by NO and none inhibited it, is reasonable to suggest that exogenously applied NO generally enhances detoxification of H_2O_2 or lipid peroxides by inducing GPX activity. In response to NaCl alone, four isoforms (GmGPX1, GmGPX2, GmGPX4, GmGPX5) were induced whereas the rest of the isoforms remained unaltered in the same treatment. The same isoforms were up-regulated in the combined treatment with NO and NaCl. The activity levels detected for these isoforms were higher than those observed for the control plants but similar to that of the NaCl treated plants. We therefore conclude that both exogenously applied NO and NaCl resulted in an increase in GPX activity as can be observed for the isoforms in this study. It is possible that this is reflective of the relationship between NaCl and NO, where NaCl triggers an increase in NO content (Neill et al. 2008) and thus the NaCl-induced elevation of NO triggers elevated GPX activity.

4.5.3 GR activity in soybean root nodules is differentially regulated by NO under salt-induced stress

As the enzyme catalyzing the rate-limiting step of the ascorbate-glutathione pathway, GR is present in the chloroplast as well as in the mitochondria and the cytoplasm. Its role in H_2O_2 scavenging in plant cells has been well established in the Halliwell-Asada pathway (Meloni et al. 2003). This enzyme is important for the

maintenance of the reduced form of the glutathione in the cell at high levels, because reduced glutathione is itself a free radical scavenger (Hussain et al. 2008). GR is responsible for recycling GSSG to GSH and controls these redox couples in plant cells. It has been observed that stress-tolerant plants tend to have high GR activities (Mittova et al. 2003; Sekmen et al. 2007). In our study, a slight but insignificant increase in GR activity (GmGR3 and GmGR4) was observed in response to 80 mM NaCl stress. This result is in contradiction to those of Bandeoglu et al (2004), who observed that the GR activity decreased under severe salt stress conditions. The reduction in GR activity in response to NaCl stress was also previously reported (Gueta-Dahan et al. 1997; Comba et al. 1998; Hernandez et al. 1999; Hernandez et al. 2000; Meloni et al. 2003;). The NO pre-treated salt-stressed plants showed no increases in GR activity (Figure 4.4). We have also observed increases in GR activity (for isoforms GmGR2 and GmGR4) in response to treatment with NO. Similar increases in the GR activity of NO-treated seedlings have also been reported (Laspina et al. 2005; Sang et al. 2008; Xu et al. 2010). It has been shown in various studies that increased GR activity by NO under salt stress maintained a higher GSH/GSSG ratio and GSH level (Hoque et al. 2007; Hossain and Fujita 2010).

In summary, this study provides insight into the role of NO in regulating the biochemical response (antioxidant capacity) of soybean plants to long-term salt stress. The results demonstrate that NO treatment in soybean plants enhances protection against salt-induced oxidative damage. These observations are mainly attributed to the protective effect of exogenously applied NO, because the components of antioxidant defense were up-regulated in the presence of a NO donor, which allowed the plants to cope better with salinity stress.

CHAPTER 5

CAFFEIC ACID REGULATES SOYBEAN NODULE FUNCTIONING VIA MODULATION OF ANTIOXIDANT ENZYMES UNDER SALT STRESS

5.1 Abstract

Caffeic acid (CA; 3,4 dihydroxycinnamic acid) is a cinnamic acid derivative occurring naturally in a variety of plant species. The effects of CA (100 µM) on soybean root nodule number, nodule dry weight, root dry weight, shoot dry weight, nitric oxide and cGMP contents were studied under salt stress. Furthermore, CA-induced changes in nodule ROS content, cell viability, lipid peroxidation and antioxidant enzyme activity as well as the genes that encode these enzymes were also investigated in the presence or absence of salt stress (70 mM NaCl). Treatment with 100 µM CA reversed the negative effects of salt stress on soybean growth parameters, restricted nodule cell death and the extent of lipid peroxidation. CA also decreased both superoxide (O_2^-) and hydrogen peroxide (H_2O_2) contents that otherwise accumulate in response to salt stress and this decrease appears to be mediated by changes in root nodule antioxidant activity via activation of ROS-scavenging enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR). We therefore suggest that CA improves soybean salt stress tolerance via signals that include NO biosynthesis to regulate accumulation of ROS during salt stress.

5.2 Introduction

Salt stress is one of the most important abiotic stresses that adversely affect crop productivity and causes significant crop losses worldwide (Sekmen et al. 2007;

Perez-Lopez et al. 2009). Salinity and osmotic stress are known to suppress growth by reduction in osmotic potential and excessive accumulation of undesirable salts in the cytoplasm, which may have direct or indirect effects on the metabolism through disruption in mineral uptake and transport (Shereen et al. 2005). Soil salinity may also limit nitrogen fixation by reducing nodule numbers and nodule functioning. When soybean is grown in saline environments, it tends to display a reduction in yield potential as well as reduced numbers and weights of root nodules (Wilson 1974; Singleton and Bohlool 1984; Snook et al. 1994; Harrison et al. 2003). This phenomenon has also been reported in chickpea (Soussi et al. 1998; Mudgal et al. 2009). Plant responses to excess salt are complex, and involve changes in their morphology, physiology, and metabolism (Pujari and Chanda 2003). According to Parida and Das (2005), salinity stress negatively affects plant growth and can lead to plant cell death and severe reduction of crop yield because of its negative effects on diverse plant biochemical and physiological processes. Salt-induced stress results in the overproduction of ROS such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) that are responsible for the oxidative damage associated with plant stress (Zilli et al. 2009).

This overproduction of ROS can initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. There is, however, a balance between the generation of ROS and removal of ROS by the antioxidant system in plants. Overproduction of ROS occurs when the endogenous antioxidant defence system is inadequate. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification of cell membranes or intracellular molecules (Duh et al. 1999; Gülçin and Küfrevioglu 2001). Various antioxidants are produced by plants to minimize ROS-induced damage. For example, phenolic compounds can trap the

free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes such as SOD, APX, GPX and GR (Rao et al. 1996). One such phenolic compound is caffeic acid (CA), which is widely present in the plant kingdom and an important component in the defense chemistry of plants (Batish et al. 2008). This phenolic compound has several biological and pharmacological properties, such as antioxidant (Iwahashi et al. 1990; Gebhardt and Fausel 1997) and anti-carcinogenic (Challis and Bartlett 1975) activities. It has been shown that CA inhibits both lipoxygenase activity and suppresses lipid peroxidation (Okutan et al. 2005; Dos Santos et al. 2011). According to Jayanthi and Subash (2010), CA also blocks the production of ROS and the xanthine/xanthine oxidase system and its derivatives are good substrates of polyphenol oxidases, and under certain conditions may undergo oxidation in plants (Kerry and Rice-Evans 1998; Bassil et al. 2005). In several plant species, the levels of CA and other phenolics appear to be related to pest resistance (Cvikrova et al. 1993; Maher et al. 1994; Snook et al. 1994) and allelopathic potential (Baghestani et al. 1999). As one of many phenolic compounds, CA is considered to be an important part of the general defense mechanism of plants against infection and predation (Faulds and Williamson 1999; Friend 1985). We therefore hypothesized that increased activity of antioxidant enzymes such as SOD, APX, GPX and GR in response to exogenously applied CA can contribute to the protection of soybean plants from salt-induced cell death. Therefore, this study aims to evaluate the effects of exogenously applied CA and salt stress on ROS production as well as the enzymatic activity of ROS scavenging enzymes. This study also explores the role of CA and salt stress on soybean biomass, coupled with the extent of lipid peroxidation and root nodule cell death in order to better understand the effects of CA on salt stress responses of plants.

5.3 Materials and Methods

5.3.1 Plant Growth

Soybean (*Glycine max* L. merr. cv. PAN626) seeds were surface-sterilized in 0.35% sodium hypochlorite for 10 minutes, followed by four washes with sterile distilled water. The seeds were sown in 2 liters of filtered silica sand (98% SiO_2, Rolfes® Silica, Brits, South Africa) that had been pre-soaked in distilled water to ensure that the sand was sufficiently moist, in 20 cm diameter plastic pots. The sand was kept moist by watering only with distilled water every two days until the seeds germinated. Germinated seedlings at the VC stage of growth (thinned to one plant per pot) were allowed to grow further in a glasshouse under natural day light in summer at the University of the Western Cape (daytime temperature range of 23-32ºC and night-time temperature range of 14-18ºC during the entire growing period). Nitrogen-free nutrient solution [1 mM K_2SO_4, 2 mM $MgSO_4$, 5 mM $CaCl_2$, 1 mM K_2HPO_4 buffer at pH 6.4, 5 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $ZnSO_4$, 1 µM $CuSO_4$, 2 µM Na_2MoO_4, 1 µM $CoSO_4$, 100 µM Fe-NaEDTA and 5 mM 2-(N-Morpholino)ethanesulfonic acid (MES) at pH 6.4] was supplied at intervals of three days to the plants when they reached the V1 stage until they developed to the V3 stage of vegetative growth.

5.3.2 Treatment of plants

Treatments were initiated on plants when they reached the V3 stage of vegetative growth on the same day. Control plants (referred to as Untreated) at the V3 stage were supplied with nitrogen-free nutrient solution containing 0.1% ethanol (since caffeic acid is dissolved in ethanol and treatments with caffeic acid would contain ethanol at a final concentration of 0.1%, all treatments contained 0.1% ethanol to normalize all samples for the effect of ethanol in the treatments) at

intervals of three days for a total of 12 days (i.e. 4 applications of nitrogen-free nutrient solution, with the last application done 3 days before harvesting the plants for assays/measurements).

For the rest of the treatments (over a period of 12 days); plants at the V3 stage were supplied with nitrogen-free nutrient solution containing 0.1% ethanol that was supplemented with either 100 µM caffeic acid (CA), 70 mM NaCl (NaCl, regarded as salt stress) or a combination of 100 µM caffeic acid and 70 mM NaCl (CA + NaCl) at intervals of three days for a total of 12 days (i.e. 4 applications of nutrient solution containing either CA, salt or a combination of CA and NaCl so that the last application is done three days before assaying). Three days after the last application of either nitrogen-free nutrient solution (containing 0.1% ethanol) devoid of or containing either CA, salt or a combination of caffeic acid and salt, all plants were used for various experiments as described below.

5.3.3 Analyses of plant growth parameters

Plants were carefully removed from the sand, avoiding any loss of shoots, roots or nodules during the up-rooting of the plants. Six plants from each treatment were divided into shoots, roots and nodules. Each of these plants was scored for nodule number and dry weight of the shoots, root and nodules.

5.3.4 Evaluation of cell viability

A modified method of Sanevas et al. (2007) was followed for the cell viability assays. Briefly, soybean root nodules (± 100 mg per treatment) from five different plants of each of the treatments were harvested and stained at room temperature with 0.25% (w/v) Evans Blue for 15 minutes. The root nodules were washed twice for 20 min each time in distilled water. Nodules were incubated for 1 hour at 55°C, after

which the Evans Blue stain was extracted from nodule tissue using 1% (w/v) SDS. Absorbance of the extract was measured at 600 nm to determine the level of Evans Blue up-take by nodules.

5.3.5 Measurement cGMP content in soybean root nodules

Frozen nodule tissue (100 mg) was ground in liquid nitrogen to a fine powder and homogenized in 1 ml of ice-cold 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 600 X *g* for 10 min. The aqueous extract was extracted four times in three volumes of diethyl ether, dried overnight under vacuum at 25°C and stored at -80°C. Cyclic guanosine monophosphate (cGMP) content was determined according to the manufacturer's instructions for the cGMP Enzyme Immunoassay Kit (Sigma) following the acetylation protocol.

5.3.6 Protein isolation from soybean root nodule tissue

Enzyme extracts were obtained from soybean nodules by grinding plant tissue into a fine powder in liquid nitrogen and homogenizing 200 mg of the tissue with 1 ml of homogenizing buffer [40 mM K_2HPO_4, pH7.4, 1 mM EDTA, 5% (w/v) polyvinylpyrrolidone (PVP) (molecular weight = 40 000] for the determination of NO content, antioxidant enzymatic activities and superoxide content, or 1 ml of 10% trichloroacetic acid (TCA) for H_2O_2 content and lipid peroxidation. The resulting homogenates were centrifuged at 12 000 X *g* for 30 min and the supernatants were used for all the assays described below. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

5.3.7 Measurement of lipid peroxidation

Lipid peroxidation was measured as the amount of MDA determined by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). An assay mixture

containing 1 ml of the supernatant and 2 ml of 0.5% (w/v) TBA in 20% (w/v) TCA was heated at 95°C for 30 min and then rapidly cooled in ice for 10 min. The absorbance of the assay mixture was read at 532 nm, and the values corresponding to nonspecific absorption (600 nm) were subtracted. Lipid peroxidation products were measured as the content of TBA-reactive substances. The MDA content was calculated based on the molar extinction coefficient of 155 mM^{-1} cm^{-1}.

5.3.8 Spectrophotometric quantification of nitric oxide content

NO content was measured by slight modification of the haemoglobin-based assay (Murphy and Noack 1994). Briefly, protein extracts were obtained from root nodule tissue (± 200 mg) by grinding the tissue into fine powder in liquid nitrogen and homogenizing the tissue with 1 ml of homogenizing buffer [40 mM K_2HPO_4, pH7.4, 1 mM EDTA, 5% (w/v) polyvinylpyrrolidone (PVP) (molecular weight = 40 000]. The resulting homogenates were centrifuged at 13 200 X *g* for 30 min and incubated with 100 Units of catalase and 100 Units of superoxide dismutase for 10 min, followed by addition of oxyhaemoglobin to a final concentration of 10 µM. The mixture was incubated for 2 min, followed by spectrophotometric measurement of NO content by following the conversion of oxyhaemoglobin to methaemoglobin at 401 and 421 nm.

5.3.9 Nitric oxide detection by confocal laser scanning microscopy (CLSM)

Measurement of NO was performed with the specific NO dye DAF-2DA, using a modified method (Zhao et al. 2004). Fresh root nodule sections of (150 µm thick) were incubated in loading buffer (0.1 mM $CaCl_2$, 10 mM KCl, 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES)-Tris, pH 7.2) and DAF-2DA at a final concentration of 20 µM for 30 min in the dark at 25°C, followed washing with loading buffer for 3 times for 10 minutes each time. NO-induced fluorescence was observed using a Zeiss confocal laser-scanning microscope (LSM 510 META, Zeiss,

Germany) (excitation at 488 nm, emission at 515 nm). Pixel intensities estimated as the level of fluorescence of DAF-2T were calculated using the AlphaEase FC imaging software V4 (Alpha Innotech Corporation, USA).

5.3.10 Detection of antioxidant enzyme activity in soybean root nodules

Protein concentrations were quantified according to Bradford (1976) prior to loading of the native polyacrylamide gels to ensure equal loading of protein for accurate comparison of isoforms between treatments.

For the detection of SOD isoforms, samples containing 150 µg protein per well were subjected to native polyacrylamide gel electrophoresis (PAGE) in 5% stacking and 10% separating gels under constant current (120 mA) at 4°C. SOD activity was detected by photochemical staining with riboflavin and nitrotetrazolium blue chloride (NBT) as described by Beauchamp and Fridovich (1973). SOD isozyme classes were determined by incubating the gels with 6 mM H_2O_2 to inhibit both Cu/ZnSOD and FeSOD, or with 5 mM KCN to inhibit only Cu/ZnSOD (Fridovich 1982). MnSOD activity is resistant to both treatments.

Electrophoretic APX separation was carried out as described by Seckin et al. (2010) and native PAGE was performed at 4°C in 10 % polyacrylamide mini gels containing 10% glycerol. Before loading samples (60 µg protein), gels were equilibrated with running buffer containing 2 mM ascorbate for 30 min at 4°C. Following electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 min and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were transferred to a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N′,N′-

Tetramethylethylenediamine (TEMED) and 2.5 mM NBT for 10-20 min with gentle agitation in the presence of light.

GPX in-gel assays were carried out as a modified method based on the method of Seckin et al. (2010) for APX isoform detection except that ascorbate was replaced with GSH in the staining solutions. Native PAGE was performed at 4°C in 10 % polyacrylamide mini gels using 80 µg of protein, gels were equilibrated with running buffer containing 2 mM GSH for 30 min at 4°C. Following electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM GSH for 20 min and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM GSH and 0.01% cumene hydroperoxide for 20 min. The gels were washed with distilled H_2O and transferred to a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.5 mM NBT for 10-20 min with gentle agitation in the presence of light.

GR activity detection was performed in 10% native polyacrylamide gels containing 10 % glycerol at 4°C. GR activity was detected according to the procedure of Lee and Lee (2000) with slight modifications. GR was visualized in the native gel by incubation in 50 ml of 0.25 M Tris–HCl buffer (pH 7.9) containing 4.0 mM oxidized glutathione (GSSG), 1.5 mM NADPH, and 2 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for 20 min. The GR activity was negatively stained in the dark with a solution containing 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1.6 mM phenazine methosulfate (PMS) for 5-10 min.

Densitometry analysis was done on all the native PAGE gels after image acquisition, using the Spot Denso tool (AlphaEase FC imaging software V4, Alpha

Innotech Corporation). The enzymatic activity (for the respective antioxidant enzymes) of each isoform in the treatments was scored as an average of the relative pixel intensities (by assigning a value of 1 for the isoform with the lowest pixel intensity in that type of isoform and expressing the rest of the pixel intensities for that type of isoform in the other treatments relative to this isoform, for example: if MnSOD1 has the lowest pixel intensity in the NaCl treatment, then this pixel intensity was assigned a value of 1 and all the pixel intensities for MnSOD1 in the rest of the treatments will be expressed relative to the pixel intensity of MnSOD1 in the NaCl treatment) from three independent gels and expressed in arbitrary units.

5.3.11 Quantification of ROS

For detection of O_2^- in soybean root nodules, a modified procedure described by Able et al. (1998) was used. Four nodules (± 100 mg) were homogenized in 500 µl of 50 mM potassium phosphate buffer (pH 8.2) containing 0.12 mM 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT). The homogenate was incubated in the dark at room temperature for 20 min and centrifuged at 12 000 X *g* for 5 min to obtain the O_2^- extract that would react with the XTT. O_2^- content was measured by monitoring absorbance at 450 nm for 30 min at 5 min intervals, using the extinction coefficient of 2.36 X 10^4 $M^{-1}.cm^{-1}$ (Sutherland and Learmonth 1997). O_2^- estimation was carried out in duplicate for all samples.

H_2O_2 content was determined based on a method adapted from Velikova et al. (2000). Root nodule tissue (100 mg) was ground to a fine powder in liquid nitrogen and homogenized in 500 µl of cold 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 13,200 X *g* for 30 min at 4°C to obtain the H_2O_2 extract for determination of H_2O_2. The reaction mixture contained 75 µl of the extract, 5 mM K_2HPO_4, pH 5.0 supplemented with KI at 0.5 M. Samples were incubated at

25°C for 20 minutes and absorbance readings of the samples were taken at 390 nm. H_2O_2 content was calculated based on a standard curve constructed from the absorbance (A_{390} nm) of H_2O_2 standards.

5.3.12 Isolation of total RNA and semi-quantitative reverse transcription PCR analysis

Total RNA was extracted from soybean root nodules (from the plants subjected to the various treatments) using the RNeasy® Plant Mini Kit (Qiagen, USA) as described by the manufacturer. The amount of RNA isolated was determined by measuring the absorbance at 260 nm. Two micrograms of total RNA were treated with RNase-free DNase I (Fermentas) according to the manufacturer's instructions, of which 200 ng was reverse-transcribed into cDNA in a 20 µl reaction mixture containing 5 X RT buffer (50 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 200 µM each of the four dNTPs (dATP, dTTP, dGTP and dCTP), 20 U of Ribolock™ RNase Inhibitor (Fermentas), 0.5 µM reverse primer (gene-specific, Table 5.1) and 200 U of Revert Aid™ Premium Reverse Transcriptase (Fermentas, USA) for 30 min at 50 ºC, followed by termination of the reaction by incubation for 5 min at 80 ºC.

A reaction mixture (25 µl) containing cDNA products from the reverse transcription reaction (2 µl), 1 X TrueStart™ Taq buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, primers (0.2 µM each) and 1.25 U of TrueStart™ Hot Start Taq DNA Polymerase (Fermentas) was used for PCR amplification. The PCR profile was set as follows: 95 ºC for 1 min, then 22 cycles of 95 ºC for 0.5 min, annealing temperature (primer specific) and72 ºC extension for 1 min, followed by a final step of 72 ºC for 5 min. Also, primers for soybean β-tubulin (Table 5.1) were used as an internal control for the amount of RNA and RT efficiency. For the internal control

gene, samples were subjected to thermocycling (95 ºC for 1 min then 22 cycles of 0.5 min at 95 ºC, 0.5 min at 60 ºC, and 0.5 min at 72 ºC, with a final extension at 72 ºC for 7 min).

Table 5.1 PCR primers used in quantitative real-time PCR for detection of antioxidant gene expression in soybean root nodules.

Antioxidant enzymes	Origin	Primer sequences	Amplicon size(bp)
GmMnSOD	glyma02g09630	ATG AAT TTG TTA TCG CAA TCC A TTA CAG CAT TGG AGT CTT CAC	654
GmFeSODI	glyma20g33880	ATG GCC TCA TTG GGT GGG TT TCA TGC ACT GGT AAT TAA AGC	747
GmFeSODII	glyma10g33710	ATG GCC TCA TTG GGT GGG TT TTA AAG CCT TGC TTG TTC AAG T	750
GmAPX1	glyma11g11460	GGA CCT AAC GGT TCG ATC CGG GCG AAC TTC ATA CAA GTA ACT	690
GmAPX2	glyma11g08320	GAG GCC CTA ATG GTT CTA TCA TCA ATT GGT TTT TTT GTT CAG T	689
GmGPX1	glyma11g02630	ATG GCT AGC CAA TCA AAC ACT AAA TC TCA TCG ATC TAG CAG CTT CTT	504
GmGPX2	glyma04g42840	ATG TCC TCC ATG GCT TCC TC TCA GGC AGC AAG TAA CTT CTG GAT	705
GmGR1	glyma02g16010	CAA AGC GCT TCC ACT ATC ATC TC GAA GGG GCG ACC TCC AAC TGC	592
GmGR2	glyma10g0370	CAA AGC GCT TCC TCT ATC TCG GAA GGG GCG ACC TCC AAC GGT	592
β-tubulin	M21297.1	CTG CGA AAG CTT GCA GTG AAC C TCT TGC CTC TAA ACA TGG CTG AGG	250

The amplified products were visualized on 1% agarose gels using GelRed™ nucleic acid stain (Biotium, USA). Stained gels were photographed (AlphaImage

2200 system) and analyzed using the Spot Denso Tool (AlphaEase FC imaging software V4, Alpha Innotech Corporation). Individual gels were scored as relative densitometry values (relative gene expression) of three independent gels used for gene expression analysis, using β-tubulin densitometry values as a reference for the relative gene expression.

5.3.13 Statistical analysis

All experiments were performed three times, independently. Samples from different replicates were stored separately at -80°C until analysed. Data were given as mean ± standard error of six independent determinations. For statistical analysis, One-way analysis of variance (ANOVA) test was used for all data and means were compared according to the Tukey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

5.4 Results

5.4.1 Caffeic acid (CA) and salt stress (NaCl) alter soybean root nodule NO and cGMP content

To date no evidence exists in the public domain that shows if exogenously applied CA and exposure to long term salt stress alters the soybean root nodule NO and cGMP contents even though both CA and NO are implicated in modulating plant ROS production and thus antioxidant activity while some NO signals are transduced via cGMP. Therefore, it was relevant to determine whether exogenously applied CA and salt stress effect changes in NO and cGMP contents. The level of NO and cGMP in root nodules was measured for plants treated at the V3 stage of vegetative growth as described in the “Materials and Methods” section.

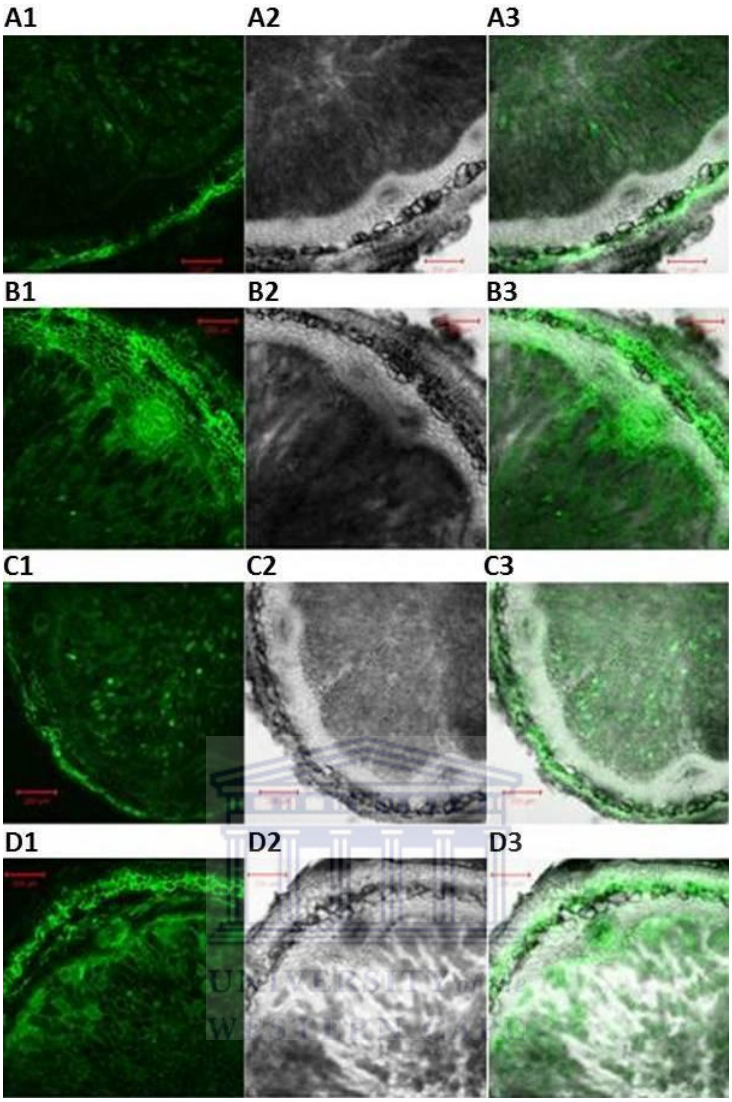

Figure 5.1 Detection of NO in soybean root nodules in response to CA and salt stress using confocal microscopy. The effect of exogenously applied CA and salt stress on soybean root nodule NO content were measured. Fluorescence (A1, B1, C1, D1), bright field (A2, B2, C2, D2) and overlay (A3, B3, C3, D3) micrographs of DAF-2DA-treated root nodule sections (150 µm) are shown. Nodule sections were obtained 12 days after treatment with CA and NaCl. DAF-2DA fluorescent triazole derivative fluorescence was observed following long-term treatment with 100 µM CA (B1), 70 mM NaCl (C1), 100 µM CA + 70 mM NaCl (D1) and was compared with the control plant (A1). Bar = 200 µm.

Soybean plants treated with CA (at a final concentration of 100 µM) for a period of 12 days showed an increase in root nodule NO content (Figure 5.1B1-3) in comparison to nodules from untreated plants (Figure 5.1A1-3). On the basis of fluorescence intensity, this CA-induced increase in root nodule NO content was ± 141% higher than the root nodule NO content of untreated plants (Figure 5.1B2 and Figure 5.2A)

but the haemoglobin-based assay showed that this CA-induced increase in root nodule NO content was ± 131% of the root nodule NO content of untreated plants (Figure 5.2B). In response to salt stress the root nodule content (on the basis of fluorescence intensity) increased (by ± 53%; Figure 5.1C1 and Figure 5.2A) to levels higher than was observed in untreated plants but lower than those observed for CA-treated plants whereas the haemoglobin-based assay showed that this salt stress-induced increase in root nodule NO content was ± 31% of the root nodule NO content of untreated plants (Figure 5.2B). Exposure of soybean plants to a combination of CA and NaCl resulted in an increase in root nodule NO content to levels that are higher than both untreated and salt-stressed plants (Figure 5.1D1, Figure 5.2A, and Figure 5.2B). Data presented here for the fluorescence intensity shows that root nodule NO content was enhanced by ± 58% (Figure 5.1D1 and Figure 5.2A) in response to treatment with both CA and NaCl whereas the haemoglobin-based assay shows an increase of ± 68% in root nodule NO content compared to the untreated plants (Figure 5.2B).

Along with the changes in NO content, soybean root nodule cGMP content was also elevated by ± 62% in response to treatment with CA whereas NaCl also increased the root nodule cGMP content by ± 32%. However, the combined treatment (CA + NaCl) resulted in a nodule cGMP content increase of ± 54%, which is higher than both untreated and NaCl-treated plants (Figure 5.1 D).

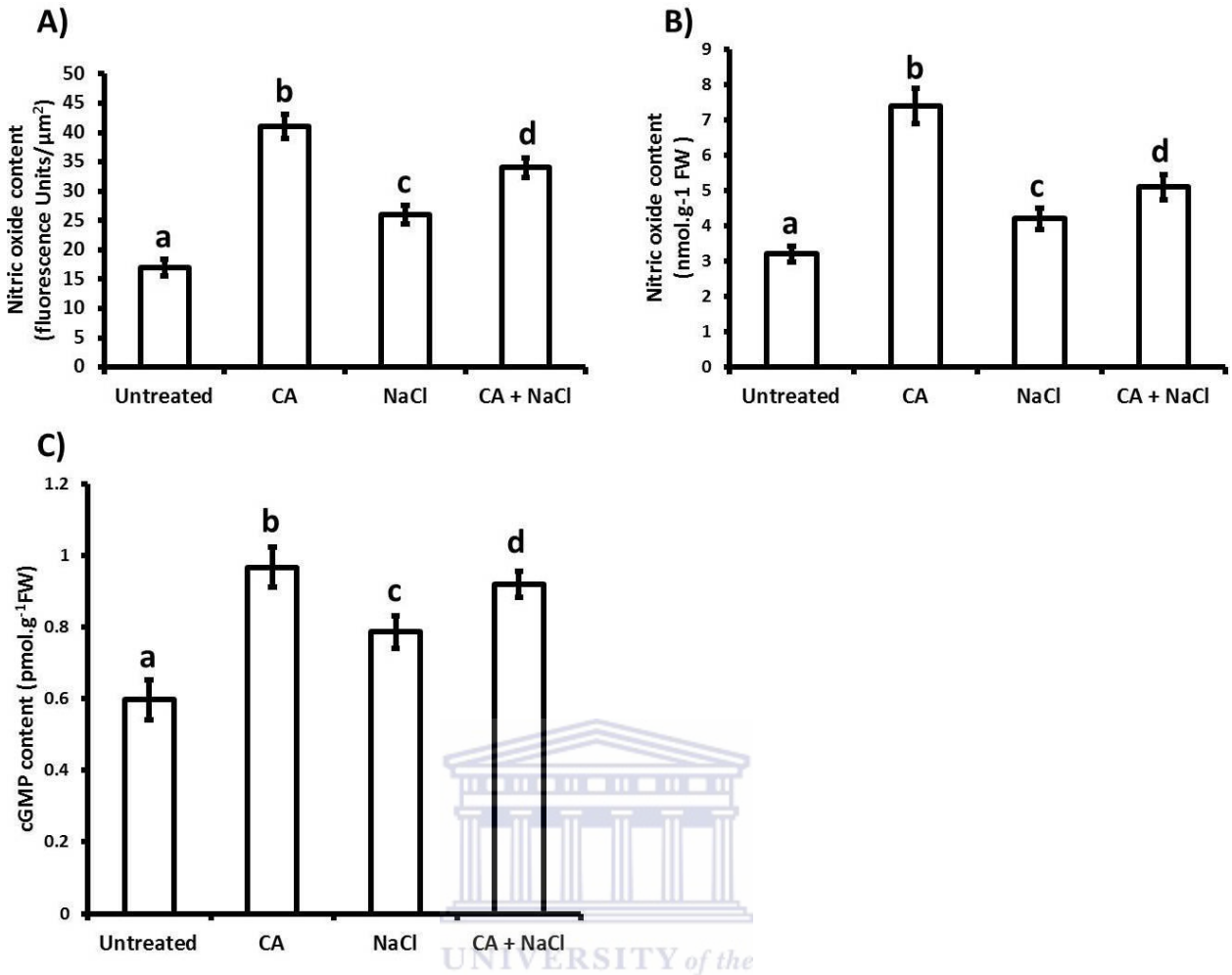

Figure 5.2 Effect of CA and salt stress on root nodule NO and cGMP contents. CA and NaCl were applied to soybean plants at the V3 stage of vegetative growth and NO and cGMP contents were measured in soybean root nodules. NO content were measured in soybean root nodules (A-B). cGMP content in root nodules was also measured (C). Error bar values represent the mean (± SE; n = 3) and the data are representative of three independent experiments.

5.4.2 CA improves soybean biomass under salt stress

The effect of the various treatments on soybean biomass was investigated because salt stress reduces plant biomass, thus the biomass should improve if CA enhances salt tolerance. Compared to untreated control plants, shoot dry weight was reduced by ± 21% in response to treatment of plants with CA (Figure 5.3 A). Treatment of soybean plants with NaCl or a combination of NaCl and CA lead to ± 58% and ± 39% reduction in shoot dry weight respectively (Figure 5.2 A) compared to untreated plants.

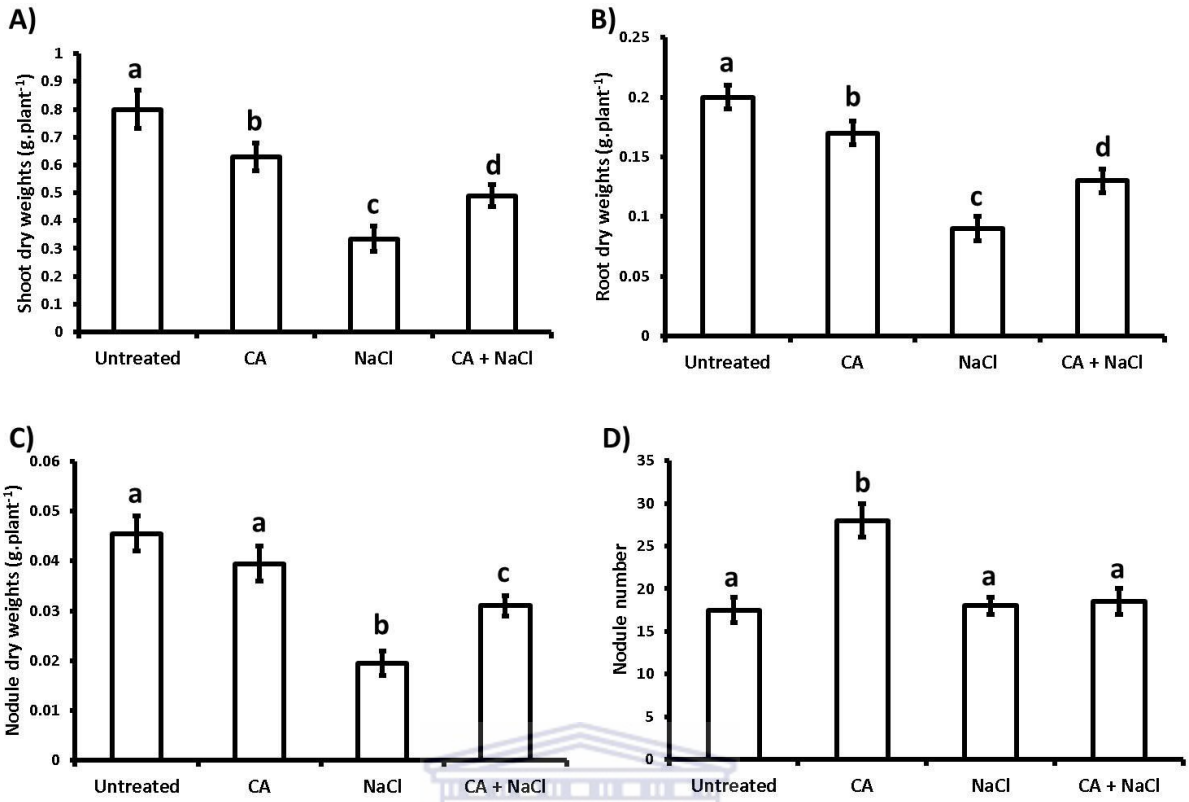

Figure 5.3 Responses of soybean biomass to exogenously applied caffeic acid and salt stress. Measurements were done on soybean plants treated at the V3 developmental stage for a period of 12 d. Biomass was evaluated by measuring shoot (A), root (B), nodule (C) dry weights and nodule number (D) at the end of the treatment period and bars are representative of the mean (±SE) of three independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

Root dry weight was reduced by ± 15% in response to treatment of plants with CA (Figure 5.2 B) compared to untreated plants. Plants treated with NaCl had root dry weights reduced by ± 55% compared to untreated plants (Figure 5.3 B). On the other hand, root dry weights in plants treated with a combination of NaCl and CA was reduced by ± 35% compared to untreated plants (Figure 5.3 B).

Nodule dry weight was slightly but insignificantly decreased in response to treatment with CA in comparison to untreated plants (Figure 5.3 C). Plants treated with NaCl had nodule dry weights reduced by ± 50% compared to untreated plants (Figure 5.3 C). On the other hand, nodule dry weights in plants treated with a

combination of NaCl and CA decreased by ± 24% when compared against untreated plants (Figure 5.3 C).

For nodule number, an increase of ± 60% was observed in response to treatment with CA compared to the untreated plants (Figure 5.3 D). No significant difference was observed for plants treated with NaCl or the combined treatment of CA + NaCl (Figure 5.3 D).

5.4.3 CA reduces salt stress-induced cell death and the extent of lipid peroxidation

The effect of the various treatments on root nodule cell death was investigated because salt stress induces PCD and such PCD is linked to enhanced lipid peroxidation. For evaluating cell death, the extent Evans Blue uptake (indicative of dead cells that take up the Evans Blue stain because of ruptured cell membranes, since living cells with intact cell membranes do not take up the stain) was measured in the root nodules of soybean at the end of the 12 d treatments. Cell death was ± 17% lower in plants treated with CA when compared to the untreated plants. On the other hand, cell death in plants treated with NaCl resulted in a huge increase in cell death (increase by ± 72%) whereas the combined treatment of CA and NaCl lead to a ± 27% increase compared to the untreated plants (Figure 5.4 A).

Root nodule lipid peroxidation was reduced by ± 21% in response to treatment of plants with CA whereas plants treated with NaCl experienced lipid peroxidation at levels ± 48% higher than nodules from untreated plants (Figure 5.4 B). On the other hand, root nodule lipid peroxidation in plants treated with a combination of CA and NaCl was restricted to an increase of ± 20% compared to lipid peroxidation in root nodules from untreated plants (Figure 5.4 C).

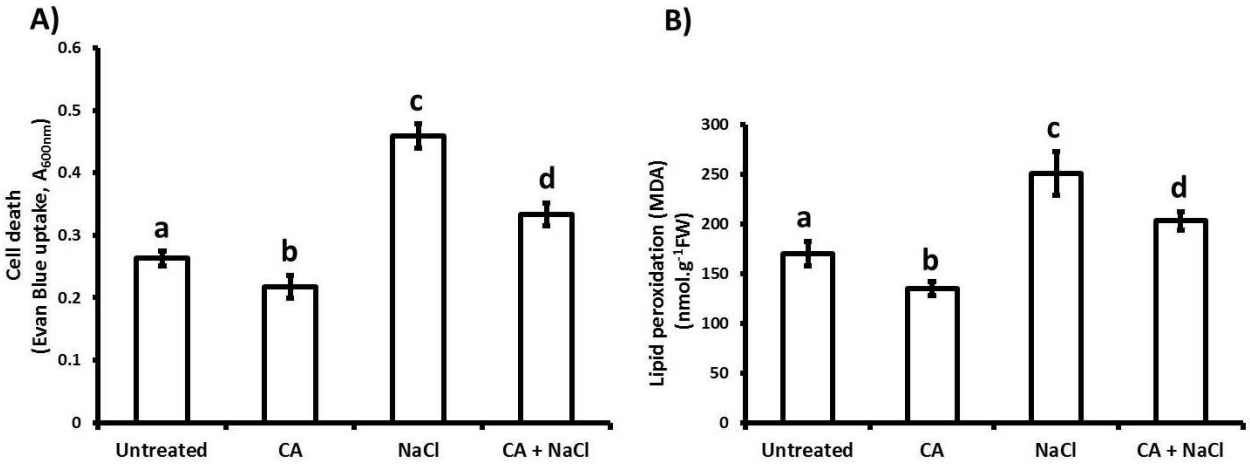

Figure 5.4 The effect of exogenous CA and salt stress on cell death (A) and lipid peroxidation (B) in soybean root nodules. Measurements were performed on soybean root nodules exposed to various treatments (100 µM caffeic acid, 70 mM NaCl and '100 µM caffeic acid + 70 mM NaCl) at the V3 stage for a period of 12 d. Data represent the mean (±SE) of three independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

5.4.4 CA restricts accumulation of ROS under salt stress

The effect of the various treatments on ROS production (O_2^- and H_2O_2) in soybean root nodules was investigated because salt stress is known to cause excessive ROS accumulation. It has been shown in literature that CA blocks oxidative stress and should therefore reverse the negative effects caused by salt stress if it were to enhance salt tolerance in soybean plants.

Root nodule O_2^- content was reduced by ± 43% in response to treatment with CA (Figure 5.5 A). However there was a massive increase of ± 229% in O_2^- content in response to NaCl treatment when compared to the untreated plants (Figure 5.5 A). On the other hand plants treated with the combination of CA and NaCl resulted in a ± 63% increase in O_2^- content when compared to nodules from untreated plants, which is far less than what was observed in the NaCl treated plants (Figure 5.5 A).

For root nodule H_2O_2 content, a similar profile to the O_2^- content was observed. Plants treated with CA showed a reduction of ± 27% in H_2O_2 content compared to the

untreated plants (Figure 5.5 B). Treatment with NaCl resulted in an increase of ± 89% whereas the combined treatment with CA and NaCl showed an increase of ± 47% compared to the untreated plants (Figure 5.5 B). The response seen in plants treated with the combination of CA and NaCl showed that CA does have a rescuing effect by reducing the amount of H_2O_2 accumulation in the root nodules compared to the plants treated with NaCl alone. The results obtained in this study are in agreement to what has previously been shown, namely that CA blocks oxidative stress.

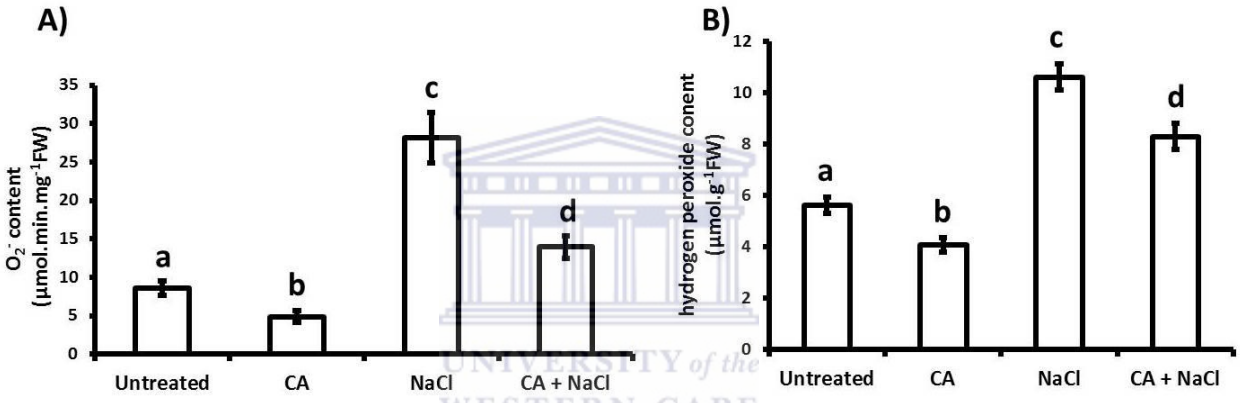

Figure 5.5 Effect of CA and salt stress on superoxide content (A) and hydrogen peroxide levels (B) in soybean root nodules. Measurements were performed on soybean root nodules that were treated at the V3 stage for a period of 12 d. Data represent the mean (±SE) of three independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

5.4.5 CA modulates soybean root nodule antioxidant activity under salt stress

5.4.5.1 The effect of CA and salt stress on root nodule SOD isoforms

Since the discovery of SOD by McCord and Fridovich (1969), the enzyme has attracted the attention of many researchers because it is an essential component in an organism's defense mechanism against oxidative stress (Hamid et al. 2004). SOD is the first enzyme involved in the antioxidative process (Lee et al. 2001, Rubio et al. 2002). This enzyme converts the superoxide radical (O_2^-) to hydrogen peroxide

(H_2O_2) and molecular oxygen (O_2) (Mhadhbi et al. 2004). This part of the study explores the effect of exogenously applied CA and long term salt stress on SOD isoforms in soybean root nodules. A total of 150 µg of nodule protein for each sample was separated on a 10% polyacrylamide gel. According to Figure 5.6 A-C both CA and NaCl differentially regulate the different isoforms detected for SOD in soybean root nodules.

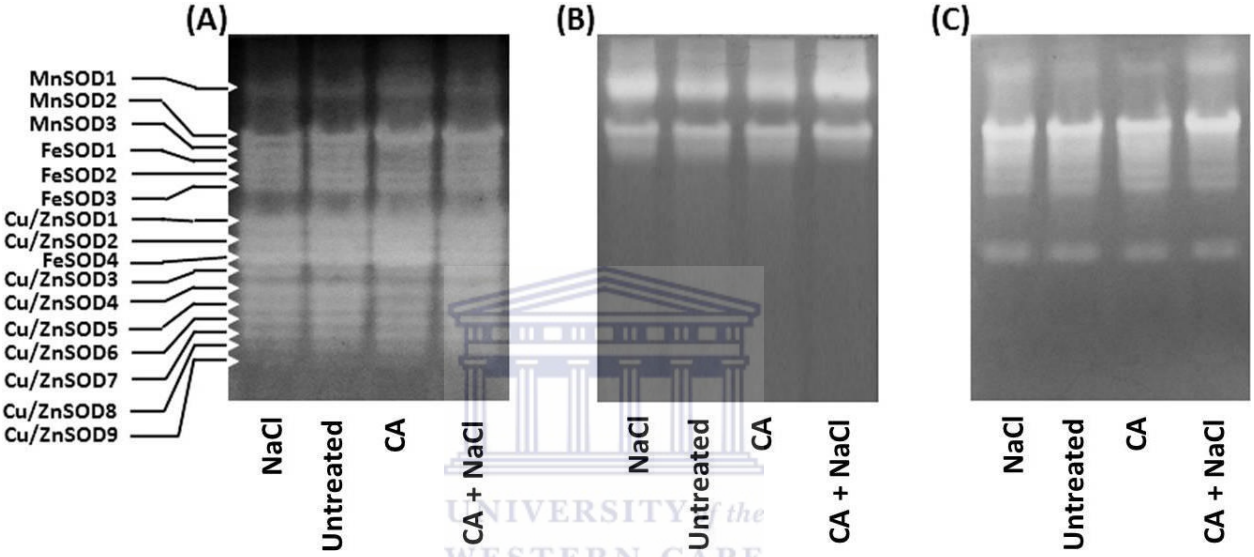

Figure 5.6 CA and salt stress differentially regulate SOD enzymatic activity in soybean root nodules. Assays were done on soybean plants that were treated (70 mM NaCl, 100 µM Caffeic acid and 100 µM Caffeic acid + 70 mM NaCl) at the V3 developmental stage for a period of 12 d (they were at the V5 developmental stage at time of harvest). The in-gels show the detection of SOD isoforms (A) with not inhibitors, (B) in the presence of 6 mM H_2O_2 and (C) in the presence of 5 mM KCN in response to the various treatments.

The existence of different SOD isoforms in soybean root nodules was determined by using two inhibitors namely H_2O_2 and KCN respectively (Figure 5.6B-C). Analysis of these results suggests the existence of three MnSOD isoforms, four FeSOD isoforms and nine Cu/ZnSOD isoforms in soybean root nodules. The activity of the first three SOD isoforms was resistant to both H_2O_2 and KCN, which suggests that they are MnSOD isoforms, hence they were named MnSOD1, MnSOD2 and

MnSOD3 respectively (Figure 5.6A-C). The SOD activity of isoforms 4, 5, 6 and 9 (numbering from the top of the gel) were sensitive to H_2O_2 and resistant to KCN, thus identifying them as FeSOD isoforms and hence referred to as FeSOD1, FeSOD2, FeSOD3 and FeSOD4 respectively (Figure 5.6A-C). Analysis of the in-gel activity of isoform 7, 8, 10, 11, 12, 13, 14, 15 and 16 revealed that the SOD activity of these bands was inhibited by both H_2O_2 and KCN, suggesting that they are Cu/ZnSOD isoforms, hence they were named Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD3, Cu/ZnSOD4, Cu/ZnSOD5, Cu/ZnSOD6, Cu/ZnSOD7, Cu/ZnSOD8 and Cu/ZnSOD9 respectively. Some of these isoforms were either induced, inhibited or unaffected by either or both CA and NaCl or a combination of CA and NaCl (Figure 5.6A).

Table 5.2 Relative SOD activity of different isoforms in soybean root nodules.

Relative SOD Activity (Arbitrary Units)	Soybean nodule SOD isoforms	Treatments			
		NaCl	Untreated	CA	CA + NaCl
	MnSOD1	**1.00 ± 0.050^a**	**1.25 ± 0.063^b**	**1.76 ± 0.088^c**	**1.39 ± 0.070^d**
	MnSOD2	**1.00 ± 0.050^a**	**1.33 ± 0.067^b**	**2.19 ± 0.110^c**	**2.05 ± 0.103^c**
	MnSOD3	1.01 ± 0.051^a	1.02 ± 0.051^a	1.00 ± 0.050^a	1.02 ± 0.051^a
	FeSOD1	1.01 ± 0.051^a	1.02 ± 0.051^a	1.00 ± 0.050^a	1.02 ± 0.051^a
	FeSOD2	1.00 ± 0.050^a	1.11 ± 0.056^a	1.02 ± 0.051^a	1.02 ± 0.051^a
	FeSOD3	1.00 ± 0.050^a	1.02 ± 0.051^a	1.00 ± 0.050^a	1.04 ± 0.052^a
	Cu/ZnSOD1	1.00 ± 0.050^a	1.00 ± 0.050^a	1.02 ± 0.051^a	1.01 ± 0.051^a
	Cu/ZnSOD2	1.00 ± 0.050^a	1.04 ± 0.052^a	1.10 ± 0.055^a	1.09 ± 0.055^a
	FeSOD4	1.01 ± 0.051^a	1.00 ± 0.050^a	1.02 ± 0.052^a	1.02 ± 0.052^a
	Cu/ZnSOD3	**1.22 ± 0.061^a**	**1.00 ± 0.050^b**	**1.51 ± 0.076^c**	**2.04 ± 0.102^d**
	Cu/ZnSOD4	1.03 ± 0.052^a	1.06 ± 0.053^a	1.00 ± 0.050^a	1.03 ± 0.052^a
	Cu/ZnSOD5	1.00 ± 0.050^a	1.03 ± 0.052^a	1.00 ± 0.050^a	1.05 ± 0.053^a
	Cu/ZnSOD6	1.00 ± 0.050^a	1.04 ± 0.052^a	1.00 ± 0.050^a	1.00 ± 0.050^a
	Cu/ZnSOD7	1.00 ± 0.050^a	1.05 ± 0.053^a	1.03 ± 0.052^a	1.03 ± 0.052^a
	Cu/ZnSOD8	1.00 ± 0.050^a	1.02 ± 0.051^a	1.03 ± 0.052^a	1.03 ± 0.052^a
	Cu/ZnSOD9	1.00 ± 0.050^a	1.00 ± 0.050^a	1.02 ± 0.051^a	1.00 ± 0.050^a

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

Densitometry analysis revealed that most of the isoforms detected in this study were unaffected in response the various treatments. However, three isoforms showed significant changes in enzymatic activity in response to the various treatments (Table 5.2). These isoforms are MnSOD1, MnSOD2 and Cu/ZnSOD3.

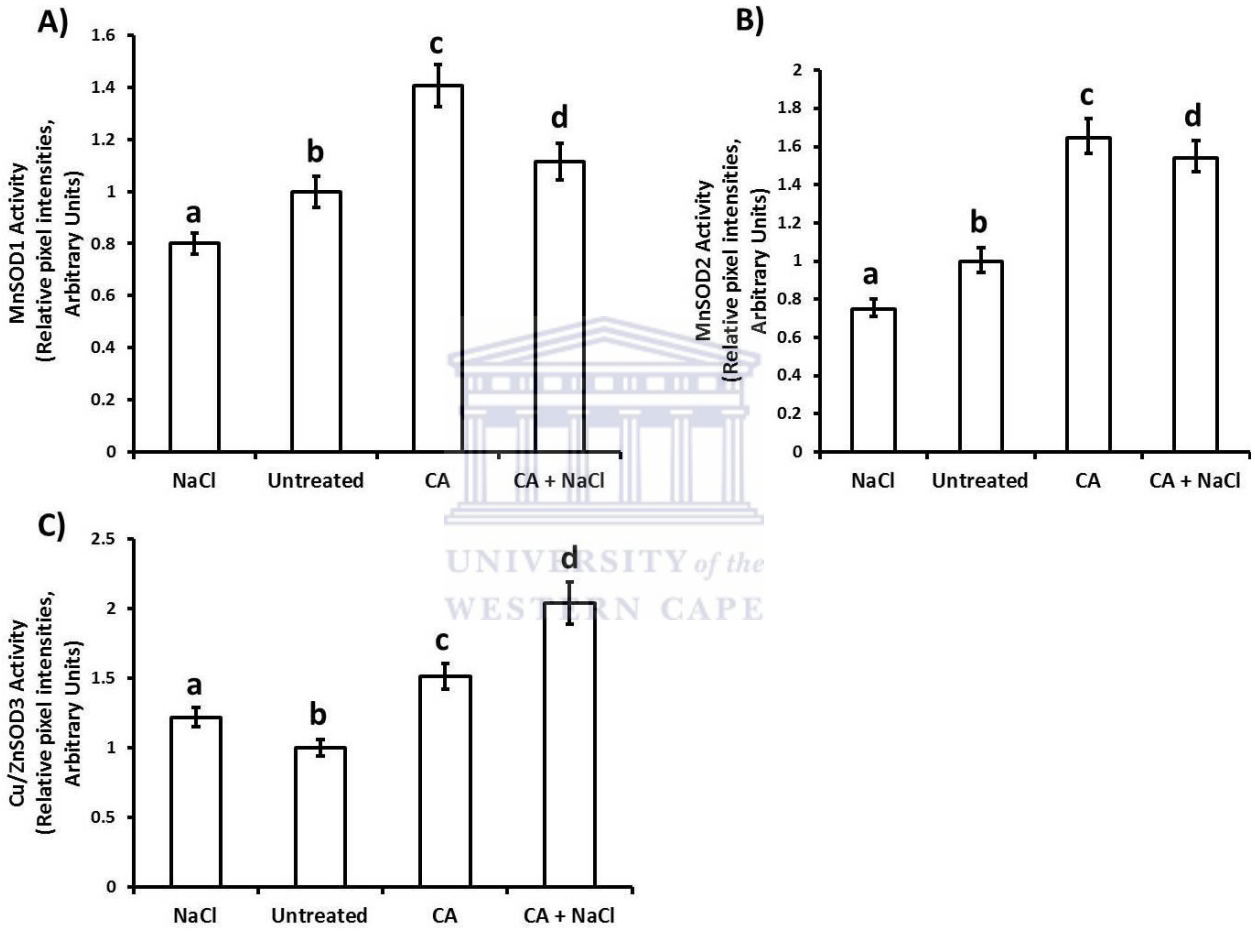

Figure 5.7 The enzymatic activity of three SOD isoforms in response to CA and salt stress. Assays were done on soybean root nodules that were exposed to the various treatments (70 mM NaCl, 100 µM Caffeic acid, '100 µM Caffeic acid + 70 mM NaCl') at the V3 stage for a period of 12 d (they were at the V5 stage at time of harvest). Relative pixel intensities signifying the level of enzymatic activity of three root nodule SOD isoforms (A-C), derived from analysis of the intensity of the bands corresponding to each SOD isoform (Figure 5.6 A-C), are indicated. Error bars represent the means (±SE; n = 4) of three independent experiments.

The analysis showed that salt stress inhibits MnSOD1 activity by ± 17% whereas CA strongly induces MnSOD1 activity by ± 54% and the inhibitory effect of salt stress

on MnSOD1 activity is reversed by CA, as signified by slight but significant elevation of MnSOD1 activity (± 13%) in response to the combined treatment of CA and NaCl (Figure 5.7A; Table 5.2). A similar trend was observed for MnSOD2 (Figure 5.7B). Treatment with NaCl inhibited MnSOD2 activity by ± 33% whereas CA increase the activity by ± 119% compared to the untreated control. Salt-stressed plants supplemented with exogenous CA showed significantly enhanced MnSOD2 activity (an increase by ± 105% compared to the untreated control). The activity of the MnSOD3 and all four FeSOD isoforms (FeSOD1, FeSOD2, FeSOD3 and FeSOD4) was not affected by the various treatments (Figure 5.6A-C and Table 5.2).

Densitometry analysis further revealed that the enzymatic activity of eight of the nine Cu/Zn isoforms (Cu/ZnSOD1, Cu/ZnSOD2, Cu/ZnSOD4, Cu/ZnSOD5, Cu/ZnSOD6 Cu/ZnSOD7, C/ZnSOD8 and Cu/ZnSOD9) did not respond to any of the treatments used in this study (Table 5.2) whereas the activity of one Cu/ZnSOD isoform (Cu/ZnSOD3) was enhanced by ± 22% in response salt stress. However, plants treated with CA had enhanced enzymatic activity of Cu/ZnSOD3 (increased by ± 51%), whereas an even higher increase in activity of Cu/ZnSOD3 (by ± 104%) was observed in salt-stressed plants supplemented with CA.

5.4.5.2 CA and salt stress alter enzymatic activity of ascorbate peroxidase and glutathione peroxidase in soybean root nodules

Soluble and cell wall-bound peroxidase activity is significantly enhanced in response to various concentrations of exogenously applied CA in soybean and this is linked to CA-induced decrease in H_2O_2 levels in soybean roots (Bubna et al. 2011). However, this study by Bubna et al. (2011) focused primarily on the role of CA on root growth of soybean seedlings in the absence of stress. To date the role of exogenous CA and salt stress on APX and GPX antioxidant activity in soybean root

nodules has not been elucidated. Therefore this part of the study focuses on the role of both CA and salt stress on the activities of the two peroxide scavenging enzymes in soybean root nodules.

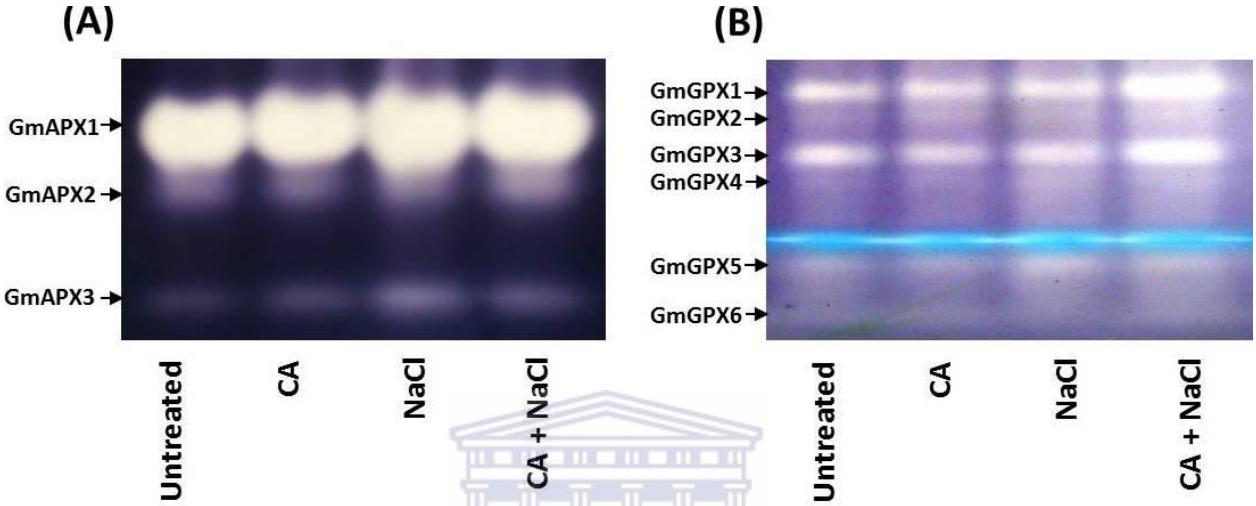

Figure 5.8 In-gel assays for (A) APX and (B) GPX in response to caffeic acid and salt stress in soybean root nodules. Assays were done on mature root nodules that were treated with the various treatments at the V3 stage for a period of 12 d (they were at the V5 stage at time of harvest). In-gel activities (as isoforms/bands on gel) in response to treatment with 100 µM Caffeic acid, 70 mM NaCl, (100 µM Caffeic acid + 70 mM NaCl) are shown for the two enzymes detected in soybean root nodules.

For the detection of APX activity in soybean root nodules, a total of 60 µg of protein for each treatment was separated on a 10% native PAGE gel at 4ºC. Three nodule APX isoforms were detected in each of the treatments (Figure 5.8A). The enzymatic activity of the APX isoforms detected in soybean root nodules is differentially regulated by exogenous CA and NaCl. Soybean plants treated with NaCl had enhanced GmAPX1 activity (an increase of ± 25%) when compared to untreated plants (Figure 5.8A and 5.9A). The enzymatic activity for GmAPX2 was slightly inhibited by ± 11% in response to CA whereas NaCl did not alter the enzymatic activity compared to the untreated plants (Figure 5.8A and 5.9B). However the combined treatment of CA and NaCl resulted in a ± 15% increase in

GmAPX2 isoform activity compared to the untreated plants. The enzymatic activity for GmAPX3 showed a significant increase of ± 100% in response to salt stress whereas treatment with CA only resulted in an increase of ± 21% when compared to the untreated control. The combined treatment (CA + NaCl) on the other hand enhanced the enzymatic activity by ± 43% which is higher than what was observed for both untreated and CA-treated plants (albeit half the intensity observed for the salt-treated plants) (Figure 5.8A and 5.9C).

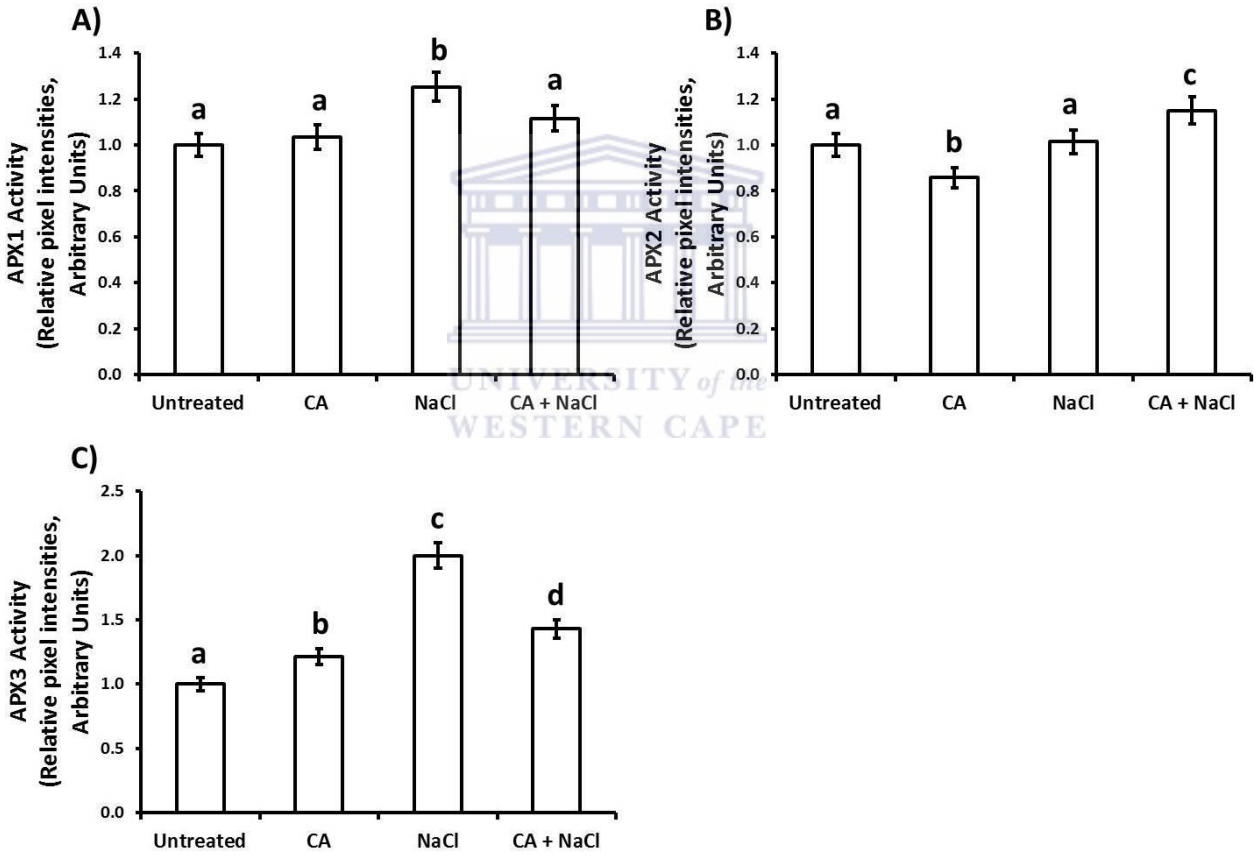

Figure 5.9 Exogenously applied CA and salt stress alter APX activity in soybean root nodules. Assays were done on soybean root nodules that were exposed to the various treatments (70 mM NaCl, 100 µM Caffeic acid, 100 µM Caffeic acid + 70 mM NaCl) at the V3 stage for a period of 12 d (they were at the V5 stage at time of harvest). Pixel intensities signifying the level of enzymatic activity of root nodule APX isoforms (A-C), derived from analysis of the intensity of the bands corresponding to each APX isoform (Figure 5.8A), are indicated. Error bars represent the means (±SE; n = 4) of three independent experiments.

For detection of GPX activity in soybean root nodules, 30 µg of protein per treatment were separated on a 10% native PAGE gel. The enzymatic activities of isoforms detected in soybean root nodules are represented as bands on the native PAGE gel (Figure 5.8B). Six GPX isoforms (Figure 5.8B) were detected in the soybean root nodules and analyzed using densitometry (Table 5.3). Plants supplemented with CA or NaCl differentially regulate the activity of the enzyme (as seen for the different isoforms on the gel). Densitometry analysis for GmGPX1 showed that the enzymatic activity was not influenced by CA or NaCl when compared to the untreated controls (Table 5.3).

Table 5.3 The effect of CA and salt stress on GPX activity in soybean root nodules.

Relative GPX Activity (Arbitrary Units)	Soybean nodule SOD isoforms	Treatments			
		Untreated	CA	NaCl	CA + NaCl
	GmGPX1	1.00 ± 0.050^{a}	1.00 ± 0.050^{a}	1.02± 0.051^{a}	1.54 ± 0.077^{b}
	GmGPX2	1.28 ± 0.064^{a}	1.85 ± 0.093^{b}	1.00 ± 0.050^{a}	2.04 ± 0.102^{d}
	GmGPX3	1.24 ± 0.062^{a}	1.00 ± 0.050^{a}	1.33 ± 0.067^{c}	1.62 ± 0.081^{d}
	GmGPX4	1.10 ± 0.055^{a}	1.00 ± 0.050^{a}	1.49 ± 0.074^{b}	1.39 ± 0.070^{b}
	GmGPX5	1.26 ± 0.063^{a}	1.00 ± 0.050^{a}	1.77 ± 0.089^{c}	1.36 ± 0.086^{d}
	GmGPX6	1.00 ± 0.050^{a}	1.02 ± 0.051^{a}	1.02 ± 0.051^{a}	1.02 ± 0.051^{a}

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

However, the combined treatment of CA and NaCl significantly elevated GmGPX1 activity by ± 54% when compared to the other treatments. The enzymatic activity for GmGPX2 in soybean root nodules was enhanced by ± 44% in response to CA while NaCl resulted in an inhibition of ± 23% and this inhibition was reversed upon supplementation of exogenous CA to levels higher than those observed for the

CA-treated plants. The enzymatic activity of GmGPX3 was inhibited by ± 20% in response to treatment with CA, whereas the combined treatment of CA and NaCl enhanced enzymatic activity ± 30% compared to the untreated control (Table 5.3). A slight increase in activity was observed for GmGPX3 in response to salt stress (± 7%), however this increase was so small that it was statistically insignificant when compared to the untreated control (Table 5.3). The enzymatic activity detected for GmGPX4 revealed that CA did not significantly alter the activity of the isoform whereas treatment with NaCl (including those NaCl-treated plants supplemented with CA) enhanced enzymatic activity between 26% and 34% (Figure 5.8B and Table 5.3). NaCl enhanced the activity of GmGPX5 whereas CA had the opposite effect on this isoform. In response to CA, the activity of GmGPX5 was inhibited by ± 21% whereas salt stress enhanced the enzymatic activity by ± 40% as compared to the untreated control (Table 5.2). A slight increase in activity (± 7%) was observed in response to the combined treatment of CA and NaCl although not significant. On the other hand no change in activity was observed for GmGPX6 in response to the various treatments used in this study (Table 5.3).

5.4.5.3 GR activity in soybean root nodules is differentially regulated by CA and salt stress

The changes in enzymatic activity of GR isoforms detected in soybean root nodules in response to treatment with exogenous CA and salt stress (NaCl) were investigated by separating 60 µg of total root nodule protein (per treatment) on a 10% native PAGE polyacrylamide gel.

Six GR isoforms were detected in response to the various treatments. For GR1 no or very low activity was detected in the salt treatments (including salt-stressed plants supplemented with exogenous CA). Enzymatic activity for GR1 was only

detected in the untreated as well as the CA-treated plants. However the activity detected in the CA-treated plants were higher (by ± 17%) than observed for the untreated plants (Figure 5.10 and Table 5.4). The enzymatic activity detected for GR2 was slightly inhibited in response to treatment with CA whereas NaCl enhanced activity to levels significantly higher than was observed for the untreated plants. However, the inhibition observed in the CA-treated plants was reversed (to levels significantly higher than the NaCl-treated plants) when plants were treated with a combination of NaCl and CA (Table 5.4). It is therefore plausible to suggest that the increase observed in the combined treatment is mainly attributed, although not limited, to NaCl in the treatment.

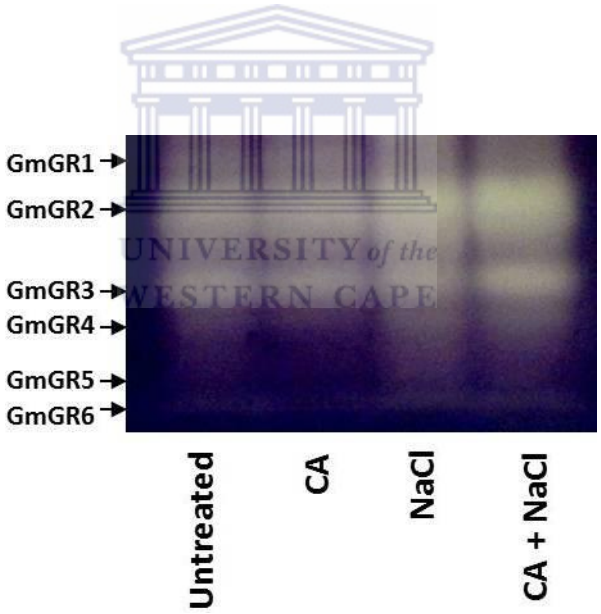

Figure 5.10 Detection of GR isoforms in response to CA and salt stress on in soybean root nodules. In-gel assay for detecting root nodule GR isoforms in response to treatment with 100 µM Caffeic acid, 70 mM NaCl and 100 µM Caffeic acid + 70 mM NaCl are shown. Assays were done on soybean plants that were treated at the V3 stage for a period of 12 d (they were at the V5 stage at the time of harvest).

For GR3, treatment with CA resulted in a slight inhibition (± 12%) of activity whereas NaCl did not significantly alter enzymatic activity compared to the untreated plants. However, the combined treatment of CA and NaCl enhanced the activity of

GR3 to levels significantly higher than was observed for the other treatments. Plants treated with CA resulted in inhibition of GR4 enzymatic activity while treatment with NaCl enhanced the activity to levels much higher than exhibited by the untreated plants. However the inhibition observed for GR4 in the CA treatment was reversed when NaCl-treated plants were supplemented with CA (Table 5.4). Again, it can be argued that this increase in activity observed in the combined treatment could be attributed to NaCl, but not limited to NaCl alone as this increase was higher than observed for the NaCl-treated plants.

Table 5.4 GR activity detected in soybean root nodules in response to CA and NaCl.

Relative GR Activity (Arbitrary Units)	Soybean nodule SOD isoforms	Treatments			
		Untreated	CA	NaCl	CA + NaCl
	GmGR1	1.00 ± 0.050[a]	1.17 ± 0.058[b]	NA	NA
	GmGR2	1.12 ± 0.056[a]	1.00 ± 0.050[b]	1.39 ± 0.070[c]	1.58 ± 0.079[d]
	GmGR3	1.12 ± 0.056[a]	1.00 ± 0.050[a]	1.14 ± 0.057[c]	1.33 ± 0.067[d]
	GmGR4	1.27 ± 0.064[a]	1.00 ± 0.050[a]	1.52 ± 0.076[b]	1.70 ± 0.085[b]
	GmGR5	1.22 ± 0.061[a]	1.00 ± 0.050[a]	1.77 ± 0.089[c]	1.22 ± 0.061[d]
	GmGR6	1.43 ± 0.072[a]	1.00 ± 0.050[b]	1.96 ± 0.098[a]	1.54 ± 0.077[a]

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test. The letters NA in the table indicate that very low or no activity was detected.

Furthermore, densitometry analysis revealed that the enzymatic activity profiles for GR5 and GR6 are similar but not identical. Similarly to what was observed for the other three GR isoforms (GR2, GR3 and GR4), the enzymatic activity of GR5 and GR6 in response to CA was inhibited whereas treatment with NaCl enhanced activity to levels well above those in the nodules of untreated plants (Figure 5.10 and Table

5.4). On the other-hand treatment with CA and NaCl (in combination) also enhanced enzymatic activity to levels higher than those in the nodules of untreated plants but less than those in NaCl-treated plants. Contrary to what was observed for the previous two isoforms (GR3 and GR4), the increase in activity of GR 5 and GR6 observed in the combined treatment can thus be solely attributed to NaCl in the treatment as CA caused an inhibition in activity and, although this inhibition was reversed in the combined treatment, it was not higher than in the NaCl-treated plants.

5.4.6 Exogenously applied CA and salt stress alter the expression of soybean root nodule antioxidant genes

Gene expression analysis plays an important role in furthering our understanding of the signaling and metabolic pathways which underlie developmental and cellular processes in various plants/animal species. Semi-quantitative reverse transcription PCR (RT-PCR) represents a particularly suitable technology platform for this purpose, thanks to its sensitivity and specificity (Huggett et al. 2005). To avoid experimental errors arising from variation in the quantity and integrity of the RNA template, as well as in the efficiency of the RT reaction used to synthesize cDNA, a normalization step is an essential pre-requisite. The most common way to achieve normalization is to include at least one, or a small number of reference genes, whose expression is assumed to be constitutive and stable (Radonic et al. 2004; Suzuki et al. 2000). Here, we have explored the effect of exogenous CA and salt stress on soybean antioxidant gene expression with the use of a soybean β-tubulin housekeeping gene. The targeted genes that code for the various antioxidant enzymes are tabulated in Table 5.1 (‘Materials and Methods’ section).

5.4.6.1 Effect of CA and salt stress on the expression of soybean root nodule SOD genes

To our knowledge this study is the first of its kind which investigates the role of exogenous CA and salt stress on soybean root nodule SOD gene expression. Three putative SOD genes namely MnSOD (glyma02g09630), FeSODI (glyma20g33880) and FeSODII (glyma10g33710) have been used for the gene expression analysis across the various treatments. They have been assigned the various names because of the presence of the various metal-cofactor binding domains. For ease of reference we have also included their original accession numbers as assigned in Phytozome (http://www.phytozome.net). The expression results obtained after PCR amplification are shown in Figure 5.11A-C.

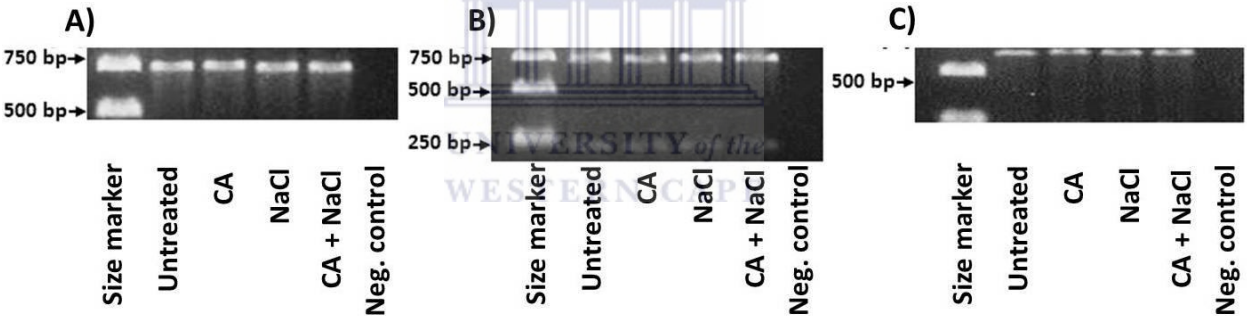

Figure 5.11 Gene expression levels of three soybean root nodules SOD genes in response to the various treatments. The effect of CA and NaCl on gene expression for three soybean root nodule SOD genes (A-GmMnSOD; B- GmFeSODI and C- GmFeSODII) was measured using semi-quantitative RT-PCR. Soybean β-tubulin (co-migrating with 250bp marker at bottom of B) was used as a reference gene. All PCR products were size fractionated with a DNA size marker on a 1% agarose gel.

The results obtained here showed that gene expression for all three SOD genes did not respond to any of the treatments (Figures 5.11A-C and 5.12). To avoid experimental errors arising from variation in the quantity and integrity of the RNA template, as well as in the efficiency of the RT reaction used to synthesize cDNA, the

soybean β-tubulin housekeeping gene was used (migrating at the bottom of Figure 5.11B with the 250bp DNA marker) as reference gene. The expression results of the β-tubulin gene in response to the various treatments remained constant with no variation.All SOD genes produced amplicons with expected sizes (Table 5.1) in response to the various treatments. We can therefore conclude that the results obtained in this assay were not due to either over/under loading of samples on the agarose gel and is thus a true reflection of the expression of these genes in response to the various treatments. The bands identified on the gel images (Figure 5.11A-C) were quantified using densitometry analysis and expressed as pixel intensities relative to the β-tubulin housekeeping gene (Figure 5.12).

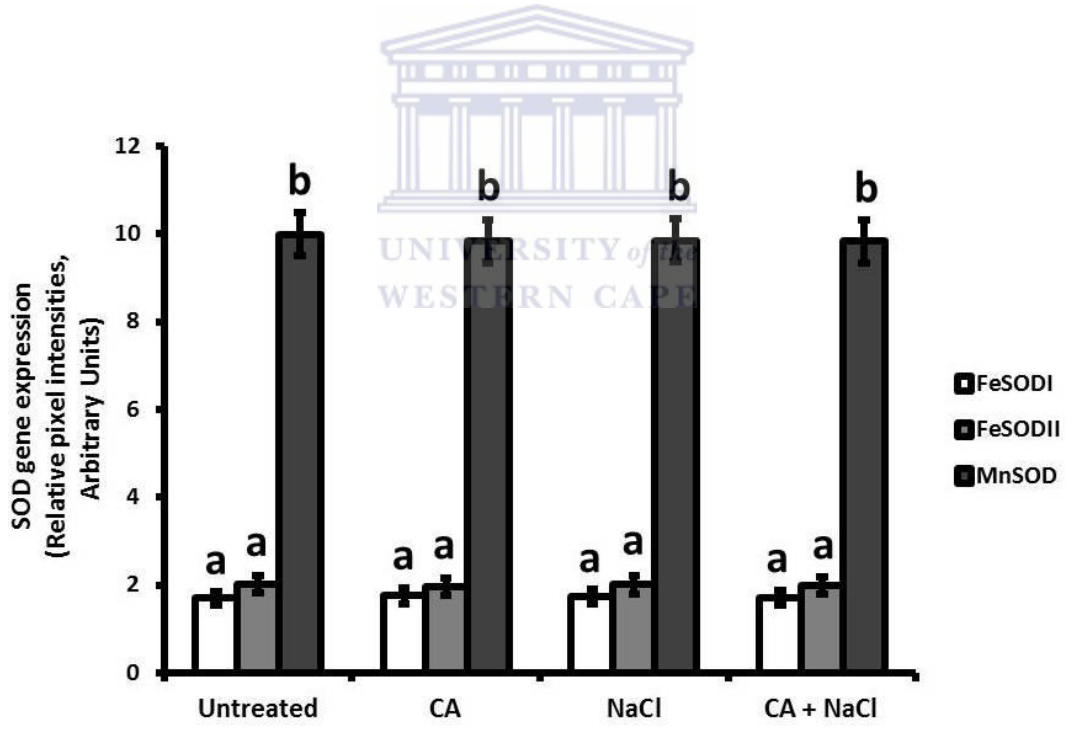

Figure 5.12 Effect of CA and NaCl on mRNA levels of GmSOD genes in soybean root nodules. Semi-quantitative RT-PCR for GmSOD genes was performed as described in Materials and methods. The expression of GmSOD genes was normalized to that of soybean β-tubulin for determination of the relative amount of mRNA of GmSOD genes (expressed as relative pixel intensities). Values are the mean of four independent experiments and bars indicate the standard error.

These analyses confirmed the results obtained on the agarose gel that none of the treatments had an effect of the level of expression of the three SOD genes.

As previously shown, 16 SOD isoforms were detected in response to the various treatments and have been classified into three groups based on the two inhibitors used (Figure 5.6). However we have only explored the gene expression of three genes, which did not respond to any of the treatments at the transcriptional level. In order to obtain a more in-depth expression profile for the isoforms identified we need to include more if not all putative SOD genes in the soybean genome.

5.4.6.2 Effect of CA and salt stress on APX and GPX gene expression

The effect of CA and salt stress on the gene expression of two ROS scavenging enzymes (APX and GPX) in soybean root nodules was investigated. The expression results obtained were quantified using densitometry analysis and expressed as relative pixel intensities relative to the housekeeping gene (β-tubulin) from soybean.

For APX mRNA expression, two genes namely GmAPX1 (glyma11g08320) and GmAPX2 (glyma11g11460) were used to test for gene expression in response to the various treatments (100 µM CA, 70 mM NaCl and 100 µM CA + 70 mM NaCl). Semi-quantitative real-time PCR was as used as tool to detect the expression levels of the two genes in response to the various treatments. The resulting PCR product was size fractionated on 1% agarose gels as shown in Figure 5.13A-B). In response to treatment with CA (CA and 'CA + NaCl') the expression levels of GmAPX1 was up-regulated by ± 71% and ± 56% respectively (Figure 5.14A) whereas treatment with NaCl enhanced the expression by ± 25% when compared to the untreated control. For GmAPX2, treatment with CA reduced the expression levels slightly (by ± 10%) whereas the combined treatment (CA and NaCl) enhanced the level of expression by

± 14% when compared to the untreated control. The expression levels in response to salt stress were not altered as it exhibited the same expression profile to that of the untreated control (Figure 5.13B and 5.14A).

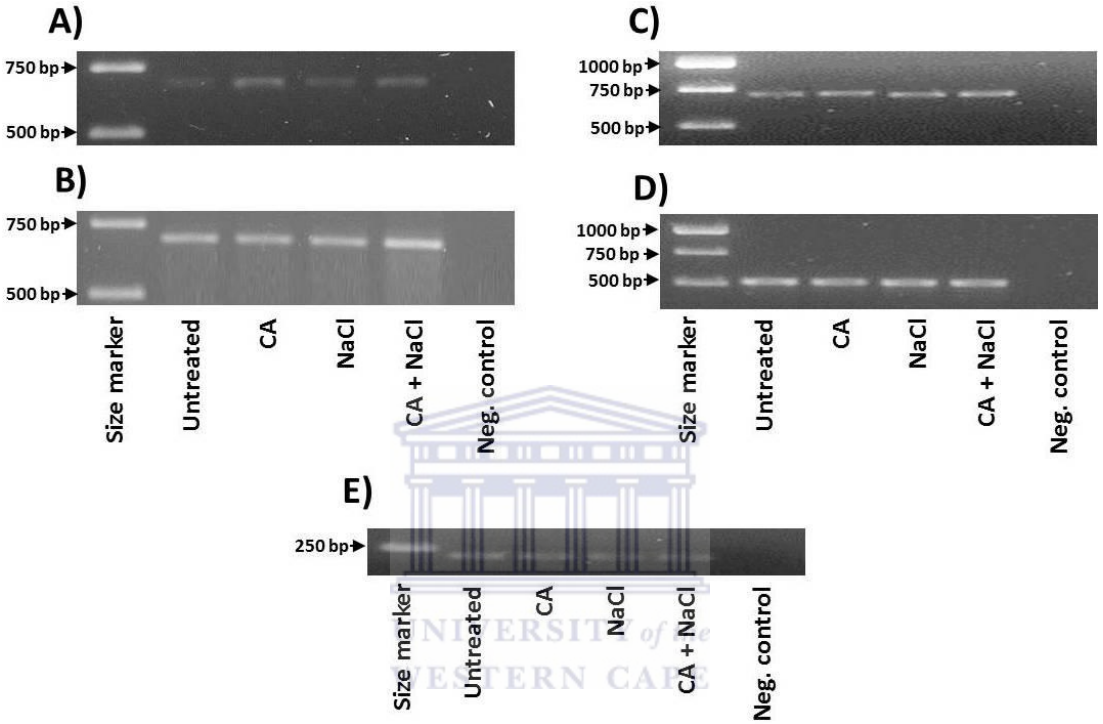

Figure 5.13 Gene expression levels of two ROS scavenging enzymes in soybean root nodules in response to CA and salt stress. The effect of CA and NaCl on gene expression for two soybean root nodule APX genes (A- glyma11g08320, B- glyma11g11460) and GPX (C- 17g34110, D- 01g42840) was measured using semi-quantitative RT-PCR. Soybean β-tubulin (E) was used to normalize the expression study. All PCR products were size-fractionated on a 1% agarose gel.

For the expression of GPX genes in soybean root nodules two genes namely GmGPX1 (glyma17g34110) and GmGPX2 (glyma01g42840) were used in response to treatment with CA and NaCl (Figure 5.13C-D and 5.14B). Similarly to what was observed for APX expression, densitometry analysis was used to quantify the expression of the genes in response to the various treatments. The expression

obtained for each treatment was expressed as relative pixel intensities relative to the housekeeping gene (β-tubulin) from soybean.

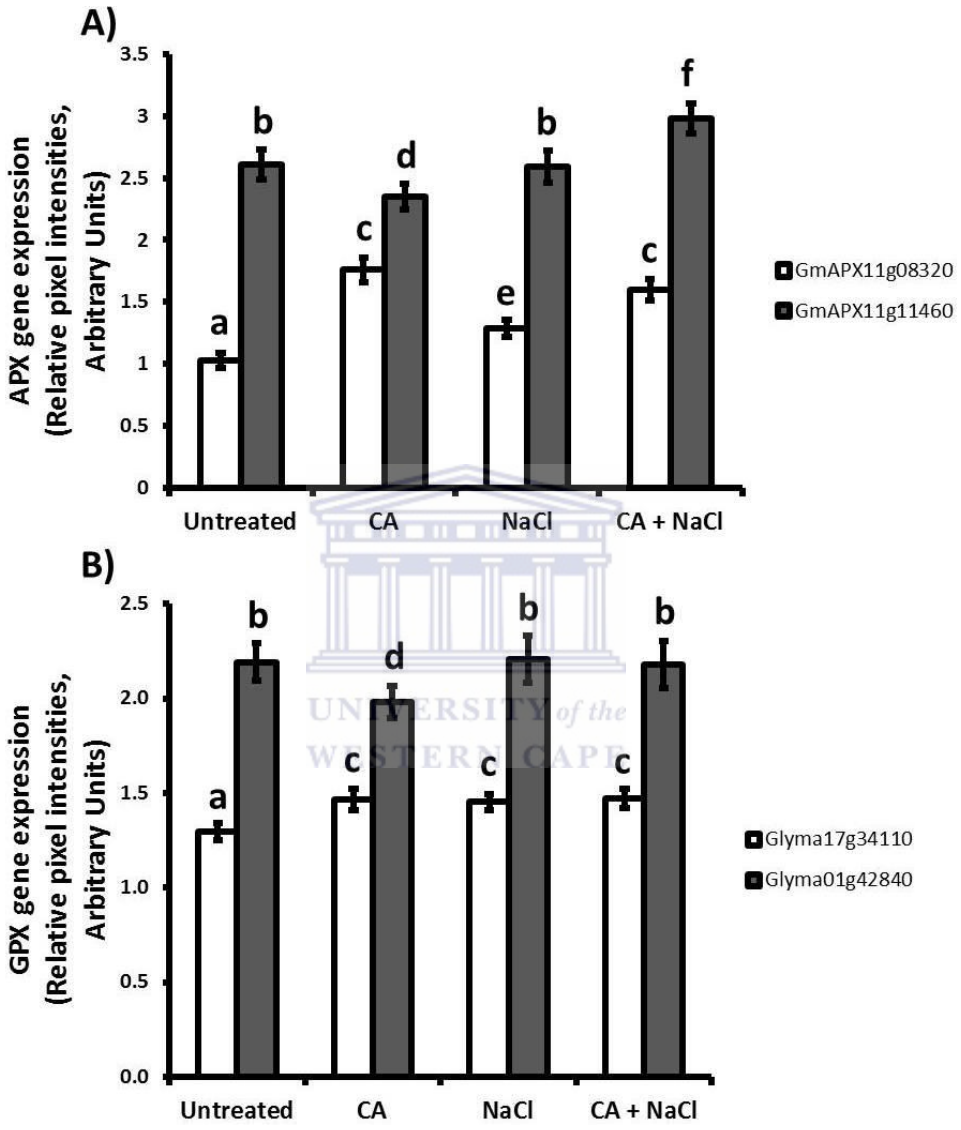

Figure 5.14 Effect of CA and NaCl on mRNA levels of GmAPX and GmGPX genes in soybean root nodules. Semi-quantitative RT-PCR for GmAPX and GmGPX genes was performed as described in Materials and methods. The expression of GmGR genes was normalized to that of soybean β-tubulin for determination of the relative amount of mRNA of GmAPX and GmGPX genes (expressed as relative pixel intensities). Values are the mean of four independent experiments and bars indicate the standard error.

For GmGPX1, the expression levels were slightly up-regulated in response to the various treatments (Figure 5.13C and 5.14B). In response to CA the level of expression of GmGPX1 was elevated by ± 13%, whereas NaCl and the combination of CA and NaCl increased the expression by ± 12% respectively. On the other hand the expression levels for GmGPX2 were not altered in response to treatment with NaCl and the combination of CA and NaCl, as shown in Figure 5.13D). However treatment with CA resulted in a slight reduction in GmGPX2 gene expression (by ± 10%) when compared to the untreated control.

5.4.6.3 Effect of CA and salt stress on GR gene expression in soybean root nodules

To determine whether the aforementioned different GR isoform patterns in root nodules (Figure 5.10) were due to transcriptional changes, GR mRNA were analyzed by semi-quantitative RT-PCR coupled with densitometry for quantification to determine the effect of exogenous CA and salt stress (NaCl) on soybean root nodule GR gene expression. Two GR genes namely GmGR1 (glyma02g16010) and GmGR2 (glyma10g0370) were used in this study. For GmGR1, the transcript levels were significantly altered after supplementation of CA or NaCl as well the combined treatment of CA and NaCl when compared to the untreated control (Figure 5.15A).

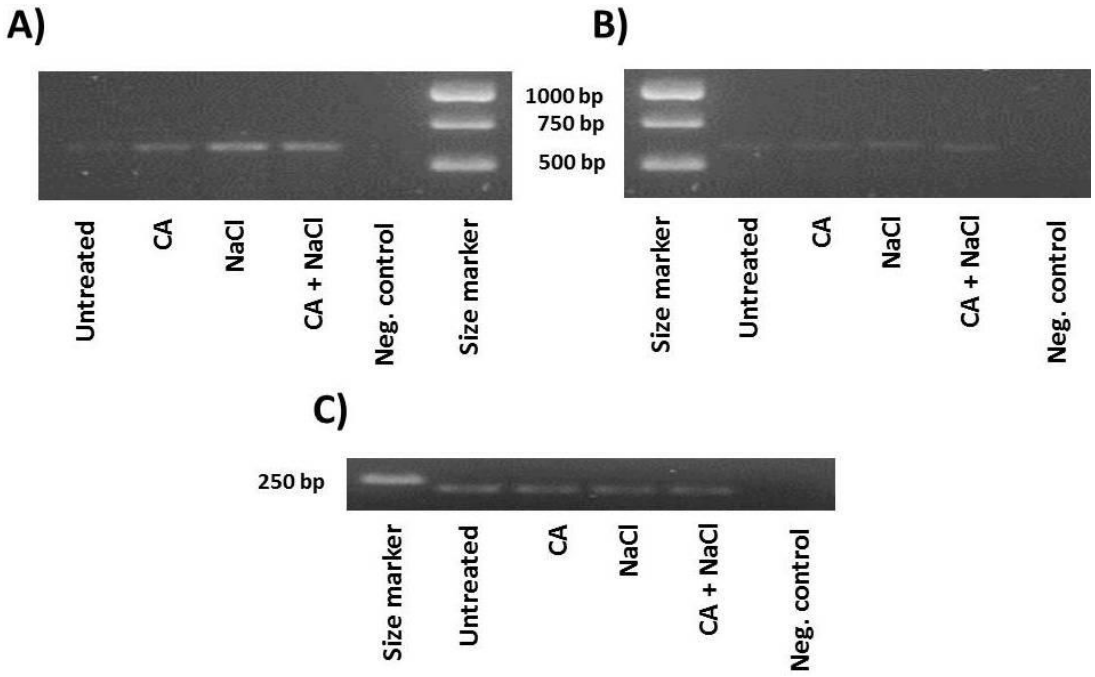

Figure 5.15 Effect of various treatments on (A) GmGR1, (B) GmGR2 and (C) soybean β-tubulin expression levels in soybean root nodules. GR gene expression was analyzed by semi-quantitative RT-PCR and densitometry analysis as described in 'Materials and Methods' section. The β-tubulin amplification bands (C) are shown to confirm equal loading of RNA and RT efficiency. All PCR products were size fractionated on a 1% agarose gel.

In response to CA, the transcript levels of GmGR1 were up-regulated by ± 41% whereas the salt stressed plants (which include NaCl-treated plants and the combination of CA and NaCl) resulted in an even higher increase in transcript levels (by ± 105% and ± 75% respectively) (Figure 5.15). The increase in GR transcript levels in response to salt stress have previously been reported in the roots of rice seedlings (Tsai et al. 2004; Hong et al. 2009). As for GmGR2, the transcript levels did not show any significant changes in response to the various treatments with respect to untreated control (Figure 5.15B). These results were confirmed by densitometry analysis of the gel images (Figure 5.16).

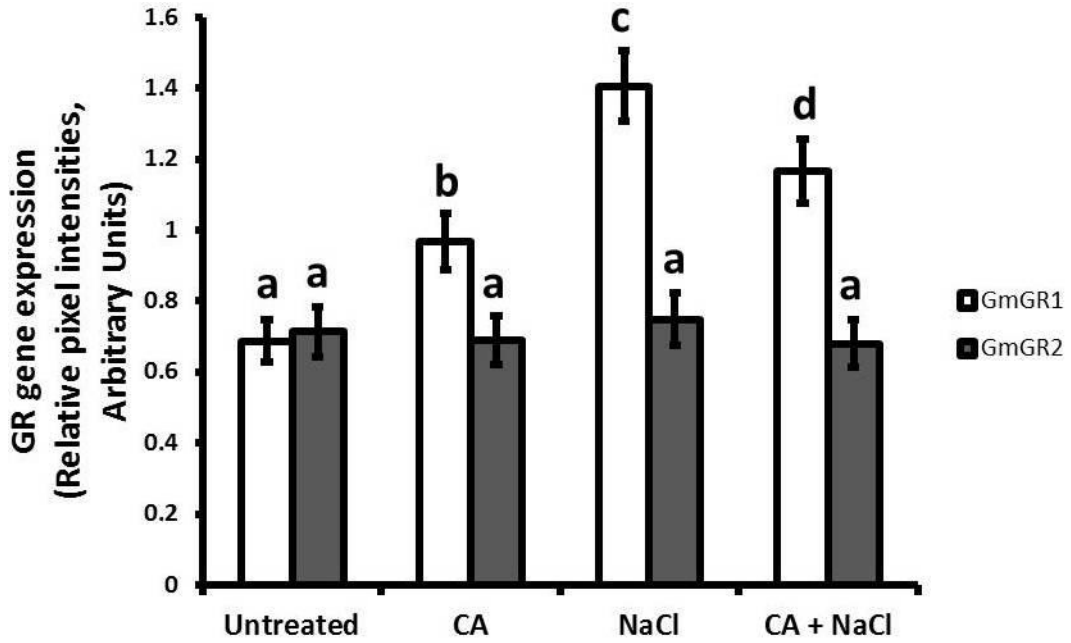

Figure 5.16 Effect of CA and NaCl on mRNA levels of GmGR genes in soybean root nodules. Semi-quantitative RT-PCR for GmGR genes was performed as described in Materials and methods. The expression of GmGR genes was normalized to that of soybean β-tubulin. Values are the mean of four independent experiments and bars indicate the standard error.

5.5 Discussion

5.5.1 CA improves soybean biomass under salt-induced stress

Exposure of soybean plants to exogenously applied CA has recently been associated with inhibition of root growth both in relation to the length and dry weight of the roots (Bubna et al. 2011). Despite the fact that such root growth-inhibiting effects were observed with CA concentrations higher than those used in this study (between 250 µM and 2 mM CA in the study by Bubna et al. (2011) compared to 100 µM CA in this study, the use of lower CA concentrations (100 µM) resulted in root dry weight inhibition similar to that obtained in the Bubna et al. (2011) study. A similar trend as observed for roots occurred in shoots in this study since shoot dry weight also decreased in a similar fashion in response to 100 µM CA. Reduction of plant growth upon exogenous application of CA, which is thought to be a result of CA-induced premature lignification of plant cell walls, has been reported for various plant

species (Baleroni et al. 2000; Batish et al. 2008; Politycka and Mielcarz 2007; Reigosa and Pazos-Malvido 2007; Vaughan and Ord 1990). Although agreeing with these reports, this is the first study showing that CA promotes nodulation because the number of root nodules formed per plant was significantly higher in CA-treated plants than in untreated plants. The mechanism by which exogenously applied CA promotes nodulation is unknown and will thus require further analysis but might involve changes in the expression of genes (rhizobial Nod factor-encoding genes and legume nodulin genes) involved in the plant-rhizobial interaction towards the development of nodules. It is believed that the expression of these genes plays an important role in nodule formation (Schultze and Kondorosi 1998). Despite the high number of nodules obtained in CA-treated plants compared to the untreated plants the data shows that the dry weight per nodule was reduced in the CA treatment. The dry weight per nodule was derived from a simple calculation by dividing the dry weight of all the nodules from a single plant with the number of nodules in that plant to obtain the average dry weight per nodule. The finding showing that dry weight per nodule is reduced by CA is substantiated by our observation that exogenously applied CA resulted in nodules that appeared visually smaller than nodules from untreated plants (data not shown) and the fact that nodule dry weight per plant was statistically similar between CA-treated and untreated plants. Therefore, the increased nodule number in response to CA does not translate to increased nodule dry weight because of the small size of the nodules that occur in plants treated with CA.

Various studies have shown that salt stress inhibits plant growth and reduces nodulation in legumes (Phang et al. 2008; Swaraj and Bishnoi 1999). This is confirmed in our study because shoot, root and nodule dry weights were drastically

reduced by salt stress and cell death in nodules was drastically increased by salt stress (signified by increased Evans Blue uptake in nodules subjected to salt stress). The fact that supplementation of CA to salt-treated soybean plants caused an improvement in the dry weights of the shoots, roots and nodules; together with the reduced cell death in nodules of salt-treated soybean when supplemented with CA; when compared to dry weights and nodule cell death of plants exposed to salt stress alone; implies that CA confers salt stress tolerance in soybean. Given the directly proportional relationship between salt-induced reduction in biomass (reflected by reduced dry weight) and salt-induced elevation of cell death (reflected by increased Evans Blue uptake), together with improved biomass and cell viability under salt treatment in the presence of exogenously applied CA, it is likely that CA improves salt tolerance in soybean via a process that acts to prevent salt stress-induced cell death.

5.5.2 CA and salt stress increases soybean root nodule NO and cGMP content

The study suggests that long-term exposure to CA leads to enhanced biosynthesis of NO in soybean nodule tissue and also that long-term treatment with NaCl elevates NO content in soybean root nodules. This result is based on both fluorescence intensity and spectrophotometric measurements to detect the levels of NO produced in the various treatments involving CA and NaCl. However, it is worth mentioning that the increase in soybean root nodule NO content in response to salt stress is far less than the increase caused by exogenously applied CA. The NO levels detected in salt-stressed plants supplemented with exogenous CA are significantly higher than those obtained for the plants treated with NaCl alone. A similar trend is observed for cGMP, which is a second messenger via which NO signals are transduced. This suggests that CA may act via induction of NO

biosynthesis, which triggers cGMP biosynthesis (possibly from NO-responsive guanylyl cyclase) that would influence plant responses to salinity stress. There is evidence for involvement of NO-mediated cGMP signaling in plant responses to salinity stress in which the involvement of H_2O_2 metabolism is implicated (Li et al. 2011; Martinez-Atienza 2007) and occurrence of NO-stimulated guanylyl cyclase activity in plants to trigger cGMP biosynthesis (Mulaudzi et al. 2011).

5.5.3 CA restricts accumulation of ROS and the extent of lipid peroxidation and cell death under salt stress

The poor growth of soybean plants under salt stress can be ascribed to excessive accumulation of ROS because H_2O_2 and O_2^- levels increased drastically in response to salt stress and this corresponded to reduced dry weights in plants subjected to salt stress. This view is supported by the observation that extensive cell death occurred in response to salt treatment and this trend corresponded to that seen for lipid peroxidation. The results therefore suggest that salt stress induces excessive accumulation of H_2O_2 and the resulting excessive ROS levels destabilize the cell membrane, leading to cell death and loss of biomass. The fact that exogenously applied CA reduced O_2^-, H_2O_2 and lipid peroxidation levels (to levels below that of untreated plants) in plants supplemented with 100 µM CA and in plants treated with the combination of CA and NaCl; together with the fact that the combined treatment reversed the extent of lipid peroxidation, cell death and biomass loss; implies that exogenously applied CA is involved in improving soybean tolerance to salt stress by regulating H_2O_2 accumulation and cell death. This confirms that the enhancing effects on salt stress tolerance in the CA treatments are conferred by the exogenously applied CA.

5.5.4 CA-induced salt stress tolerance is mediated by regulation of antioxidant enzyme activity

The involvement of ROS scavenging in the long-term salt stress tolerance in soybean mediated by CA is supported by the enhancement of antioxidant enzymatic activity in the various treatments. The enzymatic activities of ROS detoxifying enzymes were generally enhanced in response to both exogenously applied CA or salt stress but the salt stress-induced increase in these enzymatic activities was generally significantly lower than the increase in the enzymatic activities seen for treatments where salt stress was applied in combination with exogenously applied CA.

5.5.4.1 CA-induced salt stress tolerance is mediated by regulation of SOD activity

In this study the presented data is in agreement with the well-established view that excessive salt stress induces accumulation of ROS such as O_2^- (Jebara et al. 2005; Gómez et al. 1999; Hernández et al. 2001). Given the extensive evidence in the public domain, it is generally expected that salt stress would result in enhanced SOD activity to scavenge O_2^- considering that over production of such O_2^- (as a result of long-term salt stress) leads to elevation in SOD activity (Becana et al. 2000; Rubio et al. 2002). This implies that exposure to long-term salt stress in plants that are not tolerant to the salt stress, would cause accumulation of O_2^- to excessive levels that impose oxidative stress in the plant cells and this would ultimately cause cell death and loss in biomass as seen in this study. This view is supported by the data presented in this study because salt stress caused elevated nodule O_2^- levels that show a directly proportional relationship to nodule cell death and an inversely proportional relationship to soybean plant biomass (expressed as dry weight).

With the exception of Cu/ZnSOD3 activity that was enhanced in response to salt stress, the SOD activities for two of the three MnSOD isoforms (MnSOD1 and MnSOD2) was inhibited by salt whereas the rest of the SOD isoforms identified in this study were unaffected by salt stress. The observation that the enzymatic activities of MnSOD isoforms was inhibited by salt stress, with exception of only one Cu/ZnSOD3 for which the SOD activity was enhanced by salt stress, together with the observation that the SOD activities of the rest of the isoforms were not altered by salt stress, implies that total SOD activity in response to long-term salt stress in soybean nodules would be inhibited rather than augmented. This hypothesis is supported by spectrophotometric total SOD activity assays in the same nodule samples (results not shown) and observations of root SOD activity (total SOD activity assayed by a spectrophotometric method) in a salt stress-sensitive maize genotype (Keyster et al. 2012). Such net inhibition of SOD activity in response to salt stress would result in excessive accumulation of O_2^-, causing oxidative stress that leads to cell death and loss of biomass and ultimately reduction in soybean crop yield. It is important to note that the results presented in this study identify SOD isoforms that are negatively affected (i.e. inhibited) by long-term salt stress in soybean. These isoforms are potential targets for genetic improvement of salt tolerance in soybean because enhancement of their activities in response to long-term salt stress may lead to efficient scavenging of O_2^-, which would contribute towards preventing O_2^--induced oxidative stress during long-term salt stress. We propose that the degree of induction of SOD activity by salt stress, as seen here for the isoforms whose activity is induced in response to salt stress, is not sufficient to offer protection against the O_2^- levels generated in response to long-term salt stress in the range of salt concentration used in our experimental system. This would be the reason for the

observed excessive accumulation of O_2^- under salt stress in our experimental system.

In consideration of the observation that exogenously applied CA induced three SOD isoforms (MnSOD1, MnSOD2 and Cu/ZnSOD3) while the rest (MnSOD3, FeSOD1, FeSOD2, FeSOD3, FeSOD4, Cu/ZnSOD1, Cu/ZnSOD2, CuZnSOD4, Cu/ZnSOD5, Cu/ZnSOD6, Cu/ZnSOD7, Cu/ZnSOD8 and Cu/ZnSOD9) remained unchanged, taken together with the decline in O_2^- in nodules treated with CA; it is reasonable to suggest that exogenously applied CA generally enhances scavenging of O_2^- by inducing SOD activity. It is worth noting that MnSOD1 and MnSOD2 are inhibited by long-term salt stress and are induced by CA, noting as well that they are induced to levels higher than those for untreated plants when salt stress treatment is combined with CA treatment (the CA + NaCl treatment). This suggests that these SOD isoforms may be crucial in the SOD-mediated salt stress tolerance induced by CA.

We thus propose that exogenously applied CA confers salt stress tolerance in soybean and that this tolerance is mediated in part by the augmentation of SOD activity that results in detoxification of salt stress-induced O_2^-, thus preventing salt stress-induced cell death and allowing for better biomass preservation in CA-supplemented salt-stressed plants than in salt-stressed plants. Given the fact that changes in O_2^- content influence other antioxidant enzymatic pathways, we have also investigated how antioxidant enzymes such as APX, GPX and GR (involved in H_2O_2 metabolism) are affected by the treatments described in this study.

5.4.5.2 CA-induced regulation of hydrogen peroxide metabolizing enzyme activities mediate salt stress tolerance in soybean plants

Given that H_2O_2 levels remained high in the salt treated soybean plants and were reduced in the 'CA + NaCl' treatment, together with an increase in APX, GPX and GR activities in the 'CA + NaCl' treatment, it is hypothesized that the increase in activity of some APX and GPX isoforms in response to NaCl is inadequate to counteract the excessively high levels of H_2O_2 that accumulate in response to salt stress while the increase in GR activity (as shown by some isoforms) under salt stress is insufficient to cater for efficient regeneration of GSH. The result of such inefficiency in the antioxidant system is accelerated cell death under salt stress. However, the addition of CA to salt stressed plants would thus be thought to induce sufficient antioxidant activity to efficiently reduce $O_2^{\cdot-}$ and H_2O_2 to levels that are less damaging to the plant, although not down to the levels of untreated plants.

From the three GmAPX isoforms detected in soybean root nodules, one isoform (GmAPX2) remained unchanged whereas the other two were augmented (GmAPX1 and GmAPX3) in response long-term salt stress in soybean root nodules. We therefore propose that GmAPX1 and GmAPX3 are the only isoforms that make a difference to total APX activity in soybean root nodules responsible for scavenging nodular H_2O_2 in response to long term salt stress. The results further showed that CA (at a final concentration of 100 μM) altered APX activity in soybean root nodules only very slightly, which is in contradiction to what was reported by Singh et al. (2009) who showed that at the same concentration of CA total APX enzymatic activity was augmented by more than two-fold in mung bean. Apart from GmAPX1 which showed no significant changes in activity in response to the combined treatment (CA + NaCl), the activity of both GmAPX2 and GmAPX3 were augmented to levels higher than

those observed for the untreated plants. GmAPX3 could be regarded as a potential target for genetic improvement of salt tolerance in soybean because the activity of this isoform is higher than both the NaCl and the CA-treated plants in the combination treatment (CA + NaCl). We therefore propose that this isoform (GmAPX3) contributes to the total APX activity induced by CA in response to long term salt stress and may lead to efficient scavenging of H_2O_2, which would contribute towards preventing H_2O_2-induced oxidative stress during long-term salt stress.

In this study, a total of six GPX isoforms was identified from soybean root nodules in response to the various treatments. The result clearly showed that more GPX isoforms were enhanced (GmGPX3, GmGPX4 and GmGPX5) than inhibited (GmGPX2) in response to salt stress. The observation that activities of three GPX isoforms was enhanced by salt stress, with exception of only one that was reduced together with the observation that the activities of the rest (two) of the isoforms were not altered implies that total GPX activity in response to long-term salt stress in soybean nodules would be augmented rather than inhibited. However this increase in activity would thus not be sufficient to prevent the excessive accumulation of H_2O_2, causing oxidative stress that leads to enhanced cell death and loss of biomass. The results further show that in response to exogenous CA, more GPX isoforms were inhibited than induced. It is worth noting that three (GmGPX3, GmGPX4 and GmGPX5) of the six isoforms were inhibited whereas one (GmGPX2) was enhanced and the other two (GmGPX1 and GmGPX6) remained unchanged. Taking into consideration the degree of inhibition versus the degree of induction it would be reasonable to suggest that the scavenging of H_2O_2 (as shown in section Figure 5.5B) is not mediated by a CA-induced increase of GPX activity (as seen for the CA treatment for all isoforms detected). However, it is clear that the relevant nodule GPX

isoform in the CA-mediated scavenging of nodule H_2O_2 is GmGPX2 since this is the only isoform induced by exogenously applied CA. However, it is worth noting that while GmGPX2 is inhibited by salt stress and induced by CA, it is induced to levels higher than those for untreated plants when salt stress treatment is combined with CA treatment (the CA + NaCl treatment). This suggests that GmGPX2 may be crucial in the GPX-mediated salt stress tolerance induced by CA. The fact that the activity of GmGPX1 is unaltered by both salt stress and CA and enhanced by supplementing the salt treatment with CA (the CA + NaCl treatment) also implicates GmGPX1 as another GPX isoform that could have a role in CA-induced soybean tolerance to salt stress. We thus propose that exogenously applied CA confers salt stress tolerance in soybean and that this tolerance is mediated in part by the augmentation of GPX activity that results in detoxification of salt stress-induced peroxides derived from H_2O_2, thus preventing salt stress-induced cell death and allowing for better biomass protection in CA-supplemented salt-stressed plants than in salt-stressed plants.

Glutathione reductase plays a significant role in the ascorbate-glutathione cycle that controls endogenous H_2O_2 levels and maintains GSH which can scavenge ROS and maintain ascorbate pools (Gratao et al. 2005). In this study, six GR isoforms have been identified in soybean root nodules in response to exogenous CA and salt stress. From the analysis shown in this study, it is evident that more GR isoforms are augmented (GmGR2, GmGR4, GmGR5 and GmGR6) rather than inhibited (GmGR1) in response to salt stress. Apart from one isoform (GmGR1) that was completely inhibited coupled with another that remained unchanged (GmGR3), the rest (GmGR2, GmGR4, GmGR5 and GmGR6) were enhanced in response to treatment with NaCl. This observation would imply that total GR activity in response

to long-term salt stress in soybean nodules would be augmented rather than inhibited. The contrary can be said for treatment with exogenous CA. The results showed that only one (GmGR1) isoform was augmented in response to treatment with CA. This isoform could be regarded as an potential target in CA-induced GSH generation by GR in plants as this isoform is inhibited in plants treated with salt stress including the salt-stressed plants that were supplemented with CA. Apart from one (GmGR1) isoform that was enhanced the rest (GmGR2, GmGR3, GmGR4, GmGR5 and GmGR6) were inhibited in response to exogenous CA. In consideration of the observation that exogenously applied CA inhibited more nodule GR isoforms than it induced; taken together with the degree of inhibition versus the degree of induction, it is reasonable to suggest that exogenously applied CA generally inhibits GR activity. However, it is important to look at the isoforms whose activity was augmented or remained unchanged in response to CA when compared to the untreated plants. We have identified one isoform (GmGR1) that was enhanced in response to CA and therefore hypothesized that this isoform could contribute in the generation of GSH in soybean root nodules. By taking into consideration that CA alone did not significantly alter activities of both APX and GPX (apart from a few isoforms) together with GR activity it is reasonable to suggest that CA does not really influence the metabolism of both AsA and GSH in soybean root nodules as was evident in measuring total GR activity (result not shown). This result is in contradiction to what was reported by Singh et al. (2009) who showed that total GR activity in mung bean was up-regulated in response to CA at a final concentration of 100 µM. It is worth noting that GmGR3 and GmGR5 are inhibited by CA and induced by long-term salt stress, noting as well that they are induced to levels higher than those for both untreated and salt-stressed plants when salt stress treatment is

combined with CA treatment (the CA + NaCl treatment). This might suggest that these GR isoforms may be crucial in the CA-mediated regeneration of GSH in salt stressed plants.

5.5.5 CA and salt stress alter expression of a subset of antioxidant genes in soybean root nodules

Apart from the effect of long-term salt stress and CA treatments on ROS scavenging antioxidant enzymes, we have also explored the influence of the treatments on the transcript levels of different antioxidant genes. Gene expression in response to abiotic stress is usually studied at the level of mRNA abundance.

With the use the of housekeeping gene (β-tubulin) from soybean to normalize the PCR assay we found that all three GmSOD genes used in this study did not respond to any of the treatments administered in this study. However, it is still unclear whether any of the transcripts assayed in this study correspond to the isoforms detected on the native PAGE gel as none of these isoforms has been fully characterized. In order to answer this question we would be looking at the expression of soybean SOD transcripts in response to the various treatments. This will indicate whether any of the increases in enzymatic activities detected for the SOD isoforms are transcriptionally or post-translationally regulated.

The expression results have shown that GmAPX activity is differentially regulated in response to the various treatments. The level of gene expression detected for GmAPX1 showed that plants supplemented with CA increased the level of GmAPX1 expression whereas NaCl also increase the level of expression to levels higher than those in the untreated plants but not to the level of the CA-treated plants. Salt-stressed plants supplemented with CA resulted in an increase in expression of

GmAPX1 to a level higher than what was observed for NaCl. This suggests that GmAPX1 is clearly induced by CA and might be regarded as a potential marker for APX-mediated salt stress tolerance conferred by CA in soybean. Contrary to what was observed for GmAPX1, the level of expression for GmAPX2 was reduced in response to CA whereas NaCl did not alter the expression levels of the gene. However salt-stressed plants supplemented with CA increased the level of expression of the gene. This suggest that the GmAPX2 isoform may be crucial in the APX-mediated salt stress tolerance conferred by CA.

The transcript levels detected for the two GmGPX genes in soybean root nodules showed that the expression of the two genes are differentially regulated by the various treatments. GmGPX1 was slightly but significantly up-regulated in response to CA and NaCl. The same effect was observed in response to the combined treatment (CA + NaCl). GmGPX2, on the other-hand was inhibited in response to CA whereas NaCl and the combined treatment of CA and NaCl did not alter (level of expression was similar to the untreated control) the transcript levels of the gene. It is therefore unclear as to whether any of the GPX genes could be regarded as a potential candidate for GPX-mediated salt stress tolerance conferred by CA in soybean plants as the transcript level in the combined treatment are similar to that of the salt-stressed plants.

The relative gene expression for two GmGR genes was also determined by using semi-quantitative RT-PCR. It has been previously shown that the expression of GR genes in plants is enhanced by treatment with NaCl (Kaminaka et al. 1998; Tsai et al. 2005; Hong et al. 2009). In the present study we have shown that NaCl augmented the transcript levels of one of the GR genes whereas the other remained unchanged. This increase in mRNA expression in response to salt stress was also

reported for two rice GR genes by Hong et al. (2009). It is however unclear whether the increase in expression for GmGR1 in response to NaCl is associated with the increase of GR enzymatic activity in the same treatment. The present result might suggest that GR1 induced by NaCl may affect ROS scavenging properties in soybean root nodules. Plants that were supplemented with CA also showed an increase in expression for GmGR1. As was previously mentioned, it is unclear as to whether this increase is in any way associated with the increase in enzymatic activity shown in the in-gel enzymatic activity assay. On the other-hand, salt-stressed plants supplemented with CA showed an increase in GmGR1 expression to a level higher than was observed for both untreated and CA-treated plants yet still lower than the salt stressed plants. No significant change in transcript levels was observed for GmGR2 in response to the various treatments. It has been shown that tolerance to oxidative stress is enhanced in transgenic plants by overexpressed bacterial GR genes (Aono et al. 1991; Broadbent et al. 1995; Foyer et al. 1994). It is therefore apparent that more experiments regarding overexpression of GR genes in plants are needed for our understanding of its function in soybean root nodules under salinity stress conditions.

CHAPTER 6

CONCLUSION AND FUTURE OUTLOOK

Salinity stress is amongst the major negative factors hampering plant growth and contributes to crop loss especially in marginal semi-arid agricultural areas. This is a situation that will inevitably lead to a rise in demand and therefore a rise in cost of limited food resources. Food security is therefore heavily dependent on the development of crop plants with increased resistance to environmental and pathogenic factors. This continues to call for advances in the area of plant biotechnology, some of which may include overexpression of some genes that may be able to confer increased tolerance to biotic and abiotic stresses in plants. To counter the deleterious effects caused by long-term salt stress on both cereals (maize) and legumes (soybean); this study explored the effects of exogenous NO (for both maize and soybean) and CA (soybean) in response to long-term salt stress.

The results obtained in Chapter 2 and Chapter 3 explored the regulatory role of NO (DETA/NO as NO donor) in enhancing salt stress tolerance in a cereal (maize) crop. The study on salt stress tolerance established that the regulation of ROS accumulation by antioxidant enzymes is a key determinant of maize responses to salt stress. Here, we have illustrated that maize undergoes more cell death under high salinity conditions. By supplementing maize with NO under high salinity, t salt tolerance was improved via enhancement of its antioxidant capacity to restrict ROS accumulation. This in turn would limit lipid peroxidation by inducing elevated antioxidant enzyme activity to maintain redox homeostasis and limit the extent of salt stress-induced cell death.

The study extends the effect of NO beyond its regulatory role on cereal salt stress tolerance but further to its role in enhancing salt stress tolerance in legumes (using soybean as an example) by exploring the influence of exogenously applied NO on nodule antioxidant activity in response to long term salt stress (Chapter 4). The results show that although some isoforms were augmented in response to salt stress, this up-regulation was not sufficient to scavenge total ROS caused by the high salt content. The high salt levels would thus cause an increase in ROS accumulation and lipid peroxidation that would ultimately result in cell death. However, the results have further demonstrated that NO protects soybean root nodules against salt-induced oxidative damage by enhancing some antioxidant isoforms (that contribute to total enzymatic activity) to restrict ROS accumulation. These observations were mainly attributed to the protective effect of exogenously applied NO, because the components of the antioxidant defense were up-regulated in response to the NO donor, which allowed the plants to cope better with salinity stress.

This study further analyzed the role of exogenously applied CA in responses of soybean to long-term salt (Chapter 5). The results demonstrated that exposure to long-term salt stress resulted in the reduction of soybean biomass, accumulation of ROS and increased levels of lipid peroxidation that caused an increased in cell death. However the supplementation of CA to salt-stressed plants reversed the deleterious effects caused by high salt levels. We have also shown that exogenously applied CA not only enhances the NO content and cGMP content in soybean nodules but also improves soybean tolerance to long-term salt stress by inducing antioxidant enzyme activity ; resulting in restricted O_2^- accumulation, H_2O_2 accumulation and limited lipid peroxidation that limit the extent of salt stress-induced

cell death. This result is in agreement with what has been previously described by various authors, namely that CA is a potent antioxidant and ROS scavenger. Furthermore, the effect of CA on expression of antioxidant genes coding for antioxidant enzymes was also investigated. Apart from the three SOD genes that were not influenced by either CA and salt stress, the other genes coding for antioxidants such as APX, GPX and GR were differentially regulated by either/both CA and salt stress. A possible reason that no changes were detected for the SOD genes in question could be attributed to the likelihood that the changes seen on the in-gel activity assay could be as a result of post-translational modification. To conclusively say that there are no transcriptional changes for SOD in response to exogenous CA and salt stress would be inappropriate as this study on the expression of these antioxidant genes is limited to only a few of these genes.

We can therefore conclude that the roles of both NO and CA in conferring salt stress tolerance in food crop plants as reported here strengthens the foundation on which genetic engineering of crop plants for regulated NO/CA biosynthesis under salt stress can be used to improve plant performance in saline environments. The identification of the various isoforms as potential target for salt stress tolerance (as seen in this thesis) in response to NO and CA lays a foundation for performing further studies that could contribute to salt stress tolerance in salt-sensitive plant species (in particular soybean and maize). The characterization of some of the components of NO-mediated regulation of maize and soybean plant responses to salinity as well as CA-mediated regulation of soybean responses to salinity is an effort that will in future be extended to identify the rest of the molecular signaling components and their contribution to maize and soybean functioning in response to salt stress. To some extent this can be achieved by analyzing transcripts and

proteins that are differentially regulated by NO and CA, via transcriptomics and proteomics and linking these transcripts and proteins to maize and soybean functioning under salinity stress. The role of NO and CA in mediating plant tolerance to salt stress cannot be limited only to modification of the plant antioxidant system. It will thus be useful to identify other molecular pathways that are involved in NO and CA-mediated salt stress tolerance in plants. Therefore other specialized molecular techniques such as transcriptomics and proteomics would also make a meaningful contribution towards dissecting such molecular pathways. The knowledge obtained from such techniques would pave the way forward for genetic engineering of crop plants with enhanced tolerance to salt stress.

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