NITRIC OXIDE-MEDIATED SIGNALING IN LEGUMES AND ITS ROLE IN MAIZE

RESPONSES TO SALT STRESS

by

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DECLARATION

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Marshall Keyster

Signed …… ……

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Dedicated to my late mother and father, may your souls

rest in peace.

With love!

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LIST OF ABBREVIATIONS

ABA	abscisic acid
ANOVA	analysis of variance
AO	amine oxidase
AOX	alternative oxidase
APX	ascorbate peroxidase
ARA	acetylene reduction assay
AtCYS1	*Arabidopsis thaliana* cystatin 1
AtNOA1	*Arabidopsis thaliana* Nitric Oxide Associated 1
AtNOS1	*Arabidopsis thaliana* Nitric Oxide Synthase 1
BAPNA	Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride
BLAST	Basic Local Alignment Tool
BNF	biological nitrogen fixation
BSA	bovine serum albumin
CAT	catalase
cGMP	guanosine monophosphate
CPI	cysteine protease inhibitor
CYP	cysteine protease/ cysteine endopeptidase
CYPs	cysteine proteases/ cysteine endopeptidases
DETA	Diethylenetriamine
DETA/NO	2,2′-(hydroxynitrosohydrazono)bisethanimine
DNA	deoxyribonucleic acid
E64	l-trans-epoxysuccinyl-leucyl-amido-(4-guanidino)butane
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
FW	Fresh weight
GC	guanylyl cyclase

GO	glycolate oxidase
GPX	glutathione peroxidase
GSH	glutathione
GST	glutathione *S*-transferase
Kan	kanamycin
kDa	kilodalton
Lb	leghaemoglobin
LB	Luria Bertani
LbNO	nitrosylleghaemoglobin
L-NAME	NG-nitro-L-arginine methyl ester
L-NNA	N_{ω}-nitro-L-arginine
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitrotetrazolium blue chloride
NaCl	sodium chloride
NO	nitric oxide
NOS	nitric oxide synthase
NOX	NADPH oxidase
NR	Nitrate reductase
PAGE	Polyacrylamide gel electrophoresis
PCD	programmed cell death
PhyCys	phytocystatin
PMSF	phenylmethylsulfonyl fluoride
PVP	polyvinylpyrrolidone
OXO	oxalate oxidase
RNA	ribonucleic acid
ROOH	organic hydroperoxide
ROS	reactive oxygen species
SNP	sodium nitroprusside

SOD	superoxide dismutase
TCA	trichloroacetic acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TPX	thioredoxin peroxidase
VPE γ	vacuolar processing enzyme-γ
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
XO	Xanthine oxidase
XTT	3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide

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CHAPTER 5

SUMMARY

The role of nitric oxide synthase (NOS) activity and exogenous NO in regulating soybean root nodule development and nodule functioning was studied. Here, it is shown that NOS activity regulates nodule development and nodule functioning. The study shows that regulation of redox homeostasis (by regulating the production and detoxification of reactive oxygen species) through modulation of antioxidant enzymatic activities by nitric oxide (NO) produced from NOS plays a role in nodule development and nodule functioning. It is revealed that this NO-regulated redox homeostasis maintains the levels of nodule reactive oxygen species (ROS) at thresholds optimal for the molecular signaling required for nodule development and function while preventing excessive ROS accumulation that would otherwise be deleterious to nodule development/functioning. Furthermore, the study shows that excessive levels of exogenously applied NO imposes oxidative stress on legume root nodules and this oxidative stress triggers cysteine protease (CYP) activity and lipid peroxidation, ultimately leading to cell death. The study further shows that endogenous NO is required for the expression of Group-1 cystatins and that reduced endogenous NO levels lead to suppression of expression of Group-1 cystatins, which permits heightened CYP activity with the consequence of increased nodule cell death.

The study extends the role of NO beyond its regulatory function on legume development further to is role in enhancing salt stress tolerance in cereals. Investigation on salt stress tolerance is initiated with comparison of salt tolerance of two maize genotypes where it is established that the regulation of ROS accumulation by antioxidant enzymes is a key determinant of maize responses to salt stress and that central to these responses is the activity

of CYP. Here, it is illustrated that salt-sensitive maize genotypes have high CYP activity (and thus experience more cell death and yield loss) than salt-tolerant genotypes under conditions of high salinity. By supplementing the salt-sensitive genotype with NO under high salinity, the study shows that the tolerance of this genotype to salt is improved. This NO-mediated improvement is shown to be due to modulation of the plant antioxidant system to scavenge toxic ROS and the suppression of the activity of salt-responsive cysteine proteases (CYPs).

Aims of this study

The study aimed to investigate the following:

1. Regulation of legume root nodule development and functioning, using soybean as the exemplary legume, by endogenous NO produced from nitric oxide synthase activity.

2. The effect of high concentrations of exogenously applied NO on legume root nodule development and functioning, with soybean used as the legume under this study.

3. The role of reactive oxygen species and antioxidant enzymes in determining the level of salt stress tolerance in two maize genotypes with contrasting degrees of tolerance to the salt stress.

4. Investigating the molecular mechanisms, with focus on reactive oxygen species and cell death, by which nitric oxide improves maize tolerance to salt stress.

CHAPTER 1

Literature review

1.1 Introduction

Abiotic stress adversely affects plants in the natural environment because plants cannot escape contact with the stress in an affected area (Bhatnagar-Mathur et al. 2008). Abiotic stresses include extreme temperatures, high winds, drought, floods and elevated salinity (Becana et al. 2000; Maestre et al. 2008). These stresses have negative impact on agriculture and it has been estimated that abiotic stress reduces crop yield by more than 50% of the potential yield (Wang et al. 2007).

Abiotic stress leads to secondary stresses which include ionic stress, osmotic stress, hormonal disproportion and nutritional imbalance (Hamed et al. 2007). Furthermore, abiotic stress also causes oxidative stress, resulting in the formation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^.$), peroxyl radicals, organic hydroperoxide (ROOH) and peroxynitrite ($ONOO^-$) (Turkan and Demiral 2008). ROS production because of abiotic stress causes disruption to 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).

Plants have evolved different strategies to cope with ROS damage and downstream injuries. To regulate ROS levels (especially O_2^- and H_2O_2) in cells, plants attempt to modulate the activity

of ROS-producing enzymes such as NADPH oxidase (NOX), xanthine oxidase (XO), oxalate oxidase (OXO), glycolate oxidase (GO) and amine oxidase (AO) (Grant and Loake 2000; Mittler 2002). Failure to prevent excessive production of ROS triggers the activation ROS-scavenging enzymes which include superoxide dismutase (SOD) for O_2^- removal plus peroxidases and catalase (CAT) for H_2O_2 eradication (Larson 1988; Mittler 2002). Important peroxidases occurring in plants include ascorbate peroxidase (APX), glutathione peroxidase (GPX) and thioredoxin peroxidase (TPX). 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).

Modulation and scavenging of ROS needs to be optimal or else major chemical and physical changes will occur in the plant tissue, which are mainly due to macromolecular degradation in the cell (Thomas et al. 2003). Plant cysteine proteases (CYPs) are major contributors in protein degradation under ROS accumulation induced by abiotic stress (Solomon et al. 1999). A senescence-induced cysteine protease (CYP) identified in Arabidopsis, viz. vacuolar processing enzyme-γ (VPEγ) has enzymatic and structural similarities to animal caspases (Rogers 2006). Animal caspases modulate programmed cell death (PCD) in animal cells and this is crucial for regulation of developmental processes and for survival of the cell under stressed conditions. The homology between VPEγ and animal caspases has indicated the possibility of cysteine protease-mediated regulation of PCD in plants (Sanmartín et al. 2005). CYPs have also been discovered in ricinosomes of endosperms from *Ricinus communis* (Rogers 2006). Ricinosomes are organelles that develop as the cells undergo PCD where they will rupture and release proteases directly into the cytosol (Gietl et al. 1997; Schmid et al. 1999).

CYP activity has also been linked to premature cell death leading to severe crop yield reductions (Wagstaff et al. 2002). Failure to modulate CYP activity in cells after exposure to abiotic stress contributes to their sensitivity to the stresse (Habib and Fazili 2007). To regulate CYP activity in plant cells, plants synthesize cysteine protease inhibitors (CPI) (Martinez and Diaz 2008). Plant cystatins (also known as phytocystatins [PhyCys]) inhibit CYPs *in planta* by forming complexes with the enzymes. PhyCys have a molecular mass of 12-23 kDa and form a wedge within the CYP active site using a partially flexible N-terminus containing a glycine residue and two hairpin loops, one containing a highly conserved QXVXG domain and the other a tryptophan residue. Functions for PhyCys have been described and include regulation of CYP activity during seed germination and development (Arai et al. 2002), PCD *in planta* (Solomon et a. 1999), responses to invasion by pests (Alvarez-Alfageme et al. 2007) and antifungal defenses (Pernas et al. 1999).

Plants regulate PCD by either using the classical amino acid-derived hormones (Kende and Zeevaart 1997), peptidic hormones (Lindsey 2001), plant steroid hormones (Schaller 2003) and/or nitric oxide (NO) (Neill et al. 2003). NO is a small, water and lipid-soluble gas which has emerged as an important signaling molecule in plants (Durner et al. 1999). The stability of NO is quite variable and is largely dependent on reacting molecules such as oxygen (O_2) or haems (Henry et al. 1997). NO was named 'Molecule of the year' by *Science* in 1992 and has gained huge popularity in the biological field (Koshland 1992) since then. In plant science, NO emissions and its effects on plant growth were depicted in the 1970s by Anderson and Mansfield (1979), and Klepper (1979). NO signaling in plants was described in the late 1990s by Leshem and Haramaty (1996), and Laxalt and co-workers (1997). However, two publications in

1998 which describe NO as a plant defense molecule lead to drastic increase in NO studies in plants (Delledonne et al. 1998; Durner et al. 1998). Ever since, the role of NO in diverse physiological processes has been described and includes seed germination or dormancy (Beligni and Lamatina 2000; Libourel et al. 2006), regulation of senescence (Mishina et al. 2007), flowering (He et al. 2004), stomatal movements (Gracia-Mata and Lamatina 2001), regulation of responses to abiotic and biotic stresses such as drought (Gracia-Mata and Lamatina 2002), salinity (Zhao et al. 2004), heat (Uchida et al. 2002), cold (Mallick et al. 2000), UV-B radiation (Mackerness et al. 2001; Shi et al. 2005) and exposure to pathogens (Delledonne et al. 1998).

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). Shi et al. (2005) showed that NO prevented membrane peroxidation and death in plants exposed to UV-B irradiation by reducing ion leakage, chlorophyll loss, ROS accumulation and inhibition of photosystem II. Furthermore, Shi et al. (2005) demonstrated that antioxidant enzymes like SOD, CAT and APX were up-regulated by NO; thus contributing to lower levels of ROS. Evidence for regulation of antioxidant capacity by NO in cadmium (Cd^{2+})-stressed plants was provided by Hsu and Kao (2004) in rice leaves, Laspina et al. (2005) in sunflower leaves and Singh et al. (2008) in wheat roots. Qiao and Fan (2008) indicated that the antioxidant activity of NO is concentration-dependant because only low levels of NO eliminate O_2^- by interacting with the O_2^- to form $ONOO^-$. It has been revealed that $ONOO^-$ is lethal to animal cells but not toxic to plant cells (Delledonne et al. 2001).

NO also regulates cell death in plants under abiotic stress conditions by controlling CYP activity (Belenghi et al. 2003). CYP activity can be altered by S-nitrosylation of the cysteine residues and transition metals in the protein via an oxygen-dependant chemical reaction or by the transfer of NO from a nitrosothiol to a protein sulfhydyl group (transnitrosylation) (Stamler 1994). Lindermayr et al. (2005) reported the first S-nitrosylated proteins in plants using artificially released NO and a proteomic approach. The proteins identified by Lindermayr et al. (2005) were found to play a role in diverse processes in plants including: metabolism, photosynthesis, redox control, and stress responses.

Solomon et al. (1999) showed that ectopic expression of a soybean cystatin, an endogenous cysteine protease inhibitor gene, lead to suppression of ROS and subsequent PCD in soybean cell cultures. In addition, Belenghi et al. (2003) demonstrated that NO regulates PCD in *Arabidopsis thaliana* by up-regulating the expression of a specific class of cystatins, the first study to illustrate a direct effect of NO on plant cystatins. These two studies (Solomon et al. 1999; Belenghi et al. 2003) highlighted the pivotal role of cystatins in the plant PCD pathway via ROS suppression by CYP inhibition, a process which is still unclear in plants.

This review describes the recent development in the field of NO in plants and the role of NO in abiotic stress in plants with emphasis on salt stress.

1.2 NO production in plants

Two distinct NO production pathways have been elucidated in plants: the arginine pathway and the nitrite pathway (Foresi et al. 2010; Guo et al. 2003). The arginine pathway is mediated by NO synthase (NOS; EC 1.14.13.39) which utilizes the substrates L-arginine, O_2, and NADPH.

The products of this reaction are L-citrulline and NO. Studies focusing on this pathway utilizes chemical analogous of L-arginine such as NG-nitro-L-arginine methyl ester (L-NAME) to illustrate competitive inhibition and presence of a NOS enzyme in plants. However, to date, no NOS has been identified and isolated in higher plant systems. Two candidate genes have been shown to have NOS-like NO production in plants. The first enzyme was identified as a variant of the P protein of the mitochondrial glycine decarboxylase complex (Chandok et al. 2003), and utilized L-arginine as a substrate similar to mammalian NOS. The second candidate was identified in *Arabidopsis thaliana* by sequence homology to NOS from a snail (*Helix pomata*) (Guo et al. 2003), and was thought to be the first NOS in plants, hence the name AtNOS1 (*Arabidopsis thaliana* Nitric Oxide Synthase 1). However, research conducted by Zamojtel et al (2006) and Crawford et al (2006) indicated that this protein was a GTPase rather than NOS, and the name was changed to AtNOA1 (*Arabidopsis thaliana* Nitric Oxide Associated 1). Recently, Foresi et al. (2010) characterized a novel NOS gene from the single-celled alga *Osterococcus tauri* which share a common ancestor with higher plants. *O. tauri* is the smallest-known free-living photosynthetic eukaryotes which belong 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 hope in the quest to discover a NOS enzyme in higher plants.

The nitrite NO producing pathway is catalysed by plant nitrate reductase (NR; EC 1.6.6.1) and was first observed in 1986 by Dean and colleagues. NR catalyses the production of NO in plants from nitrites using NADH as an electron donor (Rockel et al. 2002). In this reaction, peroxinitrite gets synthesized simultaneously with NO. The role of NO in pathogen attack has been elucidated by Yamamoto et al. (2003) using potato tubers infected by fungus. Thus a role

for NR has been proposed under plant stress conditions (Rockel et al. 2002). NR-mediated NO synthesis has also been described in Absisic acid (ABA) signaling in guard cells where NR-deficient mutants (*Arabidopsis thaliana nia1* and *nia2*) failed to induce NO in stomatal closure (Bright et al. 2006). Rockel et al. (2002) showed that NO generation by NR is stimulated by hypoxic conditions which can be modulated by the phosphorylation status of the NR. However, the specific location (leaves and/or roots) of NO production by NR is a major draw back in plant NO biology (Gupta et al. 2005).

Several non-enzymatic reactions are also known to produce NO *in planta*. Ascorbate and nitrous acid (HNO_2) reacts under acidic conditions to produce dehydroascorbic acid and NO (Weitzberg and Lundberg 1998). Alternatively, carotinoids have been shown to transform NO_2 to NO in the presence of light (Cooney et al. 1994). Bethke et al. (2004) demonstrated non-enzymatic conversion of nitrite to NO at low pH by apoplasts in the barley aleurone layer.

1.3 NO-mediated signaling in plants

NO reacts with diverse molecules during signal transduction in plants (Neill et al. 2003; Wendehenne et al. 2001). It reacts with: superoxide to form peroxynitrite, transition metals, heme-containing proteins, and thiol groups to form S-nitrosothiols (Radi et al. 2004). However, downstream signaling targets of NO during signaling are not very well elucidated and have been made difficult by the inability to discover a specific receptor for NO in plants (Neill et al. 2007). Nevertheless, pathways for NO signaling may involve both directly activation of ion channel proteins and/or proteins that regulate their gene expression or indirect regulation of signal cascade proteins (Neill et al. 2003).

NO signaling can be via dependent or independent pathways involving the second messenger molecule cGMP (Neill et al. 2007). The enzyme soluble guanylyl cyclase (sGC) plays a key role in NO signaling in animal cells. NO activates sGC by binding to its haem domain stimulating a transient rise in cGMP levels by catalyzing the synthesis of cGMP from GTP (Pfeiffer et al. 1994). However, in plants the Arabidopsis GC, AtGC1, is actually not activated by NO (Ludidi and Gehring 2003). So, studies in this field (NO-cGMP cross-talk) have turned to the use of pharmacological reagents. Neill et al. (2003) showed by using inhibitors of NO sensitive guanylyl cyclase that NO effects downstream cGMP synthesis which in turn effects ABA signaling in guard cells. A key signaling molecule downstream of cGMP is cyclic ADP ribose (cADPR) (Wendehenne et al. 2001). In cGMP-dependent signaling, the resultant increase in cGMP levels raises cytosolic calcium levels (Durner et al. 1998) by increasing levels of activated cADPR. This cGMP pathway also activates intracellular protein kinases (Trewavas et al. 2002). Alternatively, in cGMP-independent NO signaling, proteins that contain thiol groups and metals, such as iron, copper, zinc, are among the primary targets of NO (Wendehenne et al. 2001).

1.4 A role for NO in plant growth and development

The effects of NO on plant growth and development were established as being concentration dependent (Anderson and Mansfield 1979); specifically, high concentrations of NO (40-80 ppm) inhibited growth in tomato and pea plants, whereas low concentrations (up to 20 ppm) stimulated growth (Hufton et al. 1996; Leshem and Haramaty 1996). NO donors stimulate leaf enlargement, seed germination, de-etiolation, and inhibits hypocotyl and internode growth (Beligni and Lamattina 2000). NO also increased chlorophyll content in

lettuce, red cabbage, Arabidopsis, and guard cells of pea leaves (Beligni and Lamattina 2000; Bollmann et al. 1999; Leshem et al. 1997). Graziano et al. (2002) showed that NO also inhibited chlorosis in NO-treated wild corn. NO is also an endogenous factor that delay senescence in pea leaves and *Helianthus annuus* L. cotyledons (Leshem et al. 1998). Leach et al. (2010) studied the effects of a NOS inhibitor on soybean growth parameters and nodule functioning, and suggested that NO, resulting from NOS activity, is required for development of functional soybean nodules.

1.5 Importance of NO in abiotic stress

Abiotic stresses disrupt the cellular redox homeostasis in planta leading to the onset of oxidative stress or the generation of ROS (Asada 2006). The plasma membrane (PM) NADPH oxidase has been described as a major source of ROS in plants when faced with abiotic stress (Qin et al. 2004). This enzyme can use cytoplasmic NADPH to transfer an electron to molecular O_2 to form O_2^- followed by dismutation of O_2^- to hydrogen peroxide (H_2O_2) (Van Gestelen et al. 1997). ROS gets generated in different locations of the plants cell during the period of oxidative stress (mitochondria, chloroplast, peroxisome, and nucleus) which ultimately causes injury and cell death (Mano 2002). In contrast, ROS also plays a vital role in intracellular redox signaling and the activation of antioxidant resistance pathways. An increase in NO production has been detected during both water and short-term heat stress (Leshem & Haramaty 1996; Leshem et al. 1998) indicating a role of NO in abiotic stress. NO can interact with ROS especially superoxide anion (O_2^-) due the presence of an unpaired electron within the NO molecule. The combination of NO with ROS is can either be toxic or protective, depending on the conditions in

cells. Low concentrations of ROS operate as signals for the activation of plant defense responses where higher amounts cause severe injury. NO may act as a chain breaker in a system where high ROS levels leads to toxicity, and therefore may limit the damage (Lipton et al. 1993). Plants exposed to various abiotic stresses modulates ROS via enhancing of antioxidant defense systems where it is believed that NO is involved in two respiratory electron transport pathways in mitochondria (Yamasaki et al. 2001; Zottini et al. 2002). NO also endogenously controls gibberellin induced PCD in barley eleurone cells by inducing cyto protective proteins and the production of catalase (CAT), SOD, glutathione S-transferase (GST), and alternative oxidase (AOX) (Beligni et al. 2002; Huang et al. 2002; Polverari et al. 2003). Orozco-Cardenas and Ryan (2002) demonstrated that NO inhibits H_2O_2 production induced by jasmonic acid in tomato leaves, suggesting a role as an anti-stress molecule during abiotic stress in plants.

1.6 Role of NO in salt stress tolerance

Salinity stress affects plant growth, as well as development processes such as seed germination, seedling growth, vegetative growth, flowering and fruit set (Siddiqui et al. 2008; Sairam and Tyagi 2004). Ionic imbalance and hyperosmotic stress are primary features of salt-affected plants. Massive changes in ionic and water balance cause molecular damage and growth arrest (Siddiqui et al. 2008) ultimately leading to tissue death and destruction of the whole plant. Serrano et al. (1999) showed that salt stress affects the integrity of cellular membranes, activities of enzymes and the functioning of the plant photosynthetic apparatus which might occur via production of ROS. However, less is known about NO involvement in tolerance to plants to salt stress. The NO function in salt tolerance was demonstrated in many

plant species. Exogenous application of sodium nitroprusside (SNP) (NO donor) under salt stress significantly alleviated the oxidative injury to seedling of rice (Uchida et al. 2002), lupin (Kopyra and Gwóźdź 2003), and cucumber (Fan et al. 2007), enhanced seedlings' growth (Song et al. 2009), and increased the dry weight of maize and *Kosteletzkya virginica* seedlings (Guo et al. 2009). Zheng et al. 2010 demonstrated that pretreatment with NO effectively contributed to better balance between carbon and nitrogen metabolism via increasing total soluble protein.

Studies using the Arabidopsis mutant *Atnoa1* with an impaired *in vivo* NO synthase (NOS) activity and a reduced endogenous NO level was more sensitive to sodium chloride (NaCl) stress than wild type (Zhao et al. 2007). However, treatment of *Atnoa1* plants with exogenous SNP alleviated the oxidative damage caused by NaCl stress. *Atnoa1* mutants displayed a greater Na^+/K^+ ratio in shoots than wild type when exposed to NaCl, but SNP treatment lead to a decrease of Na^+/K^+ ratio back to the levels observed in the wild type (Zhao et al. 2007). Zhang et al. (2006) reported that NO enhanced salt tolerance in maize seedlings, through increasing K^+ accumulation in roots and shoots, while decreasing Na^+ accumulation. It is therefore suggested that NO may enhance salt tolerance in plants by increasing the expression of plasma membrane Na^+/H^+ antiporter gene and which is important for Na^+ homeostasis and K^+ acquisition.

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CHAPTER 2

Nitric oxide controls root nodule functioning in soybean via modulation of antioxidant enzymes

2.1 Abstract

Soybean plants were used to analyze the molecular events through which nitric oxide synthase enzymatic activity regulates nodule functioning. In soybean plants in which inoculation and treatments with the nitric oxide donor 2,2′-(hydroxynitrosohydrazono)bis-ethanimine (DETA/NO) or the nitric oxide synthase (NOS) inhibitor N_ω-nitro-L-arginine (L-NNA) was done at the VC stage of vegetative growth, cysteine protease activity in root nodules increased in response to both DETA/NO and L-NNA but was more pronounced in the L-NNA treatments. In these plants, the elevated nodule cysteine protease activity was accompanied by increased nodule cell death, which was more severe in the L-NNA treatments than the DETA/NO treatments. In the same plants, nitrogenase activity was reduced in response to both DETA/NO and L-NNA but was more severely reduced in response to L-NNA that DETA/NO. The trend in DETA/NO and L-NNA-induced cysteine protease activity, cell death and nitrogenase activity was maintained in root nodules of soybean plants in which inoculation was done on seeds and treatments applied to plants at the V3 stage of vegetative growth. Furthermore, NO content increased in root nodules of these plants in response to treatment with DETA/NO whereas it decreased in response to treatment with L-NNA. The increased NO content caused elevation of cyclic guanosine monophosphate (cGMP) content in the nodules but L-NNA reduced the nodule cGMP content in these plants. For soybean plants in which inoculation was

done on seeds and treatments applied to plants at the V3 stage of vegetative growth, inhibition of NOS activity reduced shoot, root and nodule dry weight despite the fact that this inhibition had no significant effect on nodule number. In these plants, superoxide dismutase enzymatic activity was inhibited by suppression of NOS activity whereas NADPH oxidase enzymatic activity was increased by the same treatment and this was accompanied by an increase in superoxide content in the nodules. Despite the suppression of superoxide dismutase activity because of inhibition of NOS, hydrogen peroxide content increased in nodules of plants treated with L-NNA and this was accompanied by up-regulation of ascorbate peroxidase and glutathione peroxidase in these nodules. These results suggest that regulation of soybean nodule functioning by NOS activity occurs via modulation of antioxidant and cysteine endopeptidase enzymatic activities to maintain nodule cell viability and nodule redox homeostasis at levels that support nitrogen fixation.

2.2 Introduction

Nitric oxide (NO) is a gaseous molecule, synthesized via enzymatic and non-enzymatic mechanisms in plant cells, with diverse signaling roles in plants (reviewed in Besson-Bard et al. 2008; Hong et al. 2008; Manjunatha et al. 2010; Qiao and Fan 2008; Wilson et al. 2008; Xiong et al. 2010). It has recently been demonstrated that soybean plants with suppressed nitric oxide synthase (NOS) activity, as a result of treatment with the NOS-specific inhibitor N_{ω}-nitro-L-arginine (L-NNA), have poor nodulation intensity with restricted nodule development that impacts negatively on nodule functioning; thus suggesting a requirement of NOS activity in nodule development and functioning (Leach et al. 2010). A number of reports demonstrate the

existence of nitric oxide synthase activity in plant tissue, including legume root nodules (Corpas et al. 2006; Corpas et al. 2009; Cueto et al. 1996). However, the genes or proteins responsible for nitric oxide synthase enzymatic activity remain elusive.

Accumulation of reactive oxygen species (ROS), such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), to levels in excess of the content that is required for protective cellular signaling can lead to cell death via a necrotic or programmed cell death (PCD) pathway in plant tissue (Bailey-Serres and Mittler 2006; Becana et al. 2000; Dat et al. 2003; Epple et al. 2003; Mittler et al. 2004; Overmyer et al. 2003). In instances where the PCD pathway is responsible for the ensuing ROS-induced cell death, cysteine endopeptidases (CYP) of the caspase-like family are key executors/effectors of the PCD (Alesandrini et al. 2003; Chang et el. 2009; Groten et al. 2006; Naito et al. 2000; Zhou et al. 2008). In fact, it is likely that proper nodule development and functioning requires a basal level of ROS for signaling purposes that may trigger essential nodule developmental processes or PCD processes regulated by cysteine endopeptidases (CYPs) (Alesandrini et al. 2003; Chang et el. 2009; Naito et al. 2000; Peleg-Grossman et al. 2007; Santos et al. 2001; Zhou et al. 2008). It is thus reasonable to expect that a steady-state level of ROS would be crucial for the development of functional nodules and the maintenance of such functioning. However, it also follows that a level in any significant excess of this critical level could trigger oxidative stress, ultimately culminating in cell death (as the PCD that is characteristic of nodule senescence) and poor nodule functioning (which can be reflected by poor nitrogenase activity).

The fact that NO is intricately linked to generation and detoxification of ROS suggests that a fine-tuned system exists in plants for ensuring maintenance of basal levels of NO, coupled with interaction between NO signals and ROS signals, required for signaling purposes that ensure cellular homeostasis. For example, inhibition of NOS activity affects nodule development and nodule functioning negatively while high levels of exogenously applied NO also have negative influence on nodule development and functioning (Leach et al. 2010). In fact, NO is regarded as a signaling molecule vital to the maintenance of plant redox homeostasis and plays a pivotal role in alleviating oxidative stress that is consequent to abiotic stresses (Da Gara et al. 2010). Furthermore, NO is regarded as a component of the rhizobium-legume interaction for the establishment of the symbiotic relationship between these two symbiotic partners (Ferrarini et al. 2008). It therefore seems that NO has a role in nodule redox homeostasis. Coupled with the role of reactive oxygen species in symbiotic relations, in combination with the contribution of CYPs in the rhizobia-legume interaction, NO signaling may have implications for nodule functioning. In consideration of such implications, we engaged on a study to elucidate the contribution of NOS activity (and thus NO generated from such enzymatic activity) in nodule functioning once the rhizobia-legume symbiotic relationship is established in soybean. We thus used a specific inhibitor (L-NNA) of NOS and an NO donor (DETA/NO) that releases NO in the same redox form as that produced by NOS *in vivo* [i.e. the NO˙ redox form, as documented by Feelisch (1998)] to evaluate the effect of these compounds on various aspects of nodule functioning. Parameters evaluated here were biomass yield in nodulated soybean, nodule functioning as signified by nitrogenase activity, cell death, CYP enzymatic activity, ROS content and antioxidant enzymatic activities.

2.3 Materials and Methods

2.3.1 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 imbided in sterile distilled water for 1 hour and sown in sand for plants used in treatments at the cotyledon (VC) stage (when the unifoliolate leaves are fully expanded and there is one node) 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 three-node (V3) 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 until they reached the V3 stage (for treatments that started at the VC stage) or the five-node (V5) stage of vegetative growth (for treatments that started at the V3 stage). Plants of the same phenological stage and similar height were selected for analysis in each set of treatments.

2.3.2 Treatment of plants

Plants were treated with either nitrogen-free nutrient solution or L-NNA, which is a specific inhibitor of NOS (EC 1.14.13.39), or a combination treatment with L-NNA and the nitric oxide donor DETA/NO. 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 VC stage (plants that were inoculated with rhizobia only at the VC stage), plants (denoted 'L-NNA') were pre-treated (one day before inoculation) in the pots for 16 h by supplementing 100 ml of the nitrogen-free nutrient solution (per plant) with L-NNA (final concentration of 1 mM). The pre-treatment solution was subsequently removed and another 3-h pre-treatment with L-NNA was done, as described above. This was followed by immediate inoculation with rhizobia, after which the pre-treatment solution was replaced with 100 ml of the nitrogen-free nutrient solution (per plant) containing 1 mM L-NNA. To evaluate the effects of recovery of plants from the effects of L-NNA, another set of plants (denoted 'L-NNA + DETA/NO') was pre-treated in the pots for 16 h by supplementing 100 ml of the nitrogen-free nutrient solution (per plant) with L-NNA (final concentration of 1 mM). After the 16 h treatment of the 'L-NNA + DETA/NO' plants, the pre-treatment solution was subsequently removed and followed by another pre-treatment with L-NNA for 3 h. At this stage, the 'L-NNA + DETA/NO' plants were immediately inoculated with rhizobia and the pre-treatment solution was replaced with 100 ml of the nitrogen-free nutrient solution (per plant) containing 1 mM L-NNA and 200 µM DETA/NO. Another set of plants was treated at the VC stage with 100 ml (per

plant) of 200 µM DETA/NO in order to investigate the effect of NO on soybean nodules at concentrations used in the above recovery treatment. Another set of plants was treated with 100 ml (per plant) of 200 µM DETA as a negative control for DETA/NO (to determine if the effects of DETA/NO are truly a consequence of the NO that is released by DETA/NO instead of the by-product DETA that is also released by DETA/NO). Another batch of plants (referred to as 'L-NNA + DETA') was treated as the 'L-NNA + DETA/NO' set of plants, except that 200 µM DETA was used in the place of DETA/NO.

For treatment of plants at the V3 stage (plants for which inoculation with rhizobia was done to the seeds before sowing), a set of plants was treated with 100 ml of the nitrogen-free nutrient solution (per plant) containing 1 mM L-NNA. Another set of plants (denoted 'L-NNA + DETA/NO') was treated with 100 ml of the nitrogen-free nutrient solution (per plant) containing 1 mM L-NNA and 200 µM DETA/NO to evaluate the effects of recovering the plants from the effects of L-NNA. An additional set of plants was treated with 100 ml (per plant) of the nitrogen-free nutrient solution supplemented with 200 µM DETA/NO in order to investigate the effect of NO on soybean nodules at concentrations used in the above recovery treatment. Another batch of plants was treated with 100 ml (per plant) of the nitrogen-free nutrient solution supplemented with 200 µM DETA as a negative control for DETA/NO. The last set of plants (referred to as 'L-NNA + DETA') was treated as the 'L-NNA + DETA/NO' set of plants, except that 200 µM DETA was used in the place of DETA/NO.

Growth parameters, nodule cell viability, nodule CYP activities, nodule ROS (O_2^- and H_2O_2) levels, NADPH oxidase enzyme activity, nitrogen fixation, nitric oxide content, cGMP content, nodule antioxidant enzyme activities plus in-gel activities [superoxide dismutase (SOD) activity,

ascorbate peroxidase (APX) activity and glutathione peroxidase (GPX) activity], were evaluated. Freshly harvested plants were used for measurement of growth parameters and nodule cell viability but snap-frozen (in liquid nitrogen) tissue were used for all other assays (stored at -80°C).

2.3.3 Plant growth parameters

Plants were removed from the sand, being careful to avoid any loss of shoots or roots during the up-rooting of the plants. Twelve plants from each treatment were divided into shoots and roots, and the nodules were removed. Each of these plants was scored for dry weight of the shoots, roots and nodules in addition to nodule number.

2.3.4 Cell viability analysis

A modified method of Sanevas et al. (2007) was followed for the cell viability assays. Briefly, soybean nodules (100 mg per treatment) from five different plants of each of the treatment were harvested and stained at room temperature with 0.25% (w/v) Evans Blue for 15 minutes. The nodules were washed for 30 min in distilled water, followed by extraction of the Evans Blue stain (taken up by dead nodule cells) from nodule 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 nodule tissues.

2.3.5 Nitrogen fixation analysis

Plants from each treatment were used for nitrogenase activity assays as a measure of biological nitrogen fixation (BNF) based on an acetylene reduction assay (ARA) modified from

that described by Suganuma et al. (2003). Briefly, intact nodulated plants were placed in 1000 ml Erlenmeyer flasks and the flasks were sealed with rubbers toppers. Air was removed from the flasks by withdrawing the air several times with a 100 ml gas syringe (B&M Scientific cc, CapeTown, South Africa) fitted with a 30 gauge B-D Precision glide® needle until the syringe lever resisted manual (hand) pulling. The flasks were injected with 900ml of 10% (v/v) acetylene (Afrox Ltd., Germiston, SouthAfrica) using a 100 ml gas syringe fitted with a 30 gauge B-D Precision glide® needle. The samples were incubated for 1 h, followed by measurement of ethylene formed (sampled using a 1 ml 1000 Series Gastight® Hamilton® syringe) on a Varian 3400 gas chromatograph (Varian Inc., Palo Alto, CA) using a standard curve generated from ethylene (Afrox Ltd., Germiston, SouthAfrica) standards.

2.3.6 Cysteine Protease activity

CYPs were assayed for using a modified procedure of Zhang et al. (2008). Protein extraction was done using 100 mg of nodule tissue, homogenised in 1 ml of buffer containing 100 mM Tris-HCl (pH 6.2), 5 mM MgCl$_2$, 2 mM EDTA, 10 % (v/v) glycerol and 10 mM β-mercaptoethanol. For CYP activity 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to inhibit serine proteases in the homogenate prior to incubation of the mixture at 37°C for 5 min. Then, 0.5 mM Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) was added as substrate to the reaction mixture and incubated at 37°C for 60 minutes. CYP activity was determined by measuring absorbance of released chromophore p-nitroaniline at 405 nm every 20 min (using the extinction co-efficient of 9.6 mM^{-1}.cm^{-1}).

2.3.7 cGMP content

Frozen nodule tissue (100 mg) was ground in liquid nitrogen to a fine powder, added to 1 ml of ice-cold 5% trichloroacetic acid (TCA) and the homogenate 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. cGMP content was determined according to the manufacturer's instructions for the cGMP Immunoassay Kit (Sigma catalog number CG-200) following the acetylation protocol.

2.3.8 Protein extraction from 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]. The resulting homogenates were centrifuged at 12,000 X g for 30 min and the supernatants were used for nitric oxide content determination as well as enzyme assays.

2.3.9 Quantification of nitric oxide

NO content was measured by slight modification of the haemoglobin-based assay (Murphy and Noack 1994). Briefly, protein extracts were incubated with 100 U of catalase and 100 U 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.

2.3.10 Determination of antioxidant enzyme activities

NADPH oxidase (NOX, EC 1.6.3.1) activity was determined in the nodules using a procedure described by Tewari et al. (2009). The reaction mixture contained 50 µl of protein extract, 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM $MgCl_2$, 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 1 mM $CaCl_2$ and 0.3 mM 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT), in a 200 µl reaction. NOX activity was calculated based on the reduction of XTT by monitoring absorbance at 450 nm for 5 minutes, using the extinction coefficient of $2.16 \times 10^4 \ M^{-1}.cm^{-1}$.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined in the nodules using a procedure modified from Beyer and Fridovich (1987). The reaction mixture contained 50 mM K_2HPO_4, pH 7.8, 0.1 mM EDTA, 0.025% (w/v) Triton X-100, 0.1 mM xanthine, 6.25 nM xanthine oxidase, 0.1 mM 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) and 20 µl of enzyme extract (in a 200 µl reaction). The reaction mixture was incubated for 20 minutes at 37°C and absorbance readings were taken at 450 nm. SOD activity was calculated based on the amount of enzyme that was required to cause 50% decrease in the reduction of WST-1.

Total ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured in nodules using a procedure adapted from Asada (1984). Briefly, enzyme extracts were supplemented with ascorbate at a final concentration of 2 mM. The reaction mixture contained 10 µl of enzyme extract, 50 mM K_2HPO_4, pH 7.0, 0.1 mM EDTA, 50 mM ascorbate and 1.2 mM H_2O_2 (200 µl

reaction). APX activity was calculated based on the change in absorbance at 290 nm as ascorbate was oxidised during the reaction, using the extinction co-efficient of 2.8 $mM^{-1}.cm^{-1}$.

Total glutathione peroxidase (GPX, EC 1.11.1.9) activity was evaluated according to a method modified from Drotar et al. (1985). The reaction mixture contained 10 µl of enzyme extract, 50 mM K_2HPO_4, pH 7.0, 2 mM EDTA, 2 mM glutathione (GSH), 0.1 mM NADPH and 2.5 Units of glutathione reductase, and 0.09 mM 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.

2.3.11 Antioxidant isoform detection

Protein concentrations were quantified, using protein extracts, as described by the manufacturer for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories, Inc., Hercules, CA) prior to loading of the native polyacrylamide gels to ensure equal loading of protein for accurate comparison of isoforms between treatments.

For the separation of SOD isoenzymes, samples containing 80 µg protein per well were subjected to native Polyacrylamide gel electrophoresis (PAGE) in 5% stacking and 7.5% 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 Beuchamp and Fridovich (1973).

Electrophoretic APX separation was carried out as described by Seckin et al. (2010) and native PAGE was performed at 4°C in 7.5 % polyacrylamide mini gels containing 10% glycerol.

Before loading samples (120 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-gels was carried out as a nobel method based on the method of Seckin et al. (2010) for APX isoform detection except for altering ascorbate to GSH in the staining solutions. Native PAGE was performed at 4°C in 7.5 % polyacrylamide mini gels using 120 µg of 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 GSH for 20 min and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM GSH and 0.09 mM H_2O_2 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.

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 relative densitometry values (Pixel intensities) of three independent gels.

2.3.12 ROS quantification

For O_2^- content, a procedure described by Tewari et al. (2009) was used. Nodule tissue (100 mg) was ground in liquid nitrogen and homogenized in 400 µl of cold 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 X g for 30 min at 4°C to obtain the O_2^- extract. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 50 µl and 0.5 mM XTT. O_2^- content was calculated based on the reduction of XTT by monitoring absorbance at 450 nm for 4 min, using the extinction coefficient of 2.16 X 10^4 $M^{-1}.cm^{-1}$.

H_2O_2 content was determined in based on a method adapted from Velikova et al. (2000). Nodule tissue (100 mg) was ground to fine powder in liquid nitrogen and homogenized in 400 µl of cold 5% (w/v) TCA. The homogenate was centrifuged at 12,000 X g for 30 min at 4°C to obtain the H_2O_2 extract. The reaction mixture contained 75 µl of the 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.

2.3.13 Statistical analysis

All results are 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.

2.4 Results

2.4.1 Root nodule nitric oxide content is altered by DETA/NO and L-NNA, correspondingly influencing nodule cGMP content

Given that no report existed on the use of DETA/NO and L-NNA to alter NO content in soybean root nodules, it was relevant to first determine if DETA/NO elevates soybean nodule NO content and if L-NNA inhibits nodule NOS activity (which can be traced on the basis of a reduction in NO content in the nodules upon treatment with L-NNA). The level of NO and cGMP in root nodules was measured for plants treated at the VC stage of vegetative growth as described in 'Materials and Methods'. Treatment with L-NNA reduced nodule NO content by ± 75% whereas DETA/NO increased nodule NO content by ± 75% and the combination treatment (L-NNA plus DETA/NO) elevated nodule NO content by ± 30% compared to untreated plants (Figure 2.4.1 A). Along with the changes in NO content, nodule cGMP content was reduced by ± 42% in response to treatment with L-NNA whereas DETA/NO increased nodule cGMP content by ± 52% and the combination treatment (L-NNA plus DETA/NO) resulted in nodule cGMP content ± 35% higher than untreated plants (Figure 2.4.1 A).
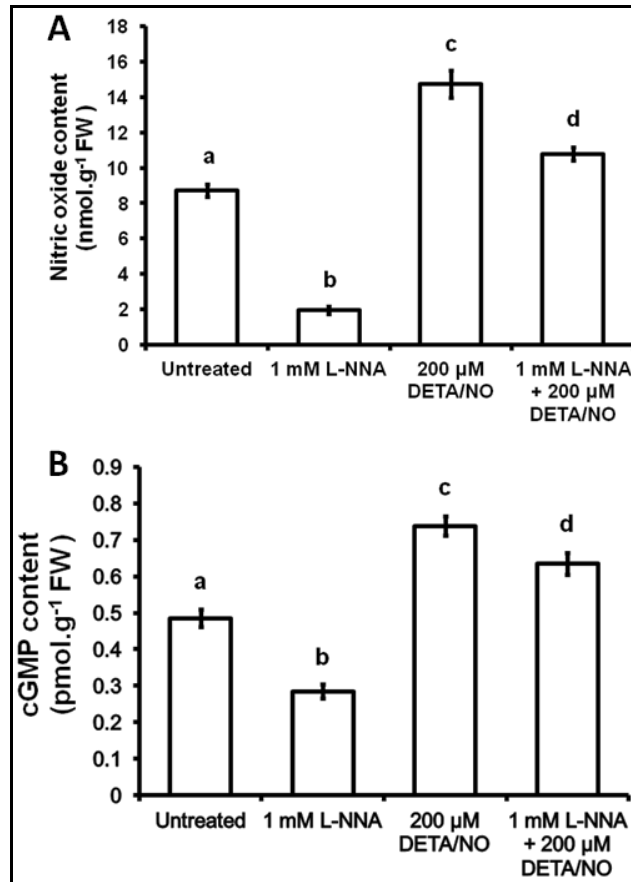
Figure 2.4.1 Effect of inhibition of NOS activity and exogenous application of NO on nodule NO and cGMP content. The NOS inhibitor L-NNA and the NO donor DETA/NO were applied to soybean plants at the V3 stage of vegetative growth and NO content was measured in the nodules (A). In the similarly treated soybean plants, cGMP content was measured in the root nodules (B). Error bar values represent the mean (± SE; n = 3) and the data are representative of three independent experiments.

2.4.2 Nitric oxide synthase activity regulates CYP activity and is required for maintenance of

nodule cell viability and functioning

It has been reported that NO can have both protective and cell death-inducing effects on

plant tissue (Beligni and Lamattina 1999; Wojtaszek 2000). Furthermore, there is evidence for

NO-induced programmed cell death (PCD) in plants and it is known that PCD in plants may also

be mediated by CYP activity (Clarke et al. 2000; Dat et al. 2003; Groten et al. 2006; Murgia et al. 2004). The effect of inhibition of NOS activity (by treatment with L-NNA) and the elevation of NO content by application of exogenous NO at high concentrations (by treatment with DETA/NO) were thus investigated. The effects of these treatments on nodule cell death, nodule CYP activity and nitrogenase activity were evaluated in root nodules of plants treated at the VC stage of vegetative growth as described in 'Materials and Methods'. Elevated cell death occurred in plants treated with L-NNA, reflected here by an increase of ± 140% in Evans Blue uptake compared to untreated plants (Figure 2.4.2.1 A). This increase in nodule cell death was reversed by the combination treatment (L-NNA plus DETA/NO) as plants from this treatment only had cell death equivalent to untreated controls (Figure 2.4.2.1 A). However, a combination treatment with L-NNA and DETA (i.e. L-NNA plus DETA) did not reverse the augmentation of nodule cell death by L-NNA (Figure 2.4.2.1 A). A less pronounced increase in nodule cell death occurred in response to 200 μM DETA/NO alone, but this increase in cell death was less than that observed in plants treated with L-NNA (Figure 2.4.2.1 A).

CYP activity in plants treated with L-NNA increased by ± 42% compared to the CYP activity of the untreated plants (Figure 2.4.2.1 B). Application of 200 μM DETA/NO elevated the nodule CYP activity by ± 21% compared to untreated plants, which was less pronounced than the CYP activity resulting from inhibition of NOS activity (Figure 2.4.2.1 B).
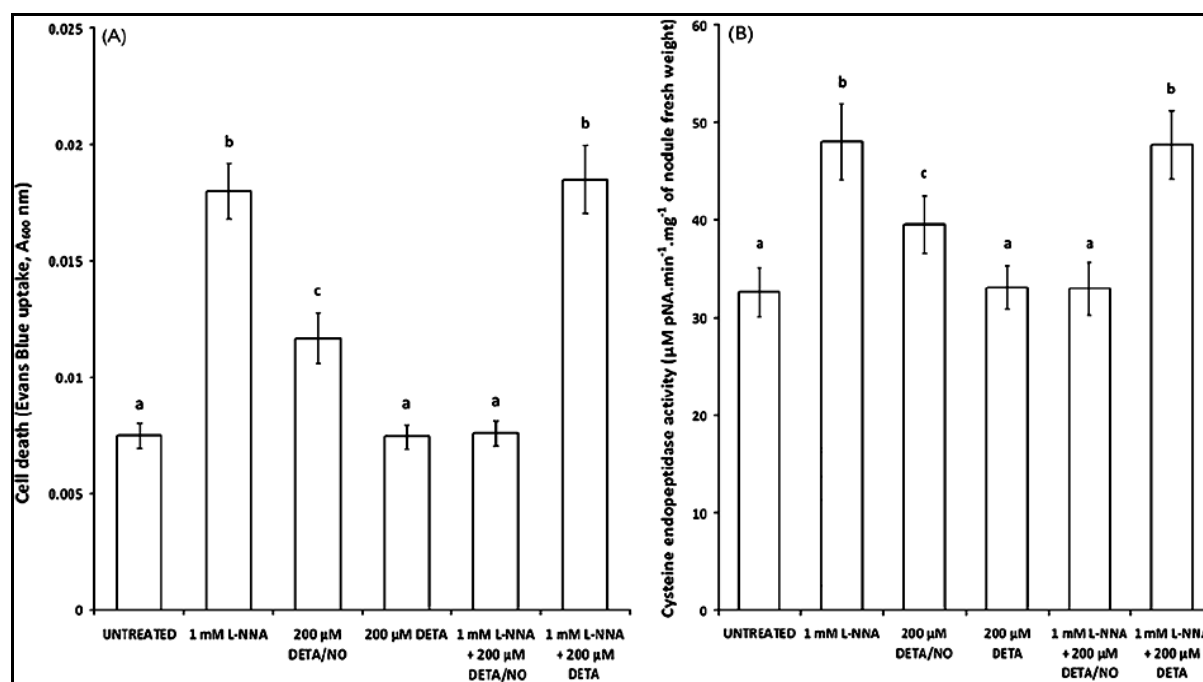
Figure 2.4.2.1 Changes in cell viability (A) and cysteine endopeptidase activity (B) in soybean root nodules. Assays were done on soybean nodules after 7 days of daily treatment with either nitrogen-free nutrient solution (UNTREATED), 1 mM L-NNA, 200 µM DETA, 1 mM L-NNA + 200 µM DETA/NO or 1 mM L-NNA + 200 µM DETA. Nodules taken from four different plants per treatment were analyzed. Data shown are the mean (±SE) of three independent experiments. Means with different letters are significantly different from each other ($p < 0.05$). This data has been published as Figure 4 in the research paper of Leach et al. (2010).

L-NNA caused a reduction of ± 95% in nitrogen fixation, presented as nitrogenase activity, relative to untreated plants, and this reduction was reversed by treatment with a combination of 1 mM L-NNA and 200 µM DETA/NO (there was no statistically significant difference in nitrogenase activity between the 'Untreated' and the 'L-NNA + DETA/NO' plants), whereas nitrogen fixation in the 'L-NNA + DETA' plants was not significantly different from the 'L-NNA' plants (Figure 2.4.2.2).
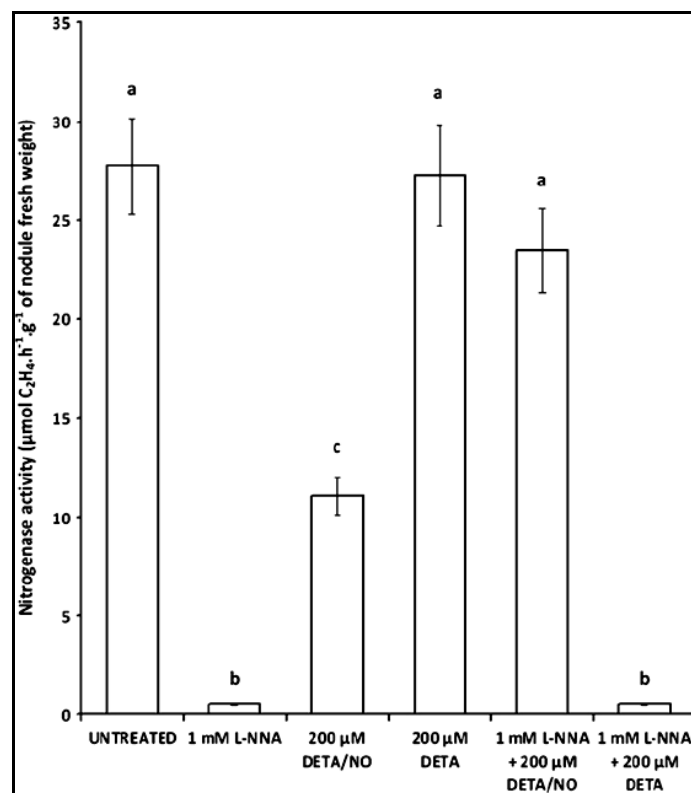
Figure 2.4.2.2. Influence of various treatments on nitrogen fixation. Evaluation of nitrogenase activity in soybean plants after 21 days of treatment with either nitrogen-free nutrient solution (UNTREATED), 1mM L-NNA, 200 µM DETA/NO, 200 µM DETA, 1 mM L-NNA + 200 µM DETA/NO or 1 mM L-NNA + 200 µM DETA. Twelve plants were analyzed for each treatment. The values are the means (±SE) of three independent experiments. Different letters indicate significantly different means between treatments ($p < 0.05$). This data has been published as Figure 3 in the research paper of Leach et al. (2010).

2.4.3 Nitric oxide synthase activity is required for maintenance of soybean biomass

Recent evidence shows that NOS activity is required for soybean nodule development and that cysteine protease-induced nodule cell death occurs if soybean NOS enzymatic activity is inhibited, with the consequence that the cell death prevents nodule development and functioning (Leach et al. 2010). It is also known that ROS such as O_2^- and H_2O_2 can trigger cysteine protease-induced PCD if the ROS accumulate to excessive levels (Bailey-Serres and Mittler 2006; Dat et al. 2003; Alesandrini et al. 2003; Chang et el. 2009; Zhou et al. 2008). It was

on this basis that the influence of L-NNA (a NOS inhibitor) and excessive NO content (by application of exogenous NO at high concentrations via treatment with DETA/NO) on soybean nitrogenase activity, nodule cell death, nodule CYP activity and O_2^- content were investigated.

No significant differences were observed in nodule number amongst the various treatments (Figure 2.4.3.1 A) but the dry weight (indicative of biomass) of nodules, shoots and roots was reduced (by ± 50%, ± 47% and ± 57% respectively) in plants treated with the NOS inhibitor L-NNA compared to untreated plants (Figure 2.4.3.1 B – D). Application of exogenous NO (as 200 µM DETA/NO) resulted in reduced dry weight in nodules, shoots and roots (by ± 28%, ± 30% and ± 40% respectively) but this reduction is less pronounced than that observed in plants treated with the NOS inhibitor L-NNA compared to untreated plants (Figure 2.4.3.1 B – D). Plants treated with a combination of both L-NNA and DETA/NO accumulated nodule, shoot and root biomass to the same extent as untreated plants (Figure 2.4.3.1 B – D).
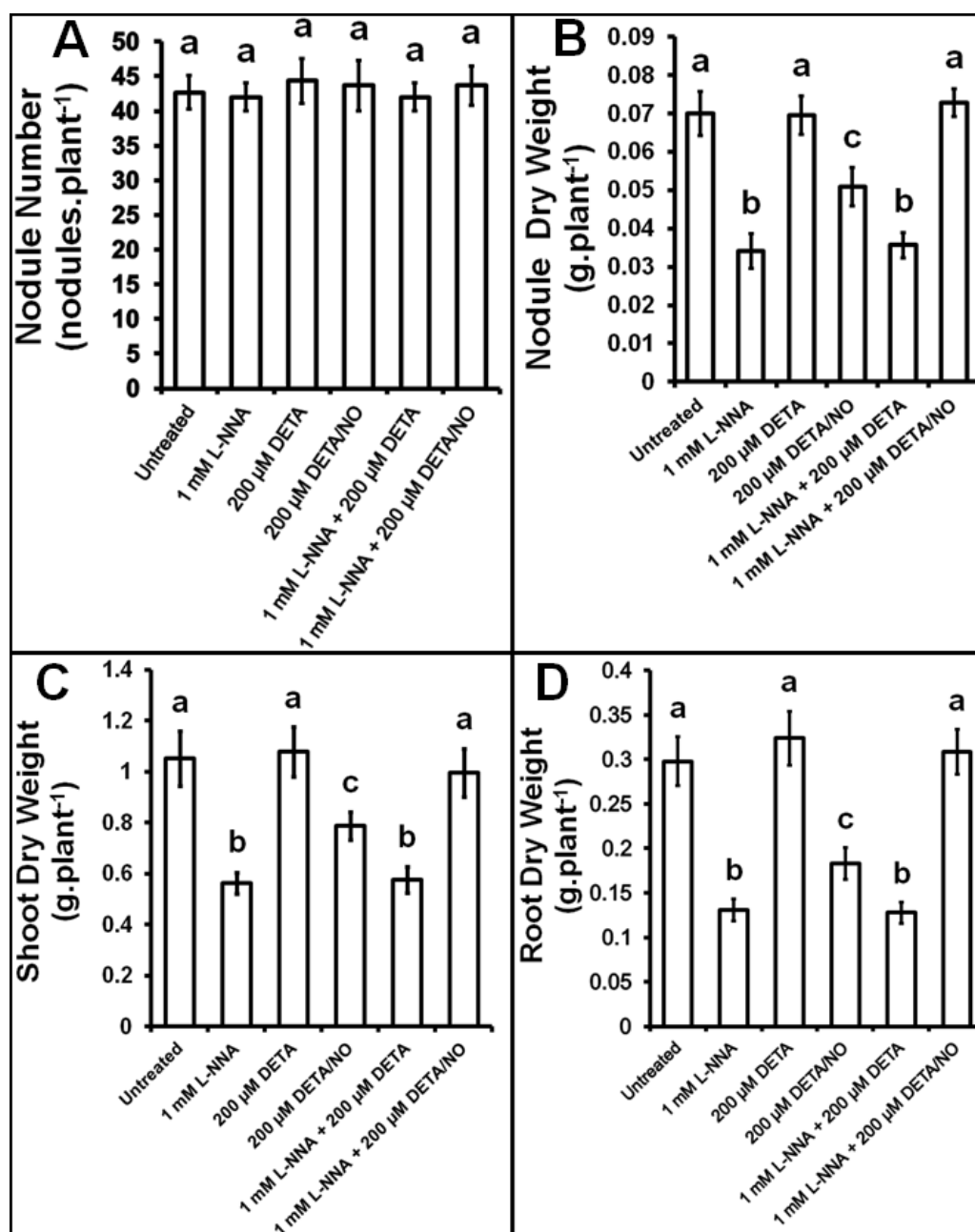
Figure 2.4.3.1 Responses of soybean growth parameters to various treatments that influence nodule NO content. Measurements were done on soybean plants that were treated at the V3 stage for a period of 21 d when they reached the V5 stage. Data represent the mean (±SE) of three independent experiments from eight plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

Upon observing changes in growth parameters (as a consequent to inhibition of NOS activity or application of excessive amounts of exogenous NO to the plants), it became necessary to evaluate nitrogenase activity (indicative of nitrogen fixation), cell death, cysteine protease/endopeptidase (CYP) enzymatic activity and O_2^- content in root nodules. This is because loss of soybean biomass could be a result of altered nitrogen fixation and this could likely be due to induction of cysteine protease-dependent PCD that could be triggered by high O_2^- content. Nitrogenase activity was diminished by ± 60% in response to treatment with 1 mM L-NNA and was reduced by ± 37% in response to treatment with 200 µM DETA/NO, compared to untreated plants (Figure 2.4.3.2 A). Treatments in which 1 mM L-NNA was combined with 200 µM DETA/NO partially reversed the inhibitory effect (on nitrogenase activity) of both 1 mM L-NNA or 200 µM DETA/NO because this combination treatment resulted in improved nitrogenase activity, albeit still lower than the nitrogenase activity of untreated plants (Figure 2.4.3.2 A).

L-NNA augmented of nodule cell death by ± 150% whereas nodule cell death induced by 200 µM DETA/NO was limited only to an increase of ± 65% compared to untreated plants (Figure 2.4.3.2 B). A combination treatment with 1 mM L-NNA and 200 µM DETA/NO reversed the cell death caused by either 1 mM L-NNA or 200 µM DETA/NO when used alone (Figure 2.4.3.2 B). Along with augmented cell death in response to either L-NNA or DETA/NO, cysteine protease enzymatic activity was ± 35% higher in nodules taken from plants treated with 1 mM L-NNA than in nodules of untreated plants whereas nodule CYP enzymatic activity increased by ± 19% in response to treatment of soybean plants with 200 µM DETA/NO (Figure 2.4.3.2 C). A

combination treatment with both 1 mM L-NNA and 200 µM DETA/NO resulted in CYP activity that is similar to that of untreated plants. (Figure 2.4.3.2 C).
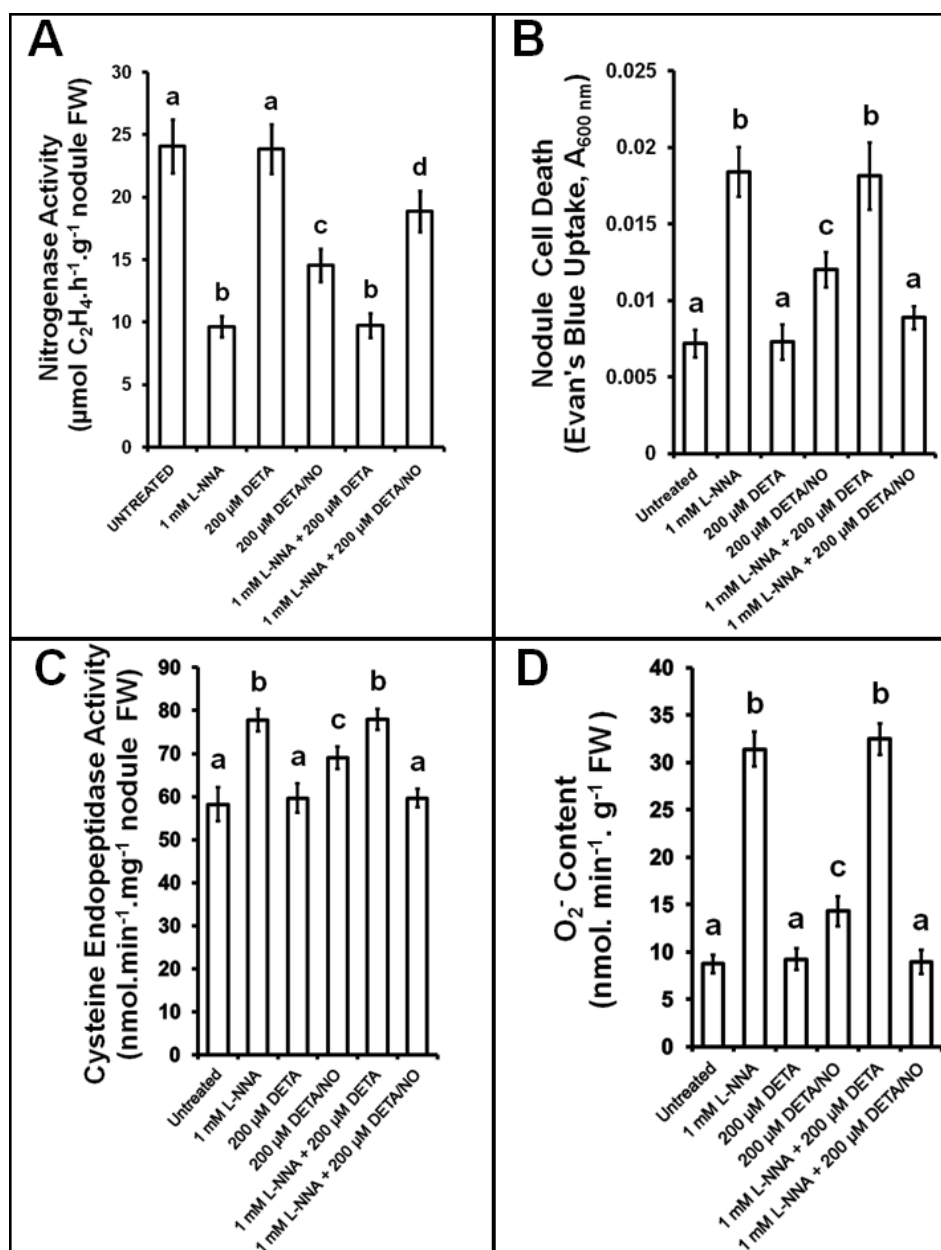
Figure 2.4.3.2 Influence of various treatments on nitrogen fixation, cell death, cysteine protease activity and O_2^- content in soybean nodules. Nitrogenase activity (A), nodule cell death (B), cysteine protease enzymatic activity (C) and O_2^- content (D) were measured in soybean plants (at the V5 stage of vegetative

growth) after 21 days of treatment that was initiated at the V3 stage of vegetative growth. Treatments were with either nitrogen-free nutrient solution (UNTREATED), 1 mM L-NNA, 200 μM DETA/NO, 200 μM DETA, 1 mM L-NNA + 200 μM DETA/NO or 1 mM L-NNA + 200 μM DETA. Four plants were analyzed for each treatment. The values are the means (±SE) of three independent experiments. Different letters indicate significantly different means between treatments ($p < 0.05$).

O_2^- content was drastically increased (by ± 300% compared to untreated plants) in nodules of plants treated with L-NNA and this increase was reversed to levels similar to that of untreated plants when 1 mM L-NNA was used in combination with 200 μM DETA/NO (Figure 2.4.3.2 D). Treatment with 200 μM DETA/NO alone caused an increase of ± 85% in nodule O_2^- content compared to the O_2^- content of nodules from untreated plants.

2.4.4 Nitric oxide synthase regulates soybean nodule functioning by modulating antioxidant enzymatic activity

Consequent to discovering that inhibition of NOS enzymatic activity or application of excessive amounts of exogenous NO severely impairs nitrogenase activity (indicative of negative effects on nodule functioning) and noting that NOS inhibition or excessive NO levels result in highly elevated O_2^- content in root nodules of soybean, it was became relevant to investigate the influence of L-NNA (a NOS inhibitor) and excessive NO content on soybean nodule NADPH oxidase enzymatic activity because NADPH oxidase is one of the major sources of O_2^- in plants (Jones et al. 2007; Potocky et al. 2007).

Treatment with DETA had no influence on any of the parameters investigated (Figure 2.4.4.1). No significant differences were observed in nodule NADPH oxidase enzymatic activity between untreated plants and plants treated with the combination of 1 mM L-NNA and 200 μM DETA/NO (Figure 2.4.4.1 A) but NADPH oxidase enzymatic activity in nodules of plants treated

with 1 mM L-NNA and those treated with 200 µM DETA/NO was increased (by ± 65% and ± 35% respectively) compared to untreated plants (Figure 2.4.4.1 A).
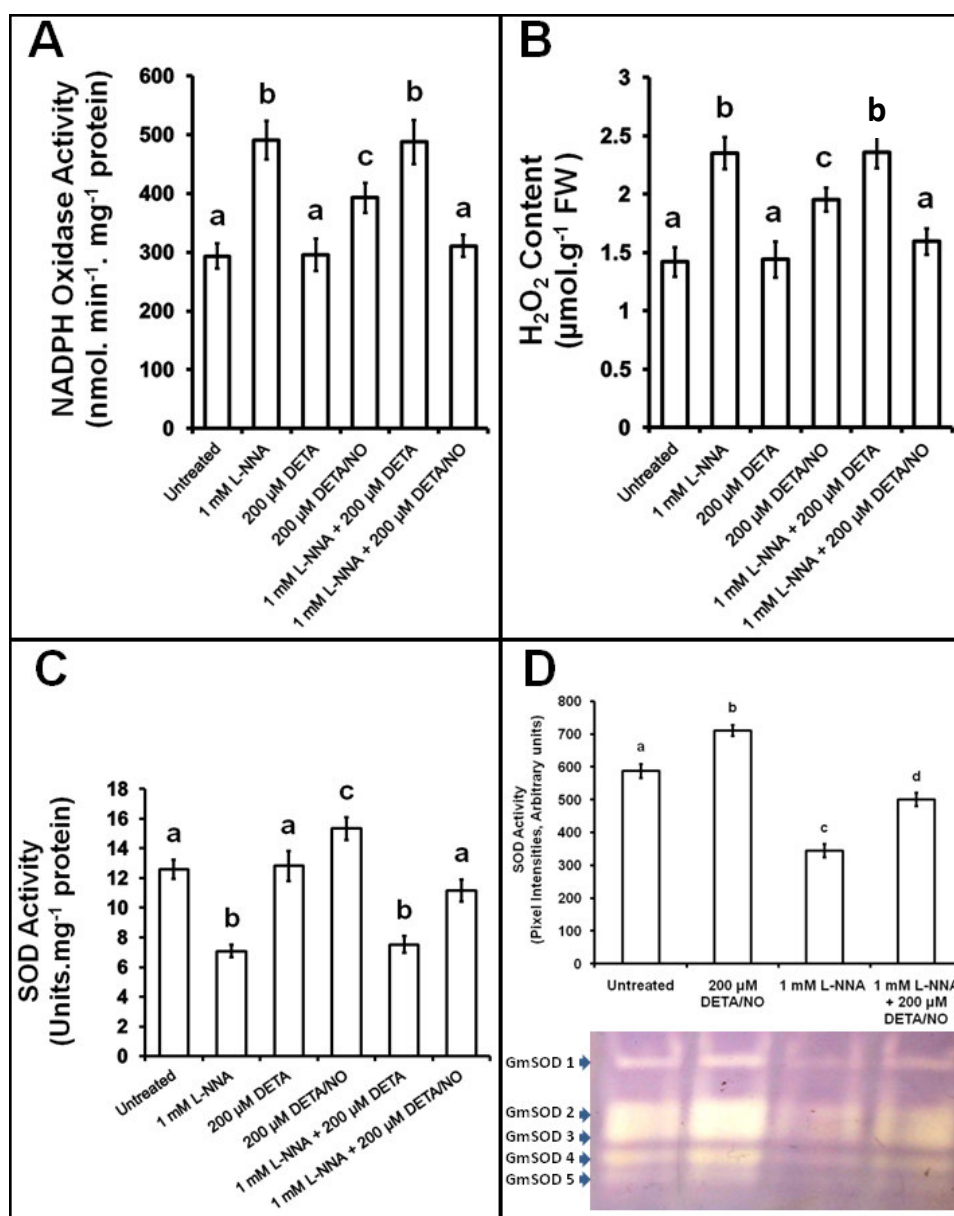
Figure 2.4.4.1 Changes in soybean NADPH oxidase enzymatic activity, SOD enzymatic activity and H$_2$O$_2$ content in response to various treatments that influence nodule NO content. Assays were done on soybean plants that were treated at the V3 stage for a period of 21 d when they reached the V5 stage. Data represent the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

Given that an increase in nodule NADPH oxidase activity occurs in response to treatment of the soybean plants with either 1 mM L-NNA or 200 µM DETA/NO and that these treatments result in elevated O_2^- content in the nodules, SOD enzymatic activity was investigated because SOD is the key enzyme in the detoxification of O_2^- by converting it to H_2O_2 (Becana et al. 2000; Mittler et al. 2004).

Application of 1 mM L-NNA lead to significant reduction of nodule SOD activity whereas application of exogenous NO (as 200 µM DETA/NO) resulted in enhanced nodule SOD enzymatic activity and the combination treatment (1 mM L-NNA + 200 µM DETA/NO) resulted in restoration of the SOD activity and the trend in all treatments was maintained for all SOD isoforms (five isoforms were detected) as shown in the in-gel assays (Figure 2.4.4.1 C – D).

Surprisingly, H_2O_2 content in nodules of plants treated with 1 mM L-NNA increased by ± 65% compared to H_2O_2 content of nodules from untreated plants despite the fact that 1 mM L-NNA suppressed nodule SOD activity (Figure 2.4.4.1 B). The H_2O_2 content in nodules of plants treated with 200 µM DETA/NO increased by ± 30% compared to that of nodules from untreated plants whereas the combination treatment (1 mM L-NNA + 200 µM DETA/NO) resulted in nodule H_2O_2 content similar to that of nodules from untreated plants (Figure 2.4.4.1 B).

Changes in H_2O_2 content are likely to trigger alterations in the enzymatic activities of H_2O_2-scavenging enzymes such as APX and GPX and these altered enzymatic activities would determine the H_2O_2 content of plant tissues, depending on the efficiency of H_2O_2-scavenging enzymes in detoxification of H_2O_2 to H_2O (Mittler et al. 2004). Based on the observed changes in

H$_2$O$_2$ content in response to 1 mM L-NNA and 200 M DETA/NO, the response of APX and GPX to these treatments was investigated.

Treatment with 1 mM L-NNA up-regulated nodule total APX enzymatic activity but APX in-gel assays (which identified three APX isoforms) revealed that only the two smaller (based on migration on the PAGE gel) isoforms appear to be up-regulated by L-NNA whereas the larger (in the upper portion of the PAGE gel) isoform appears to be unresponsive to 1 mM L-NNA (Figure 2.4.4.2 A – B). The 200 µM DETA/NO treatment also up-regulated nodule total APX enzymatic activity in a manner similar to that seen for 1 mM L-NNA for but the APX up-regulation caused by 200 µM DETA/NO was less pronounced than that caused by 1 mM L-NNA, whereas the combined treatment (1 mM L-NNA + 200 µM DETA/NO) had a similar level of APX activity as untreated plants (Figure 2.4.4.2 A – B). The nodules from both the plants treated with 1 mM L-NNA and those treated with 200 µM DETA/NO had higher GPX enzymatic activity than nodules from untreated plants but the increase in GPX activity in response to 1 mM L-NNA was more pronounced that the increase in response to 200 µM DETA/NO, as reflected by both spectrophotometry-based and gel-based enzyme activity assays (Figure 2.4.4.2 C – D). Simultaneous treatment with both 1 mM L-NNA and 200 µM DETA/NO partially restored GPX activity as it was at a level only slightly above that of GPX activity in nodules from untreated plants (Figure 2.4.4.2 C – D).
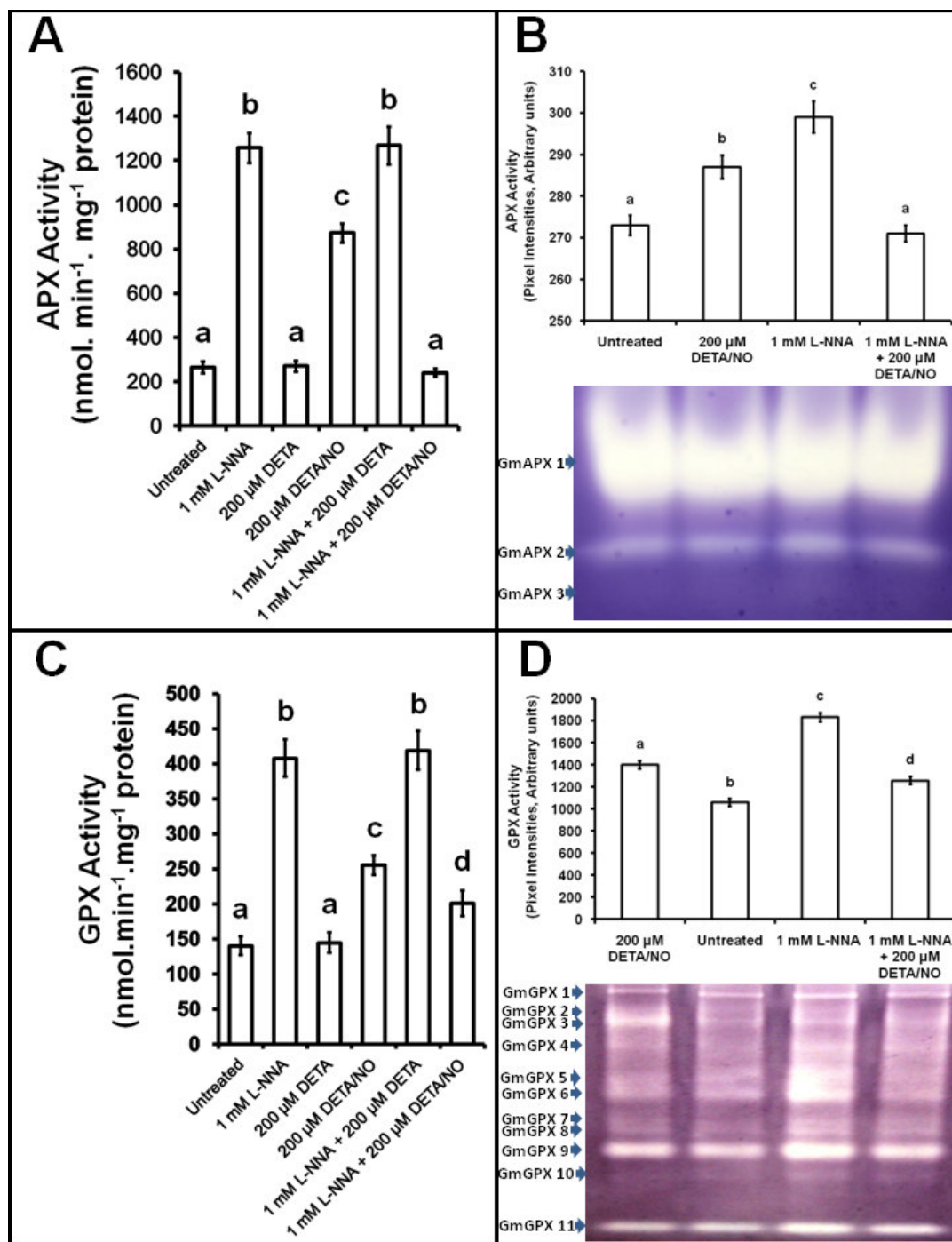
Figure 2.4.4.2 Changes in soybean APX and GPX enzymatic activity in response to various treatments that alter nodule NO content. Total APX enzymatic activity (A) was measured spectrophotometrically in root nodules of soybean plants, whereas isoform-specific APX activity (B) was detected using in-gel assays in total soluble protein extracts of soybean nodules. Total GPX enzymatic activity (C) was measured spectrophotometrically in soybean root nodules and isoform-specific GPX activity (B) was detected using in-gel assays in total soluble protein extracts of the nodules. Assays were done on soybean plants that were

treated at the V3 stage for a period of 21 d (they were at the V5 stage at that time). Data represent the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

2.5 Discussion

The work reported here investigated the effect of inhibition of NOS enzymatic activity (using the NOS-specific inhibitor L-NNA) and the effect of exogenous application of excessive levels of NO (generated from the NO donor DETA/NO) on the functioning of mature but non-senescent nodules (N_2 fixing) by evaluating various molecular and physiological parameters. In order to ensure that any effects observed are a consequence of either NOS inhibition (and thus reduction of NO content *in planta*) or elevation of plant tissue NO content, the NO content in nodules was measured in nodules after treatment of plants with either the NO donor, the NOS inhibitor or a combination of both the NO donor and the NOS inhibitor. The by-product (DETA) generated from the breakdown of the NO donor was used a control in various treatments to ensure that effects observed in the treatments with the NO donor (DETA/NO) are truly caused by NO and not the by-product DETA because DETA does not release NO.

The fact that the level of nodule NO was significantly diminished by treatment with the NOS inhibitor L-NNA is evidence to the fact that L-NNA is an efficient inhibitor of nodule NOS activity and that a significant level of nodule NO is synthesized by NOS enzymatic activity. This notion is supported by the observation that simultaneous treatment of soybean plants with L-NNA and the NO donor DETA/NO restores nodule NO content to levels even slightly above those of the untreated plants. Furthermore, the fact that application of DETA/NO to soybean plants results in significantly elevated NO content in root nodules is testimony to the fact that

DETA/NO releases NO under the experimental conditions used here and the released NO enters the nodule tissue. It is known that NO stimulates soluble guanylate cyclase activity (Stone et al. 1995), leading to elevated cGMP levels in plant tissue (Pfeiffer et al. 1994). The observation that L-NNA inhibits NOS activity in root nodules and this reduced NOS activity coincides with reduction of cGMP content in root nodules implies that nodule NOS activity synthesizes NO that is required for maintenance of basal levels of nodule cGMP content. Evidence for NO-induced regulation of cGMP content emanates from the fact that NO (as the nitric oxide donor DETA/NO) applied exogenously to nodulated soybean plants results in a significant increase in cGMP levels in the root nodules. NO-specificity of the NOS-dependent induction of cGMP elevation in root nodules is evidenced by the fact that simultaneous treatment of nodulated soybean plants with DETA/NO reverses the L-NNA-induced reduction in nodule cGMP content to a level even slightly above the level of cGMP in untreated plants.

The work presented here indicates that inhibition of NOS enzymatic activity causes loss in shoot, root and nodule biomass even though nodule number is not reduced (for plants generated from rhizobium-inoculated seeds; that had fully developed and mature nodules, i.e. plants that were assayed at the V5 stage after treatment was initiated at the V3 stage). In the same plants, DETA/NO applied at a concentration of 200 µM had a similar effect on all these biomass parameters but the effect of DETA/NO was less pronounced than that of L-NNA. This indicates that NOS activity is required for maintenance of nodule and plant biomass and that excessive NO content is detrimental to plant biomass. The study also shows that nitrogenase activity is inhibited if NOS activity is reduced by treatment with the NOS inhibitor L-NNA. Given that reduced NOS activity leads to reduced NO content, it follows that endogenous NO

produced from NOS activity in soybean nodules is required for efficient nitrogen fixation. A significant but less severe decrease (in comparison to the effect of L-NNA) in nitrogen fixation occurred in response to 200 μM DETA/NO, suggesting that high concentrations of exogenously applied NO in the absence of NOS inhibitors has a negative influence on nitrogen fixation in legumes. Overall, the study suggests that endogenous NO from NOS activity is required for maintenance of nitrogen fixation and that suppression of NOS activity causes loss of plant biomass because of limited nitrogen fixation under nitrogen-deficient growth conditions. Similarly, the study shows that excessive levels of exogenous NO suppress nitrogen fixation and causes loss of plant biomass because of limited nitrogen fixation under nitrogen-deficient growth conditions. The fact that DETA on its own and DETA in combination with L-NNA did not alter the pattern seen for untreated samples and the pattern of L-NNA-treated samples, respectively, confirms that effects seen in the DETA/NO and L-NNA treatments are a consequence of alterations in nodule NO content rather than the by-product produced during breakdown of DETA/NO.

The fact that the L-NNA treatment and the DETA/NO treatment induce cell death suggests that loss of nodule biomass results from nodule cell death in these two treatments. In addition, it was noted here that the control combination treatment (L-NNA + DETA) did not reverse the inductive effect of L-NNA on nodule cell death. This implies that the inhibition of NOS activity in soybean roots inoculated with rhizobia promotes nodule death and thus implicates NO in maintaining cell viability in these nodules. A less pronounced increase in nodule cell death was also observed in response to 200 μM DETA/NO alone, but the increase in cell death was less than that observed for L-NNA, suggesting that high concentrations of exogenously applied NO

in the absence of NOS inhibitors induce nodule cell death, despite to a less extent than when NOS is inhibited. Whether the loss of cell viability in the nodules occurs via necrotic or programmed cell death remains unknown and could be an important area of future investigation.

Furthermore, inhibition of NOS triggered an increase in nodule CYP activity and a similar but less pronounced effect was observed in nodules of plants treated with high levels of exogenously applied NO (200 µM DETA/NO). From these observations, it can be concluded that application of high concentrations of exogenous NO triggers CYP activity in the absence of NOS inhibitors, but it must be noted that this was less potent in inducing CYP activity compared to the induction caused by inhibition of endogenous NOS alone. It thus appears that inhibition of NO production from nodule NOS triggers activation of soybean nodule CYP activity and thus implicates endogenous NO in regulating CYP enzymatic activity. The pathway for such NOS-dependent regulation of CYP activity remains to be elucidated.

The molecular signals that are essential for maintaining cell viability in the nodules via NO signaling are yet to be elucidated. However, CYP activity could be one of the targets for such signaling because these proteases can be instrumental in the execution of cell death, especially via programmed cell death (Naito et al. 2000; Vincent and Brewin 2000; Groten et al. 2006). The fact that increased levels of CYP activity in plants treated with L-NNA were observed suggests that NOS-derived NO could play a role in regulating the activity of CYP in root nodules. NO is known to induce the expression of an Arabidopsis cystatin, resulting in inhibition of hypersensitive cell death, and this is thought to occur via the inhibition of CYP activity by the cystatin (Belenghi et al. 2003). It is thus likely that inhibition of NOS-dependent NO production

by L-NNA could allow for enhanced CYP activity in the nodules, leading to pronounced cell death that is presented as loss of nodule cell viability. The fact that similar trends occur in all the parameters evaluated here, irrespective of whether treatments were done concurrently with inoculation with rhizobia on seedlings at the VC stage for assays on plants that are at the V3 stage of vegetative growth or treatments were done on plants at the V3 stage (which were generated from seeds that were inoculated before sowing) for which assays were done at the V5 stage, implies that the role of NOS-derived NO in nodule functioning is consistent throughout the various developmental stages of the nodules.

It can be suggested that the level of NO produced by root nodules under conditions that support optimal nodule function is possibly kept at beneficial levels [possibly via the action of leghaemoglobin (Lb) to produce nitrosylleghaemoglobin (LbNO)], and it is only when NO levels rise above this beneficial threshold that such high NO levels may be detrimental to nodule functioning processes such as nitrogen fixation. It has, in fact, been observed that application of DETA/NO to soybean (in the absence of NOS inhibitors) at a concentration of 200 μM has detrimental effects on nodule functioning, as this relatively high concentration reduces nitrogen fixation, nodule cell viability and plant growth parameters (Leach et al. 2010).

The effects of NO on plant cell functioning can be dictated by the concentration of both NO and other reactive oxygen species (Delledonne et al. 2001). In fact, high concentrations of NO derived from the NO donor sodium nitroprusside (in the range of between 1 mM and 10 mM sodium nitroprusside) inhibit nitrogen fixation, while low concentrations of NO derived from sodium nitroprusside (0.1 mM sodium nitroprusside) enhance nitrogen fixation (Kato et al. 2010). DETA/NO generates between 25 and 50 times more NO than an equivalent

concentration of sodium nitroprusside (Murgia et al. 2004) and it can therefore be expected that the amount of NO produced from 200 µM DETA/NO would be equivalent to that produced by at least 5 mM sodium nitroprusside under the same conditions. The results from this study are thus in agreement with the observation of Kato et al. (2010) that exogenously applied NO at high concentrations inhibits nitrogen fixation (under conditions of normal NOS activity). The fact that L-NNA has a much more severe negative impact on the same physiological aspects suggests that endogenous NO is required for soybean nodule functioning under optimal growth conditions. In agreement with this conclusion, Kato et al. (2010) showed that exogenously applied NO at lower concentrations (as derived from 0.1 mM sodium nitroprusside) has no detrimental effect on nitrogen fixation, but rather enhances nitrogen fixation.

It is unlikely that plants would produce the same level of NO under unstressed conditions as that produced by treatment with an equivalent of 5 mM sodium nitroprusside. This hypothesis is based on the observation that plant tissue synthesizes 0.2 ± 0.1 µM NO min^{-1} in the absence of sodium nitroprusside, whereas treatment of plant tissue with 5 mM sodium nitroprusside leads to an NO production rate of 0.75 ± 0.12 µM NO min^{-1} within 1 h of application to plant tissue (Ederli et al. 2009). This is 70% more than the NO level produced in the absence of sodium nitroprusside. The level of NO synthesized by stressed root nodules compared to unstressed nodules remains to be determined, and it would be important to investigate whether NO has any role in regulating nodule stress responses, as this would provide new strategies for regulating nodule responses to stress. Critical to such strategies would be to determine the level and redox state of NO that confers protection against such stresses. The use of DETA/NO as NO donor could be useful for such endeavours, as it releases

NO in its uncharged (NO$^{\cdot}$) redox state. This may be attractive for efforts to decipher plant signaling pathways that are mediated by NO-activated soluble guanylate cyclase because only the NO$^{\cdot}$ redox form of NO is believed to activate soluble GC and is the *bona fide* product of NOS (Dierks and Burstyn 1996; Goyal and He 1998).

There is a common trend between cell death, CYP activity, O_2^- content and H_2O_2 content in root nodules in response to treatment of plants with 1 mM L-NNA. The same common trend is maintained for cell death, CYP activity, O_2^- content and H_2O_2 content in root nodules in response to treatment of plants with 200 µM DETA/NO except the fact that the L-NNA-induced responses are more pronounced than the DETA/NO responses. Given that it is well established that excessive levels of ROS trigger PCD (Bailey-Serres and Mittler 2006; Becana et al. 2000; Mittler et al. 2004) and the fact that plant PCD is mediated by CYP activity (Alesandrini et al. 2003; Chang et el. 2009; Dat et al. 2003; Groten et al. 2003; Zhou et al. 2008), it can be suggested that the cell death observed in response to the L-NNA or DETA/NO treatments is likely to be PCD resulting from excessively accumulated O_2^- and H_2O_2 that trigger cysteine protease-mediated PCD.

The fact that NADPH oxidase (an O_2^- generating enzyme) activity is up-regulated in response to 1 mM L-NNA, coupled with the fact that L-NNA leads to augmented O_2^- content in the nodules, implies that NOS regulates NADPH oxidase activity and also implies that the reduction of endogenous NO levels (by inhibition of NOS) triggers enhanced NADPH activity, thus leading to overproduction of O_2^-. On the other hand, the 200 µM DETA/NO treatment causes a similar response for nodule NADPH oxidase and O_2^- content as the 1 mM L-NNA treatment but the degree of up-regulation in NADPH oxidase activity and O_2^- content is

significantly less than that seen in response to 1 mM L-NNA. This suggests that excessive NO content (because of high levels of exogenously applied NO, in the form of 200 µM DETA/NO in this case) enhances NADPH activity and this leads to overproduction of O_2^-.

It would be expected that high levels of O_2^- should trigger SOD activity in order to remove the excessive O_2^- since SOD is a O_2^--scavenging enzyme (Mittler et al. 2004). This is true for the treatment with 200 µM DETA/NO because SOD is up-regulated in response to treatment with this concentration of NO donor. However, it would be expected that the augmented SOD activity should reduce the O_2^- content. The fact that this does not occur despite enhanced SOD activity suggests that excessive NO (as occurs for treatment with 200 µM DETA/NO) causes O_2^- accumulation to levels beyond a certain threshold for SOD scavenging capacity (perhaps because SOD has limited efficiency beyond which it cannot remove any further O_2^- if the O_2^- level is beyond a certain limit). The fact that 1 mM L-NNA inhibits SOD activity; together with the fact that the same treatment enhances NADPH oxidase activity; implies that SOD cannot scavenge the O_2^- produced from the enhance NADPH oxidase activity and it thus can be concluded that inhibition of NOS activity causes O_2^- overproduction by enhanced NADPH oxidase and suppressing SOD activity. In conclusion, this study shows that NOS activity is required for maintaining O_2^- levels within the homeostatic range in nodules under normal growth conditions and this is a breakthrough discovery as it has implications for the manipulation of plant redox status.

Enhanced O_2^- production would activate O_2^- detoxifying enzymes such as SOD, resulting in conversion of O_2^- to H_2O_2 (Mittler et al. 2004). The results of this study show that treatment of nodulated soybean plants with 200 µM DETA/NO up-regulates SOD and also results in high

H_2O_2 content in soybean root nodules. The increased H_2O_2 content may thus be a result of enhanced SOD activity. On the other hand, the fact that the L-NNA treatment inhibits root nodule SOD activity and yet the same treatment yields augmented H_2O_2 content in the nodules suggests that other sources of H_2O_2 could be generating the H_2O_2 instead of SOD. Such other sources of H_2O_2 could include oxalate oxidase (Lane et al. 1993) and polyamine oxidase (Augeri et al. 1990; Federico et al. 1988). It will thus be important in future studies to investigate if endogenous NO regulates oxalate oxidase or polyamine oxidase enzymatic activities. In any case, it is clear that both sub-optimal (resulting from inhibition of NOS) and excessive (consequent to high concentrations of exogenously applied NO) levels of NO cause excessive accumulation of both O_2^- and H_2O_2. High levels of H_2O_2 trigger augmented activities of H_2O_2 detoxifying enzymes (Mittler et al. 2004) and this is confirmed in this study by the fact that both the inhibition of NOS enzymatic activity and the exogenous application of high concentrations of NO cause highly augmented activities of APX and GPX (the two enzymes that detoxify H_2O_2 into H_2O). The fact that the H_2O_2 levels in nodules taken from plants treated with either 1 mM L-NNA (NOS inhibitor) or 200 μM DETA/NO do not decrease despite the highly augmented APX and GPX activities suggest that the H_2O_2 generated in these treatments is beyond the capacity of these H_2O_2-scavenging enzymes to efficiently remove the H_2O_2. It is possible that it is not only the activities of the two APX isoforms, namely GmAPX 2 and GmAPX 3, that are influenced by altered nodule NO content (see in-gel assay for APX in Figure 2.4.4.2 B) but also GmAPX 1 considering that the in-gel assay appears saturated with GmAPX1 and so this saturation masks any changes that might be detectable if less GmAPX 1 had been loaded on the gel. However, loading less protein would have made the other two APX isoforms undetectable from the in-gel

assay. Future study of the isoforms in which they are analyzed separately will resolve this shortcoming.

It is reasonable to conclude that accumulation of both O_2^- and H_2O_2 beyond the levels that the plant can tolerate will trigger extensive cell death. Given that the treatments causing accumulation of these two ROS are the same treatments that cause augmented cysteine protease (CYP) activity and cell death, taken together with the fact that CYP activity is instrumental in the execution of PCD, it is proposed here that these treatments trigger increased nodule NADPH oxidase activity and this leads to generation of excessive levels of nodule O_2^-. This excessive O_2^- production is coupled with excessive production of nodule H_2O_2. The excessive ROS then trigger PCD-inducing nodule CYP activity, which is manifested as extensive nodule cell death. The consequence of the extensive nodule cell death is impaired nitrogen fixation capacity and loss of plant biomass. This study provides a foundation for further study of other nodule redox pathways that are regulated by NO and may ignite interest in other biochemical/physiological processes, in root nodule functioning, regulated by NO beyond just redox-mediated signals. Such studies can be effectively performed by analyzing global transcriptomic and proteomic analysis of nodule responses to alterations in nodule NO content.

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2.7 Research Outputs

The work reported in this chapter has been published [***Leach J, Keyster M, Du Plessis M, Ludidi N (2010) Nitric oxide synthase activity is required for development of functional nodules in soybean. J Plant Physiol 167: 1584-1591*** and ***Keyster M, Klein A, Ludidi N (2010) Endogenous NO levels regulate nodule functioning: Potential role of cGMP in nodule functioning? Plant Signal Behav 5: 1-3***]. However, the results presented for cell death, cysteine protease activity and nitrogen fixation in the article by Leach et al. 2010 is entirely my own work. Furthermore, the results presented in this thesis for the haemoglobin-based nitric oxide content and the cGMP content is exclusively my own work and excludes the DAF-FM-based fluorescence intensities for estimation of NO content in nodule tissue (which is described in Keyster et al. 2010 and is part of Mr. Ashwil Klein's work).

CHAPTER 3

Recombinant expression, purification and functional analysis of a novel cystatin from Soybean

3.1 Abstract

Recent studies have identified a novel class of plant cystatins [proteins that inhibit cysteine protease (CYP) enzymatic activity] and this class of cystatins has been assigned to Group-1 of plant cystatins. In this study, a novel member of Group-1 cystatins (named here as GmCYS1p626) has been identified in soybean. Recombinant expression of GmCYS1p626 as a GST-fusion protein showed that it is a cystatin with inhibitory activity against papain and against the CYP activity of nodule extracts. Furthermore, the effects of a nitric oxide synthase (NOS) inhibitor on CYP activity and cell death in soybean root nodules were investigated. Inhibition of NOS activity triggered enhanced root nodule CYP activity along with increased nodule cell death. Exogenously applied nitric oxide (NO) donor reversed the increase in nodule CYP and cell death that was caused by inhibition of NOS. No study of expression of any Group-1 cystatin has been reported in root nodules in response to endogenously synthesized NO, despite the importance of cystatins in regulating CYP activity, which is key to regulation of programmed cell death (PCD). The study thus analyzed of the expression of GmCYS1p626 in root nodules in response to alteration in NO content, achieved via the use of a NOS inhibitor and NO donor. Inhibition of NOS activity suppressed expression of GmCYS1p626. The NO donor reversed the suppression of GmCYS1p626 expression that was caused by the NO inhibitor.

3.2 Introduction

The study presented in Chapter 2 of this dissertation shows that inhibition of nitric oxide synthase (NOS) activity leads to an increase in the enzymatic activity of cysteine proteases (CYPs). It may be possible that this increased activity in the enzymatic activity of CYPs is a result of a reduction in the inhibitory activity of plant cystatins (PhyCys) in nodules, allowing for higher cysteine protease (CYP) activity in nodules that are exposed to NOS inhibitors. PhyCys are proteins involved in the reversible inhibition of papain-like plant CYPs (Arai et al. 2002). Phylogenetic analysis shows that cystatins are widespread in nature because they occur in vertebrates, invertebrates and plants (Kordis and Turk 2009). Cystatins are classified on the basis of molecular weight, number of disulfide bonds, primary structure characteristics and subcellular localization (Abe et al. 1987; Megdiche et al. 2009). PhyCys lack disulfide bonds and have been subdivided into three groups, based on sequence homology, molecular weight and domain organization (Martinez et al. 2009). Group-1 cystatins have a molecular weight of between 10 kDa and 16 kDa and show high homology with chicken egg white cystatin (Megdiche et al. 2009). Group-2 cystatins have an approximate molecular weight of 25 kDa and a repetitive peptide at the C-terminus (Shyu et al. 2004). Group-3 cystatins are called the multi-cystatins due to the presence of eight cystatin domains and have a molecular weight of ca. 85 kDa (Waldron et al. 1993; Wu and Haard 2000). The N-terminal region of PhyCys contains two important regions viz. a glycine residue and a conserved motif namely [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N (Margis et al. 1998; Zhang and Xinxin 2008). Other conserved regions include a QxVxG (x is any amino acid) motif centrally located in the protein sequence and proline-tryptophan residues within the C-terminal region (Margis et al. 1998).

PhyCys inhibit CYPs by forming a tight, reversible equimolar complex by acting as pseudo-substrates that penetrate the active site thus blocking access to protein substrates (Barrett et al. 1998). Cystatins utilizes three signature elements to form a tripartite wedge which fit into the CYPs active site during inhibition. The first structural element which makes contact with the CYPs active site is a hairpin loop which contains the conserved QxVxG domain (Turk et al. 1997). The second structural element to enter the active site is a hairpin loop situated in the C-terminal part of the cystatin protein, containing the proline-tryptophan residues (Bode et al. 1988). The third element located in the N-terminal region is a conserved glycine residue which is essential for strong inhibition of CYPs (Barrett et al. 1998).

PhyCys have become scientifically important because of the regulatory and protective effects in plant tissues (Megdiche et al. 2009). Specifically, PhyCys control proteolytic processes that regulate plant development and responses to biotic and abiotic stimuli (Belenghi et al. 2003; Felton and Korth 2000; Gaddour et al. 2001; Valdés-Rodriguez et al. 2007). These proteolytic processes are a consequence of activation of CYPs, which may ultimately lead to programmed cell death (PCD). Belenghi et al. (2003) demonstrated that an *Arabidopsis thaliana* PhyCys (AtCYS1) inhibits pathogen-induced programmed cell death and that short-term exogenous application of the nitric oxide (NO) donor sodium nitroprusside (SNP) at a concentrations currently considered as very high [on the basis of the level of NO produced by such concentrations of SNP versus the endogenous levels of NO that plant cells can produce (Ederli et al. 2009; Keyster et al. 2010; Leach et al. 2010)] induced expression of AtCYS1. The SNP-induced expression of AtCYS1 suggests that exogenously applied NO regulates CYPs activity by controlling cystatin gene expression. However, the effect of changes in endogenously

synthesized NO on cystatin gene expression and CYP activity in plants has not been reported. Furthermore, we recently demonstrated a role for endogenously synthesized NO (via nitric oxide synthase) in regulating cell death and have linked this role to CYP activity in soybean root nodules (Chapter 2; Leach et al. 2010). It is noteworthy that there is evidence for negative influence of SNP, especially at high concentrations, on plant metabolism and physiology (Wodala et al. 2010; Murgia et al. 2004) and so the use of SNP at elevated concentrations as NO donor is questionable from a physiological perspective. This casts doubts on assignment of SNP effects on molecular/physiological functions as being reflective of *bona fide* effects of physiologically attainable NO levels *in planta*. We thus investigated the role of nitric oxide synthase (NOS)-derived NO on the expression of a novel soybean cystatin (homologous to AtCYS1 and referred to here as GmCYS1p626) and the influence of this cystatin on CYP activity.

The aim of the work reported here includes the following:

a) Identification of GmCYS1p626

b) Characterization of the cystatin activity of recombinant GmCYS1p626

c) Determination of the influence of nitric oxide synthase enzymatic activity on gene expression of GmCYS1p626

3.3 Materials and Methods

3.3.1 Identification of GmCYS1p626

The ACBU1760 (renamed GmCYS1p626) EST was obtained from the SoyBase (http://soybase.org) database using the Basic Local Alignment Tool (BLAST) with AtCYS1 as the

query sequence. Amino acid sequences of GmCYS1p626 and AtCYS1 was aligned using the Geneious software version 4.8.3.

3.3.2 Plant Growth

Soybean (*Glycine max* L. merr. cv. PAN 626) seeds were surface-sterilized in 0.35% sodium hypochlorite for 10 min, followed by five washes with sterile distilled water. The seeds were imbided in sterile distilled water for 1 hour. The seeds and inoculated with *Bradyrhizobium japonicum* supplied as the commercial peat-based HiStick2 Soybean Inoculant (Becker Underwood Ltd., West Sussex, United Kingdom). The *G. max* seeds were sown in 1 litres 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). 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 until the V4 stage. Plants of the same phenological stage and similar height were selected for all experiments.

3.3.3 Treatment of plants

Treatments were initiated at the V3 stage and done for 24 hours. For these treatments, plants were supplied with either nitrogen-free nutrient solution or nitrogen-free solution containing the nitric oxide synthase (NOS) (EC 1.14.13.39) inhibitor N-ω-nitro-L-arginine [L-NNA

(1 mM)] and the nitric oxide donor 2,2'-(hydroxynitrosohydrazono)bisethanimine [DETA/NO (200 µM)] in combination with 1 mM L-NNA. Nodules were harvested, frozen in liquid nitrogen, and stored at -80°C until used for protein and RNA extraction.

3.3.4 Molecular cloning of GmCYS1p626

Snap-frozen nodule tissue was milled to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted as previously described (Gasic et al. 2004). The total RNA was treated with RNase-free DNase to remove any remaining DNA. This was followed by cDNA synthesis with reverse transcription (using the reverse primer) on 0.2 µg of the total RNA using the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's protocol. The reverse transcription products (2 µl of each) were used in a polymerase chain reaction (PCR) using the following oligonucleotides (10 pmol each): forward (5'-GTTGAG**GGATCC**ATGGCAGCACTTGGTGGCAATC-3') and reverse (5'-GTCAAT**GTCGAC**CTATGCAGGTGCATCTCCAAC-3'). PCR conditions were as follows: 3 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 30 sec at 62°C and 1 min at 72°C, then 10 min at 72°C catalysed by 1 U of *Pfu* DNA Polymerase (Fermentas).

The amplification product was cloned in frame in the restriction sites (bold underlined in oligonucleotide sequences) BamHI and SalI of pET-41a(+) (Novagen) and chemically transformed into *E.coli* KRX (Promega). The recombinant clones were sequenced on an ABI Prism 377 DNA sequencer using the Big Dye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems).

3.3.5 Purification of recombinant GmCYS1p626

Single colonies of KRX cells harboring T7 promoter-based pET-41a(+) expression vectors were grown overnight in Luria Bertani (LB) supplemented with kanamycin (kan) (30 µg/ml) at 37 °C. Overnight cultures were diluted 1:100 into auto-induction media (LB, 0.05% glucose, 0.1% rhamnose and 30 µg/ml kan) and grown for 18 h at 25 °C. After induction, the cells were harvested and lysed by sonication in 10 mM phosphate buffer (pH 8); the lysate was fractionated into soluble and precipitate fractions by centrifugation at 12,000 X g and then subjected to SDS−PAGE.

Pellets from 1 ml of each expression culture were resuspended in 0.5 ml of 50 mM HEPES (pH 7.5), frozen for 20 min at −70 °C, thawed at room temperature and lysed by addition of 0.5 ml lysis buffer [1X FastBreak Cell Lysis Reagent (Promega), 0.2 mg/ml lysozyme (Sigma) and 20 U RQ1-DNase (Promega)] for 30 min with slow rotation mixing. These crude lysates were used as total expression fractions, and soluble expression fractions were prepared from the crude material by high speed centrifugation (12,000 X g) at 4 °C for 30 min. Total and soluble fractions were analyzed by 12% SDS-PAGE (BioRad) and proteins were detected by SimplyBlue staining (Invitrogen). PageRuler^{TM} Prestained Protein Ladder (Fermentas) was used as reference marker to estimate size and expression levels.

Protein purification was done by applying the supernatants directly onto glutathione agarose (Sigma) purification resin at a ratio of 50:1 original culture volume to settled resin. Binding to the resin was conducted at 4°C for 1 h with constant end-over-end gentle rotation, followed by a wash of the resins with 10X resin volume of the appropriate purification buffer

(50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT and 0.5 mM EDTA). The target protein was recovered by eluting the bound proteins off of the glutathione agarose resin with 5 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0). Protein yields were quantified as described by the manufacturer for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories, Inc., Hercules, CA).

3.3.6 Assay for cystatin activity of GmCYS1p626

Purified recombinant GmCYS1p626 was tested for cystatin activity using papain (Sigma, EC 3.4.22.2) as the model CYP enzyme. Papain (20 µg) was prepared in buffer containing 100 mM Tris-HCl (pH 6.2), 5 mM MgCl$_2$, 2 mM EDTA, 10 % (v/v) glycerol and 10 mM β-mercaptoethanol. Recombinant GmCYS1p626 (20 µg) was added to the mixture followed by incubation for 10 min at 25°C. As substrate, 0.5 mM Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) was added to the reaction mixture and incubated at 37°C for 60 minutes. CYP activity was determined by measuring absorbance at 405 nm after every 20 min. As controls, 20 µg of either boiled recombinant GmCYS1p626 (boiled at 95°C for 20 min), GST or 10 µM l-trans-epoxysuccinyl-leucyl-amido-(4-guanidino)butane (E64) was added to the reaction mixture to substitute the active recombinant GmCYS1p626.

GmCYS1p626 specificity to CYPs only was tested using 20 µg trypsin (Sigma, EC 3.4.21.4) (serine protease) prepared in a buffer containing 100 mM Tris-HCl (pH 8) and 50 mM CaCl$_2$. Recombinant GmCYS1p626 (20 µg) was added to the reaction mixture followed by incubation for 10 min at 25°C. The reaction was initiated by addition of 0.5 mM Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) to the reaction mixture and incubated at 37°C for 60

minutes. Serine protease activity was determined by measuring absorbance at 405 nm after every 20 min.

GmCYS1p626 was tested for cystatin activity on soybean nodule CYPs using a modified procedure of Zhang et al. (2008). Protein extraction was done using 100 mg of nodule tissue, homogenised in 1 ml of buffer containing 100 mM Tris-HCl (pH 6.2), 5 mM $MgCl_2$, 2 mM EDTA, 10 % (v/v) glycerol and 10 mM β-mercaptoethanol. For CYP activity 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to inhibit serine proteases in the homogenate prior to incubation of the mixture at 37°C for 5 min. To test for inhibition, 20 μg of recombinant GmCYS1p626 was added to the mixture and incubated for 10 min at 25°C. Then, 0.5 mM Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) was added as substrate to the reaction mixture and incubated at 37°C for 60 minutes. CYP activity was determined by measuring absorbance at 405 nm every 20 min. As controls, 20 μg of either boiled recombinant GmCYS1p626 (boiled at 95°C for 20 min) or GST was added to the reaction mixture as substitute for the active recombinant GmCYS1p626.

3.3.7 GmCYS1p626 gene expression studies

Snap-frozen nodule tissue was ground in liquid nitrogen to a fine powder using a mortar and pestle. Total RNA was extracted as described by Gasic et al. (2004). The total RNA was treated with RNase-free DNase to remove residual DNA. This was followed by cDNA synthesis with reverse transcription (using the reverse primer) on 0.1 μg of the total RNA using the RevertAid[TM] First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's protocol. The reverse transcription products (2 μl of each) were used in a polymerase chain

reaction (PCR) using the following oligonucleotides (10 pmol each): β-tubulin (forward primer: 5'-CTGCGAAAGCTTGCAGTGAACC-3'; reverse primer: 5'-TCTTGCCTCTAAACATGGCTGAGG-3') and GmCYS1p626 forward primer: 5'-GTTGAGGGATCCATGGCAGCACTTGGTGGCAATC-3'; reverse primer: 5'-GTCAATGTCGACCTATGCAGGTGCATCTCCAAC-3'). PCR conditions were as follows: 2 min at 95°C, followed by 25 cycles of 20 sec at 95°C, 20 sec at 62°C and 30 sec at 72°C, then 7 min at 72°C catalysed by 1 U of TrueStart^{TM} Hot Start *Taq* DNA Polymerase (Fermentas). The PCR products (10 µl of each) were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide (EtBr), followed by image acquisition on the AlphaImage 2200 system (Alpha Innotech Corporation).

Densitometry analysis was done using the Spot Denso tool (AlphaEase FC imaging software, Alpha Innotech Corporation). Individual gels were scored as relative densitometry values (Pixel intensities) of three independent gels used for expression analysis.

3.3.8 Cysteine protease assay

Soybean nodules were assayed for CYP activity using a procedure of Zhang et al. (2008) with slight modification. For this assay, 100 mg of nodule tissue was homogenised and extracted in 1 ml of buffer containing 100 mM Tris-HCl (pH 6.2), 5 mM $MgCl_2$, 2 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated at 37°C for 5 min. Then, 0.5 mM Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) was added as substrate to the reaction mixture and incubated at 37°C for 60 minutes. CYP activity was determined by measuring absorbance at 405 nm every 20 min (using the extinction co-efficient of 9.6 $mM^{-1}.cm^{-1}$).

3.3.9 Cell viability analysis using Evans Blue staining

Cell viability assays was done using a modified method of Sanevas et al. (2007). Briefly, soybean nodules (100 mg per treatment) from five different plants of each of the treatment were harvested and stained with 0.25% (w/v) Evans Blue for 15 minutes at room temperature. The nodules were washed for 30 min in distilled water, followed by extraction of the Evans Blue stain (taken up by dead nodule cells) from nodule 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 taken up by the nodule tissue.

3.3.10 Statistical analysis

One-way analysis of variance (ANOVA) test was used to analyze all data and means were compared by the Tukey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

3.4 Results

3.4.1 Identification of GmCYS1p626

Homology searches, using AtCYS1 protein sequence as a query against the soybean EST database (SoyBase), identified a soybean protein consisting of 97 amino acids with 65% similarity to AtCYS1 (Figure 3.4.1). The conserved N-terminal glycine residue and N-terminal motif, namely [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N, were identified in GmCYS1p626 together with the centrally located QxVxG motif (Figure 3.4.1). However, the

prolilne residue in the C-terminal just before the tryptophan residue is substituted by alanine whereas the tryptophan is still conserved (Figure 3.4.1).


```
Identities = 45/103 (43%), Positives = 67/103 (65%), Gaps = 8/103 (7%)

NP_196775,AtCYS1     :   1 MADQQAGTIVGGVRDIDANANDLQVESLARFAVDEHNKNENLTLEYKRLLGAKTQVVAGT  60
                           +GG RD+  + N L+++ LARFAV+EHNK +N  LE+++++ AK QVV+GT
ACBU1760,GmCYS1p626  :   1 ------MAALGGNRDVAGSQNSLEIDGLARFAVEEHNKKQNALLEFEKVVSAKQQVVSGT  54


NP_196775,AtCYS1     :  61 MHHLTVEVADGETNKVYEAKVLEKAWENLKQLESFNHLHDV--  101
                           ++ +T+E  DG   KVYEAKV EKAW N K+++ F  + D
ACBU1760,GmCYS1p626  :  55 LYTITLEAKDGGQKKVYEAKVWEKAWLNFKEVQEFKLVGDAPA   97
```

Figure 3.4.1 Alignment of AtCYS1 to GmCYS1p626. Arrows signify conserved amino acid residues in plant cystatins. The conserved N-terminal glycine is in red underlined font. The conserved N-terminal motif is in red font, whereas the conserved centrally located motif is signified by the blue font. The conserved tryptophan residue in the C-terminus is denoted by the underlined blue font.

3.4.2 Cloning, recombinant expression and purification of GmCYS1p626

RT-PCR amplification using GmCYS1p626-specific primers yielded an amplicon of 320 bp (Figure 3.4.2 A), which corresponds to the expected size for the coding region in the GmCYS1p626 cDNA inclusive of the additional nucleotides added to facilitate restriction endonucleases digestion for subsequent cloning purposes. The encoded protein was recombinantly expressed as a glutathione *S*-transferase (GST) fusion protein in pET-41a(+) to produce a protein (GST-GmCYS1p626) of ± 35 kDa (Figure 3.4.2 B), corresponding to the expected size of the recombinant protein. Upon purification of the recombinant protein on a glutathione agarose column (Figure 3.4.2 C), the recombinant protein was used for assays to determine its cystatin activity.
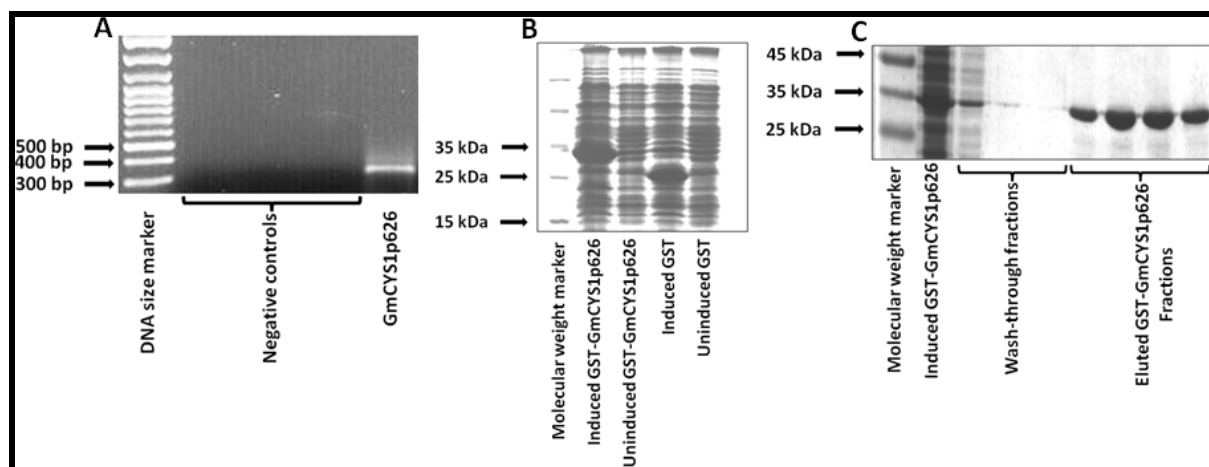
Figure 3.4.2 Cloning, recombinant expression and purification of GmCYS1p626. (A) Isolation of GmCYS1p626 by RT-PCR. Arrows indicate DNA size (in bp). (B) Expression of GmCYS1p626 as a recombinant protein in *E. coli* on pET-41a (+). Recombinant GmCYS1p626 is represented by the thick band at ± 35 kDa in the lane denoted as 'Induced GST-GmCYS1p626' and arrows indicate protein size (in kDa). Recombinant GST is represented by the thick band at ± 25 kDa in the lane marked 'Induced GST'. (C) Purification of recombinant GST-GmCYS1p626 using glutathione agarose, followed by elution of the purified protein with glutathione. Purified recombinant GST-GmCYS1p626 is represented by the thick bands denoted 'Eluted GST-GmCYS1p626 Fractions'.

3.4.3 Assay for cystatin activity of GmCYS1p626

Purified recombinant GmCYS1p626 was tested to determine if it has cystatin activity. For this assay, papain was used as it is the best studied representative of enzymes belonging to the CYP family (Czaplewski et al. 1999). On the other hand, GST was used as a negative control to determine if any cystatin activity of the recombinant protein was attributable to the GST fusion partner. Furthermore, the irreversible cysteine protease inhibitor (CPI), E64, was used as a positive control for efficient cysteine protease inhibition (Figure 3.4.3 A).

The recombinant GmCYS1p626 reduced the CYP activity of papain by ± 70%, whereas boiled recombinant GmCYS1p626 failed to inhibit papain's protease activity (Figure 3.4.3 A).

Recombinant GST alone had no effect on the CYP activity of papain but E64 suppressed the CYP activity of papain by ± 95% (Figure 3.4.3 A). On the other hand, protease activity detected for trypsin (a serine protease) was ± 30% higher than that of papain and was not affected by GmCYS1p626 (Figure 3.4.3 A). To test the efficacy of GmCYS1p626 as cysteine protease inhibitor in soybean, the cystatin activity of the recombinant protein was evaluated on nodule extracts. Recombinant GmCYS1p626 reduced the CYP activity of the nodule extracts by ± 40% (Figure 3.4.3 B). However, boiled recombinant GmCYS1p626 and recombinant GST had no effect on nodule extract CYP activity (Figure 3.4.3 B).
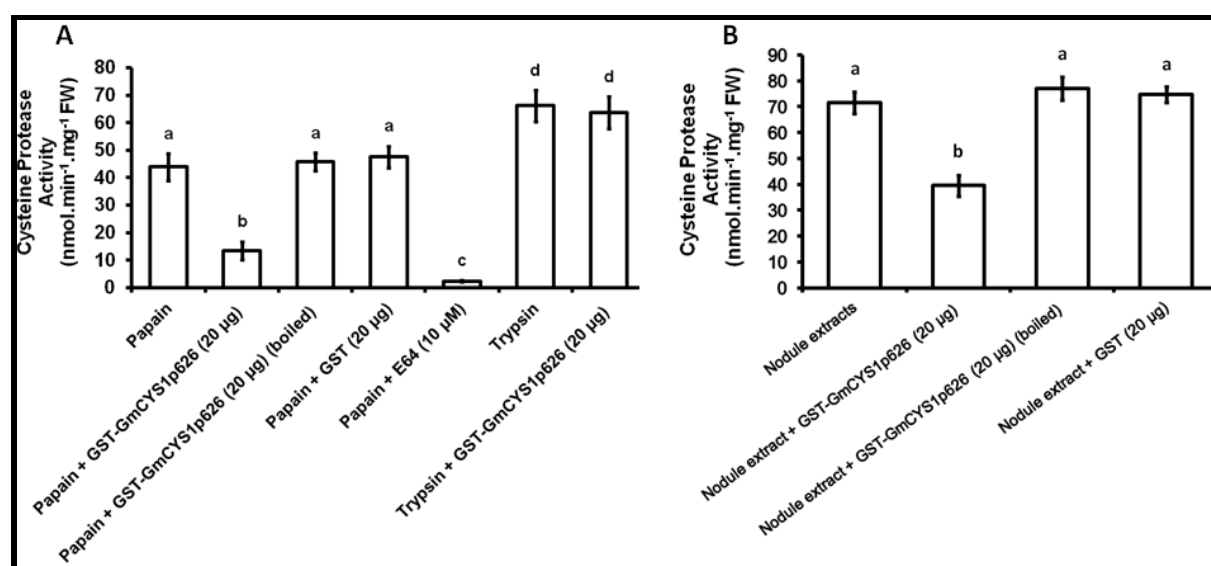
Figure 3.4.3 Assay for cystatin activity of GmCYS1p626. (A) Cystatin activity of GmCYS1p626 on papain and trypsin. Recombinant GmCYS1p626 was tested for its ability to inhibit the enzymatic activity of a *bona fide* cysteine protease, papain. Recombinant GST was also used as a negative control whereas the irreversible cysteine protease inhibitor, E64, was used as a positive control for the cystatin activity assays. Trypsin, a serine protease, was used to ascertain the specificity of GmCYS1p626 for cysteine proteases instead of serine proteases. (B) Cystatin activity of GmCYS1p626 on soybean nodule extracts. Recombinant GmCYS1p626 was tested for its ability to inhibit the enzymatic activity of cysteine proteases contained in soybean nodule protein extracts. Recombinant GST and boiled recombinant GmCYS1p626 were also used as negative controls for the cystatin activity assays. Error bars represent the mean (± SE; n = 3) and the data are representative of three independent experiments.

3.4.4 Regulation of the expression of the gene encoding GmCYS1p626 by nitric oxide synthase

Since GmCYS1p626 is a homologue of AtCYS1 and the expression of the gene encoding AtCYS1 is induced by exogenously applied NO (as SNP) at fairly high concentrations (Belenghi et al. 2003) but it is unknown if NOS activity is required for basal levels of expression of genes encoding proteins belonging to this family of small (Group-1) cystatins, it should be important to determine how NOS enzymatic activity influences the expression of these cystatins. Semi-qRT-PCR was thus performed on RNA from soybean root nodules upon treatment of nodulated soybean plants with the nitric oxide synthase inhibitor L-NNA and a combination treatment with L-NNA plus the nitric oxide donor DETA/NO.

Visual inspection of the semi-qRT-PCR amplicons on ethidium bromide-stained agarose gels revealed that none of the treatments affected the expression of β-tubulin significantly (Figure 3.4.4 A) but L-NNA reduced the expression of GmCYS1p626 and the L-NNA-induced decline in GmCYS1p626 gene expression was reversed by DETA/NO to a level similar to the untreated control (Figure 3.4.4 B). Densitometry analysis showed that the expression of β-tubulin is not altered by any of the treatments (Figure 3.4.4 C) but L-NNA reduces the expression of GmCYS1p626 by ± 30% compared to the untreated control and the L-NNA-induced decline in GmCYS1p626 gene expression was reversed by DETA/NO to a level similar to the untreated control (Figure 3.4.4 D).
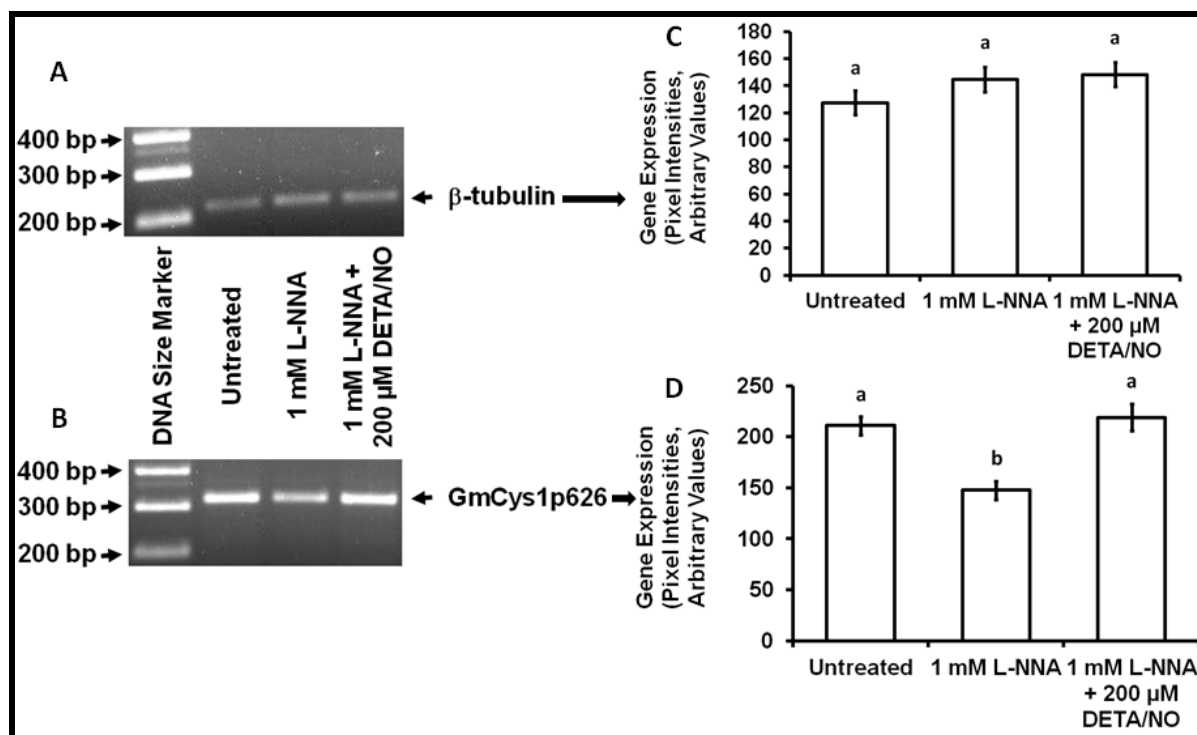
Figure 3.4.4 Changes in the expression of the gene encoding GmCYS1p626 in response to inhibition of NOS enzymatic activity. Gene expression for β-tubulin in root nodules in response to inhibition of NOS enzymatic activity by L-NNA and in response to restoration of nodule NO content in L-NNA-treated soybean by application of DETA/NO as observed in ethidium bromide-stained agarose gels (A) and analyzed by densitometry (C). Effect of L-NNA and the combination of L-NNA plus DETA/NO on the expression of GmCYS1p626 in root nodules as noted visually in ethidium bromide-stained agarose gels (B) and analyzed using densitometry (D). Gel images are representative examples of out of three independent experiments and error bars signify the means (± SE; n = 3) from three independent experiments.

3.4.5 Regulation of nodule cysteine protease activity and cell death by nitric oxide synthase

Given that GmCYS1p626 inhibits nodule CYP activity and the maintenance of optimal basal

levels of expression of the gene encoding GmCYS1p626 requires NO derived from NOS, it was

deemed appropriate to determine how NOS enzymatic activity influences root nodule CYP

activity. Furthermore, given that programmed cell death is regulated by CYP activity (van der

Hoorn et al. 2004), it would be important to determine how the influence of NOS enzymatic

activity on CYP activity affects cell death in the soybean root nodules. The efficiency of L-NNA to inhibit NOS enzymatic activity in soybean nodules was discussed in chapter 2. CYP activity and cell death were thus evaluated in root nodules upon treatment of nodulated soybean plants with the nitric oxide synthase inhibitor L-NNA and a combination treatment with L-NNA plus the nitric oxide donor DETA/NO.

L-NNA increased the CYP activity in root nodules by ± 65% compared to the untreated control and the L-NNA-induced increase in nodule CYP activity was reversed by DETA/NO to a level similar to the control (Figure 3.4.5 A). Along with changes in CYP activity, L-NNA augmented cell death in the soybean root nodules by ± 120% compared to the untreated control but this increase in cell death was reversed by DETA/NO to the same level as that determined for the untreated control (Figure 3.4.5 B).
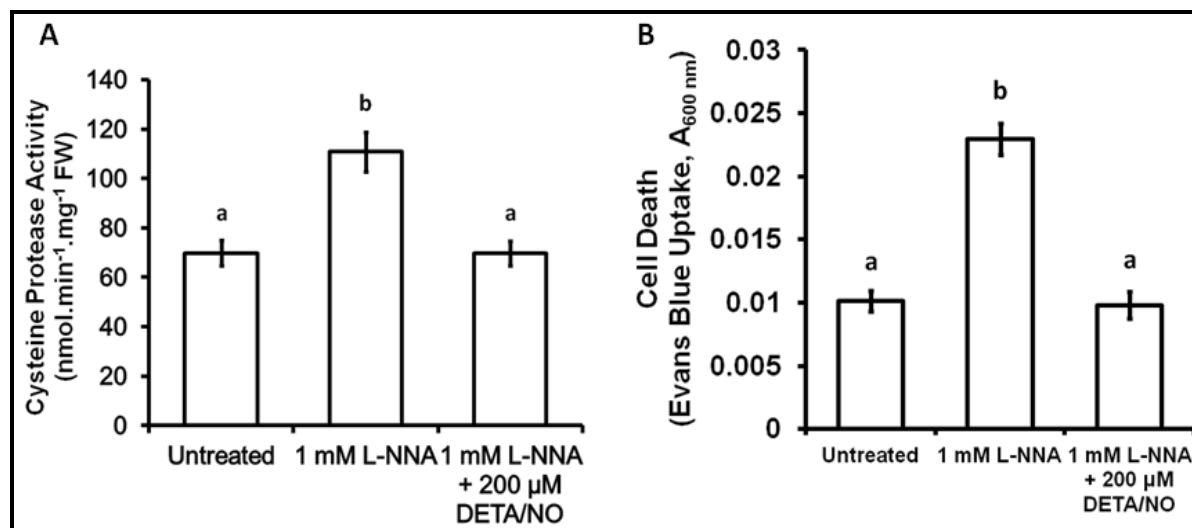
Figure 3.4.5 Cysteine protease activity and cell death in soybean root nodules in response to inhibition of NOS enzymatic activity. (A) Cysteine protease activity in root nodules in response to inhibition of NOS enzymatic activity by L-NNA and in response to restoration of nodule NO content in L-NNA-treated soybean by application of DETA/NO. (B) Effect of L-NNA and the combination of L-NNA plus DETA/NO on nodule cell death. Error bars represent the mean (± SE; n = 4) and the data are representative of three independent experiments.

3.5 Discussion

This study has identified a novel cystatin (GmCYS1p626) with significant primary sequence homology to AtCYS1 and belonging to Group-1 of the PhyCys family. Besides GmCYS1p626, several other soybean sequences with significant homology to AtCYS1 and belonging to either Group-1 or Group-2 of PhyCys were identified but the study presented here focuses only on GmCYS1p626 because AtCYS1 belongs to Group-1 PhyCys, was amongst PhyCys that had the highest homology to AtCYS1 and was the only sequence not deposited in the GenPept database of NCBI. On this basis and the study's search for a novel soybean cystatin belonging to Group-1 PhyCys, GmCYS1p626 was chosen for further analysis.

Conserved residues diagnostic of plant cystatins were also identified in GmCYS1p626. The fact that GmCYS1p626 has a molecular weight of 11 kDa and contains all the conserved amino acid residues found in Group-1 PhyCys, except that the proline residue (at the conserved Pro-Trp position in the C-terminus) is substituted with alanine, confirms that GmCYS1p626 belongs to this group of plant cystatins (Figure 3.4.1). The proline-alanine substitution appears to be the norm in several other Group-1 cystatins (Dutt et al. 2010).

Further evidence confirming that GmCYS1p626 is a cystatin comes from the fact that recombinant GmCYS1p626 inhibits the CYP activity of both papain and soybean nodule extracts but fails to inhibit the proteolytic activity of the serine protease trypsin; whereas boiled GmCYS1p626 has no inhibitory activity against the CYP activity of papain or soybean nodule extracts (Figure 3.4.3). The inhibitory effect of recombinant GmCYS1p626 must be from

GmCYS1p626 and not the GST fusion partner because no cystatin activity could be detected for GST (Figure 3.4.3).

The fact that GmCYS1p626 is a homologue of the AtCYS1 and the expression of this Arabidopsis cystatin (AtCYS1) is induced by the NO donor SNP (at concentrations ranging from 0.5 mM to 5 mM SNP); and appears not to be expressed in some organs without exogenously applied NO or biotic/abiotic stress (Belenghi et al. 2003); prompted us to investigate if the expression of GmCYS1p626 is regulated by NOS enzymatic activity since NOS is one of the sources of NO in plants. Given that recent studies suggest that SNP has negative effects on plant molecular and physiological processes (Murgia et al. 2004; Wodala et al. 2010), together with the fact that DETA/NO releases NO in the same redox state as the direct *bona fide* product of NOS enzymatic activity demonstrated to stimulate guanylate cyclase (GC, EC 4.6.1.2) activity (Dierks and Burstyn 1996; Goyal and He 1998), it is undesirable to use SNP to study the effects of NO on plant molecular and physiological processes. Furthermore, SNP concentrations as high as those used by Belenghi et al. 2003 may lead to nitrosative stress on the plant as a result of excessive NO levels and impair plant redox homeostasis as a consequence of high cyanide levels accumulated as a by-product of breakdown of SNP. DETA/NO is thus one of the ideal NO donors for such investigations.

The results presented in this study indicate that GmCYS1p626 is expressed in soybean root nodules at basal levels; however, the expression of GmCYS1p626 is reduced when NOS enzymatic activity is inhibited. This indicates that NOS activity is required for optimal expression of GmCYS1p626 in soybean root nodules. The expression of GmCYS1p626 was restored by the

addition of DETA/NO to the L-NNA-treated soybean nodules, confirming that it is the NO produced from NOS activity that regulates GmCYS1p626. The fact that only a reduction of ± 30% in the expression of GmCYS1p626 occurs upon inhibition of NOS activity by L-NNA is likely related to the efficiency of L-NNA in inhibiting NOS in the period of the treatment with L-NNA but may also be an indication that NO from other sources besides NOS (e.g. nitrate reductase) could still maintain expression of GmCYS1p626, albeit at lower levels than when NOS activity is not inhibited.

It is important to note that inhibition of NOS enzymatic activity increases CYP activity in soybean nodules and that this is associated with increased nodule cell death (Figure 3.4.5). Given that cysteine proteases (CYPs) are instrumental in the execution of PCD (Solomon et al. 1999) and the fact that the increase in nodule CYP activity caused by inhibition of NOS enzymatic activity is accompanied by an increase in nodule cell death (Figure 3.4.5) suggests that the cell death is triggered by increased CYP activity and may perhaps be PCD, but it remains to be determined if the observed cell death is truly via a PCD pathway. Nonetheless, it is clear from the study here that NOS enzymatic activity is required for maintenance of cell viability/because reduced NOS activity triggers cell death and it is probable that inhibition of NOS enzymatic activity triggers a PCD pathway (mediated via augmented CYP activity) presented as elevated cell death in the nodules. Evidence that such a cell death pathway is due to NO from NOS enzymatic activity comes from the fact that the cell death reverts to levels similar to untreated plants if the L-NNA (NOS inhibitor) treatment is supplemented with the nitric oxide donor DETA/NO. The discovery that endogenously synthesized NO is required for maintenance of plant cell viability is novel. The fact that this study shows that NOS enzymatic

activity is required for optimal basal level of expression of the cystatin GmCYS1p626, coupled with the fact that the same study shows that inhibition of NOS enzymatic activity increases CYP activity, suggests that maintenance of optimal basal cystatin gene expression (such as that seen for GmCYS1p626) requires NO (synthesized from NOS enzymatic activity) and regulates cysteine protease-dependent cell death. Future experiments to determine if endogenously synthesized NO regulates plant cell death via a PCD pathway should be important in designing biotechnological interventions that may alleviate PCD that occurs in plants during biotic and abiotic stresses as this could be a tool for enhancing plant tolerance to such stresses.

3.6 References

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CHAPTER 4

Contrasting ROS metabolism in two maize genotypes determines their tolerance to salt stress

4.1 Abstract

The response of two maize (*Zea mays* L.) genotypes, namely Grace (salt-tolerant) and Silverking (salt-sensitive), to salt stress (50 mM incremental increase from 0 mM to 150 mM NaCl) were investigated under controlled environmental growth conditions. NaCl reduced various growth parameters and altered the levels of reactive oxygen species along with antioxidant enzyme activities in both genotypes. The salt-tolerant Grace genotype had higher antioxidant capacity and superior cell viability compared to the salt-sensitive Silverking genotype in the presence of salt stress. Furthermore, the Grace genotype had lower cysteine endopeptidase (CYP) enzymatic activity than the Silverking genotype under salt stress. Our results suggest a relationship between salt stress tolerance and antioxidant capacity in maize, while also indicating that salt stress triggers CYP activity. We conclude that superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and CYP enzymatic activities are critical determinants of the contrasting responses of these two genotypes to salt stress tolerance and suggest that comparison of superoxide dismutase enzymatic activity between different genotypes can be used as a screening marker for sensitivity or tolerance to salt stress.

4.2 Introduction

Salinity stress adversely 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 (Parida and Das 2005). The effects of salinity on these processes is partly due to generation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which trigger augmented antioxidant enzyme activities as a defence mechanism against the oxidative damage that would be caused by the ROS if the antioxidant defences are poor (Sairam et al. 2005). Current opinion is that plants with enhanced antioxidant capacity and improved ability to prevent cell death in response to salinity stress may have enhanced tolerance against salt stress (Miller et al. 2010; Tseng et al. 2007; Wu et al. 2008).

Antioxidant enzymes that control the biosynthesis and utilization of antioxidant metabolites such as glutathione and ascorbate to detoxify ROS (Foyer and Noctor 2005; Miller et al. 2010) intricately regulate antioxidant capacity. Furthermore, programmed cell death (PCD) that may be triggered by salt stress-induced ROS overproduction (and thus oxidative stress) may in part be controlled by CYP activity (Miller et al. 2010; Solomon et al. 1999; Wang et al. 2010). Cysteine endopeptidase-specific inhibitory proteins known as cystatins (Solomon et al. 1999) can control the ROS-activated CYP activity and these cystatins thus present a mechanism by which ROS-mediated programmed cell death can be regulated (Chapter 3).

Recent evidence suggests that plant genotypes with contrasting tolerance to some abiotic stresses have contrasting antioxidant enzyme activities when exposed to these stresses. This has been suggested for cowpea (*Vigna inguiculata* L.) and turnip (*Brassica rapa* L.) cultivars during

salinity stress (Maia et al. 2010; Noreen et al. 2010), salt-tolerant *Hordeum marinum* Huds versus salt-sentitive *Hordeum vulgare* L. exposed to salinity stress (Seckin et al. 2010), maize (*Zea mays* L.) seedlings during cadmium stress (Ekmeçi et al. 2008), wheat (*Triticum aestivum* L.) exposed to salt stress (Mandhania et al. 2006), rice (*Oryza sativa* L.) during salt stress (Vaidyanathan et al. 2003) and cotton (*Gossypium hirsutum* L.) seedlings exposed to salt stress (Gossett et al. 1994).

Notwithstanding the extensive number of reports highlighted above on the role of these antioxidant enzymatic activities in regulating plant responses to abiotic stresses, the role of glutathione peroxidase enzymatic activity in abiotic stress responses remains largely ignored and reports on CYP activity as key regulators of abiotic stress responses are limited. Furthermore, short-term effects of salt stress on maize biochemical and physiological responses are well-documented but the long-term effects of salt stress (which are more reflective of field conditions) on such processes are scarce. It was on this basis that we investigated growth responses (by comparing shoot and root fresh weights), antioxidant enzyme activities, ROS accumulation, CYP enzymatic activities and cell death in two maize genotypes with contrasting levels of tolerance (one sensitive and the other tolerant) to salt stress to establish if any relationship exists between the level of salt stress tolerance and the physiological/biochemical processes studied in this report.

4.3 Materials and Methods

4.3.1 Plant growth

Maize (*Zea mays* L.) seeds of commercial proprietary genotypes (kindly donated by Capstone Seeds Pty Ltd, Howick, South Africa) Grace and Silverking were surface-sterilized in 0.35% sodium hypochlorite for 10 minutes, followed by five washes with sterile distilled water. The maize seeds were imbibed in sterile distilled water for 1 hour 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 19.5 cm diameter plastic pots. The sand was kept moist by watering only 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, in a randomized design. 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 (when the collar of the first leaf was visible). Plants of the same phenological stage and similar height were selected for all experiments.

4.3.2 Treatment of plants

Control plants were supplied with nutrient solution every third day. For treatment with NaCl to impose salt stress, 100 ml of 50 mM NaCl in nutrient solution were applied to each plant by adding the solution directly to the sand at the base of the stem of the plant in the pot every

three days during a period of 7 days. The 50 mM NaCl treatment was followed by a similar treatment with a higher NaCl content by applying 100 ml of 100 mM NaCl every three days covering 7 days. This was finally followed by application of 100 ml of 150 mM NaCl every three days during 7 days to complete the incremental increase in the NaCl concentration applied to the plants during the 21-day period. The nutrient solution was supplemented with an additional 5 mM $CaCl_2$ for the 50 mM NaCl treatment, 10 mM $CaCl_2$ for the 100 mM NaCl treatment and 15 mM $CaCl_2$ for the 150 mM NaCl treatment to compensate for the inhibitory effect of NaCl on calcium uptake that could otherwise occur in such salt treatments.

Growth parameters, root cell viability plus shoot and root CYP activities, shoot and root ROS (O_2^- and H_2O_2) levels, shoot and root antioxidant enzyme activities [superoxide dismutase (SOD) activity, ascorbate peroxidase (APX) activity and glutathione peroxidase (GPX) activity], were evaluated. These growth and molecular/biochemical parameters were evaluated after 21 days of salt treatment. Freshly harvested plants were used for measurement of growth parameters and root cell viability but snap-frozen (in liquid nitrogen) tissue were used for all other assays (in which case the tissue was stored at -80°C and used within 2 days).

4.3.3 Analysis of plant growth parameters

Plants were removed from the sand, being careful to avoid any loss of shoots or roots during the up-rooting of the plants. Ten plants from each treatment (nutrient solution only or nutrient solution supplemented with NaCl) were divided into shoots and roots. Each of these plants was scored for fresh weight of the shoots and roots.

4.3.4 Evaluation of cell viability

We determined if the two maize genotypes differed in their ability to maintain root cell viability upon salt stress, as an indication of sensitivity or tolerance of each genotype to the salt stress. For this determination, roots from each genotype were assayed for cell viability as described for plant tissue (Sanevas et al. 2007) at the end of the salt treatment (i.e. after 21 days from the fist salt treatment).

For the cell viability assays, lateral roots (100 mg per treatment) from five different plants of each of the treatment (nutrient solution only or nutrient solution supplemented with NaCl) were harvested and stained with 0.25% (w/v) Evans Blue for 15 minutes at room temperature. The roots were then washed for 30 min in distilled water, 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 taken up by the root tissue.

4.3.5 Measurement of cysteine endopeptidase activity

Cysteine endopeptidases (CYPs) (EC 3.4.22) are vital for the execution of programmed cell death in plant tissue (Naito et al. 2000; Vincent and Brewin 2000; Groten et al. 2006). CYP activity that is instrumental in the execution of programmed cell death in plants in response to salt stress includes caspase 3-like activity (Wang et al. 2010). Given that programmed cell death occurs during plant responses to salt stress (Katsuhara 1997; Wang et al. 2010) and evidence for the involvement of caspase 3-like activity in the regulation of programmed cell death in plant

responses to salt stress (Wang et al. 2010), we investigated if CYP activity differed between the two maize genotypes amongst the salt treatments.

For assaying CYP activity, leaves (only the third youngest leaf of each plant to ensure uniformity and sufficient plant material for the rest of the assays) and lateral roots of each genotype were used at the end of the 21 days of salt treatment. The leaves and roots were assayed for CYP activity using a procedure modified from that of Zhang et al. (2008). For this assay, 100 mg of leaf or root tissue was homogenised and extracted in 1 ml of buffer containing 100 mM Tris-HCl (pH 6.2), 5 mM $MgCl_2$, 2 mM EDTA, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated at 37°C for 5 min. Then, 0.5 mM Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) was added as substrate to the reaction mixture and incubated at 37°C for 60 minutes. CYP activity was determined by measuring absorbance at 405 nm every 20 min.

4.3.6 Measurement of ROS content

In consideration of the fact that one of the responses of plants to salt stress is the accumulation of ROS such as O_2^- and H_2O_2 (Sairam et al. 2005), we investigated if O_2^- and H_2O_2 content differed between the two maize genotypes upon treatment with NaCl.

For O_2^- content, leaves and lateral roots of each genotype were used at the end of the 21 days of salt treatment. The leaves and roots were assayed for O_2^- content using a procedure described by Tewari et al. (2009). Plant tissue (100 mg) was ground in liquid nitrogen and homogenized in 400 μl of cold 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 X g for 30 min at 4°C to obtain the O_2^- extract. The reaction mixture

contained 50 mM Tris-HCl, pH 7.5, 50 µl and 0.5 mM 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT). O_2^- content was calculated based on the reduction of XTT by monitoring absorbance at 450 nm for 4 minutes, using the extinction coefficient of 2.16×10^4 $M^{-1}.cm^{-1}$.

H_2O_2 content was determined in leaves and lateral roots of each genotype at the end of the 21 days of salt treatment. The leaves and roots were assayed for H_2O_2 content based on a method adapted from Velikova et al. (2000). Plant tissue (100 mg) was ground into a fine powder in liquid nitrogen. The tissue was homogenized in 400 µl of cold 5% (w/v) TCA. The homogenate was centrifuged at 12,000 X g for 30 min at 4°C to obtain the H_2O_2 extract. The reaction mixture contained 50 µl of the 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.

4.3.7 Measurement of antioxidant enzyme activities

Differential responses of antioxidant enzymes between salt stress-sensitive and salt stress-tolerant wheat genotypes suggest that antioxidant enzyme activities are crucial in controlling plant responses to salinity stress (Sairam et al. 2005). To determine if a relationship exists between antioxidant enzyme activities and the differential responses of the two genotypes to salt stress; we evaluated total superoxide dismutase activity, total ascorbate peroxidase activity and total glutathione peroxidase activity in leaves and roots of the two maize genotypes in response the 21 days of salt treatment.

Enzyme extracts were obtained from the leaves (only the third youngest leaf of each plant to ensure uniformity and sufficient plant material for the rest of the assays) and lateral roots 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 (molecular weight = 40 000]. The resulting homogenates were centrifuged at 12 000 X g for 30 min and the supernatants were used for enzyme assays.

For total superoxide dismutase (SOD, EC 1.15.1.1) activity, leaves and lateral roots of each genotype were used at the end of each salt (50, 100 and 150 mM NaCl) treatment. The leaves and roots were assayed for SOD activity using a procedure modified from Beyer and Fridovich (1987). The reaction mixture contained 50 mM K_2HPO_4, pH 7.8, 0.1 mM EDTA, 0.025% (w/v) Triton X-100, 0.1 mM xanthine, 6.25 nM xanthine oxidase, 0.1 mM 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) and 10 µl of enzyme extract. The reaction mixture was incubated for 20 minutes at 37°C and absorbance readings were taken at 450 nm. SOD activity was calculated based on the amount of enzyme that was required to cause 50% decrease in the reduction of WST-1.

For total ascorbate peroxidase (APX, EC 1.11.1.11) activity, leaves and lateral roots of each genotype were used at the end of the 21 days of salt treatment. The leaves and roots were assayed for ascorbate peroxidase activity using a procedure adapted from that of Asada (1984). For this assay, enzyme extracts were supplemented with ascorbate at a final concentration of 2 mM. The reaction mixture contained 10 µl of enzyme extract, 50 mM K_2HPO_4, pH 7.0, 0.1 mM EDTA, 50 mM ascorbate, 1.2 mM H_2O_2 in a 200 µl reaction. APX activity was calculated based on

the change in absorbance at 290 nm as ascorbate was oxidised during the reaction, using the extinction co-efficient of 2.8 mM^{-1}.cm^{-1}.

For total glutathione peroxidase (GPX, EC 1.11.1.9) activity, leaves and lateral roots of each genotype were assayed for GPX activity at the end of the 21 days of salt treatment. The assays were carried out according to a method modified from Drotar et al. (1985). The reaction mixture contained 10 µl of enzyme extract, 50 mM K$_2$HPO$_4$, pH 7.0, 2 mM EDTA, 2 mM glutathione (GSH), 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 2.5 Units of glutathione reductase and 0.09 mM H$_2$O$_2$. 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. Nonspecific oxidation of NADPH was determined in the absence of H$_2$O$_2$ and subtracted from the enzyme activity to ensure that only enzymatic (GPX-dependent) oxidation of NADPH was reported.

Protein concentrations for all antioxidant enzyme activities was measured in the extracts used for the antioxidant enzyme activities as described by the manufacturer for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories, Inc., Hercules, CA).

4.3.8 Statistical analysis

One-way analysis of variance (ANOVA) test was used to analyze all data and means were compared by the Tukey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

4.4 Results

*4.4.1 Plant growth parameters*

In order to evaluate the differences in tolerance to salt stress between the two genotypes, we compared the fresh weights of the shoots and roots of the two genotypes at the end of the salt treatment. The growth of both genotypes was negatively affected by salt treatment but reduction in both the shoot and root fresh weight was more severe in the salt-treated Silverking genotype than the salt-treated Grace genotype (Figure 4.4.1 A and Figure 4.4.1 B). Salt treatment resulted in reduction of shoot fresh weight by ± 20% in the Grace genotype compared to its untreated control, in contrast to the ± 65% loss in the shoot fresh weight in salt-treated Silverking compared to its untreated control (Figure 4.4.1 A). Root growth was negatively affected by salt treatment in both genotypes as demonstrated by a ± 30% loss in root fresh weight for salt-treated Grace (in comparison to untreated Grace); which was significantly less than the ± 50% reduction in the root fresh weight that was observed for salt-treated Silverking when compared to the untreated Silverking control (Figure 4.4.1 B).

4.4.2 Cell viability

Upon observing that fresh weight in shoots and roots is more drastically affected by salt treatment in Silverking than in Grace, we postulated that Silverking could be undergoing more cell death than Grace under the salt treatment. We thus measured cell viability at the end of the salt treatment to compare the level of cell death in these two genotypes. Plants treated with salt suffered a loss in cell viability, as indicated by an increase in the uptake of Evans Blue (which is indicative of cell death) in leaves and roots of both genotypes (Figure 4.4.1 C and

Figure 4.4.1 D) upon salt treatment. The level of cell death was higher in salt-treated Silverking

(cell death was increased by ± 100% compared to controls in the leaves and roots of Silverking)

than in salt-treated Grace (cell death was increased by ± 38% compared to the control in the

leaves and roots of Grace); as revealed by cell death estimation in Figure 4.4.1 C and Figure

4.4.1 D.
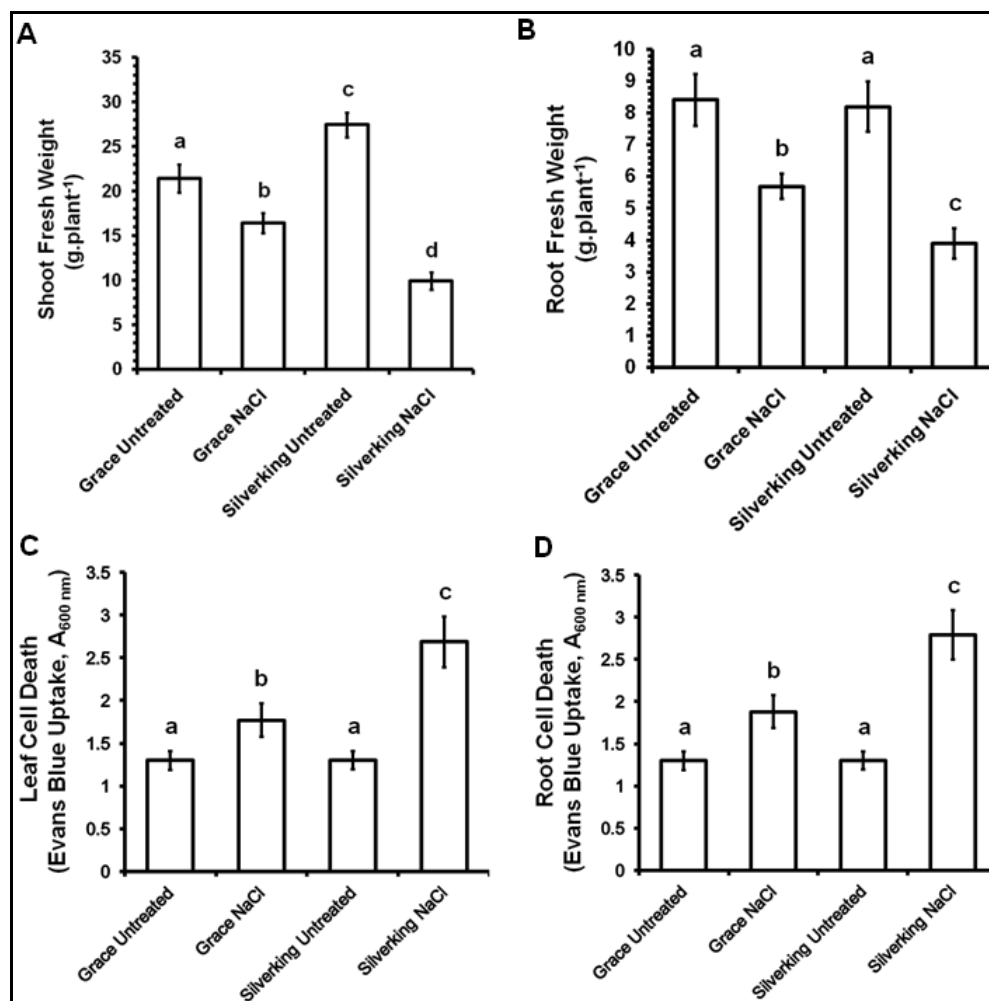
Figure 4.4.1 Effect of salt stress, resulting from treatment with increasing salt concentrations, on shoot (A) and root (B) fresh weight and on cell death in leaves (C) and roots (D) in two maize genotypes (Grace and Silverking). Plants were treated every third day. The first treatment covered a period of 7 days with 50 mM NaCl for the first instance, and then the salt treatment was continued with 100 mM NaCl over the next 7 days, finally followed with 150 mM NaCl over 7 days. Data represent measurements at the end of the entire salt

treatment (i.e. covering a total treatment period of 21 days) and are means ± standard error of ten different plants, representing three independent experiments.

4.4.3 Cysteine endopeptidase enzymatic activity

Abiotic stresses such as salt stress cause generation of ROS, which result in oxidative stress and ultimately cell death if the plant cannot present efficient defences against the stress (Miller et al. 2010; Wang et al. 2010). Such cell death can occur via a PCD pathway triggered by the accumulated ROS via activation of CYP enzymatic activity (Solomon et al. 1999; Wang et al. 2010). It was on this basis, and upon observing differences in the extent of cell death between Grace and Silverking, that we investigated if the level of CYP activity differed between these two maize genotypes.

CYP enzymatic activity increased in leaves and roots for both the Grace and Silverking in response to salt treatment compared to untreated controls (Figure 4.4.2 A and Figure 4.4.2 B). However, the leaf and root CYP enzymatic activity in salt-treated Grace was only ± 65% more than that of the untreated Grace control, in contrast to ± 270% more CYP enzymatic activity for salt-treated Silverking in comparison to the corresponding Silverking control (Figure 4.4.2 A and Figure 4.4.2 B).
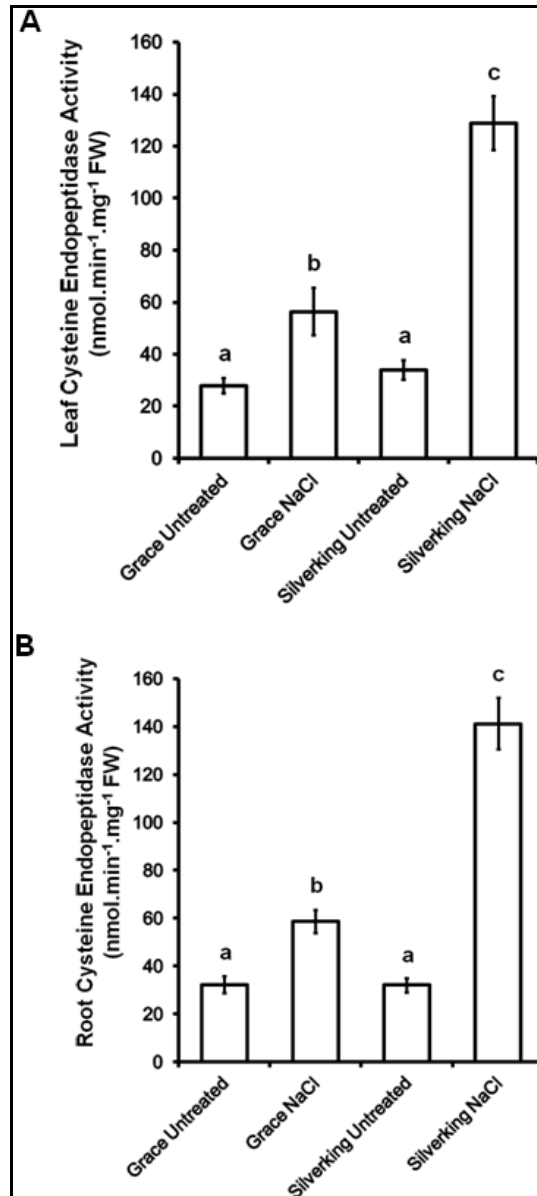
Figure 4.4.2 Changes in cysteine endopeptidase activity in leaves (A) and roots (B) in Grace and Silverking. Data represent measurements at the end of the entire salt treatment (see detailed description of the treatment in the legend to Figure 4.4.1) and are means ± standard error of three different plants for each treatment, representative of three independent experiments.

4.4.4 Superoxide anion content

The observation that salt treatment triggered an differential increases in both cell death and cysteine endopeptidase activity in a genotype-specific manner prompted us to investigate if the salt treatment altered O_2^- content in a similar genotype-specific trend, given that ROS can trigger cysteine endopeptidase-mediated programmed cell death (Solomon et al. 1999; Wang et al. 2010).

Leaf and root O_2^- content increased only slightly (± 55% more leaf and root O_2^- content in salt-treated than untreated control plants) in Grace in response to salt treatment (Figure 4.4.3 A and Figure 4.4.3 B). However, leaf and root O_2^- content increased drastically (± 280% more for leaf and ± 195% more for root O_2^- content in salt-treated than untreated control plants) in the Silverking in response to salt (Figure 4.4.3 A and Figure 4.4.3 B). The fact that the O_2^- content of Grace increases less dramatically in response to salt treatment than observed for the O_2^- content of Silverking (in response to salt) suggests that Grace may have more efficient O_2^- detoxification systems than Silverking.

4.4.5 Total superoxide dismutase enzymatic activity

Superoxide dismutase enzymatic activity is one of the major routes for the detoxification of O_2^- (Beyer and Fridovich 1987; Foyer and Noctor 2005) and is augmented in response to various abiotic stresses in plants, including salt stress (Miller et al. 2010; Mittler et al. 2004). Upon observing that O_2^- accumulation in Grace and Silverking occurs differentially in a genotype-specific manner, we set out to establish if superoxide dismutase enzymatic activity in these two genotypes differs and if it follows the same trend as the O_2^- content.

Leaf and root SOD enzyme activity increased in both the Grace and Silverking genotypes in response to salt treatment. Leaf SOD activity in Grace increased by ± 139% in response to salt treatment compared to the untreated Grace control (Figure 4.4.3 C) whereas root SOD activity for this genotype increased by ± 133% in response to salt treatment when compared to the corresponding control (Figure 4.4.3 D). However, the increase in SOD activity for Silverking was less pronounced than that of Grace, as demonstrated by the fact that SOD enzymatic activity in salt-treated Silverking was limited only to an increase by ± 40% in leaves and ± 50% in roots compared to untreated Silverking controls (Figure 4.4.3C and Figure 4.4.3 D).

Noteworthy is the finding that leaf and root SOD enzymatic activities were significantly different between the two genotypes even in the absence of any salt treatment. This is demonstrated by the fact that the leaf SOD enzymatic activity in Silverking under control conditions (i.e. nutrient solution without NaCl) was only ± 50% of the SOD enzymatic activity observed for Grace (Figure 4.4.3 C), whereas the root SOD enzymatic activity in Silverking under control conditions was ± 70% of the SOD enzymatic activity in Grace (Figure 4.4.3 D).
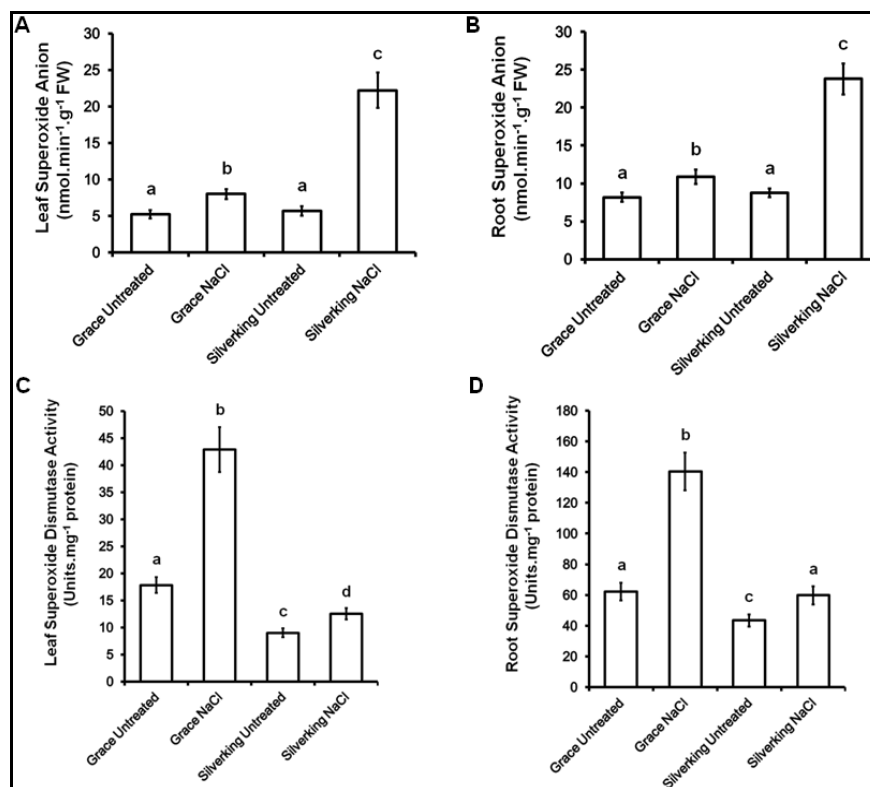
Figure 4.4.3 Superoxide anion content and superoxide dismutase activity of two maize genotypes (Grace and Silverking) in response to salt treatment. Superoxide anion content was determined in both the leaves (A) and the roots (B) of the two maize genotypes. Superoxide dismutase enzymatic activity was measured in leaves (C) and roots (D) of the two maize genotypes. Superoxide anion content and superoxide dismutase activities are reported for plants that were treated every third day (see detailed description of the treatment in the legend to Figure 4.4.1). Data represent measurements at the end of the salt treatment (i.e. covering a total treatment period of 21 days) and are means ± standard error of three different plants, representing three independent experiments.
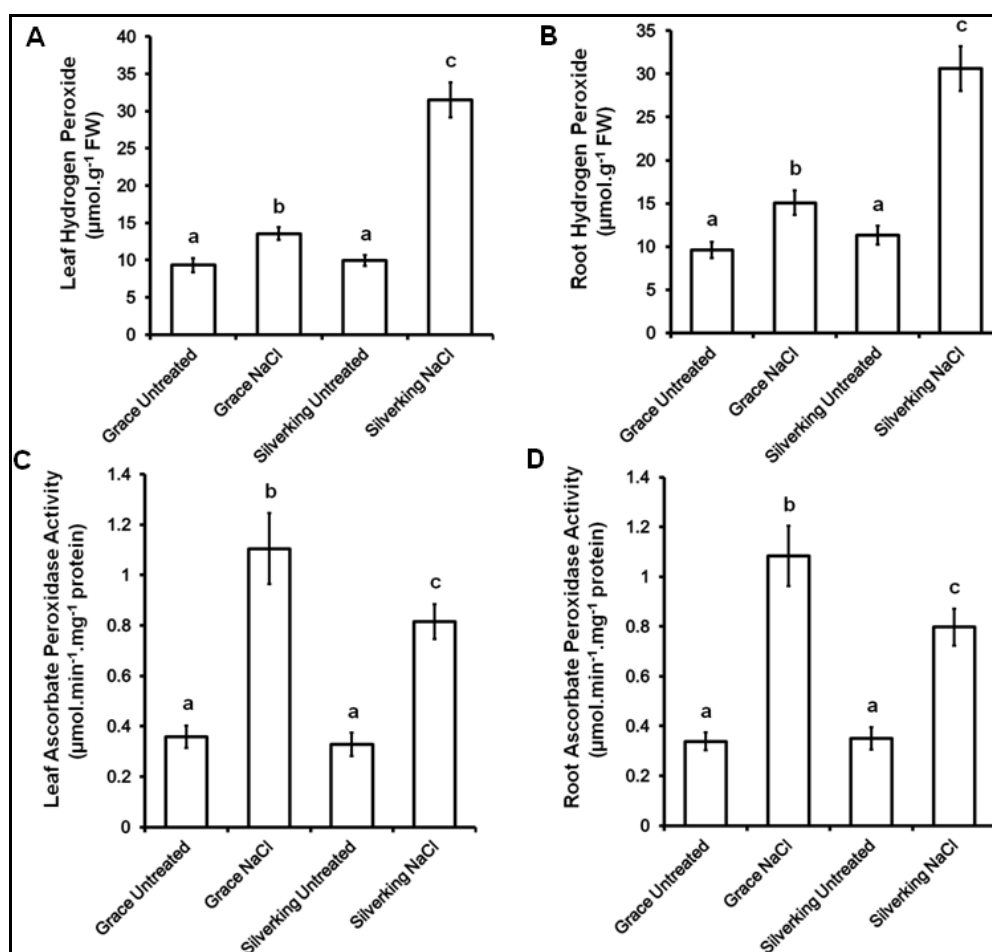
4.4.6 Hydrogen peroxide content

A consequence of detoxification of O_2^- by SOD is the production of H_2O_2 (Beyer and Fridovich 1987; Foyer and Noctor 2005). On the basis that we observed higher SOD activity in Grace than in Silverking in response to salt treatment, we were interested in establishing if the H_2O_2 content in the two genotypes differed in response to salt treatment.

Leaf and root H_2O_2 content increased by ± 55% in salt-treated Grace compared to the corresponding controls, whereas the leaf and root H_2O_2 content increased much more drastically (± 250% increase) for Silverking in response to salt (Figure 4.4.4 A and Figure 4.4.4 B).

4.4.7 Total ascorbate peroxidase enzymatic activity

Given that SOD acts to convert O_2^- into H_2O_2 and O_2 (Beyer and Fridovich 1987; Foyer and Noctor 2005), it would be expected that elevated SOD activity would lead to accumulation of H_2O_2. Accumulation of H_2O_2 can trigger augmented ascorbate peroxidase (APX) activity as an attempt by the cells to detoxify the H_2O_2. We thus measured APX enzymatic activity to establish if the trend of this enzymatic activity observed for SOD would be maintained for APX under the same conditions.

The degree of Increase in APX enzymatic activity in leaves was more pronounced in Grace (an increase of ± 200% in salt-treated Grace compared to untreated Grace) than in Silverking (an increase of ± 125% in salt-treated Silverking compared to untreated Silverking) in response to salt treatment (Figure 4.4.4 C). The level of APX enzymatic activity in roots was more elevated in Grace (an increase of ± 210% in salt-treated Grace compared to untreated Grace) than in Silverking (an increase of ± 120% in salt-treated Silverking compared to untreated Silverking) in response to salt treatment (Figure 4.4.4 D).

Figure 4.4.4 Changes in hydrogen peroxide content and ascorbate peroxidase (APX) activity in Grace and Silverking in response to salt treatments. Leaf H_2O_2 and root H_2O_2 content (Figure 4.4.4 A and Figure 4.4.4 B respectively), together with leaf (Figure 4.4.4 C) and root (Figure 4.4.4 D) APX enzymatic activities are reported for plants treated every third day as described (see detailed description of the treatment in the legend to Figure 4.4.1). Data represent measurements at the end of the entire salt treatment and are means ± standard error of three different plants for each treatment, representative of three independent experiments.

4.4.8 Total glutathione peroxidase enzymatic activity

Although popular opinion suggests that *bona fide* glutathione peroxidase (GPX) enzymes may not exist in plants but proteins thought to be responsible for GPX enzymatic activity are thioredoxin peroxidases/peroxiredoxins (Jung et al. 2002; Navrot et al. 2006), several lines of

evidence indicate that GPX activity exists in plants (Chang et al. 2009; Chen et al. 2004; Drotar et al. 1985; Halušková et al. 2009; Herbette et al. 2002) and may be important for development (Faltin et al. 2010) and responses to abiotic stress (Chang et al. 2009; Chen et al. 2004; Halušková et al. 2009). Similarly to APX (which uses ascorbate to convert H_2O_2 to H_2O), GPX detoxifies H_2O_2 to H_2O but uses glutathione (GSH) instead of ascorbate.

A noteworthy difference in the response of GPX was observed between the two genotypes, namely that the GPX activity of Grace was elevated to a much higher magnitude in response to salt in both leaves and roots whereas that of Silverking was increased only slightly in leaves and roots in response to salt (Figure 4.4.5 A and Figure 4.4.5 B). Figure 4.4.5 A and Figure 4.4.5 B show that leaf and root GPX enzymatic activity increased drastically (± 65% increase in GPX enzymatic activity for salt treatments compared to controls) in response to salt treatment in Grace, whereas the increase in GPX enzymatic activity in response to salt treatment for Silverking was very limited (only ± 33% increase in GPX enzymatic activity for salt-treated Silverking compared to controls for Silverking).
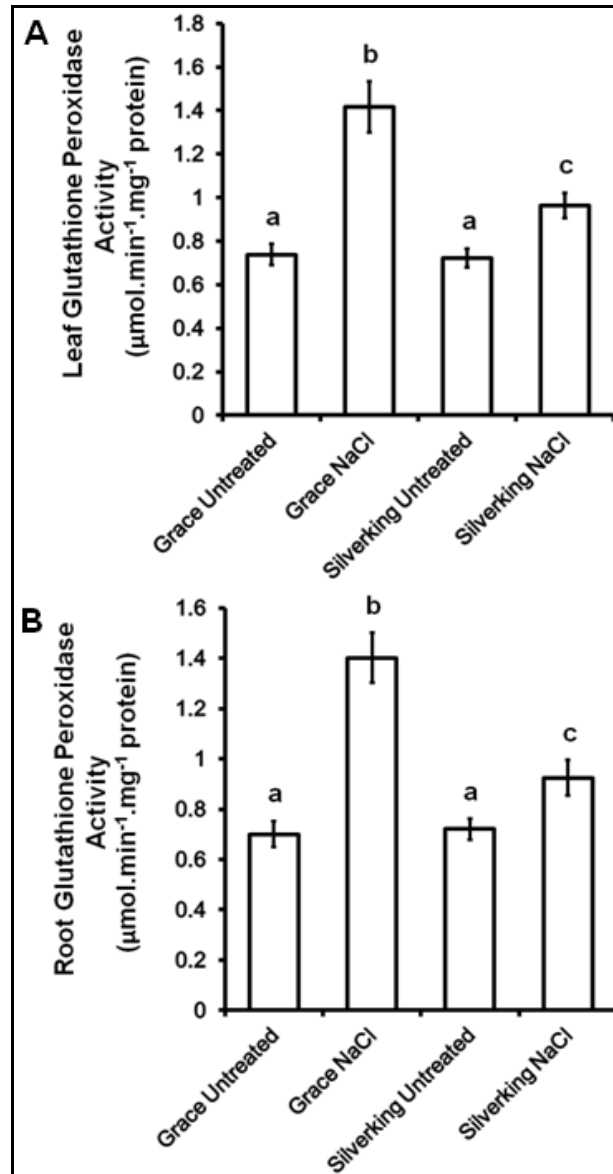
Figure 4.4.5 Glutathione peroxidase (GPX) enzymatic activity of Grace and Silverking in response to salt treatment. GPX enzymatic activity was determined in both the leaves (A) and the roots (B) of the two maize genotypes. GPX enzymatic activities are reported for plants that were treated every third day (see detailed description of the treatment in the legend to Figure 4.4.1). Data represent hydrogen peroxide contents measured at the end the entire salt treatment (i.e. covering a total treatment period of 21 days) and are means ± standard error of three different plants, representing three independent experiments.

4.5 Discussion

Despite the fact that effects of salt stress on growth parameters reported here for the Grace and Silverking genotypes are based on fresh weights of roots and leaves rather than dry weights (indicative of biomass accumulation), the data is sufficient to give an indication of changes in growth parameters because the fresh weights would not be altered if biomass accumulation was not affected. On this basis, the observation that salt treatment induces more extensive loss in growth of Silverking than it does for Grace implies that Silverking can be regarded as more sensitive to salt stress than Grace, whereas Grace can be regarded as more tolerant to salt stress than Silverking. Furthermore, the extent of cell death (loss of cell viability as indicated by the extent of Evans Blue uptake) was more severe and a more pronounced loss in shoot length was observed in Silverking than Grace in response to salt stress (results not shown). We thus refer to Silverking as salt stress-sensitive and Grace as salt stress-tolerant.

The observed increase in cell death in response to salt stress in the two genotypes can either be necrotic death or programmed cell death (PCD) and this remains to be investigated. However, the fact that strong evidence exists for the involvement of PCD in plant responses to salt stress (Katsuhara 1997; Wang et al. 2010) implies that it is highly likely that the cell death observed here for the maize genotypes could be resulting from a PCD pathway. We are currently studying these maize genotypes to investigate if such cell death in response to salt stress is truly a consequence of a PCD process, by examining features that are hallmarks of PCD (DNA fragmentation presented as ladders on agarose gels, cytochrome *c* release and TUNEL assays). A preliminary indication that the cell death is likely to be via a PCD pathway, although

necrotic death cannot be ruled out at this stage, is that CYP activity was augmented in the two maize genotypes in response to salt stress. It has been demonstrated that increased CYP activity (in the form of caspase 3-like activity) in salt-stressed plants is indicative of PCD (Wang et al. 2010). It is thus appropriate to expect that the cell death observed for the two genotypes in response to salt is PCD. Similarly to the results of the cell death assay, CYP activity in the Silverking genotype is higher than that in the Grace genotype in response to salt stress. The involvement of CYP in the response to salt stress was also demonstrated in *Mesembryanthemum crystallinum* leaves in which both mRNA and protein expression was strongly induced by salt (Forsthoefel et al. 1998). In addition, expression of CYP in transgenic Arabidopsis plants affected salt tolerance (Chen et al. 2010).

Our observation that Silverking accumulates higher levels of O_2^- than Grace implies that O_2^- would impose a higher extent of oxidative stress on Silverking than Grace, making Silverking more salt stress sensitive than Grace. Because Silverking accumulates more O_2^- than Grace and that Silverking has less SOD activity in response to salt stress, we conclude that regulation of SOD activity is the one of the most crucial steps in determining the level of salt stress tolerance in the two maize genotypes (and also in the light of differences in SOD activities between the two genotypes even in the absence of salt stress, Figure 4.4.3). Similarly to the present study, the activity of SOD exhibited a greater increase following salt stress in the tolerant maize genotypes than in the sensitive one using other genotypes (de Azevedo Neto 2005). From comparison of pea genotypes with different salt tolerance, it turned out, that SOD was induced both at transcriptional and enzymatic activity level in the tolerant genotype, but it was not affected in the sensitive one (Hernandez et al. 2000). Enhanced salt tolerance was observed in

transgenic tobacco overexpressing SOD (Badawi et al. 2004), which also corroborates the significant role of SOD in the response to salt stress.

The salt-induced changes in SOD enzymatic activity resulted in altered H_2O_2 content in the present study which was also observed in rice (Lee et al. 2001), and the importance of H_2O_2 in the stress response was indicated by its different concentrations in the two maize genotypes after salt stress. In detoxification of H_2O_2 both APX and GPX activity are important and the extent to which the H_2O_2 is removed contributes to the determination of extent of salt tolerance (higher APX and GPX activity results in more efficient removal of H_2O_2 and thus lower H_2O_2 in the salt-tolerant genotype than in the salt-sensitive genotype). The involvement of APX in the response to salt stress was also demonstrated in rice, in which salt treatment resulted in greater APX activity, and certain isoforms were preferentially induced (Lee et al. 2001). In addition, in a salt-tolerant tomato accession, the APX activity was inherently higher than in a salt-sensitive cultivar, and this difference was also observed following salt stress (Shalata and Tal 2002). The participation of GPX in the protection against salt stress was shown in *Oryza sativa* and *Lotus japonicus* at the gene expression level (Kang et al. 2003; Rubio et al. 2009). In addition, overexpression a cDNA encoding an enzyme with both glutathione S-transferase/GPX activity resulted in increased activity of both enzymes and the transgenic tobacco plants grew faster under salt stress than the control ones (Roxas et al. 1997). We acknowledge that the role of GPX enzymatic activity in plants is questionable because a number of plant proteins previously thought to belong to the GPX family of enzymes (on the basis of sequence homology to animal GPX molecules) were proved to be peroxiredoxins/thioredoxin peroxidases (Jung et al. 2002, Navrot et al. 2006). However, it is important to note that GPX activity has been

reported in a number of plant species and the encoding proteins have been confirmed to have GPX activity, shown to be important in plant development and plant responses to abiotic stress (Chang et al. 2009; Chen et al. 2004; Faltin et al. 2010; Halušková et al. 2009; Herbette et al. 2002). Furthermore, the fact that the differences in the degree of change in GPX activity between the two genotypes in response to salt stress was more pronounced than seen for APX may suggest that GPX activity has a higher contribution to the determination of salt stress tolerance than APX in these two genotypes. However, the contribution of catalase enzymatic activity to H_2O_2 removal may also be important.

We thus conclude that antioxidant capacity (i.e. the extent to which antioxidant enzymes detoxify/scavenge ROS) and CYP activity play a crucial role in regulating plant tolerance against salt stress. We extend this conclusion to emphasize the role of superoxide dismutase enzymatic activity as a key indicator of the level of tolerance against salt stress in the two maize genotypes studied here. Furthermore, we show that contrary to current thought, GPX activity exists in plants and is a crucial component of the plant antioxidant system in determining the level of tolerance to salt stress.

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NO confers salt stress tolerance on maize by restricting lipid peroxidation induced by excessive O_2^- and H_2O_2

5.1 Abstract

Salt stress causes accumulation of reactive oxygen species (ROS) to levels that are toxic to plants if the ROS are not efficiently scavenged by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). Recent studies suggests that nitric oxide (NO) can enhance plant tolerance to salt stress but the molecular mechanism for the NO-mediated salt stress tolerance is only partially understood. To understand these molecular mechanisms, maize seedlings were used to analyze the molecular events through which NO regulates plant responses to long-term salt stress. Treatment of maize seedlings with 150 mM NaCl caused inhibition of growth and this was manifested as reduction in shoot and root biomass. Along with the loss in shoot biomass, cell death, cysteine protease enzymatic activity, H_2O_2 content, O_2^- levels and lipid peroxidation were increased in response to salt stress. Application of exogenous NO as the nitric oxide donor 2,2′-(hydroxynitrosohydrazono)bis-ethanimine (DETA/NO) at a final concentration of 10 μM in combination with the salt stress lead to improved shoot and root biomass. The treatment in which DETA/NO was simultaneously combined with 150 mM NaCl resulted in less cell death, reduced cysteine protease enzymatic activity, less H_2O_2 content, reduced O_2^- levels and less lipid peroxidation than those observed in plant treated with salt alone. Taken together, these

results suggest that exogenously applied NO improves plant tolerance to salt stress by scavenging ROS.

5.2 Introduction

Plant tolerance to salt stress is dependent on how plants respond to both osmotic and ionic stress because salt stress causes disruption of osmotic and ionic balance in plant cells (Ashraf and Harris 2004; Hare et al. 1998; Niu et al. 1995; Yeo 1998; Zhu 2003). One of the consequences of salt stress in plants is oxidative stress in the plant cells, caused by accumulation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) that damage cellular macromolecules (Alscher et al. 1997; Bailey-Serres and Mittler 2006; Mittler 2002; Neill et al. 2002). Cellular macromolecules that can be damaged by ROS-induced oxidative stress include lipids, proteins and nucleic acids (Alscher et al. 1997; Imlay 2003; McKersie and Leshem 1994). Plants use an intricate mechanism (known as the plant antioxidant system) as a defence mechanism against oxidative stress; with low-molecular weight antioxidants such as ascorbate (AsA) and glutathione (GSH) together with antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) as components of the plant antioxidant system (Bailey-Serres and Mittler 2006; Drotar et al. 1985; McKersie and Leshem 1994; Noctor and Foyer 1998; Yang et al. 2006). Scavenging of O_2^- by the plant antioxidant system is done mainly by enzymatic conversion of O_2^- to H_2O_2 and O_2 via a reaction catalyzed by SOD (Giannopolitis and Ries 1977). The consequence of the SOD reaction

is elevation of H_2O_2 content in plant tissue and this triggers peroxidases (such as CAT, APX and GPX) to detoxify the H_2O_2 into H_2O (Bailey-Serres and Mittler 2006; Drotar et al. 1985; McKersie and Leshem 1994; Noctor and Foyer 1998; Yang et al. 2006). Ascorbate serves as the electron donor in the reaction catalyzed by APX for the detoxification of H_2O_2 and is suggested to be the most important peroxidase in plants (Noctor and Foyer 1998). GPX consumes GSH to detoxify H_2O_2 and lipid peroxides (Drotar et al. 1985; Halušková et al. 2009; Yang et al. 2006). MDHAR and DHAR catalyse enzymatic reactions that regenerate AsA using NADPH and GSH, respectively, producing NADP and glutathione disulfide (GSSG) as the respective oxidized by-products (Dalton et al. 1986). GR is the key enzyme in the regeneration of GSH from GSSG by consuming of NADPH (Dalton et al. 1986; Edwards et al. 1990). Several studies suggest that plant genotypes that are more efficient in restricting excessive ROS accumulation tend to be more salt tolerant than those that have poor capacity to limit excessive ROS production (Badawi et al. 2004; Gosset et al. 1994; Gueta-Dahan et al. 1997; Hernández et al. 2000; Mittova et al. 2002). Oxidation of cell membrane lipids by ROS (such as O_2^-, $OH^{\cdot}$ and the peroxyl radical) can be used as a measure of salt tolerance because it leads to the production of malondialdehyde (MDA), the content of which is indicative of the level of oxidative stress and membrane stability (Dionísio-Sese and Tobita 1998; Hernández and Almansa, 2002; Luna et al. 2000; Meloni et al., 2003; Shalata et al. 2001). Accumulation of reactive oxygen species (ROS), such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), to levels in excess of the content that is required for protective cellular signaling can lead to cell death via a necrotic or programmed cell death (PCD) pathway in plant tissue (Bailey-Serres and Mittler 2006; Becana et al. 2000; Dat et al. 2003; Epple et al. 2003; Mittler et al. 2004; Overmyer et al. 2003). In

instances where the PCD pathway is responsible for the ensuing ROS-induced cell death, cysteine endopeptidases of the caspase-like family are key executors/effectors of the PCD (Alesandrini et al. 2003; Chang et al. 2009; Groten et al. 2006; Naito et al. 2000; Zhou et al. 2008).

Evidence pointing to a role of NO in enhancing plant tolerance to salt stress has recently been reviewed (Molassiotis et al. 2010) and includes NO-mediated regulation of antioxidant enzyme activity (Tanou et al. 2009), reduction of NaCl-induced oxidative stress (Liu et al. 2007) and modulation of ion/proton pump activities (Zhang et al. 2006). Limitations on these studies are that several of them use detached leaves instead of intact whole plants or use callus/cell suspensions or they are done over a short period of time (minutes up to 4 days) or else they use NO donors [e.g. sodium nitroprusside (SNP)] known to perturb physiological processes that are crucial to optimal plant metabolism (Wodala et al. 2010; Murgia et al. 2004). Furthermore, none of the studies investigated the role of cysteine protease activity in NO-induced salt tolerance despite there is an established link between salt stress and cysteine protease activity (Katsuhara 1997; Solomon et al. 1999; Wang et al. 2010). In an attempt to further understand the molecular mechanisms by which NO regulates whole plant tolerance to long-term salt stress, the effect of DETA/NO on maize NO content, growth parameters, cell death, cysteine protease activity, O_2^- content, H_2O_2 content and lipid peroxidation (as MDA content) was studied in plants exposed to salt stress over a period of 21 d (regarded as long-term salt stress).

5.3 Materials and Methods

5.3.1 Plant Growth

Silverking [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, followed by four washes 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 19.5 cm diameter plastic pots. The sand was kept moist by watering only 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.

5.3.2 Treatment of plants

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), combination of 150 mM NaCl and 10 µM DETA/NO, and combination 150 mM NaCl and 10 µM DETA were supplimented 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 during a period of 7 days. Nutrient solution

containing NaCl was supplemented with an additional 15 mM $CaCl_2$ to compensate for the inhibitory effect of NaCl on calcium uptake that could otherwise occur in such salt treatments.

Growth parameters, shoot cell viability plus shoot CYP activities, shoot NO content, shoot ROS (O_2^- and H_2O_2) content, and shoot lipid peroxidation levels (as MDA content) were evaluated. These growth and molecular/biochemical parameters were evaluated after 21 days salt treatment. Freshly harvested plants were used for measurement of growth parameters and shoot cell viability but snap-frozen (in liquid nitrogen) tissue were used for all other assays (in which case the tissue was stored at -80°C and used within 2 days).

5.3.3 Analysis of growth parameters

Plants were removed from the sand, being careful to avoid any loss of shoots or roots during the up-rooting of the plants. Twelve plants from each treatment were divided into shoots and roots. Each of these plants was scored for dry weight of the shoots and roots.

5.3.4 Evaluation of cell viability

A modified method of Sanevas et al. (2007) was followed for the cell viability assays (i.e. after 21 days from the fist treatment). Briefly, leaf tissue (100 mg per treatment) from five different plants of each of the treatment were harvested and stained at room temperature with 0.25% (w/v) Evans Blue for 15 minutes. The leaves were washed for 30 min in distilled water, followed by extraction of the Evans Blue stain (taken up by dead leaf cells) from leaf 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 leaf tissues.

5.3.5 Determination 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 leaf 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 K2HPO4, pH7.4, 1 mM EDTA, 5% (w/v) polyvinylpyrrolidone (PVP) (molecular weight = 40 000]. The resulting homogenates were centrifuged at 12,000 X *g* for 30 min and were incubated with 100 U of catalase and 100 U 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.

5.3.6 Cysteine endopeptidase activity

For assaying CYP activity, leaves (only the third youngest leaf of each plant to ensure uniformity) were used at the end of the 21 days. CYPs were assayed for using a modified procedure of Zhang et al. (2008). Protein extraction was done using 100 mg of leaf tissue, homogenised in 1 ml of buffer containing 100 mM Tris-HCl (pH 6.2), 5 mM $MgCl_2$, 2 mM EDTA, 10 % (v/v) glycerol and 10 mM β-mercaptoethanol. For CYP activity 1 mM phenylmethylsulfonyl fluoride (PMSF) in the homogenate prior to incubation of the mixture at 37°C for 5 min. Then, 0.5 mM Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) was added as substrate to the reaction mixture and incubated at 37°C for 60 minutes. CYP activity was determined by measuring absorbance of released chromophore p-nitroaniline at 405 nm every 20 min (using the extinction co-efficient of 9.6 $mM^{-1}.cm^{-1}$).

5.3.7 Super oxide content

For O_2^- content, a procedure described by Tewari et al. (2009) was used. Leaf tissue (100 mg) was ground in liquid nitrogen and homogenized in 400 µl of cold 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 X g for 30 min at 4°C to obtain the O_2^- extract. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 50 µl and 0.5 mM XTT. O_2^- content was calculated based on the reduction of XTT by monitoring absorbance at 450 nm for 4 min, using the extinction coefficient of 2.16 X 10^4 $M^{-1}.cm^{-1}$.

5.3.8 Hydrogen peroxide content

H_2O_2 content was determined in based on a method adapted from Velikova et al. (2000). Leaf tissue (100 mg) was ground to fine powder in liquid nitrogen and homogenized in 400 µl of cold 5% (w/v) TCA. The homogenate was centrifuged at 12,000 X g for 30 min at 4°C to obtain the H_2O_2 extract. The reaction mixture contained 75 µl of the 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.

5.3.9 Lipid peroxidation levels

Lipid peroxidation (reflective of MDA content) was measured in leaf tissue as follows: Plant tissue (100 mg) was ground into a fine powder in liquid nitrogen. The tissue was homogenized in 400 µl of cold 5% (w/v) TCA. The homogenate was centrifuged at 12,000 X g for 30 min and further processed based on the method of Buege and Aust (1978).

5.3.10 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. All results are the mean of at least three replicates (n=3).

5.4 Results

5.4.1 DETA/NO and salt stress increases maize NO content

Studies on the use of DETA/NO as NO donor in which the role of NO in salt tolerance is evaluated are rare and there are contradicting reports regarding changes in plant NO content in response to salt stress. This study thus determined if the NO donor DETA/NO and salt stress (as 150 mM NaCl) alter maize NO content under long-term salt stress. DETA/NO caused an increase of ± 80% in leaf NO content, whereas DETA did not alter leaf NO content and salt stress resulted in ± 35% elevation of leaf NO content in comparison to leaf NO content of untreated control plants (Figure 5.4.1). Simultaneous treatment of maize plants with a combination of both DETA/NO and salt stress augmented leaf NO content by ± 160% compared to that of leaves from untreated controls (Figure 5.4.1). On the other hand, there were no significant differences between leaf NO content of untreated plants and that of plants that were treated simultaneously with a combination of both DETA and salt stress (Figure 5.4.1).
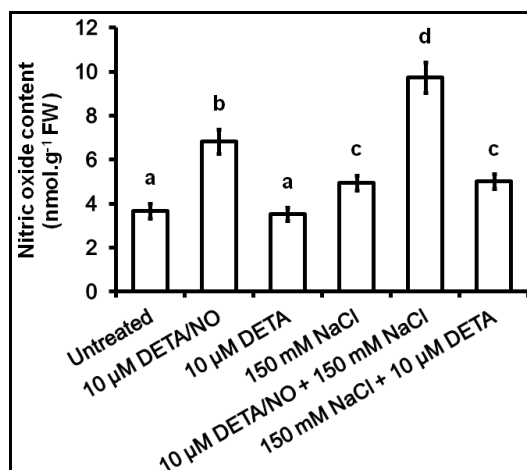
Figure 5.4.1 Nitric oxide content of leaves taken from maize plants exposed to various treatments that may alter NO content. Measurements were taken on leaves of plants that were treated at the V1 stage for a period of 21 d, with treatments done every three days. Data represent the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

5.4.2.1 NO improves maize biomass under salt stress

The effect of the various treatments on maize biomass was investigated because salt stress reduces plant biomass, thus the biomass should improve if NO enhances salt tolerance. For evaluating biomass responses to the treatments, dry weights were measured for shoots and roots of the maize plants at the end of the 21 d treatments. Compared to untreated control plants, shoot dry weight was increased by ± 30% in response to treatment of plants with 10 µM DETA/NO whereas 10 µM DETA did not affect shoot dry weight (Figure 5.4.2.1 A). However, treatment of plants with 150 mM NaCl or a combination of 150 mM NaCl and 10 µM DETA lead to ± 50% reduction in shoot dry weight (Figure 5.4.2.1 A) compared to untreated controls. Compared to plants treated with 150 mM NaCl, shoot dry weight increased by only ± 40% in response to treatment of plants with a combination of 150 mM NaCl and 10 µM DETA/NO, even

though the increase was not sufficient to reach the dry weights of shoots from untreated plants (Figure 5.4.2.1 A).

Root dry weight was increased by ± 45% in response to treatment of plants with 10 µM DETA/NO whereas 10 µM DETA did not affect root dry weight in comparison to untreated plants (Figure 5.4.2.1 B). Plants treated with 150 mM NaCl or a combination of 150 mM NaCl and 10 µM DETA had root dry weights reduced by ± 50% compared to untreated plants (Figure 5.4.2.1 B). On the other hand, root dry weights in plants treated with a combination of 150 mM NaCl and 10 µM DETA/NO increased by ± 50% compared to plants treated with 150 mM NaCl but this increase resulted in root dry weights that were still less than the dry weights of roots from untreated plants (Figure 5.4.2.1 B).
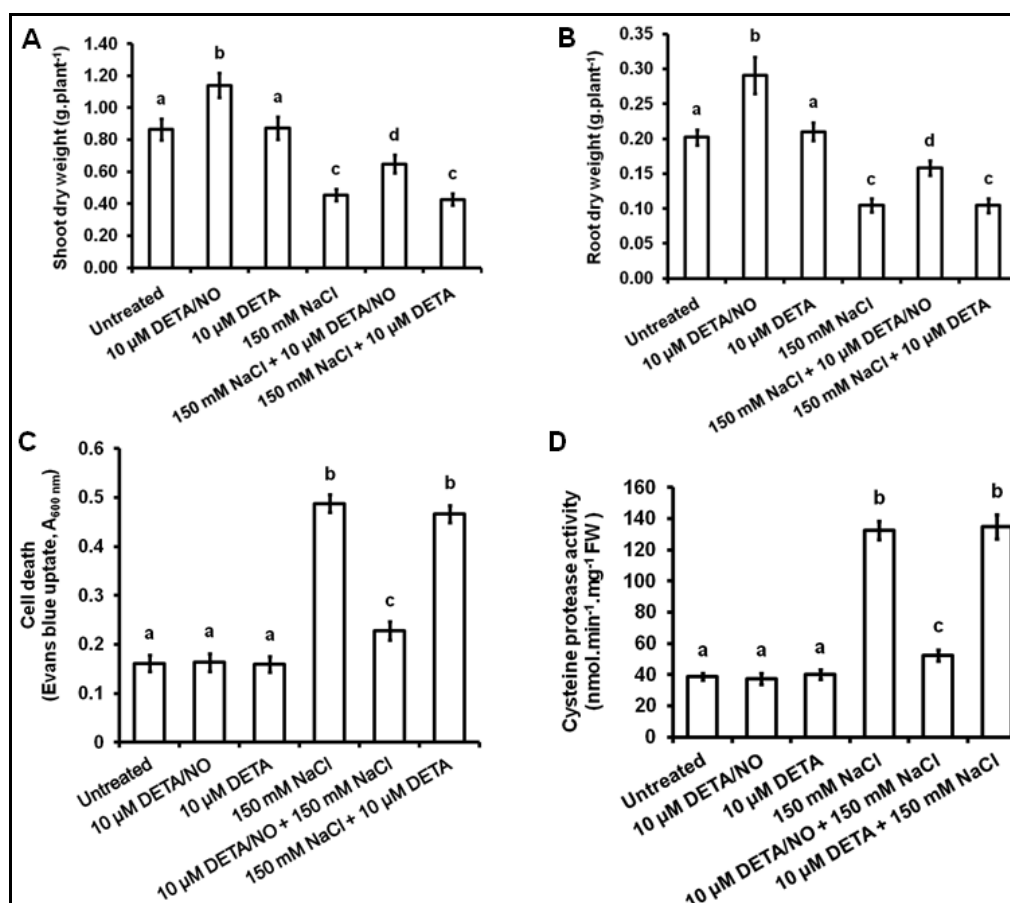
Figure 5.4.2.1 Responses of maize biomass, cell death and cysteine protease enzymatic activity to exogenously applied NO and salt stress. Measurements were done on maize plants treated at the V1 stage for a period of 21 d. Biomass was evaluated by measuring shoot (A) and root (B) dry weights at the end of the treatment period and bars are representative of the mean (±SE) of three independent experiments from 10 plants per treatment in each experiment. Cell death (A) and cysteine protease enzymatic activity (B) were assayed at the end of the treatment period and bars represent the mean (±SE) of three independent experiments from three plants in each treatment per experiment. Means with different letters are significantly different from each other ($p < 0.05$).

5.4.2.2 NO reduces salt stress-induced cell death and cysteine protease activity

The effect of the various treatments on cell death was investigated because salt stress to induce PCD and such PCD is linked to enhanced cysteine protease activity. 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 shoots of the maize plants at the end of the 21 d treatments. Cell death was 3-fold higher in salt-treated plants than in untreated plants (Figure 5.4.2.1 C) but was not affected by neither 10 μM DETA/NO nor 10 μM DETA (Figure 5.4.2.1 C). On the other hand, cell death in plants treated with a combination of 150 mM NaCl and 10 μM DETA/NO lead to reduction in the extent of salt stress-induced cell death because this treatment resulted in cell death limited to only 1.4-fold higher than untreated controls (Figure 5.4.2.1 C). DETA, as seen in the treatment in which 10 μM DETA was combined with 150 mM NaCl, did not reduce the salt stress-induced cell death (Figure 5.4.2.1 C). A similar trend for all treatments was seen for cysteine protease activity as the trend for cell death (Figure 5.4.2.1 D).

5.4.3 NO restricts accumulation of ROS and the extent of lipid peroxidation under salt stress

The effect of the various treatments on leaf content for two ROS, namely O_2^- and H_2O_2, was investigated along with lipid peroxidation (as MDA content) because salt stress is known to cause excessive ROS accumulation, leading to lipid peroxidation in plants that cannot scavenge these ROS efficiently. NO should thus reverse the effects of salt stress if NO enhances salt tolerance. Compared to untreated control plants, leaf O_2^- and H_2O_2 contents were reduced by ± 38% and ± 60% respectively in response to treatment of plants with 10 μM DETA/NO whereas 10 μM DETA did not affect leaf O_2^- and H_2O_2 contents (Figure 5.4.3 A and Figure 5.4.3 B). However, treatment of plants with 150 mM NaCl (or a combination of 150 mM NaCl and 10 μM DETA) increased leaf O_2^- and H_2O_2 contents by ± 340% and ± 650% respectively (Figure 5.4.3 A

and Figure 5.4.3 B) compared to untreated controls. Compared to untreated plants, the increase in leaf O_2^- and H_2O_2 contents was limited only to ± 40% and ± 47% respectively in response to treatment of plants with a combination of 150 mM NaCl and 10 µM DETA/NO (Figure 5.4.3 A and Figure 5.4.3 B).

Leaf lipid peroxidation was reduced by ± 30% in response to treatment of plants with 10 µM DETA/NO whereas 10 µM DETA did not affect leaf lipid peroxidation in comparison to untreated plants (Figure 5.4.3 C). Plants treated with 150 mM NaCl or a combination of 150 mM NaCl and 10 µM DETA experienced lipid peroxidation at levels ± 250% higher than leaves from untreated plants (Figure 5.4.3 C). On the other hand, leaf lipid peroxidation in plants treated with a combination of 150 mM NaCl and 10 µM DETA/NO was restricted to an increase of only ± 60% compared to lipid peroxidation in leaves from plants treated with 150 mM NaCl (Figure 5.4.3 C).
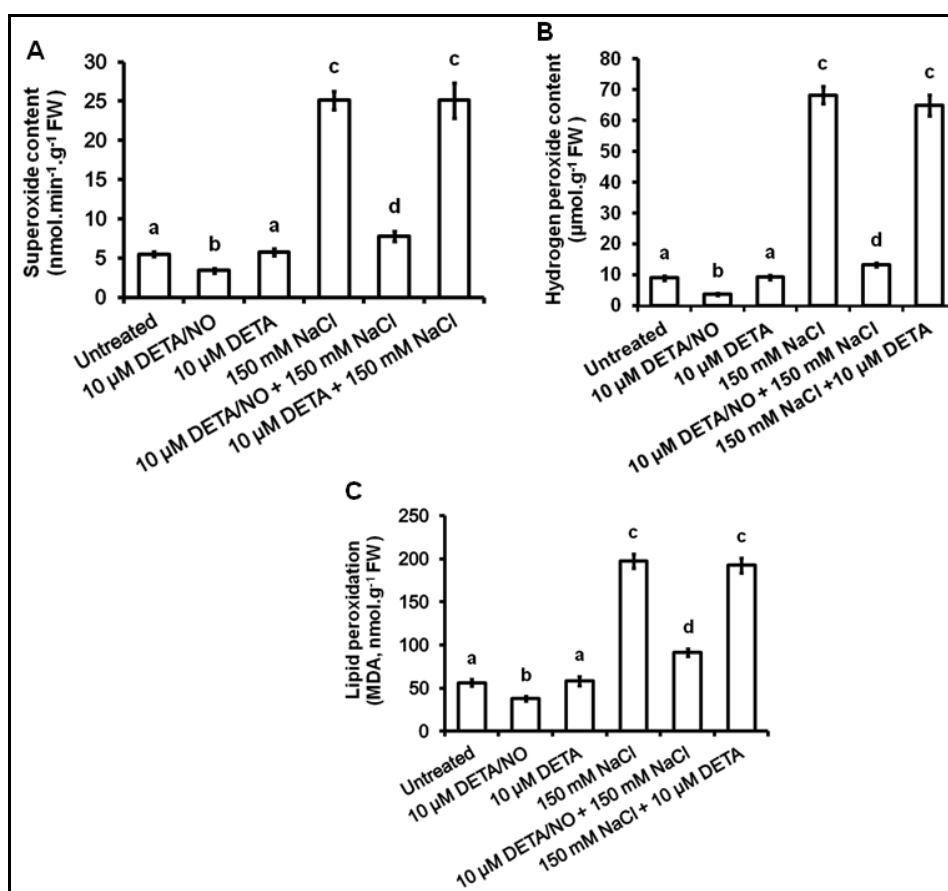
Figure 5.4.3 Effect of NO and salt stress on superoxide content (A), hydrogen peroxide levels (B) and lipid peroxidation (C) in maize. Measurements were performed on leaves taken from maize plants that were treated at the V1 stage for a period of 21 d. Data represent the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

5.5 Discussion

The work reported here investigated the effect of exogenously applied NO (using 10 µM DETA/NO as NO donor) and long-term salt stress (imposed by application of 150 mM NaCl) on growth (biomass measured as shoot and root dry weight), cell death, cysteine protease enzymatic activity, ROS levels (as O_2^- and H_2O_2) and lipid peroxidation (as MDA content) in maize. A complex signaling network has been suggested that involves intricate balance

between NO production and ROS accumulation in relation to how the interaction between NO and ROS regulate PCD (Zhao 2007; Zhao et al. 2007). The role of this NO-ROS interaction in salt stress tolerance has been studied (albeit in callus) to some extent (Zhang et al. 2007), but less attention has been paid to how stress-induced cysteine protease activity forms part of the stress-induced network leading to ROS-regulated PCD in relation to the role of NO in conferring plant tolerance to salt stress.

Analysis of the results obtained from measurement of NO content shows that long-term salt stress increases maize NO content and this is contrary to some reports that show that NO content increases in response to salt stress in the short term but eventually yields reduced NO content after several hours/few days (Tanou et al. 2009; Unchida et al. 2002; Zhang et al. 2006). This result shows that there is variation in NO content in response to salt stress, depending on the duration of the salt stress. This reinforces the notion that plants respond differently to short, medium and long-term salt stress, which in turn implies that results from long-term salt stress experiments may be more reflective of field conditions because salt stress in the field is a long-term phenomenon. Furthermore, the study shows that DETA/NO as NO donor releases nitric oxide into maize tissue and that the level of nitric oxide released by 10 µM DETA/NO elevates maize tissue NO content to levels that are higher than those attained from treatment with 150 mM NaCl. However, the NO content of maize tissue when the plants are treated simultaneously with both 150 mM NaCl and 10 µM DETA/NO is the highest amongst all the treatments. The significance of these observations is their implication for the level of NO that is required for protection of maize against salt stress. It would appear that salt-sensitive plants respond to salt stress by raising their NO levels as a defense strategy but these plants fail to

elevate NO levels to a specific threshold needed for protection and thus the plants do not tolerate the salt stress. In a case where the salt stressed plant is supplemented with exogenous NO to allow tissue NO content to reach the required threshold for NO-mediated protection against salt stress, the capacity of the plant to tolerate the salt stress is increased (manifested as improved biomass in salt-treated plants with NO supplementation compared to salt-treated plants without NO supplementation). For maize under salt stress caused by 150 mM NaCl, the protective threshold for maize tissue NO content would thus be ± 10 nmol of NO per gram of fresh weight (as measured by the haemoglobin assay for maize leaves) according to the results obtained for maize NO content under 150 mM NaCl stress with a supplementation 10 µM DETA/NO. It is appropriate to conclude that the effects seen in response to DETA/NO in these treatments are due to NO because DETA (which does not release NO and is a breakdown by-product of DETA/NO) did not alter NO content in maize tissue.

The fact that salt stress caused increased cell death cysteine protease activity suggests that salt stress induces high cysteine protease activity and this elevated cysteine protease activity possibly triggers PCD, evident as increased Evans blue uptake. Reversal of both the cysteine protease activity and cell death by NO would mean that NO regulates salt stress-responsive cysteine proteases so that these proteases are prevented from triggering cell death. Reduction of salt stress-induced excessive ROS levels by exogenously applied NO has been demonstrated previously (Liu et al. 2007) and the results of this study confirm this phenomenon. It is proposed here that salt stress triggers excessive production of ROS (with O_2^- and H_2O_2 as examples studied here) and salt stress-sensitive plants (the sensitivity being manifested as loss of biomass) would be those that do not have sufficient capacity to scavenge the ROS efficiently.

The excessive ROS then trigger events such as lipid peroxidation (seen here as elevated MDA content) and PCD-inducing high cysteine protease activity, consequently leading to cell death and loss of biomass/poor plant performance/reduced crop yield. Supplementation of such sensitive plants with NO likely reduces the extent of excessive ROS accumulation, possibly by activating ROS-scavenging systems (such as the components of the plant antioxidant system that includes SOD together with the ascorbate-glutathione cycle and its corresponding enzymes), which would result in less lipid peroxidation and reduction of the activity of salt-responsive cysteine proteases. As a result, the extent of cell death would be less when NO is supplemented to salt-stressed plants than when no exogenous NO is applied to the salt stressed plants and this would improve plant performance in saline soils.

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CONCLUSION AND FUTURE PERSPECTIVES

This study has established that nitric oxide synthase (NOS) activity regulates nodule development and nodule functioning. This is achieved via regulation of redox homeostasis (by regulating the production and detoxification of reactive oxygen species) through modulation of antioxidant enzymatic activities by nitric oxide (NO) produced from NOS. The NO-regulated redox homeostasis is aimed at maintaining nodule reactive oxygen species (ROS) content at levels optimal for signaling that is required for nodule development and function while preventing excessive ROS accumulation that would otherwise be deleterious to nodule development/functioning. Furthermore, the study shows that excessive levels of exogenously applied NO (at levels too high to be beneficial to plants, as seen for example in treatments with 200 µM DETA/NO) imposes oxidative stress on legume root nodules (as seen for soybean treated with 200 µM DETA/NO, in which the treatment leads to excessive accumulation of both O_2^- and H_2O_2) and this oxidative stress triggers cysteine protease activity and lipid peroxidation, ultimately leading to cell death. The study highlights the role of NOS activity, and thus endogenously synthesized NO, in regulating nodule cysteine protease activity by modulating the expression of Groups-1 cystatins. In essence, the study shows that endogenous NO is required for the expression of cystatins (the proteins that inhibit cell death-regulating cysteine proteases) and that reduced endogenous NO levels lead to suppression of expression of Group-

1 cystatins, which permits heightened cysteine protease activity with the consequence of increased nodule cell death.

The study extends the role of NO beyond its regulatory role on legume (soybean as an example) development but further to is role in enhancing salt stress tolerance in cereals (using maize as an example). The study on salt stress tolerance is initiated with comparison of salt tolerance of two maize genotypes where it is established that the regulation of ROS accumulation by antioxidant enzymes is a key determinant of maize responses to salt stress and that central to these responses is the activity of cysteine proteases. In this study, it is illustrated that salt-sensitive maize genotypes have high cysteine protease activity (and thus experience more cell death and yield loss) than salt-tolerant genotypes under conditions of high salinity. By supplementing the salt-sensitive genotype with NO under high salinity, the study shows that the tolerance of this genotype to salt is improved. This NO-mediated improvement is shown to be due to reduction in the accumulation of ROS and the suppression of the activity of salt-responsive cysteine proteases.

The characterization of some of the components of NO-mediated regulation of nodule development and functioning is an effort that will in future be extended to identify the rest of the molecular signaling components in NO-mediated regulation of nodule development and functioning. This can be achieved to a great extent by analyzing transcripts and proteins that are differentially regulated by NO, via transcriptomics and proteomics and linking these transcripts and proteins to nodule functioning. The role of NO in mediating plant tolerance to salt stress cannot be limited only to modification of the plant antioxidant system and cysteine

protease activity. It will thus be useful to identify other molecular pathways that are involved in NO-mediated salt stress tolerance in plants. To this end, transcriptomics and proteomics would also make a meaningful contribution towards dissecting such molecular pathways. Knowledge from such efforts would be useful for genetic engineering of crop plants with enhanced tolerance to salt stress.