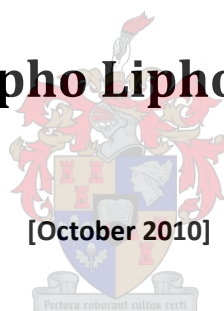


Modulation of root nodule antioxidant systems by nitric oxide: Prospects for enhancing salinity tolerance in legumes

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Submitted in fulfillment of the academic
requirements for the degree of Doctor Philosophy
(Agric) at the Institute for Plant Biotechnology,
Department of Genetics, University of Stellenbosch

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Declaration of originality

The work presented in this work is entirely mine unless otherwise acknowledged. This report has not been presented anywhere previously to any institution.

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Date: 14 October 2010

Acknowledgements

I would like to pass my sincere gratitude to my supervisor for the continuous assistance he provided during the course of this project. I am grateful for all the support he gave.

My IPB colleagues, for the technical assistance they provided, and also for the laughter at all the times.

I would also like to thank my loved ones; family and friends for being so dear to me and the support they provided when I needed it the most.

Lastly, I would like to give all the honour to The Almighty God, for his wisdom, guidance and assurance at all the times.

Abstract

Salinity is one of the major limiting abiotic stresses on legume plant yield, leading to early senescence of root nodules. This occurs because of accumulation of reactive oxygen species (ROS) in plant cells under salinity stress. Concurrent with the increase in cellular reactive oxygen species levels is the increase in cellular antioxidants and corresponding antioxidant enzymes. This feature is observed mostly in the shoots and roots of more tolerant genotypes compared to the susceptible genotypes. It is accepted that the mechanism of plant tolerance to stress is dependent upon the response of the antioxidant systems. Most studies carried out on shoot tissues suggest that scavenging of ROS by the plant antioxidant system is modulated by nitric oxide (NO). However, the pathways by which NO mediates such antioxidant responses are not fully understood. For legumes, salinity stress has adverse effects on yield and this is in part due to inhibition of nitrogen fixation in the root nodules of the legumes, which causes severe nitrogen starvation in nitrogen-deficient soils. Nodules are specialized organs comprising of both the rhizobia and the plant tissue, hence the physiological aspects may vary from the findings from the leaves. It was therefore deemed necessary to establish the role of NO on the nodule antioxidant system in the absence and presence of salinity stress.

For the purposes of this study, the effect of both exogenously applied NO and endogenous NO on superoxide dismutase, glutathione peroxidase and glutathione content was determined. The studies involved the use of nitric oxide donors like sodium nitroprusside (SNP) and diethylenetriamine/nitric oxide adduct (DETA/NO), their respective fixed controls potassium ferricyanide and diethylenetriamine (DETA), plus a

nitric oxide synthase inhibitor (to inhibit nitric oxide production by the enzyme nitric oxide synthase) on nodulated roots.

The data obtained in this work points out specifically at roles played by nitric oxide in regulating superoxide dismutases, glutathione peroxidase and glutathione during salinity stress and proposes a link between nitric oxide-mediated changes in these antioxidant systems and salinity stress tolerance. Both the exogenously applied and endogenous nitric oxide increases the enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GR). However, there is both time dependency and nitric oxide concentration dependency on the enzyme activities. The total SOD enzyme activity increases upon nitric oxide exposure and with time of exposure. The individual SOD isoforms identified and studied in the root nodules all contribute to this increase in SOD activity upon nitric oxide treatment except for MnSOD I. This increase in activity is regulated at transcriptional level as the RT-PCR results targeting the individual isoforms reveals an increase in transcript levels after 6 hours of nitric oxide treatment. However, the CuZn SOD I isoform transcripts are reduced upon nitric oxide treatment. A similar response was also observed in GPX enzyme activity in which nitric oxide increased the GPX activity above all the controls. The GR enzyme activity exhibits an opposite response because the activity decreases with time of exposure to NO and concentration of NO.

In order to determine the effect of NO under saline conditions, an experiment was set up that involved incubation of nodulated roots in solutions containing 150 mM NaCl. The stressed nodules exhibited generally higher levels of enzyme activities than the non-stressed nodules. Furthermore, exposure to nitric oxide donor in combination with NaCl induced even higher activities of SOD and GPX than NaCl or nitric oxide donor alone.

There were also higher levels of reduced glutathione and total glutathione recorded under stress compared to optimal conditions. Nitric oxide increased the concentration of these forms of glutathione, suggesting an improved redox status based on the GSH/GSSG ratios under salinity stress in the presence of nitric oxide.

Attenuation of nitric oxide synthesis with L-N_ω-Nitroarginine methyl ester (L-NAME) reverses all the recorded effects of nitric oxide on antioxidant enzymes and glutathione pool. This was observed in salinity stressed nodules and non-stressed nodules.

This work further establishes that NO plays a pivotal role in modulating the enzymatic activities through a pathway that is mediated by guanosine 3',5'-cyclic monophosphate (cGMP). The experiment involving the inhibition of soluble guanylyl cyclase (sGC) (an enzyme that catalyzes the biosynthesis of cGMP), cell-permeable cGMP analogue and L-NAME revealed that GPx activity is modulated through a cGMP-dependent pathway and NO is positioned up-stream of cGMP in the pathway leading to improved GPX activity. Cyclic GMP also modulates the GPX activity in a concentration dependent manner.

NO improves the redox status of the cell under both saline conditions and non-saline conditions and this effect is modulated through a cGMP-dependent pathway. It is thus rational to conclude that; in the root nodules of legumes, like in other plant tissues, the increased accumulation of antioxidants and the increased activity of their corresponding enzymes, as modulated through the cGMP-dependent pathway by nitric oxide, confer root nodule tolerance to salinity. This concept directly points out at an attractive strategy for developing legumes that are genetically improved for enhanced root nodule tolerance to salinity; via differential regulation of antioxidants and antioxidant enzyme genes in the root nodules under abiotic stress. Towards attaining the goal for such

genetic improvement, experiments involving construction of an abiotic stress-responsive and nodule-specific chimeric promoter were carried out. By fusing the 5'-untranslated (5'-UTR) region of the LEA gene that contains an abiotic stress-responsive *cis*-acting element (from the GmPM9 promoter) to the nodulin N23 promoter bearing the highly functional cluster of motifs for nodule specificity, the candidate nodule specific promoter that is abiotic stress responsive (ASREF/NSP) was constructed. The construct harbouring this ASREF/NSP chimeric promoter was fused to the β -glucuronidase (GUS) reporter gene so as to study the functionality of the promoter in *Medicago truncatula* plants. The construct was delivered into the Medicago plants through *Agrobacterium rhizogenes* mediated transformation to produce composite Medicago plants. The transgenic roots have been cultured for further manipulation and to confirm the functionality of the promoter.

Furthermore several strategies can be deployed via the use of this chimeric promoter so as to enhance the nodular antioxidant system. This would involve either gene regulator-chimeric promoter fusion or the use of a single gene approach. As part of this work, the MtNOA gene homologous to AtNOAs, has been cloned from *Medicago truncatula* and put as ASREF/NSP fusion in a binary vector pBINPLUS and delivered into *Medicago truncatula* for nodule-specific and abiotic stress-induced nitric oxide synthesis. Since there is no plant NOS identified to date, the possibility of the use of a regulatory gene in this aspect is still limited. There are other options involving the use of the chimeric promoter with the individual genes encoding the antioxidant enzyme genes such as genes encoding SOD, GPX and the glutathione synthetase to enhance the plant antioxidant system during abiotic stress.

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List of Abbreviations

α	alpha
μl	microliter
β	beta
μg	microgram
A_{600}	Absorbance at 600 nm
A_{412}	Absorbance at 412 nm
A_{450}	Absorbance at 450 nm
A_{340}	Absorbance at 340 nm
ABA	Abscisic acid
APS	Ammonium persulfate
BA	Benzyladenine
bp	Base pair
BSA	Bovine serum
cDNA	Complementary deoxyribonucleic acid
hr	Hour
hrs	hours
dNTP	Deoxyribonucleoside triphosphate
dH ₂ O	distilled water
ddH ₂ O	double distilled water (milliQ water)
DETA/NO	Diethylenetriamine/nitric oxide adduct
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothretiol
EDTA	Ethylene diamine tetracetic acid

EGTA	Ethylene glycol <i>bis</i> (β -aminoethyl ether) NNN'N-Tetraacetic acid
FW	Fresh weight
g	gram
G	guanine
C	cytosine
T	thiamine
A	adenine
RNA	Ribonucleic acid
IPTG	Isopropyl-1-thio- β -D-galactose
kb	kilobase
kDA	kilodalton
LEA	Late embryogenesis abundant
MDA	Monodehydroascobate
mg	milligram
ml	milliliter
min	minute
mmol	millimole
mRNA	massanger ribonucleic acid
ng	nanogram
OD	Optical density
PCR	Polymerase chain reaction
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcription
SDS	Sodium dodecyl sulfate

SDP-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
TBE	Tris/borate elctrophoresis (buffer see appendix A)
TE	Tris/EDTA (buffer see appendix)
T _m	Melting temperature
Tris	2-amino-2(hydroxymethyl)-propane-1,3- diol(Tris(hydroxymethyl) aminomethane
Tris-HCl	Tris hydrochloride
TEMED	NNN'N' tetramethylethylenediamine
U	Unit
V	volt
v/v	Volume/Volume
w/v	Weight/volume
NOC-18	DETA/NO
DETA	Diethylenetriamine
SNP	Sodium nitroprusside
L-NAME	L-N _ω -Nitroarginine methyl ester
L-NNA	N-nitro-L-arginine
GSSG	Oxidized glutathione
GSH	Reduced glutathione
NEM	N- ethylmaleimide
DTNB	5'5'-Dithiobis (2-nitro-benzoic zcid)
H ₂ O ₂	hydrogen peroxide
O ^{·-}	Superoxide radical
SOD	Superoxide dismutases
GPX	Glutathione peroxidases
GR	Glutathione reductatse

cGMP	guanosine 3',5'-cyclic monophosphate
sGC	soluble guanylyl cyclase
TBE	TRIS borate-EDTA

Chapter 1 General introduction

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

On the other hand, nitric oxide, which is a diatomic molecule, is a signaling molecule involved in various cellular processes and in particular the abiotic stress responses of plants (Niell et al, 2008 & Wendehenne et al, 2005). To be more specific, it has been reported that, nitric oxide interacts with antioxidants to scavenge ROS, thus protecting plants under stress (Niell et al, 2008). Most of these studies, focused mainly on other plant tissues rather than legume nodules, reveal that nitric oxide delays ROS-induced cell death (Beligni et al, 2002 and Hung and Kao, 2004). The legume root nodule is a symbiotic tissue which is both structurally and physiologically different from the other legume plant organs (Crespi and Ga'avez, 2000). Hence one cannot assume the absolute similarity in antioxidant responses to stress with the rest of the plant tissues and/or organs. The unique nature of nodule physiology presents an opportunity to study and modify the legumes for improved nitrogen use efficiency and improved productivity under growth-limiting environmental conditions.

This is part of a study relating to two major questions pertaining legume plant production. Firstly, the agricultural community now expects biotechnology techniques that target the regulation of a number of genes at the same time. Such transgenic plants are thus expected to exhibit more improved quantitative traits such as abiotic stress tolerance and yield (Chua & Tingey, 2006). Secondly, in addition to the growing problem of soil salinisation (Söderström, 1992; Volschenk and de Villiers, 2000, and Machacha et al, 2000 and Karlberg & de Vries 2004.), nitrogen is one of the limiting nutrients in the soils to-date (Maiangwa, 2009). Thus there is a need for development of plant legume genotypes with nitrogen fixing organs (root nodules) that are tolerant to salinity stress.

This work thus contributes to the scientific knowledge on legume antioxidant responses to salinity and how the stress effect can be counteracted with the ultimate purpose of delaying nodule senescence under saline conditions.

This report is divided into several chapters that are aimed at understanding the role played by nitric oxide in antioxidant system responses under saline conditions in the root nodules. It further examines some of the possible strategies that can be deployed to induce salinity tolerance in the nodules through genetically controlled regulation of antioxidant metabolite production and antioxidant enzymes. Hence the chapters that make up this study include:

- Chapter 1 General Introduction
- Chapter 2 Literature review
- Chapter 3 Effect of nitric oxide on superoxide dismutase in root nodules

- Chapter 4 Effect of nitric oxide on glutathione peroxidase activity in root nodules
- Chapter 5 Effect of nitric oxide on glutathione reductase activity in root nodules
- Chapter 6 Modulation of nodule glutathione levels and redox state by nitric oxide
- Chapter 7 Modulation of glutathione peroxidase activity through cGMP pathway
- Chapter 8 Construction of an abiotic stress-inducible nodule-specific promoter and prospects for abiotic stress tolerance
- Chapter 9 General conclusion

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Chapter 2 Literature review

2.1.0.0. Abiotic stress

2.1.1.0. Types of abiotic stress

Plants often encounter a period of environmental stress, sufficient to limit plant growth and reproductive capacity. This abiotic stress is one of the main detrimental factors affecting crop productivity worldwide. Some examples of abiotic stresses include drought, dehydration, UV-B irradiation, salinity, low and high temperatures. This work the will focus on salinity stress imposed on plants in the form of high NaCl concentration.

Salinity refers to high salt concentration, within the rooting zone, to the levels that impair plant growth and development. This high salt concentration decreases the osmotic potential of the soil solution, thus inhibiting water uptake by plant roots and also causes ion toxicity in plant cells. Plants adjust to salinity stress either by limiting uptake of the salt, compartmentalizing the salt within specialized organelles in the plant cell (e.g. vacuole), or through synthesis of organic solutes that rescue the plant cells from ion toxicity (Jacoby, 2002). Plants that can tolerate high salt concentrations are called halophytes; on the other hand glycophytes are those that will respond by synthesizing organic solutes (Volkmar et al, 1998). However more often than not, plants concurrently utilize these two coping mechanisms against high salt concentrations (Jacoby, 2006).

Elevated uptake of Na⁺ by the plant roots causes replacement of K⁺ and Ca⁺ with Na⁺ in the cell compartments where they are required (Marschner, 1995). Consequently as Ca⁺ ions are replaced by Na⁺, proper cell membrane functioning may be affected, leading to increased cell leakage (Orcutt & Nilsen, 2000; Jacoby, 2002; Todd, 2006). It has also been established the Na⁺ also decreases the activity of nitrate reductase, therefore affecting nitrogen metabolism of the cell (Orcutt & Nilsen, 2000). Hence some of the symptoms of NaCl toxicity would include chlorosis of the older leaves leading to necrosis and cell death (Xu et al, 2000; Kurniadie & Redmann, 1999).

2.1.2.0. Molecular basis of abiotic stress tolerance in plants

2.1.2.1. Sensors of abiotic stress in plant cells

HIS Kinases (HIK) are found in all photosynthesizing organisms and are located on the extracellular space. They function in perceiving abiotic stress and use a response regulator (re) to transduce signals to other cell compartments (Hwang et al, 2002). There are different HIK for different stress types, for instance HIK 33 perceives both the hyper-osmotic stress and cold stress; whereas salt stress is perceived by HIK 33, HIK 34 and HIK 16. Different classes of HIS Kinases regulate genes that are responsive to different types of abiotic stress (Murata & Los, 2006, review).

Different sets of genes that may be simultaneously activated by the sensors may at times contribute to one function (Figure 2-1). For instance, transcription of stress-responsive genes requires the activation of transcription factors like drought response binding proteins; DREB1 and DREB2. These transcription factors are differentially induced by

cold and osmotic stress respectively (Knight & Knight, 2001). However, both DREB1 and DREB2 bind to the DRE motif of stress-inducible genes (Knight & Knight, 2001).

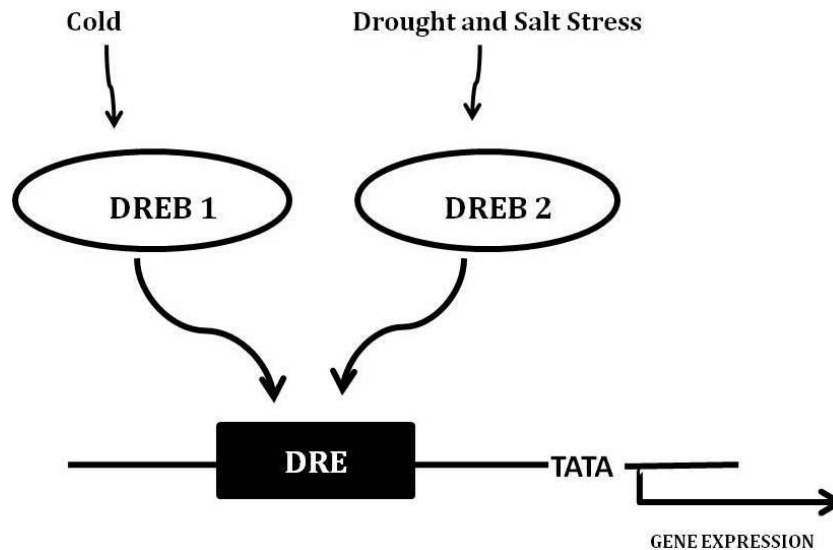


Figure 2- 1 The figure illustrating DREB1 and DREB2 transcription factors as key components in cross talk between cold and osmotic stress. Both transcription factors bind to DRE *cis*-acting elements found on the promoter regions of abiotic stress-inducible genes like rd29A from Arabidopsis. (Adapted and modified from Knight & Knight, 2001)

2.1.2.2. Biochemical pathways involved in abiotic stress signaling

The function of response regulators (re) is to relay the perceived signal by HIK through a stress signal pathway leading to physiological changes. There are several molecules involved in the cascade of events leading to such physiological changes, leading to up-regulation of transcription factors and genes responsible for acclimatization. The biochemical pathway leading to gene responses is not always linear but there is a complex cross talk between and among various pathways (Figure 2- 1 & 2- 2). Drought stress and salinity stress trigger the transcription of drought response binding proteins; DREB 1 and DREB2 respectively and these two transcription factors bind to the DRE

element of the stress responsive gene. Both MAPK and CDPK pathways function through the activation of the transcription factors.

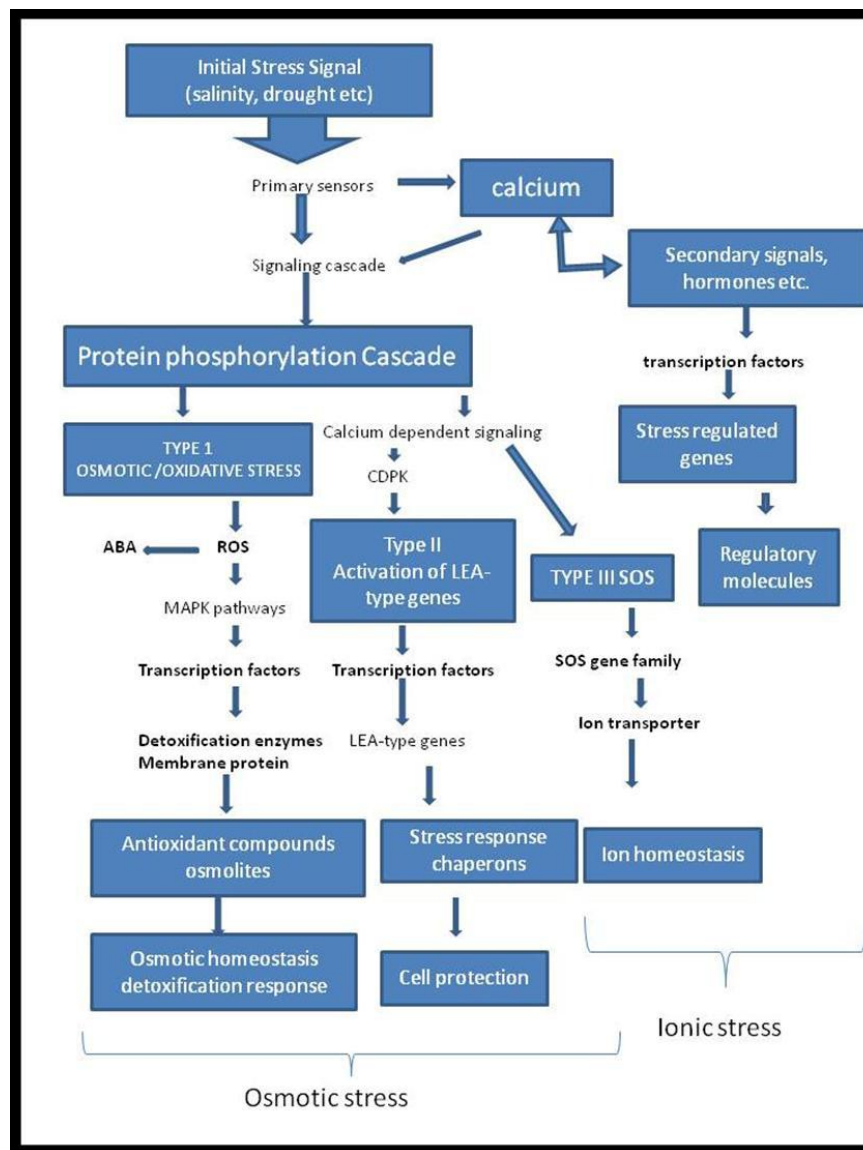


Figure 2- 2 Schematic representation of abiotic stress signaling (adapted from Rodríguez et al, 2005). Different signaling pathways are responsible for osmotic and ionic stress responses. Both water deficit and salinity can cause osmotic stress but salinity also causes ionic stress.

2.1.2.3. Signaling molecules

Calcium (Ca^+) is a secondary signaling molecule involved in most stress responses, but the physiological responses as a consequence of different stress are not the same. The calcium signature (that is dependent upon the type of stimuli), the duration and sub-cellular localization determines the type of the physiological response (Malho et al, 1998 & Bush et al, 1996). Thus manipulation of the down-stream processes in the calcium signaling pathway may alter the biological response of plants to the abiotic stress stimuli. For instance calcium accumulation in response to cold acclimatization is of cytosol and intra cellular localization (Knight et al, 1996); anoxia in maize induced the mitochondrial calcium accumulation (Subbaiah et al, 1998). Ca^+ accumulation is involved in regulating various stress-regulated proteins including calmodulin, calcium-dependent protein kinases (CDPKs) and Mitogen Activated Protein Kinase (MAPK) (Figure 2- 2).

Several calmodulins and their isoforms are expressed differentially in response to abiotic stresses such as cold and heat shock (Yang and Poovaiah, 2003). Furthermore there are many identified calmodulin binding proteins identified and these are known to bind to mammalian nitric oxide synthases (NOS) that synthesize NO from L-Arginine, a known stress-related molecule in plants. Although a nitric oxide synthase has not been identified in plants, its activity has been detected in various plants including lupine roots (Cueto et al, 1996).

Another class of stress signaling molecules is the calcium dependent protein kinase (CDPK). It is involved in induction of stress-inducible genes. For instance AtCDPK1 and

AtCDPK2 are implicated in drought and salinity stresses responses respectively (Knight & Knight, 2001).

Mitogen activated kinases (MAPK) are serine/threonine specific protein kinases and are activated by phosphorylation and eventually regulate several cellular processes, including apoptosis (Peason et al, 2001). The activation of MAPK occurs as a consequence of the stimuli activating the mitogen-activated protein kinase kinase kinase (MAPKKK) that activates mitogene-activated protein kinase kinase (MAPKK) which in turn activates MAPK. The MAPKs have been reported to activate the genes that have hydrogen peroxide-responsive promoters (Knight & Knight, 2001), thus implicating them in functions related to oxidative stress.

2.1.2.4. Abiotic stress-responsive transcription factors

DNA-binding proteins synthesized as part of the early response to abiotic stress can be regarded as abiotic stress-responsive transcription factors. They bind to cis-acting elements in promoters of stress responsive genes and thus regulate their transcription. Several of these have been identified in abiotic stress signaling and include DREB1 and DREB2 (CBF), DREB2A, DREB2B. These proteins, commonly known as *trans*-acting factors, bind to specific conserved motifs on the promoter region of the stress-inducible gene called *cis*-acting elements. Expression of the transcription factors is influenced by stress-regulated molecules like ABA and function to up-regulate gene expression (Knight & Knight, 2001).

2.2.0.0. Senescence and programmed cell death (PCD)

2.2.1.0. PCD definition

Cell death is an integral part of plant growth and development. During senescence the internal membranes of the chloroplast become disorganized and lipid globules accumulate in the chloroplast. The internal components, including macro molecules get destroyed by the cell itself. These macro molecules (lipids, nucleic acids, proteins and polysaccharides) are digested into small molecules, followed by disintegration of the cell. This process is called senescence (Todd, 2006 and Chrispeels & Sadavan, 2003) and is a form of programmed cell death (PCD), regulated by the expression of specific genes. Premature senescence can occur as a result of abiotic stresses like salinity and drought.

2.2.2.0. Role of oxidative stress in senescence

Reactive oxygen species (ROS) are often generated during photosynthesis by singlet oxygen formation as well as oxygen photo-reduction (Yuanbin, 2002). Generation of these molecules is increased under stress. ROS include superoxide, hydroxyl radical and hydrogen peroxide. The interaction of these oxidative species with the cellular molecules often results in destructive oxidative stress (Todd, 2006). ROS modifies various bio-molecules, causing cell death; it causes lipid peroxidation and thus damages lipids. The damage of lipid membranes due to peroxidation leads to increased cell permeability that in turn causes cell swelling (Todd, 2006) (Figure 2-3).

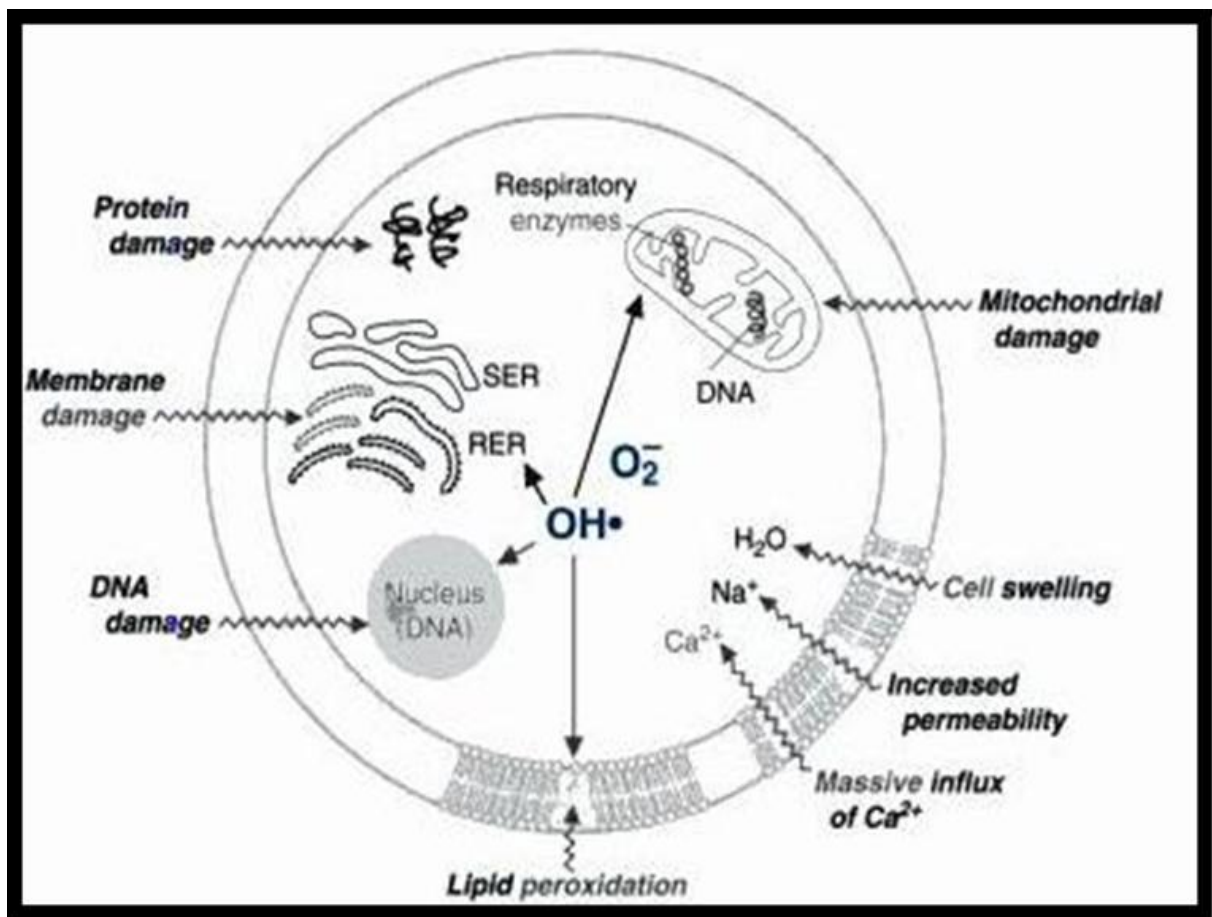


Figure 2- 3 Cell damage caused by various forms of reactive oxygen species (adapted from Todd, 2006). Lipid peroxidation by hydrogen peroxide results in lipid membrane damage, increasing cell membrane permeability to both calcium and sodium cations. Hydrogen peroxide also results in mitochondrial damage as it suppresses activities of some antioxidant enzymes. There is further DNA and protein damage as a result of accumulation of ROS.

H_2O_2 is perceived as a signaling molecule that regulates the expression of stress-related genes (Rodríguez et al, 2005). The accumulation or excessive production of H_2O_2 , which can occur in the cell during stress, triggers the expression of stress genes and changes in the level of cellular antioxidants (Rodríguez et al, 2005.). However there is mounting evidence that the destructive potential of H_2O_2 depends to a large extent on the ability of the plant antioxidant system to respond positively to the stress (Chrispeels & Sadavan, 2003).

2.2.3.0. Causes of ROS accumulation in cells

The generation of ROS is induced by a number of abiotic and biotic factors. Such factors include salinity, drought, UV-B, air pollutants (e.g. ozone, sulphur dioxide) (del Rio et al, 1993), redox-active herbicides (e.g. paraquat) (Dannahue et al, 1997; Morita et al, 1999) and phytotoxic metals (e.g. Zn, Cu, Cd) (Foyer et al, 1994). The invasion of plant cells by pathogens also results in generation of ROS (Torres et al, 2006.), causing the hypersensitive response (HR). These bursts of ROS upon stress induction, if not scavenged by plant antioxidants or buffered by redox buffers (Asada, 1992 and Guo & Crawford, 2005), trigger cell/tissue senescence (Kubo et al, 1995; Morita et al, 1999 and Neil et al, 2002a & b).

2.2.4.0. Regulation of ROS accumulation in the cell by abscisic acid (ABA)

Abscisic acid, a well known stress-related hormone, is involved in plant stress responses. There is evidence that ABA is involved in superoxide (Jiang & Zhang, 2001) and hydrogen peroxide generation (Jiang & Zhang, 2001 and Zhang et al 2001). It has been established that ABA triggers ROS generation that precedes the activation of antioxidant enzyme activities in maize leaves, including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX) (Jiang & Zhang, 2002). It further induces the expression of genes encoding antioxidants enzymes such as Cu-Zn-SOD, Mn-SOD, Fe-SOD involved in hydrogen peroxide generation (Kamanika et al, 1999) and catalase involved in scavenging of hydrogen peroxide (Guan et al, 2000).

2.3.0.0. Biochemistry of nitric oxide (NO)

2.3.1.0. Enzymatic sources of NO in plants

Nitric oxide is endogenously synthesized in plant cells (Guo et al, 2003 and Zhao et al, 2007). There are two suggested enzymatic sources of nitric oxide in plants; the nitrite-dependent pathway and arginine-dependent pathway.

Mammalian NO is synthesized enzymatically from L-Arginine in a process catalyzed by nitric oxide synthase (NOS). NOS genes have been isolated in mammals and sequenced. The existence of NOS in plants was first detected biochemically in lupine roots (Cueto et al, 1996). This arginine-dependent nitric oxide synthesis has also been detected in peas (Corpas et al, 2006). However, no gene homologue of mammalian NOS has been identified and isolated to date in plants. Several attempts have been made to isolate genes encoding plant NOS, but all have been unsuccessful. AtNOS1 (Guo et al, 2003), which was earlier reported to be a “novel plant NOS”, was found not to have NOS activity (Zamojtel et al, 2006; Crawford et al, 2006) and was later concluded to be a GTPase (Zamojtel et al, 2006).

Nitrate Reductase (NR; EC1.6.6.1) is a central enzyme in nitrogen assimilation in plants. It has two main forms depending of its cellular location; plasma membrane (PM-NR) and cytosolic (cNR) form. It was observed more than two decades ago that NR catalyzes the generation of nitric oxide from nitrites (Dean et al 1986). NR catalyzes NO generation from nitrites in a reaction that consumes NADPH as an electron donor (Rockel et al, 2002). Other studies have also reported the simultaneous generation of nitrous oxide, a pre-cursor of peroxynitrite and NO (Dean et al, 1986). In agreement with these findings

is the fact that NR activity using nitrites as a substrate under aerobic conditions yields NO and peroxynitrite (Yamasaki & Sakihama, 2000). This NR-dependent NO generation is nitrite-concentration-dependent. NO is generated from nitrites only if the nitrite levels exceed those of nitrates (Keiser et al, 2002). This occurs under dark conditions or anoxic conditions.

2.3.2.0. Nitric oxide signaling

2.3.2.1. Introduction

Nitric oxide is a highly versatile molecule involved in several metabolic processes. Due to its soluble nature in both aqueous and lipid medium, it can easily diffuse through the cell membranes (Hakim et al, 1996). It has a half-life of 445 seconds in aqueous solutions and disappears rapidly in solutions depending on the available proportion of haemoglobin. Its rate of decrease in the presence of haemoglobin is $2 \times 10^{-5} \text{M}^{-1} \text{s}^{-1}$. Its short half-life permits it to be an effective signaling molecule (Hakim et al, 1996). NO signaling is mediated through various pathways including cGMP-dependent pathways, S-nitrosylation, ABA-dependent pathways, and calcium dependent pathways.

2.3.2.2. Modulation of the NO signal through cGMP-dependent pathways.

NO acts through cGMP-dependent pathway by interacting with soluble guanylyl cyclases (Gorbe et al, 2007) and thus stimulates the synthesis of cGMP. A novel protein with guanylate activity has been identified in plants although it lacks the nitric oxide-dependent activity (Ludidi & Gehring, 2003). Despite the fact that no protein with guanylate activity that is nitric oxide-dependent has been identified to date in plants, it

has been established that NO functions to affect some metabolic activities through cGMP-dependent pathways in plants (Szmidt-Jaworska et al, 2003 and Salmi et al, 2007). Some of the effects of cGMP are exerted via activation of protein kinase K. This protein kinase K is involved in posttranslational modifications of proteins and functions to regulate various cellular responses (Szmidt-Jaworska et al, 2004 & Szmidt-Jaworska et al, 2009) (Figure 2- 4)

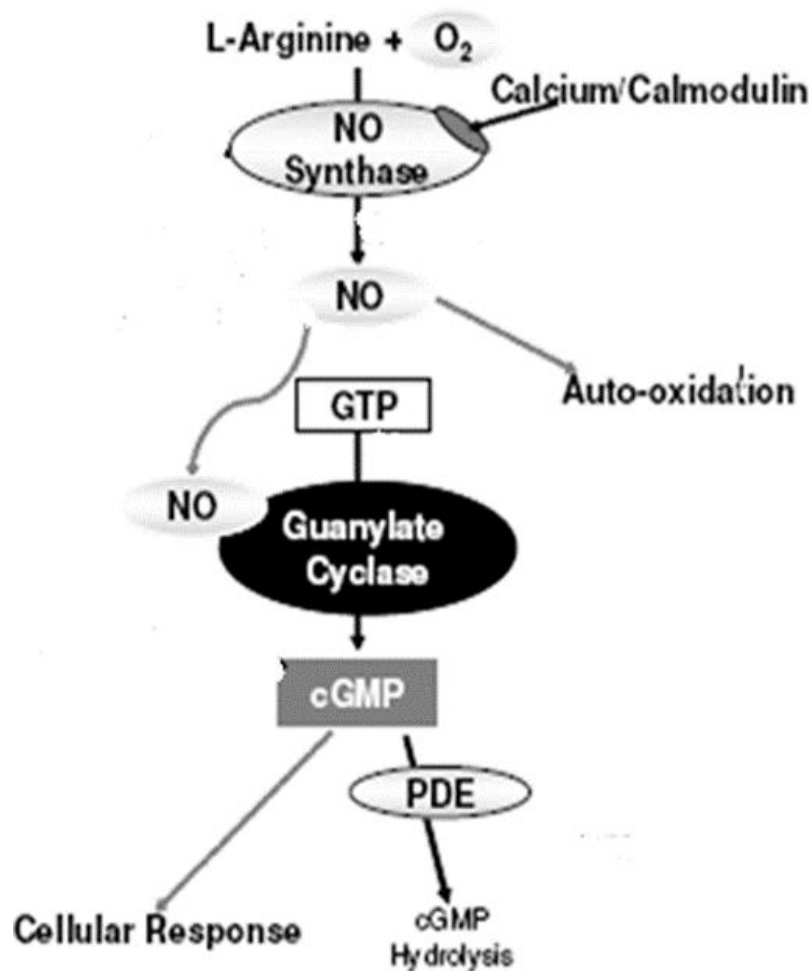


Figure 2- 4 Illustration of NO signaling cascade. Nitric Oxide Synthase (NOS) is activated by the Ca²⁺-calmodulin complex. NOS converts L-Arginine and oxygen to citrulline and NO. Guanylate cyclase catalyzes the formation of cGMP from GTP in a reaction that is NO-dependent. The PDEs are phosphodiesterases that modulate downstream responses to cGMP accumulation through hydrolysis of cGMP. Sometimes the cGMP activates protein kinases and affects cellular responses. (Adapted and modified from Jacobi et al 2006)

2.3.2.3. Modulation of the NO signal through S-nitrosylation of proteins

Nitric oxide may also be involved in regulating protein function through s-nitrosylation. S-nitrosylation occurs when nitric oxide reacts with the cysteine residue to form S-NO bonds. This is increasingly recognized as a ubiquitous control of protein function in both animals and plants. For instance, nitric oxide is thought to react with GSH to produce S-Nitrosoglutathione (GSNO). GSNO functions as a bio-transporter of the NO group to thiols in cellular compartments to form S-nitrosothiols (SNOs) that participate in a nitrosation reaction (Liu et al 2001). GSNO does not only transport NO but participates in regulating protein activity in processes such as resistance to pathogens (Belenghi B et al, 2007; Perazzolli et al, 2004 and Grennan, 2007). This nitrosylation process is reversible and regulates reactive nitrogen species (RNS) and nitroso species quantities in the cell (Ji et al, 1999).

2.3.2.4. Calcium-dependent pathways

Amongst various other roles, Ca^{2+} is involved in signal mediation of Ca^{2+} -dependent protein kinase (CDPK) during early stages of plant development (Anila & Rao, 2001). In order for signal transduction to occur, Ca^{2+} transporters, namely ATPase and $\text{H}^+/\text{Ca}^{2+}$ antiporters help transport the calcium cations through calcium channels. Ca^{2+} binding proteins and protein kinases sense, amplify and transduce the calcium-dependent signal further downstream the pathway. On the other hand there is evidence that NO usually accumulates in the cells in response to NaCl stress and both NO and NaCl increase the activity of antioxidant enzymes (Zhang et al, 2006). Furthermore, studies on NaCl-stressed maize seedlings revealed a signaling role for NO and this involves increasing the activities of vacuolar H^+ -ATPase and H^+ -PPase, which provide the driving force for

Na⁺/H⁺ exchange (Zang et al 2006). This indicates that the NO signaling role under saline conditions may be modulated through-calcium dependent pathways.

2.3.2.5. *Abscisic acid (ABA)-dependent pathways*

ABA is a stress-related hormone involved in several physiological responses including the closing of the stomata and leaf abscission. This hormone is normally secreted in larger quantities in response to stress in plants. There is increasing evidence suggesting a link between NO-regulated plant physiological processes and those regulated by the ABA pathway. Firstly Zhao et al, (2001) demonstrated that both NO and ABA levels are increased under drought and that NO functions upstream of ABA since blocking the NOS activity also blocked ABA accumulation. NO and gibberellic acid (GA) have also been found to interact with ABA upstream of the pathway that leads to vacuolation (disassembly of proteins in the vacuoles) of protein storage that occurs during programmed cell death (Paul et al, 2007 and Guo et al, 2006). Nitrate Reductase (NR)-dependent NO biosynthesis is also responsible for ABA mediated stomatal closure during dehydration (Bright et al, 2006).

2.3.3.0. **The role of NO in plant growth and development**

Plant growth and development are affected by NO concentration. Higher cellular NO concentrations are detrimental to plant health (Anderson and Mansfield, 1979). Nitrites can be a source of NO under anoxic conditions in the enzymatic reaction catalized by NR (Morot-Gaudry et al, 2002). Under these conditions where nitrite reduction by nitrite

reductase is limited, NO produced from NR inhibits photosynthesis and this is thought to be via control of chlorophyll formation by NO (Beligni, Lamattina, 2000).

Exposure of pea leaves to nitric oxide reduced the generation of endogenous ethylene, the hormone required in for senescence (del Río et al, 2004). NO in this case delayed leaf and flower senescence. Guo et al, (2005) also confirmed the role of NO in inhibiting senescence. Nodule senescence is a form of programmed cell death and occurs as part of normal plant growth and development. However, it is not known if the observed effects of NO in plant tissues as described above would be similar in the complex nitrogen fixing nodule tissue from leguminous plants.

The studies related to induction of seed germination by NO treatment (Bethke et al, 2007; Madolo, 2003) only adds to the list of the already known effects of NO on promoting plant growth. It has also been observed that NO has a role in promoting root growth and acting as an important player in promoting root gravitropism (Xiangyang, et al, 2005).

2.3.4.0. Nitric oxide and stress responses

Nitric oxide is involved in modulating pathways related to plant responses to stress (Neil et al, 2002a; Guo et al, 2005; Wang et al, 2006). Thus NO accumulation in the cells induce various processes in plants, including the expression of defense-related genes (Rusterueci et al, 2007), programmed cell death (PCD) (de Pinto et al, 2002) and stomatal closure (Niell et al, 2007).

NO has been established as a signaling molecule involved in plant abiotic stress responses (Neill et al, 2002b; Zhao et al, 2004). Water stress in plant cells occurs as a consequence of several abiotic stresses in plants, including salinity, cold and drought. Plant leaves protect the plant from dehydration through closure of stomata upon receiving the drought stress stimulus. The physiological processes leading to this stomatal closure in response to dehydration are modulated by nitric oxide (Bright et al, 2006; Neil et al, 2008). Furthermore NO is endogenously generated in plant cells in response to salinity stress and this NO activates the antioxidant system of the plant, and thus provides protection against oxidative stress (Neill et al, 2007; Zhao et al 2004; Zhang Y et al, 2006). Nitric oxide induces salt tolerance by increasing vacuolar H⁺-ATPase activity and its synthesis (Zhang et al, 2006 and Zhao et al 2004). Drought also increases NO generation in the cells and this NO protects cells against oxidative stress in the same manner as in NaCl stress (Hao, et al, 2008). There is a detailed account on NO as an antioxidant and a protective molecule against abiotic stress (Neill et al, 2008).

2.4.0.0. Biochemistry of plant antioxidants

2.4.1.0. The role of Antioxidants in plants

2.4.1.1. Introduction

The term 'antioxidant' encompasses those biological chemicals that the plant cell uses to counteract oxidative process. In plants, oxidative stress occurs as a consequence of the accumulation of reactive oxygen species (ROS) in the cell (Ahmad et al, 2008). Certain concentrations of ROS trigger expression of genes that lead to deamination of proteins, oxidation of DNA and initiation of senescence-related processes (Ahmad et al, 2008).

Since these ROS are produced as a consequence of many metabolic processes and also under stress conditions, the plant cell has evolved to have an array of antioxidant enzymes working together with redox buffers to maintain a non-toxic balance in ROS concentration, thereby mitigating the undesirable impact of ROS. One of the first set of responses to exposure to stress is an increased generation of superoxides. Superoxide leads to the activation of superoxide dismutase (SOD, EC: 1. 15.1.1). SOD works to dismutate superoxide in a process that consumes NADPH under anaerobic conditions to produce hydrogen peroxide (McCord & Fridovich, 1969). Hydrogen peroxide can then be detoxified by either catalase (CAT, EC; 1.11.1.6), glutathione peroxidase (GPx, EC: 1.11.1.9) or ascorbate peroxidase (Figure 2-4) (Møller, 2001). The two metabolites ascorbate and glutathione are also involved in detoxification of hydrogen peroxides.

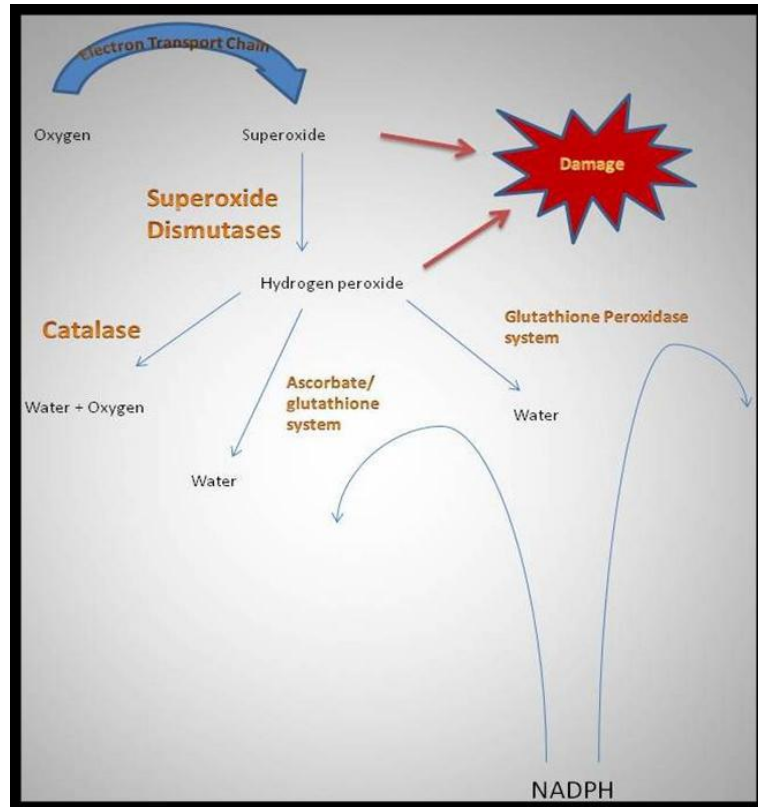


Figure 2- 5 Summary of antioxidant enzyme systems that are important for scavenging ROS in plant mitochondria (adapted and modified from Møller, 2001).

2.4.1.2. Superoxide Dismutases (SOD, EC: 1. 15.1.1)

Superoxide dismutases are a group of metallo-proteins that catalyze the dismutation of superoxide (O_2^-) to molecular oxygen and hydrogen peroxide (H_2O_2). Copper-zinc SOD from bovine erythrocytes was isolated first (McCord and Fridovich, 1969), followed by manganese-containing superoxide dismutase that was found in prokaryotes and the mitochondria of eukaryotes. Lastly, the iron-containing forms of superoxide dismutase were first identified in algae and *E. coli*. All these forms were proved to occur in higher plants (Giannopolitis and Ries, 1997). In higher plants, SOD seems to be more concentrated in the shoots than in the roots (Giannopolitis & Ries, 1997). CuSOD and ZnSODs are glycosylated tetramers that serve to protect the cell against O_2^- (Fridovich, 1995). CuZn SOD is also located in the cytosol and chloroplast (Ogawa et al, 1996). Mn SOD is located in mitochondria and peroxisomes (Wolfe-Simon et al, 2006) whereas Fe SOD is found in the chloroplast (Salin, 1988) (Figure 2- 6).

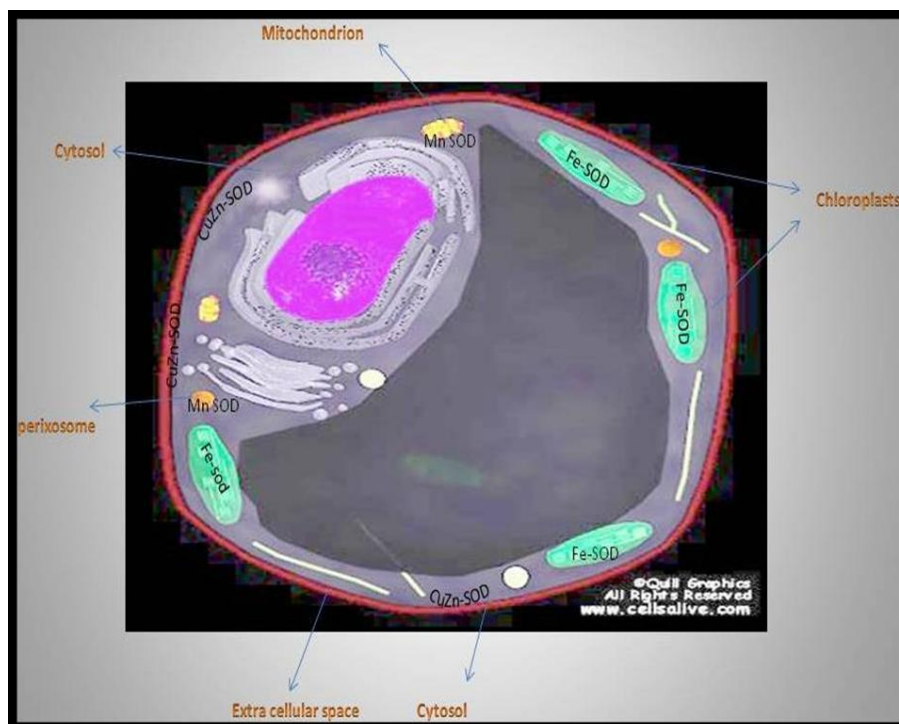


Figure 2- 6. Localization of SOD isoforms in various compartments of the plant cell. SOD; Superoxide dismutase (adapted and modified from www.cellsalive.com 02/07/09)

2.4.1.3. Catalase (CAT, EC; 1.11.1.6)

Catalases (H_2O_2 : H_2O_2 Oxidoreductase, EC 1.11.1.6) aid in decomposition of H_2O_2 in a process that yields water by aerobic organisms. CATs are mostly heme-containing tetramers of about 240 kDa. However, there are exceptions of non-heme proteins (Switala and Leuven P.C, 2002) and dimers (Putnam et al 2002). This group of enzymes has been identified in plants and is very heterogeneous. Catalase activity has been detected in abundance in glyoxysomes of germinating seeds and in leaf peroxisomes (Weiting et al, 1990). Little is known about the existence of these enzymes in roots and root nodules. Leaves of pea plants have been reported to have only one isoform whereas cotyledons of germinated cotton (*Gossypium hirsutum L.*) have about five distinct isoforms (Weiting et al, 1990).

Characterization of catalases from *Nicotiana* shows a new dimension in the heterogeneity of catalases. Based on electrophoretic mobility, it has been observed that different catalase isoforms are activated at different stages of plant development, with specific inhibition of the various isoforms, indicating functional specificity (Havir & Mchale, 1987). CAT activity is controlled both at transcriptional and posttranslational level (Ni & Trelease, 1991).

2.4.1.4. Ascorbate-Glutathione cycle Antioxidant enzymes

Ascorbate peroxidase (EC: 1.11.1.11) is the most important antioxidant enzyme in the chloroplast. It uses ascorbate as the reductant to scavenge H_2O_2 in a process that forms water as a by-product and dehydroascorbate (DHA) as the final product ((Møller, 2001). A certain level of ascorbate has to be maintained in the cell and this is done by

dehydroascorbate reductase (DHAR; EC 1.8.5.1) via recycling of DHA back into ascorbate by oxidizing reduced glutathione (GSH) (Møller, 2001). Furthermore, glutathione reductase (GR; EC 1.6.4.2) recycles oxidized glutathione (GSSG) back to GSH in a process that uses NADPH as an electron donor (Bowler et al, 1992). This specific controlled generation of metabolites (also known as redox buffers) and antioxidant enzymes could help to maintain the levels of ROS below toxic concentrations.

2.4.1.5. Glutathione peroxidase (GPx; EC 1.11.1.9)

GPx, characterized by the possession of three conserved cysteine residues in the coding region, are a family of multiple isozymes involved in scavenging hydrogen peroxide (Rouhier & Jacquot, 2005). Glutathione peroxidase detoxifies hydrogen peroxide and alkyl hydroxyls in a biochemical reaction that utilizes glutathione as a reductant. In plants, several isoforms have been identified including one from *Aloe vera* that is 16 kDa (Sabeh et al, 1993) and another 22 kDa isoform from *Citrus sinensis* (Ben-Hayyim et al, 1993).

The GPXs in plants are not only different structurally from animal glutathione peroxidase but also functionally. Animal GPx uses glutathione only as a reductant while the plant GPx shows an alternative pathway. This is evidenced by glutathione peroxidase proteins identified in *Arabidopsis thaliana* and Chinese cabbage that had glutathione-dependent peroxidase activity and showed high affinity to thioredoxin as a reductant (Mittler et al., 2002). These enzymes are localized in all the plant parts and different cell compartments (Rouhier & Jacquot, 2005, and Lee et al, 2002). Plant GPxs are responsive to ROS accumulation induced by both biotic and abiotic stress. They also

catalyze the reduction of peroxynitrite and reactive nitrogen species (RNS) (Rouhier & Jacquot, 2005).

2.4.2.0. Antioxidants and stress-induced responses.

2.4.2.1. Redox buffer responses

The ascorbate-glutathione pathway comprises two main metabolites, glutathione and ascorbate. Studies show that, depending on the concentration of ROS or extent of the oxidative stress, the ascorbate and glutathione pool can be reduced (Foyer et al, 1994). For instance, the aging process is facilitated by oxidative stress and this observation is supported by evidence showing a decline in concentrations of ascorbate in aging leaves (Foyer et al, 2004). Furthermore GSH content decreases with senescence in pea leaf mitochondria and this is mainly attributed to a decrease in the activity of GR. (Jimezen et al, 1998). Total glutathione pool also decreases with nodule senescence (Dalton et al, 1993; Evans et al, 1999 and Puppo et al, 2005). Early responses of GSH and ascorbate to abiotic stress involve an increase in transcripts of the enzymes involved in the biosynthesis of these metabolites, with prolonged exposure leading to senescence associated with a decrease in the levels of the metabolites themselves (Loscos et al, 2008).

2.4.2.2. Antioxidant enzyme responses

Various abiotic stress conditions have been studied and have been found to activate different antioxidant enzymes. NaCl-induced abiotic stress triggers the plant antioxidant system in defense against the oxidative stress that results from exposure to elevated NaCl concentrations. The activation of the antioxidant system by NaCl is tissue-

specific (Hamed et al, 2007). Arbona et al, (2003) demonstrated the differential response of both non-enzymatic and enzymatic antioxidants to different concentrations of salt. It is important to note that both abiotic and biotic stress trigger the accumulation of ROS and hence the activation of the antioxidant system under such conditions.

2.5.0.0. Legume root nodules and nitrogen fixation

2.5.1.0. Introduction

Leguminosae represent the family of plants found on both temperate and tropical regions, and are believed to originate in the tropics. They range from small annuals to large shrubs. The majority of the known legumes have a potential to fix nitrogen from the symbiotic associations with *Rhizobium/Bradyrhizobium* in their roots.

In this symbiotic relationship, bacteria fix atmospheric nitrogen in an enzymatic reaction catalyzed by nitrogenase from rhizobia (Gordon et al, 1997.). During nitrogen fixation in the root nodules, nitrogenase catalyzes the reduction of nitrogen to form hydrate (Gordon et al, 1997). This process is costly and the energy demand for the reaction is satisfied by the release of chemical energy from hydrolysis of 16 molecules of ATP to form ADP. Photosynthesis products, mainly glucose, are required in large quantities to meet this high ATP demand for nitrogen fixation. Magnesium ions are also important as co-factor for nitrogenase enzyme (Gordon et al, 1997).

2.5.2.0. Nodulation signals from root exudates of legumes and symbiotic bacteria

Signal exchange leading to nodule development begins with the secretion of flavonoids by the plant roots in recognition of a compatible symbiosis partner. These flavonoids act as signal molecules that activate the expression of specific genes in symbiotic bacteria. There are three main flavonoids that have been found to accumulate in roots during nodulation, namely; genistein, daidzein and coumestrol (Dakora, 2000). These molecules are relatively similar in structure (Figure 2- 7) allowing less specificity of the genotypes that can be infected (Table 2- 1). Upon recognition to the plant flavonoids, bacteria then respond by releasing oligosaccharides known as nodulation (NOD) factors. Consequently the bacteria get adsorbed to the root hair causing it to curl and engulf the adsorbed bacterial (Dakora, 2000).

Infection begins with the formation of infection threads that penetrate the centre of the root cortex. In this symbiotic relationship, the bacteria provide the plant with useable nitrogen, while in return the bacteria benefit from plant-derived carbon supply in the form of sugars from the products of photosynthesis (Rawsthorne et al, 1980).

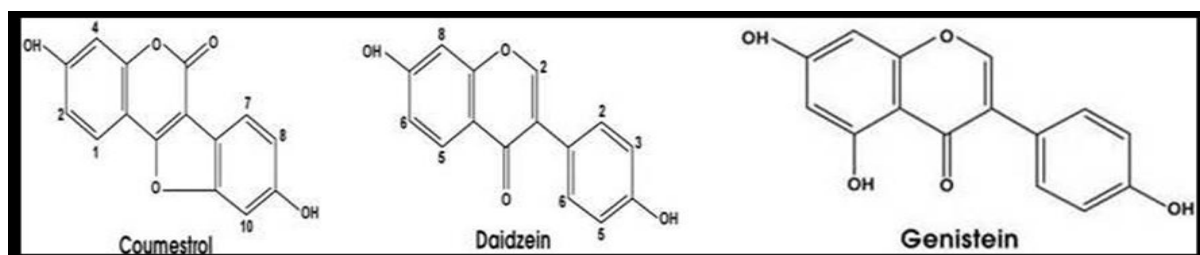


Figure 2- 6 Chemical structures of nodule inducing molecules (flavonoids) (Adapted from Dakato, 2000)

2.5.3.0. Classification of Legume Nodules

Generally, nodules are grouped into determinate and indeterminate nodules. This characterization is mainly based on the anatomy and developmental system of the nodules. The indeterminate nodules have a persistent apical meristem in mature nitrogen fixing nodules. Their central cortex is made up of the vascular bundles that are surrounded by pericycle cells and endodermis cells. These cells continue to differentiate throughout the life of the nodule (Sprent, 1980 and Dakora, 2000). On the other hand the determinate nodules have their central part as the active nitrogen fixing region enveloped by the inner cortex and also consisting of vascular bundles. The vascular tissue in this case is also enveloped by pericycle cells and endodermis. Nitrogen fixation occurs at the center of the cortex. However the nodules of determinate nodules are spherical in shape, unlike the oval indeterminate nodules, and have a non-persistent meristem (Sprent, 1980 and Dakora, 2000).

Table 2- 1 Nodulation gene-inducing molecules from root exudates of some tropical grain legumes belonging to *Phaseoleae*

Legume species	nod-Gene inducer	Bacterial strain	Reference
Bambara groundnut	Daidzein Genistein Coumestrol	Rhizobium NGR234	Dakora & Muofhe 1996
Common bean	Daidzein	R.l. bv. phaseoli 4292	Dakora et al. 1993 Genistein Hungria et al. 1991 Coumestrol Dakora et al. 1993
Soybean	Daidzein Genistein Coumestrol	<i>B. japonicum</i> USDA123	Kosslak et al. 1987
Cowpea	Daidzein Genistein Coumestrol	Rhizobium NGR234	F. D. Dakora, unpublished results
Kersting's bean	Daidzein Genistein Coumestrol	Rhizobium NGR234	F. D. Dakora, unpublished results

Adapted from Dakora (2000).

2.5.4.0. Nodule development and survival mechanisms

2.5.4.1. ROS signaling and antioxidant metabolism during nodulation

Hérouart et al, (2002) have reviewed possible roles of ROS and NO in the nodulation process and in nodule development in legumes. They suggest the occurrence of an oxidative burst accompanied by the increased levels of nitric oxide in the early stages of the infection-thread development. In order to facilitate nodulation, the legume root hairs first perceive the *Rhizobium spp* as a virulent pathogen thus followed by oxidative burst. This could possibly trigger activation of antioxidant enzymes, glutathione biosynthesis and the expression of genes involved in nodulation. It is thought that this process is modulated by nitric oxide.

2.5.4.2. Nodule biochemistry and physiology

Although symbiotic nitrogen fixation contributes a lot to plant nitrogen economy, nodule development and survival costs the plant a large proportion of the plants' total energy demand. Such demand is catered for by the product of photosynthesis of which the surplus products are translocated to other sink organs for storage (Nelson et al, 1984). There is also a need for specific regulation of oxygen in the nodules so as to protect the nitrogenase enzyme complex and simultaneously to provide the medium for enough ATP synthesis that is required for nitrogen fixation and nodule maintenance in the nitrogen fixing tissue (Gordon et al 1997). The reaction for nitrogen fixation can be represented by equation (1) as follows:



The nitrogen fixation reaction utilizes sucrose as a source of ATP and yields ammonia as the first product. Ammonia is further assimilated by incorporation into organic compounds like amino acids or ureides in the presence of carbon skeletons generated from photosynthates. These carbon skeletons are required for transport of this fixed nitrogen (Rawsthorne et al, 1984). The most common source of carbon for nitrogen fixation is sucrose. The sucrose supply to the nodules is dependent upon several environmental factors and the photosynthesis rate in the leaves. Glutamine and glutamate synthase are the main enzymes catalyzing the assimilation of ammonia in the root nodules. This pathway itself yields important molecules, including amino acids and nucleotides (ureides) (Lancern et al, 2000).

There are several environmental factors that affect nodule formation and survival. One of the most studied factors is salinity. Generally salt stress causes a decrease in plant growth parameters, e.g. shoots, roots and nodule biomass. Reduced biomass in response to salt stress has been reported (López et al, 2007) in both the determinate and indeterminate nodules. In line with these findings, (L'taiefa et al, 2006) concurs with the remarkable decrease in growth parameters due to salt stress, with some genotypes exhibiting no nodule formation under highly saline conditions. Salah et al (2008) agreed with these findings and even suggested an absolute and definite decrease in nodule biomass and number under stress regardless of the nodule morphology or genotype.

The malfunctioning of the nodules under high NaCl conditions is attributed to several factors including nitrogenase activity, nodule conductance, nodule carbon metabolism and Ca⁺/P⁺ metabolism. These factors affect the nitrogen fixation process. Bacteria fix

atmospheric nitrogen into nitrates/ammonium ions in a process that requires the supply of oxygen by leghemoglobin and the presence nitrogenase enzyme to catalyze the formation of nitrates/ammonia. The nitrogen fixation rate and amount of leghemoglobin are reduced under salinity (Comba et al, 1998). With the amount of leghemoglobin reduced due to high salt concentrations, the oxygen conductance is perturbed; hence nitrogen fixation rate will be reduced. López, et al (2007) further reports both morphology- and genotype-specific decreases in both nitrogenase activity and leghemoglobin accumulation. Salt sensitive genotypes seem to exhibit more decreased nitrogen fixation rates (Ben et al, 2008). It is further established that nodule stability and formation under saline condition is highly dependent on nodule oxygen conductance (L'taief et al, 2007 and Ben et al, 2007).

High salt concentrations also perturb the sucrolytic activities, thus affecting nodule survival negatively. Such stress conditions have been reported to inhibit sucrose synthase and inhibits the transport of sucrose to the root nodules (Ben et al, 2008), decrease the activities of trehalose-6-phosphatase (TPS), trehalase (TPE) and nodule sucrose content (López et al, 2007). Similar to the growth parameters and nitrogen fixation rate, these decreases are genotype-specific.

2.5.4.3. Transcriptomics of nodule development

Root nodule developmental stages are characterized by several polypeptides accumulating in a temporal and specific manner. These polypeptides are essential for nodule development and function. They are called nodulins and are categorized as early nodulins and late nodulins (Verma et al, 1986). Nodulins were first identified in soybean (Verma et al, 1992) and their discovery led to isolation and characterization of

several nodulins. The early nodulins are thought to be essential for infection process. While the late nodulins are expressed prior to nitrogenase activity and nitrogen fixation commencement, and are essential for nodule developmental process and nitrogen fixation (Verma et al, 1986). The late nodulin genes comprise polypeptides like legheamoglobins, sucrose synthases and glutamate synthases. Nodulins exist throughout the species of nitrogen-fixing legumes and are expressed in the nodules (Verma et al, 1992).

Further characterization of the regulation of nodulins and their families in the 20th century revealed the conserved organ specific (OSE) and nodule infected cell expression (NICE) motifs (Metz et al, 1998 and de Bruijn et al, 1994). These motifs include the well known nodule specific consensus sequences, AATGAT and CTCTT, present in all the nodule specific promoters (Fehlberg, et al, 2004 and Hohnjec et al, 2000).

2.6.0.0. Problem statement and objectives

Drought and salinity are amongst the most detrimental factors affecting crop productivity. Several approaches to improve crop tolerance to abiotic stress have been employed and legume crop varieties with a certain level of tolerance to stress have been bred, and yet the need for abiotic stress-tolerant crop development has not been met. There is also mounting evidence that these abiotic stress-tolerant crop genotypes, just like halophytes, have differential elevated activities of antioxidant enzyme activities under such stresses (Koca et al, 2006; Neto et al, 2005; Shivakumar et al, 2003). In concurrence with these findings, there is also accumulating evidence that the destructive nature of ROS that accumulate in response to abiotic stress depends to a large extent on the response of the antioxidant system (Rodriquez et al, 2005 and Neil

2007). Antioxidants are the scavengers of reactive oxygen species; however the orchestration of events leading to the scavenging process is not fully understood, especially in legume root nodules.

Nitric oxide is an endogenous signaling molecule involved in the regulation of stress responses (Yamasaki & Sakihama 2000 and Neil et al, 2008). The abiotic stress signaling pathway is very complex and involves many molecules and stress responsive genes (Bhatnagar-Mathur et al, 2008). In this pathway, nitric oxide functions upstream of Ca^{+} in the dehydration response pathway leading to stomatal closure (Neill et al, 2008). NO has also been identified as a signaling molecule involved in plant response to salinity stress (Zhang et al, 2006 and Zhao et al 2004). Despite the fact that NO is denoted as a ROS scavenger, protecting cells from oxidative damage (Beligni et al , 2002), it is still not clear as to how NO protects root nodules against oxidative stress. Direct or indirect interactions of NO with antioxidants and biochemical pathway models are not well elucidated.

Both NO and antioxidant systems are implicated in detoxification of ROS and thus alleviate oxidative stress and hence delay early senescence. It was established that NO modifies protein functions through the cGMP pathway in mammals. Despite the fact that the proteins with guanylate activity that have been identified in plants, they are independent of the nitric oxide (Ludidi & Gehring, 2003). Cyclic GMP is a signaling molecule that is involved in plant abiotic stress responses (Donaldson et al, 2004). However there is no evidence established so far that links the signaling functions of NO in modulating the antioxidant responses in root nodules to cGMP pathway.

Just like any plant root tissue, root nodules are the first sensors of the abiotic stress and are prone to oxidative stress. Hence development of legume plant genotypes with increased tolerance to abiotic stress should be of prime interest. Development of abiotic stress-tolerant legumes is still an on-going process and requires rational design that involves regulating gene expression in the root nodules, coupled with efficient legume genetic transformation procedures. Despite the significance of nodule tissue in legume nitrogen economy, strategies involving development of more abiotic stress tolerant nodules have not been employed.

Nitrogen fixation at the early stages of the plant life cycle yields nitrogen that is important for grain yield. On the other hand, at later stages of plant development, fixed nitrogen contributes more to the grain's crude protein content (Hungira & Neves, 1987). Both crude protein and yield are important attributes in determining crop productivity. These higher yields and improved protein contents from nitrogen fixing legumes come with a price of a very high total ATP demand on net products of photosynthesis (Rawsthorne et al, 1980). Expressing abiotic stress-regulated genes constitutively has often resulted in stunted plants with rather abnormal growth, reduced number of seeds and reduced fruit set (Bhatnagar-Mathur et al, 2008). This might be happening as a consequence of increased energy demands for the plant. Hence a potentially useful attempt to develop legume plants with nodules more tolerant to abiotic stress would involve differentially regulating the expression of stress-related genes in the nodules in response to abiotic stress. However, there is no identified abiotic stress-responsive nodule-specific promoter currently used in biotechnology.

Nitric oxide functions in plants have been elucidated to some extent, its functions and roles in biochemical process discovered and scrutinized (Niell et al, 2008). However,

there has not been any direct role of NO linked to abiotic stress tolerance in root nodules, secondly the pathway in which NO affects nodule enzyme activities leading to abiotic stress tolerance have not been identified. It is in this background that this work aims specifically at:

1. Evaluating the effect of nitric oxide on
 - a. Superoxide dismutase enzyme activity and transcripts
 - b. Glutathione peroxidase activity
 - c. Glutathione reductase activity
 - d. Glutathione levels and redox status
2. Establish the functionality of NO as oxidative stress protectant mediated through the cGMP pathway.
3. Construct a nodule-specific promoter that is responsive to abiotic stress.
4. Design a strategy for differentially regulating the expression of a nitric oxide synthesizing gene in root nodules.

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Chapter 3 Effect of nitric oxide on superoxide dismutase

3.1.0.0. Introduction

Superoxide dismutases (SOD, EC 1.15.1.1) represent the first line of plant defense against reactive oxygen species (ROS) in the array of enzymes that function to protect the plant cells against oxidative stress.

For this reason SOD are classified as a chain breaking group of enzymes since they scavenge superoxide and they yield another form of reactive oxygen species; hydrogen peroxide (H₂O₂).

SOD has different isoforms namely; CuZn SOD, Mn SOD and Fe SOD. These isoforms have different nodule tissue localization (Rubio et al, 2004). Classification of the SOD isoforms is based on their metallic cofactors and have specific functions related to oxidative stress. The CuZn SOD is the most abundant isoform in the pea nodules. They are located in the nodule meristems, nitrogen fixing region, infected zone and the distal part of the nitrogen fixing zone. While Mn SODs are found in the distal part of the nitrogen fixing zone (Rubio et al, 2004), Fe SOD has not been studied in the pea nodules, its activity has not been detected in the nodules thus FeSOD may not be important in nodule oxidative stress related pathways (Becana et al, 1989 and Puppo et al, 1987).

The sub-cellular location of the SOD isoforms also determines the function of the isoform. The plastid SOD exhibit the vital role in early chloroplast development. For

instance, double mutants defective in this Fe SOD in Arabidopsis exhibited the albino phenotype and increased sensitivity to oxidative stress caused by methyl viologen (Myougu et al, 2008). The Mn SOD located in the mitochondria of Arabidopsis functions to promote proper root growth and to maintain the redox balance in the plastids (Morgan et al, 2008). Lastly CuZn SOD located in the chloroplast serves to protect the chloroplast against oxidative stress by enhancing the antioxidant system (Badawi et al, 2003).

Studies carried out so far show that the destructive nature of abiotic stress exists as a consequence of ROS accumulation resulting in oxidative stress. Thus the development of any mechanism in plants that would inhibit excessive generation of ROS in plants or decrease their accumulation in response to abiotic stress may enhance plant tolerance to such stresses. It was in the light of this evidence that ever since the 20th century, transgenic plants over-expressing different isoforms of SOD were generated and conferred different levels of tolerance to different abiotic stresses (van Camp et al, 1994; Gupta et al, 1993; Wang et al, 2004). Furthermore, these transgenic plants with increased SOD activity conferred high tolerance to salinity and exhibited normal growth and development under saline conditions (Wang et al 2004). Hence, one method of increasing plant tolerance to salt stress could involve increased SOD activity (Alscher et al, 2002). It should be noted that there is differential regulation of SOD isoform expression in response to the type and the intensity of the stress. For instance, FeSOD and Mn SOD are regulated differentially in response to different NaCl concentrations in the pea chloroplasts (Gomez et al, 2003).

It is also noteworthy to point out that plant tissues defend themselves against oxidative stress through increasing the scavenging capacity of antioxidant enzymes (Gomez et al,

1999; Hernandez et al, 1993). It is further observed in several plant species and other plant tissues that antioxidant enzyme activity (SOD included) following exposure to abiotic stress are modulated by nitric oxide (Neill et al, 2008). SOD enzyme activity is always up-regulated in response to salinity (Hernandez et al, 1993; Qiu-Fang et al, 2005; Slesak et al, 2003; Gomez et al, 2004 and Goreta et al, 2007). Lastly, there is accumulating evidence that NO acts as a signaling molecule involved in antioxidant response to salinity (Li et al 2008) and protects cells against oxidative stress, as evidenced by delayed cell senescence in plant tissue treated with nitric oxide donors (Neill et al, 1999).

The role of nitric oxide as a ROS scavenger is mostly established in the shoot system of the plants. Nodule tissue is structurally and physiologically different from the rest of the legume plant. Thus, the complex physiological nature of this tissue may suggest a possibility of existence of a different response mechanism to NO treatment.

This chapter therefore intends to study the contribution of NO in ROS-scavenging processes in root nodules and to establish its effects on the various SOD isoforms. It focused on determining the effect of NO on SOD enzyme activity and goes further to look into changes that might be brought about by NO on the transcription of these isoforms.

3.2.0.0. Materials and methods

3.2.1.0. Materials

Medicago and pea seeds used for this work were kindly provided by Agricol (Brackenfell, South Africa) and all the chemicals were purchased from Sigma-Aldrich,

unless otherwise stated. The Rhizobium inoculum *Rhizobium leguminosarum* bv. *Viciae* as the commercial inoculant 'Stimulym Peas and Vetch Inoculant' was supplied by Stimuplant CC, Zwavelpoort, Pretoria; South Africa.

3.2.2.0. Plant tissue generation and nitric oxide treatments

Pisum sativum variety Crusader and *Medicago truncatula* variety Parabinga were used for this work. The seeds were planted in pre-soaked vermiculite until plant emergence. After 6 days of initial seeding date, the seedlings were inoculated with *Rhizobium spp* and re-planted back into the pots and watered with nitrogen free Hoagland nutrient solution. The 4 weeks old plants with mature nitrogen fixing nodules were used for experimental purposes.

For transcriptome analysis, the nodulated roots were cut from the main root system and immersed into 10 mM potassium phosphate buffer containing 50 μ M of diethylene triamine/nitric oxide adduct (DETA/NO) and the negative control without any nitric oxide donor respectively. The nodulated roots were left immersed in the different solutions for 6 hours.

For enzyme activity assays on pea plants, the nodulated roots were treated in 10 mM potassium phosphate buffer, 50 μ M diethylenetriamine (DETA, similar to DETA/NO except that it lacks the NO moiety), 1 mM N (G)-nitro-L- arginine methyl ester (L-NAME, a nitric oxide synthase inhibitor, Connors et al, 2005), 50 μ M DETA/NO (a nitric oxide donor) for 3 hours, 6 hours and 24 hours. The nodulated root bunches were snap frozen immediately in liquid nitrogen following the treatments. A weight of 20 mg of nodules

from each treatment were harvested and used for protein extraction in 10 times dilution extraction buffer and subsequent enzyme activity assay per replicate.

The treatment in *M. truncatula* was as follows: sodium nitroprusside (SNP) was used as nitric oxide donor, potassium ferricyanide was used as fixed control and nutrient solution at pH 7.0 was used as a negative control. The treatments were assigned as above and the nodules were harvested at intervals of 1 hour, 3 hours, 6 hours and 24 hours.

All the above mentioned treatments were undertaken under optimal plant growing conditions and also in 150 mM NaCl in the treatment solutions.

3.2.3.0. Superoxide dismutase enzyme activity assay.

The total cellular superoxide dismutases were extracted in 10 times volume of ice-cold 20 mM HEPES buffer supplemented with 1mM EGTA as a chelating agent and 70 mM sucrose at pH 7.2. The nodule tissue was mechanically homogenized for 25 seconds using an electrical grinder (Black and Decker, KC 9036, type1; 3.6V, 180/min, England SLI, 3YD). The homogenate was then centrifuged at 4 °C using a table top centrifuge at 2000 *g* for 3 minutes (Mattiuzzi et al, 2002). The supernatant was then transferred to a clean 1.5 ml Eppendorf tubes and kept on ice for the assay. All the activities were carried out at 4 °C. SOD activity was assayed spectrophotometrically in a reaction that contained 50 mM TRIS-HCl, pH 8, 0.1 diethylenetriaminepentaacetic acid (DTPA), 0.1 mM hypoxanthine, 0.03 units xanthine oxidase per 230 µl reaction (Fridovich, 1971), 10% volume of the protein extract and 0.25 mM WST tetrazolium salt (Tominaga et al, 1998) and was incubated for 20 minutes at 37 °C prior to assaying. Then the absorbance

of the reaction was measured at 450nm. The SOD activity was calculated as per Giannoplitis and Ries (1977). SOD activities were expressed as Units milligram of protein extracted. The protein content was measured from all the samples as per Bradford et al (1971).

In order to determine the SOD activity on native polyacrylamide gels, the gels were stained in riboflavin-nitroblue tetrazolium salt as in Xiaojing et al (2009) with some modifications. Briefly, 140 µg of total protein was separated on 8 % native-PAGE gels. Then the gel was stained in the a dark at room temperature in 50 mM PBS solution containing 0.2 % nitroblue-tetrazolium salt, 0.028 M TEMED and 2.8×10^{-5} riboflavin for an hour. The staining solution was immediately rinsed to stop excessive staining. The intensity of the bands was determined with densitometry using the ImageJ gel evaluation software.

3.2.4.0. Determination of metallic cofactor of SOD.

In order to determine the specific activities of SOD isorforms, the specific inhibition technique of different isoforms with H₂O₂ and KCN was used. The MnSOD is insensitive to KCN and H₂O₂ hence to identify the expression of Mn SOD the gels were pre incubated in a solution containing 6 mM potassium cyanide and 5 mM hydrogen peroxide. Fe SOD is KCN insensitive and hydrogen peroxide sensitive and thus was used to identify the band of Fe SOD. CuZn SOD is inhibited by both the hydrogen peroxide and potassium ferricianide, hence the corresponding band was identified (Rubio et al, 2004).

3.2.5.0. Total RNA source, RNA extraction and first strand cDNA synthesis

The total RNA was extracted from both the DETA/NO treated and untreated nodules of peas at 6 hours of incubation. Five different RNA extractions were done from nodules using QIAgen RNA extraction kit. DNase treatment was repeated twice using nuclease-free DNase from invitrogen, as per manufacturers' protocol. First strand cDNA was synthesized using reverse transcriptase from Transcriptor High Fidelity cDNA Synthesis sample kit from Roche. The 3' primer for each SOD isoform was used to generate the first strand cDNA. The three pea SOD isoforms whose sequences were available from sequence database; CuZn SOD I (chloroplastic SOD), CuZn SOD (cytosolic) and Mn SOD were used for this purpose (appendix B).

3.2.6.0. Reverse-transcriptase-Polymerase Chain Reaction optimization

Highly purified salt free primer set for target gene 1 (CuZn SOD: forward ATC CAT GCC TTG GGA GAC ACC A, and reverse primer, AGT CTC ATC CTC AGG GGC ACC A), target gene 2 (Mn SOD forward primer, TTG GAG CCT GTC ATT AGC GGC GA, reverse primer, ACG GCA TCG TGA AGC TGT TCG AG) and reference gene (β -TUB forward primer, TAG GTG GAG GAA CTG GAT CTG G, β TUB reverse primer CAA GCT GGT GAA CCG AGA GAG T) were generated and synthesized by Whitehead Scientific (PTY) Ltd, South Africa. All SOD isoforms primers were annealed at 68 °C and β -tublin primers were annealed 58 °C. Polymerase chain reaction conditions were all optimized in a 96 well GeneAmp PCR system 9700 (applied biosystem, USA) using Taq DNA polymerase. The various concentrations of MgCl₂, dNTPs and primers were optimized and analyzed on 2% agarose gels and stained with ethidium bromide. The optimized results were then used in runs for the actual PCR.

3.2.7.0. Semi-quantitative polymerase chain reaction analysis of CuZn SOD and Mn SOD mRNA expression

The PCR was carried out in a total reaction volume of fifty microlitres, containing MgCl₂, 200 μM dNTP, 0.5 μM forward and reverse primers, and 4 μl of first strand cDNA synthesized from 100 ng of total RNA. The cycling conditions were as follows; first denaturing step for 4 minutes at 95 °C followed by 35 cycles of 95 °C for 1 minute, and 30 seconds of annealing and extension at 72°C for 2 minutes, then the final extension at 72°C for 6 minutes. The primers were annealed at different temperatures; β-tublin primer set was annealed at 58 °C, two isoforms of CuZn SOD and Mn SOD primers were annealed at 68 °C. The band intensities from the internal reference, β-tublin, were used to standardize the transcript levels from different treatments. The transcripts intensities on the agarose gels were measured using densitometry as per ImageJ software.

3.3.0.0. Results

3.3.1.0. Effect of exogenous nitric oxide on SOD activity

Medicago nodules treated with 50 μM SNP exhibited increased total SOD activity across all time periods compared to controls and KCN treated nodules (Figure 3-1). SNP showed the highest effect of SOD activity at 3 hours then remains stable until the 6th hour of exposure to SNP. The SOD activity from the SNP treated nodules remains above the activity levels of both the FCN and control, implying that nitric oxide released from SNP modulates the SOD activity in this experiment by increasing the activity (Figure 3-1).

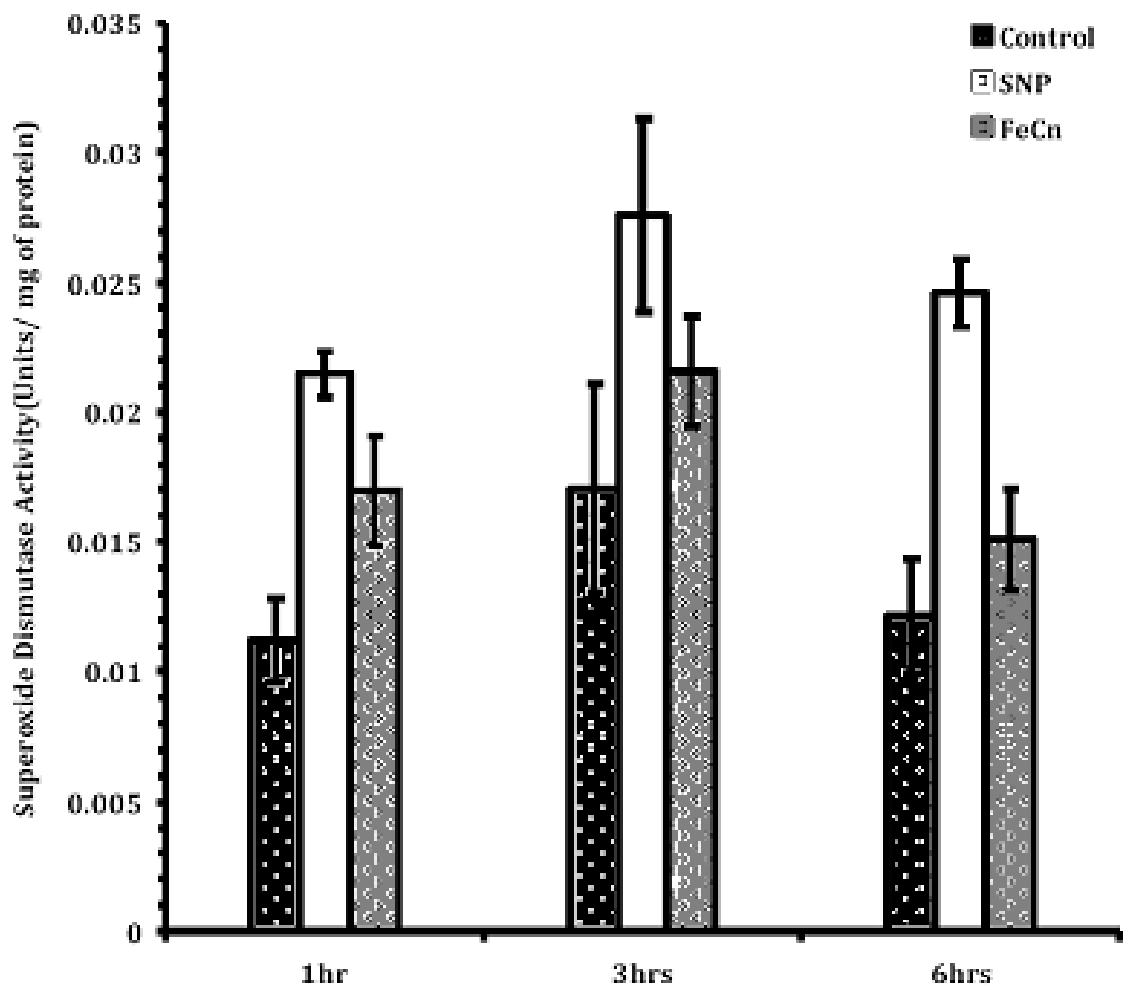


Figure 3- 1 Superoxide dismutase activities in the root nodules of *Medicago truncatula* over a period of time. The control experiment was made up of nodules incubated in nitrogen-free Hoagland solution only, while in SNP treatment, nodules were incubated in nutrient solution supplemented with sodium nitroprusside at 50 μ M. In FeCn treatment, the nodules were incubated in potassium ferricyanide at a concentration of 50 μ M in nutrient solution. The bars represent the mean SOD activities where $n \geq 3$, the error bars represent the standard errors of the means, $p < 0.05$.

A similar pattern in SOD activity in *Medicago truncatula* nodules is maintained by the treatments under saline conditions (Figure 3- 2). However, the total SOD activities under saline condition are generally higher than under normal conditions. Thus the salinity stress combined with exogenous NO increases SOD activity. Unlike under optimal conditions (Figure 3- 1), the SOD activity in NO treatments is more or less the same after the 1st hour and the 3rd hour then it decreases drastically. However, nitric

oxide donor treatments still have the highest SOD activities compared to all other treatments at 6 hours of incubation (Figure 3- 2).

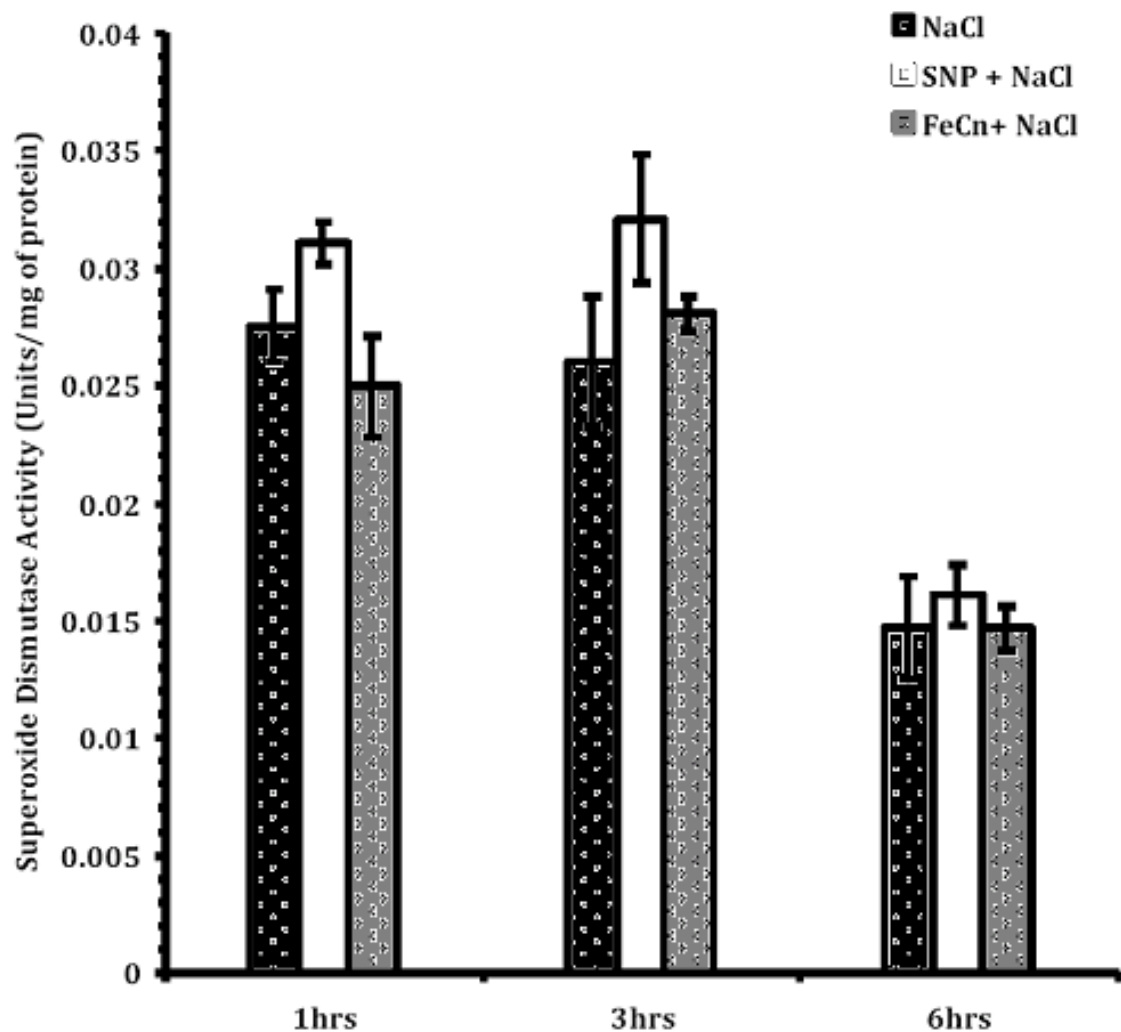


Figure 3- 2 Superoxide dismutase activities in the root nodules of *Medicago truncatula* subjected to different treatments in saline conditions. The NaCl experiment had nitrogen free Hoagland solution supplemented with 150 mM sodium chloride, while NaCl+SNP had sodium nitroprusside at 50 μ M in 150 mM NaCl solutions, lastly NaCl + FeCn is potassium ferricyanide at a concentration of 50 μ M dissolved in 150 mM NaCl. The bars represent the mean SOD activities where $n \geq 3$, the error bars represent the standard errors of the means, $p < 0.05$.

Nitric oxide also modulates the SOD activity in *Pisum sativum* nodules. Figure 3- 3 shows an obvious effect of nitric oxide released from DETA/NO on total SOD activity. There is an increase in the SOD activities in the nodules subjected to NO treatment as compared to the negative control. This is a steady increase until the 24th hour. The phosphate buffer does not have any significant effect on SOD activities across all time

periods in the pea nodules in this particular experiment. DETA/NO is a nitric oxide donor and releases nitric oxide in solutions at pH 7 (Murgia et al, 2004).

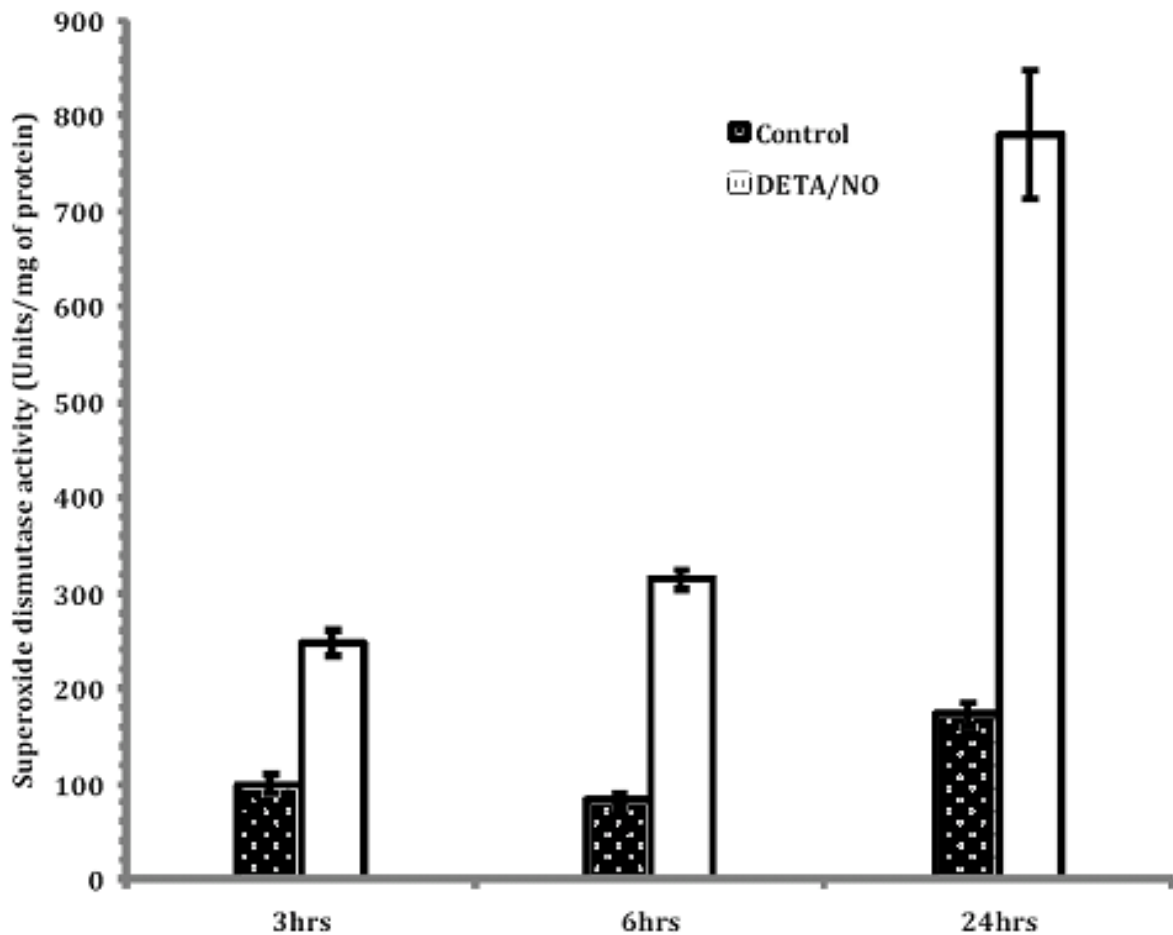


Figure 3- 3 Superoxide dismutase activity in the root nodules of *Pisum sativum* subjected to NO treatment in 10 mM potassium phosphate buffer at pH 7.2. DETA/NO represents a nitric oxide donor and was administered at 50 μ M. The control treatment represents the nodulated roots immersed in 10 mM phosphate buffer. The bars represent the mean SOD activities and the error bars represent the standard errors of the mean, $P < 0.05$, $n \geq 3$.

Salinity also increases the SOD activity in *Pisum sativum* as in *Medicago truncatula*. DETA/NO increases SOD activity at all time periods, however the activity decreases after 24 hours relative to all other time periods. Both control experiments show lower SOD activities compared to the DETA/NO experiments (Figure 3- 4).

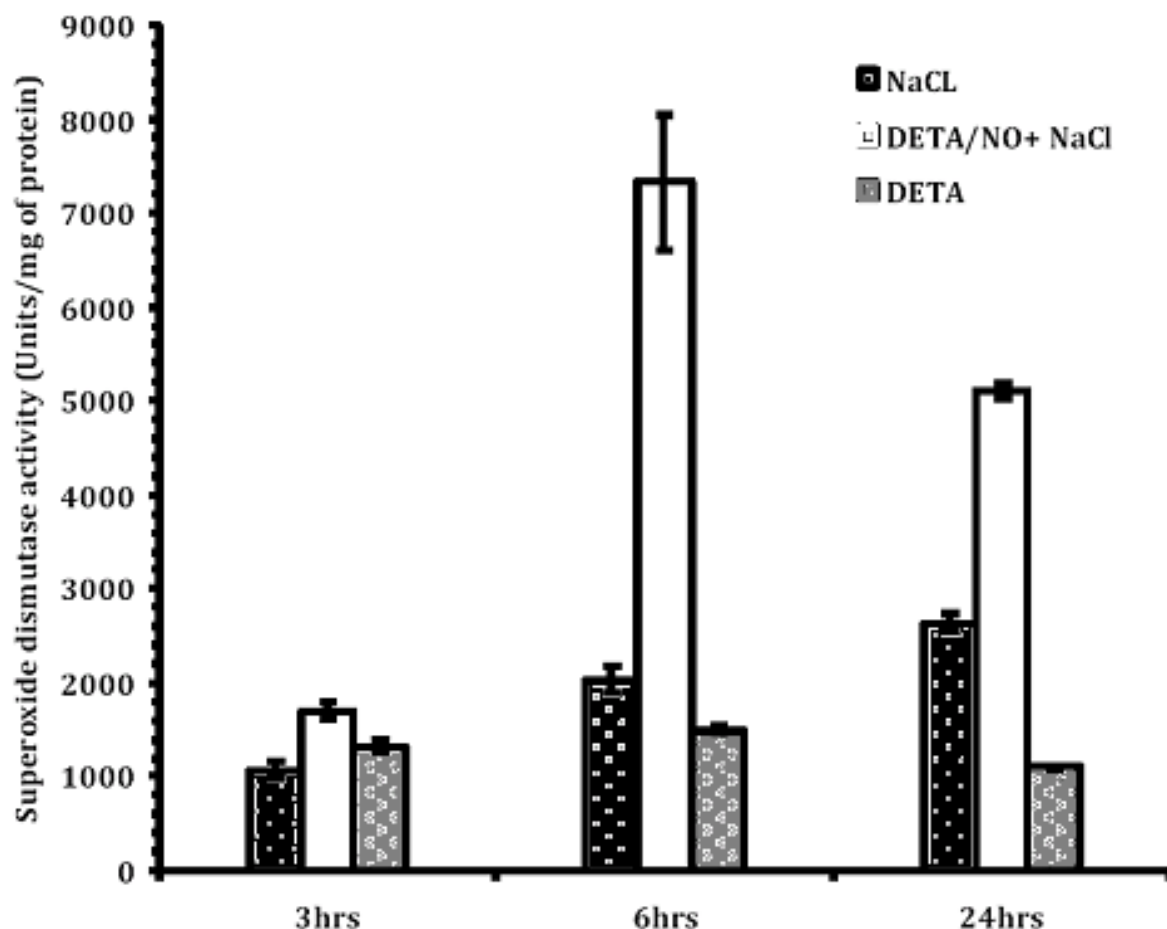


Figure 3- 4 Superoxide dismutase activities in the root nodules of *Pisum sativum* subjected to different treatments under salinity conditions. DETA/NO represents 50 μ M of diethylenetriamine/nitric oxide adjunct, a nitric oxide donor; DETA represents 50 μ M of diethylenetriamine, an analogue of DETA/NO without nitric oxide moiety. NaCl is the control experiment represents 150 mM concentration of NaCl in 10 mM phosphate buffer. The bars represent the means of SOD activity per treatment and the error bars represent the standard error of the means, $P < 0.05$; $n \geq 3$.

3.3.2.0. Effect of endogenous nitric oxide on SOD activity

L-NAME is a nitric oxide synthase inhibitor and addition of this substance to the solution inhibits the activity of SOD and this inhibition is observed after 3 hours. In this case, nitric oxide increases the SOD activity and its effect is reversed by L-NAME (Figure 3- 5). This effect is more pronounced at 6 hours of treatment. At 24 hours of incubation, the difference between the control and NO-treated plants diminishes. Seemingly there is no

time course-dependent increase in the SOD activity in response to DETA/NO because there is no significant difference between the means at different time intervals under NO treatment. The SOD activity also increases in the control with time, implying that the incubation period in the solution also has an effect on the total SOD activity.

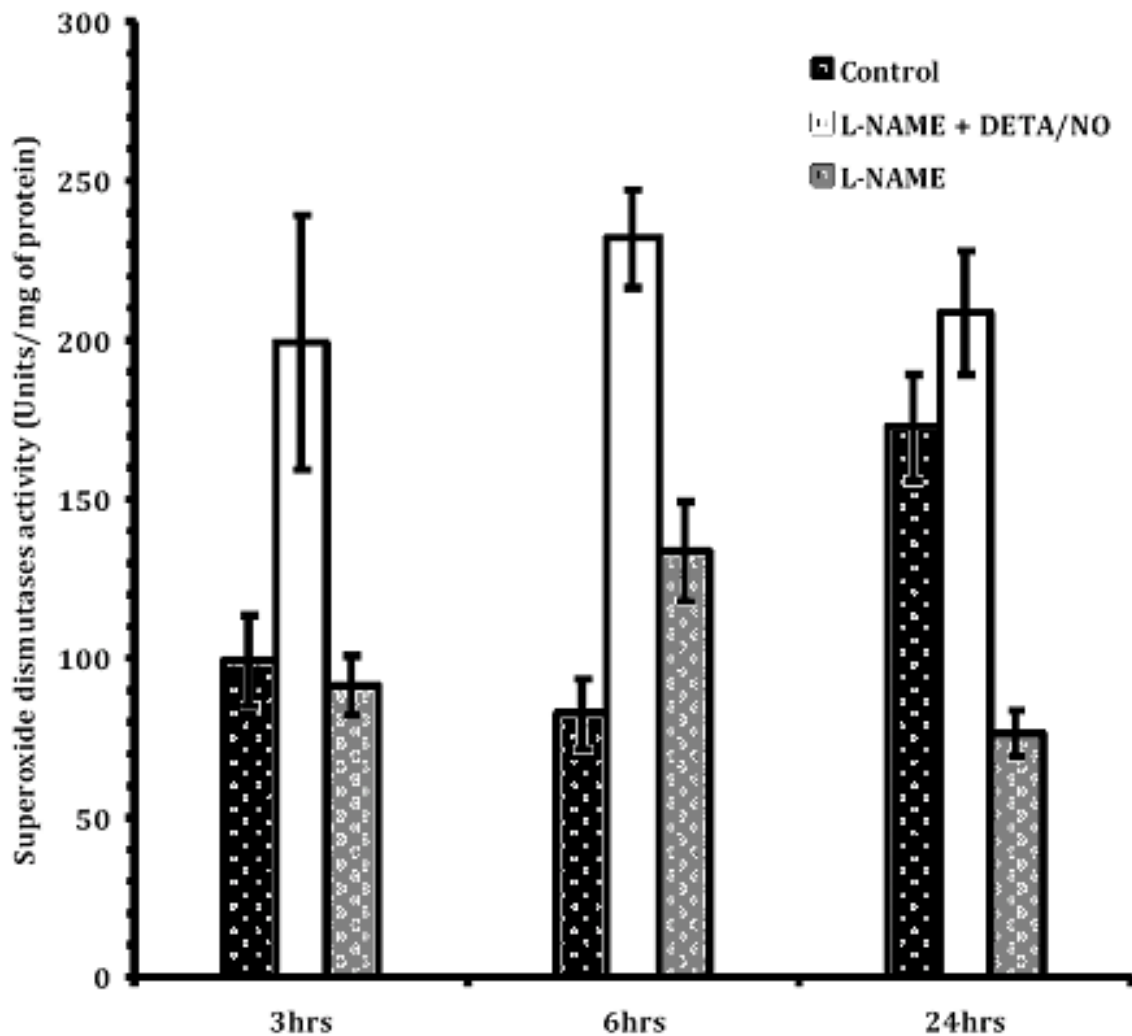


Figure 3- 5 Superoxide dismutase activities in the root nodules of *Medicago truncatula* subjected to different treatments. DETA/NO represents 50 μ M of diethylenetriamine/nitric oxide adduct, L-NAME is a nitric oxide synthase inhibitor (N_{ω} -Nitro-L-arginine methyl ester). The control treatment represents the nodulated roots immersed in 10 mM phosphate buffer. The bars represent the mean SOD activities and the error bars represent the standard errors of the mean, $P < 0.05$; $n \geq 3$.

3.3.3.0 Effect of endogenous nitric oxide on SOD activity under salinity stress

Under salinity stress, endogenous nitric oxide seems to regulate the activity of superoxide dismutases in a different manner. The total SOD activity is initially increased above the two controls at 3 hours of treatment. This is followed by a decrease that is visible after 6 hours and 24 hours. However the decrease in SOD activity due to NOS inhibition does not change significantly after 6 hours. There is a steady increase in SOD activity in response to 150mM NaCl treatment only (Figure 3-6).

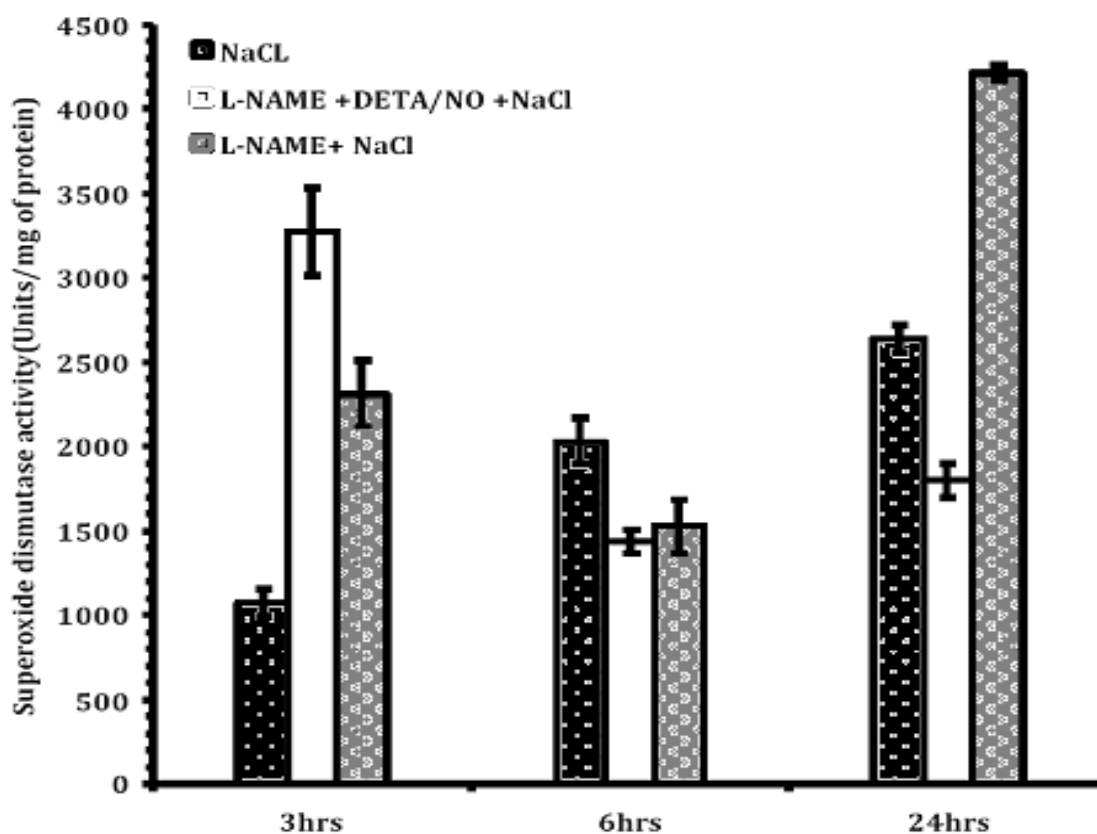


Figure 3- 6 Superoxide dismutase activities in the root nodules of *Pisum sativum* subjected to various treatments under salinity. 50 μ M of DETA/NO, a nitric oxide donor, diethylenetriamine/nitric oxide adduct was used; L-NAME (N_{ω} -Nitro-L-arginine methyl ester) represents a nitric oxide synthase inhibitor and NaCl represents 150 mM sodium chloride. The bars represent the mean activity of SOD per treatment and the error bars represent the standard errors of the means. $P < 0.05$; $n = 3$.

3.3.4.0. Determination of the effect of nitric oxide on SOD isoforms

The chloroplastic CuZn SOD isoform is inhibited by hydrogen peroxide and potassium cyanide (Xiaojing et al, 2009). In an experiment that involved the identification of SOD isoforms from the nodular protein extract, only two isoforms were identified on 8 % native acrylamide gels. Based on inhibition assays, two Mn SOD and two CuZn SOD isoforms we identified (Figure 3- 7). Based on the separation on the native acrylamimide gels, the protein band corresponding to Fe SOD (based on the fact that it is only sensitive to hydrogen peroxide but resistant to KCN) was not observed.

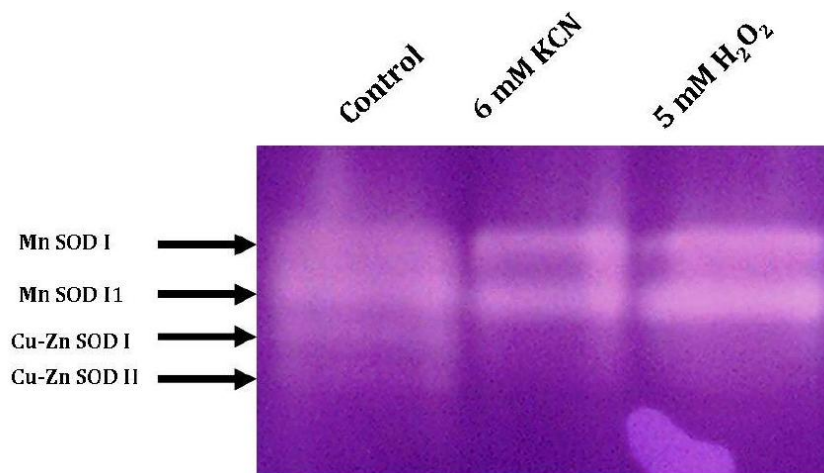


Figure 3- 7 Electrophoretic mobility of different SOD isozymes extracted from pea nodule tissue on 8% native acrylamide gels. 140 µg of total protein extracted was loaded on the wells. Potassium cyanide and hydrogen peroxide were used to specifically inhibit different isoforms.

3.3.4.0. Differential activities of individual SOD isoforms

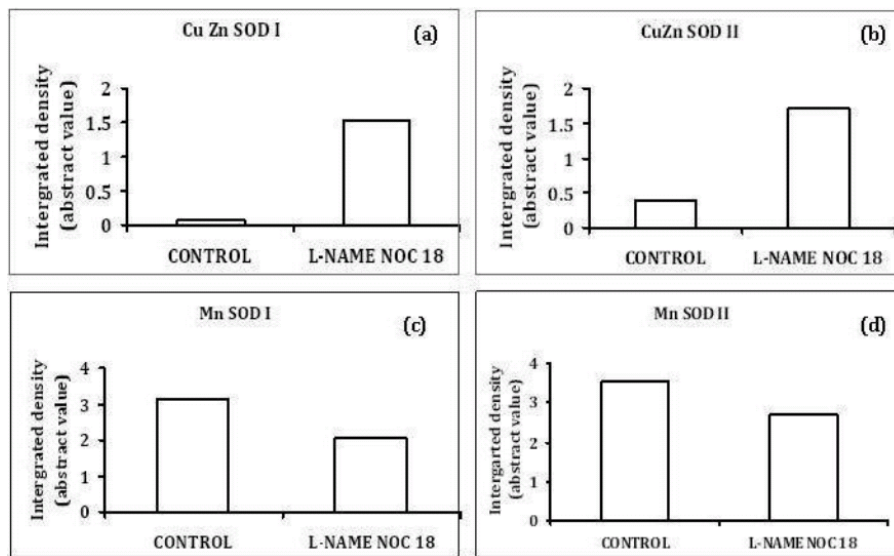


Figure 3- 8 Response of the four isoforms of SOD to endogeneous nitric oxide after 6 hour of exposure to treatments. L-NAME is a nitric oxide synthase inhibitor (L-arginine methyl ester) while NOC 18 represents DETA/NO (diethylenetriamine/nitric oxide adduct), a nitric oxide donor. Control treatment represents the treatment with only the nutrient solution. (a) CuZn SOD I isoform, (b) CuZn SOD II isoform ; (c) Mn SOD I isoform and (d) is the activity resulting from Mn SOD II isoform. The bars represent the integrated density as determined by ImageJ 1.42(rsbweb.nih.gov/ij/)software. The data was generated from two replicates and two technical repeats.

When supplementing the L-NAME solution with 50 μM DETA/NO so as to supply nitric oxide to the incubated nodules, the activities of all the two CuZn SOD isoforms increase to about 300%. However the addition of NO to L-NAME-treated nodules does not restore the SOD activities of the manganese SOD isoforms to levels higher than the controls. This amongst other results presented in this chapter shows that endogenously synthesized nitric oxide increases the activity of individual pea SOD isoforms (Figure 3-8c and d).

Pea nodules exhibit higher CuZn SOD I, CuZn SOD II and Mn SOD 2 activities when exposed to nitric oxide treatment in comparison to the untreated nodules. Mn SOD I is

an exception in this case, with a reduced activity of about 60% compared to the control.

CuZn SOD shows the highest contribution to total SOD activity. (Figure 3- 9)

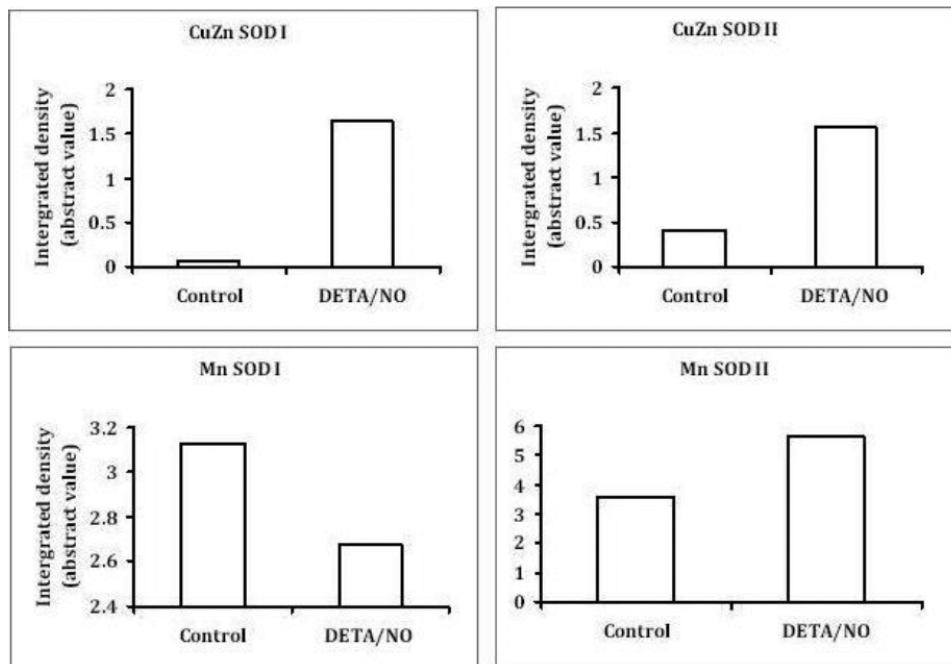


Figure 3- 9 Integrated pixel density of the superoxide dismutases isoforms as observed on 8% native acrylamide gels. The control treatment represents the activity from the pea nodules incubated for 6 hours in nutrient solutions only; the DETA/NO is a diethylenetriamine/nitric oxide adduct and it was added at a concentration of 50 μ M. The bars represent the integrated pixel density as determined by ImageJ 1.42 (rsbweb.nih.gov/ij/) software. The bars represent the intergrated pixel density value.

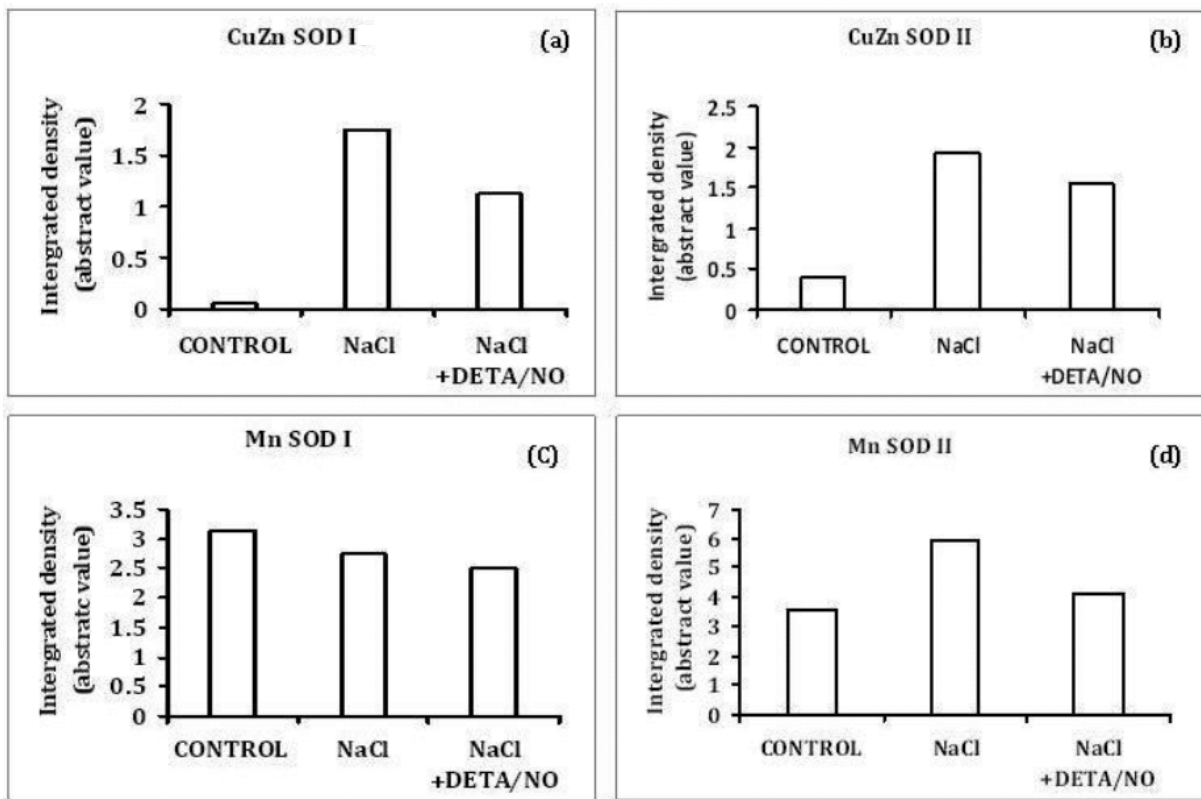


Figure 3- 10 Responses of the four isoforms of SOD to nitric oxide under saline conditions after 6 hours of exposure to treatments. NaCl represents 150 mM sodium chloride in a nutrient solution, while DETA/NO represents diethylenetriamine/nitric oxide adjunct, a nitric oxide donor. Control represents a treatment with only the nutrient solution. The bars represent the integrated pixel density as determined by ImageJ 1.42 (rsbweb.nih.gov/ij/) software.

Concerning nodule MnSOD, only the MnSOD II isoform is induced by high salinity conditions. Mn SOD I is highly sensitive to salt stress, and addition of the DETA/NO exacerbates the decrease in this SOD activity (Figure 3-10). Addition of DETA/NO to the saline solution inhibits the SOD activity of all the four SOD isoforms relative to NaCl (Figure 3- 10).

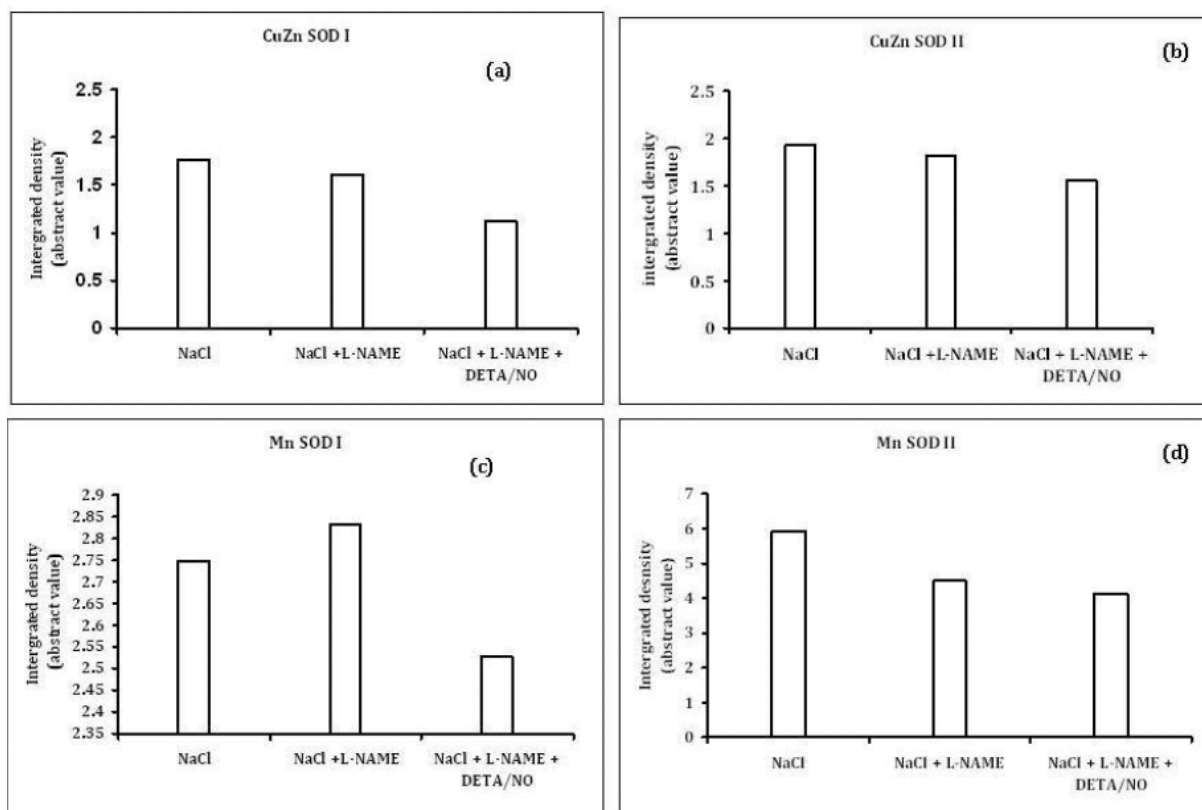


Figure 3- 11 Response of the four isoforms of SOD after 6 hour of exposure to treatments. L-NAME is a nitric oxide synthase inhibitor while DETA/NO represents diethylenetriamine/nitric oxide adduct, a nitric oxide donor. The bars represent the integrated pixel density as determined by ImageJ 1.42(rsbweb.nih.gov/ij/) software.

When L-NAME is added in combination with 150 mM NaCl to the nodules, the two CuZn SOD isoforms exhibit reduced activity (Figure 3-11). This effect is also observed in Mn SOD II. However, Mn SOD I isoform exhibits increased activity as NO synthesis is inhibited in the nodules. This is simply an indicator that unlike Mn-SOD II, Mn SOD I activity is not induced by nitric oxide. The supply of nitric oxide through the addition of nitric oxide donor for 6 hours time period in saline solution, does not reverse the effect of L-NAME. These results correspond to the total SOD enzyme activity results obtained in this chapter (Figure 3-11). On the other hand addition of DETA/NO to saline nutrient solution reduces the activity of Mn SOD I drastically (Figure 3-11).

3.3.5.0. **Semi-quantitative PCR for SOD isoforms and the reference gene β -Tublin**

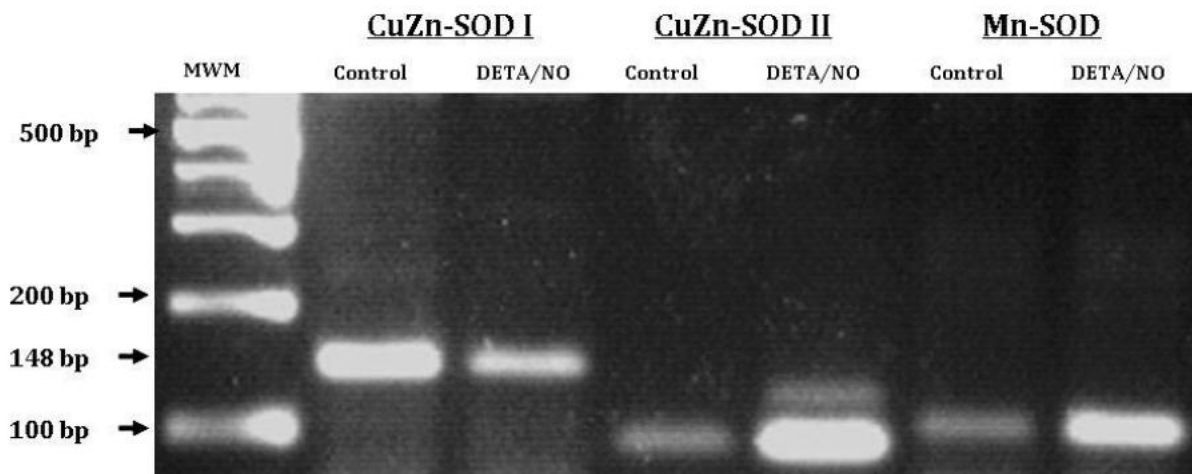


Figure 3- 12 Semi-quantitative RT-PCR of superoxide dismutase isoforms from the pea root nodules exposed to 50 μ M DETA/NO in nutrient solution. The control represents the treatment with nutrient solution only. Lane 1 is the DNA molecular weight maker (1kb DNA ladder from fermentas). The RT-PCR image was acquired with a high resolution Camera under U.V. illumination following staining of the 2% agarose gel with ethidium bromide.

The three isoforms targeted for semi-quantitative PCR were chosen based on sequence availability from DNA sequence databases. The three isoforms of SOD, as shown on the agarose gel (Figure 3-12) were successfully detected by PCR from the nodules of pea. CuZn SOD I seems to be negatively regulated by nitric oxide. Just like CuZn SOD II, Mn SOD transcripts are increased by treatment with nitric oxide donor (Figure 3-12).

3.3.5.0. Enhancement of superoxide dismutase expression by nitric oxide

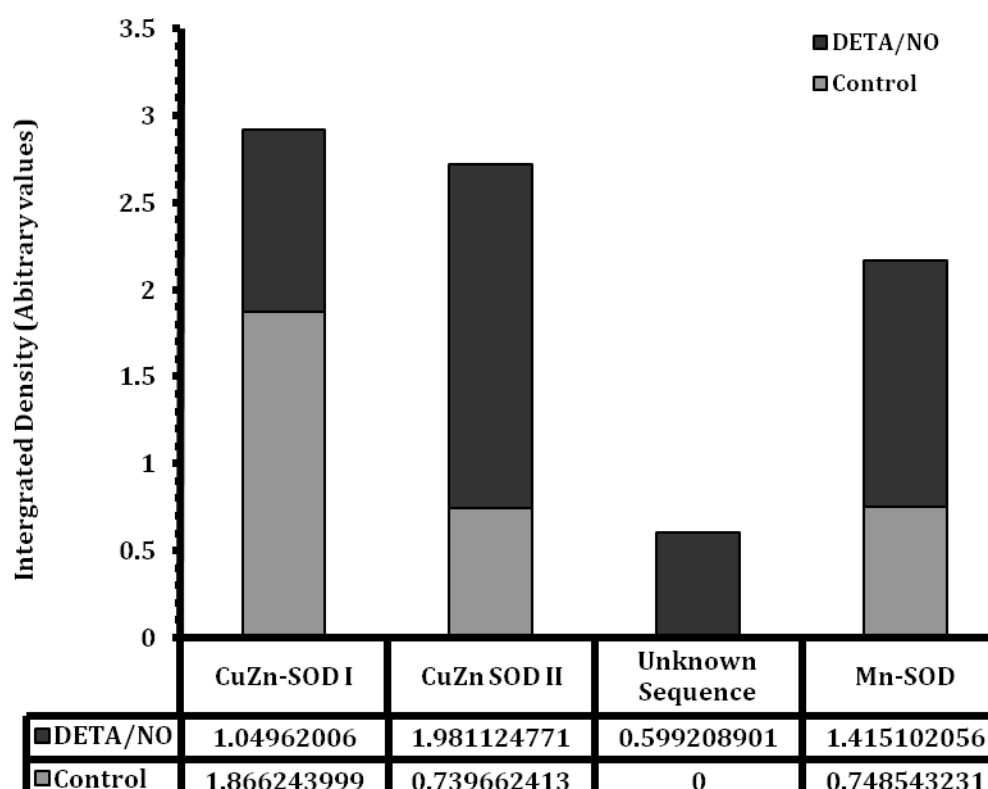


Figure 3- 13 RT-PCR normalized densitometry results from the amplified SOD isoforms. DETA/NO is a nitric oxide donor and was added at a final concentration of 50 μ M. In Control treatments, the nodulated roots were incubated in nutrient solution for six hours.

The densitometry results (Figure 3.13) show that almost all the SOD isoforms from the pea nodules are up-regulated by nitric oxide. With the exception of CuZn SOD I isoform, all other SODs are increased by NO; CuZn SOD II exhibits about 20 fold increase at the transcript level and Mn SOD II exhibits 10 folds increase in transcript levels compared to the control.

3.4.0.0. Discussion

The phenomenon of oxidative stress as a consequence of abiotic stress has been studied over decades and various components of the oxidative stress process have been identified. Abiotic stress triggers accumulation of superoxide radicals and other ROS (Rodriquez et al, 2005). This upsurge of ROS in response to abiotic stress is accompanied by induction of the antioxidant defense system. It is therefore agreed that the degree of plant cell tolerance to oxidative stress is more dependent upon the response of the antioxidant enzymes to their accumulation, including superoxide dismutases (Neill et al 2007, 2008).

Modulation of superoxide dismutase activity by nitric oxide

The data from the biochemical assays shows a significant increase in total SOD activity in response to nitric oxide treatment compared to other treatments. This increase concurs with the findings that nitric oxide from SNP increases antioxidant enzyme activities in leaves (Neill et al, 2007; Zhao et al 2004 and Zhang et al, 2006), protects the cells from oxidative stress by reducing lipid peroxidation (Neill et al, 2008), reducing monodehydroascorbate (MDA) and thus scavenging ROS. As stated earlier, SOD dismutates superoxide radicals to yield hydrogen peroxide. This accumulated hydrogen peroxide, more specifically in the chloroplast, inhibits the SOD activity (Perl et al, 1993; Tepperman & 1990). It is thus relevant to speculate that inhibition of CuZn SOD activity in response to either exogenous or endogenous nitric oxide, observed at longer hours of incubation and even under salt stress, is due to the accumulated hydrogen peroxide produced with lengthy exposure to both stress and NO.

Effect of NaCl on total superoxide dismutase activity

NaCl at higher concentration triggers NO accumulation in plant cells (Lie et al, 2008; Zhao et al, 2007). It has also been established that this accumulated NO is the one responsible for the increased antioxidant activities in the cells (Lie et al, 2008). NaCl is toxic to plant cells and causes oxidative stress; however, the degree of cell tolerance to salinity is still dependent upon the response of the antioxidants.

Under situations of low salt tolerance, plants exhibit SOD activity that is lower than under situations where the cells are tolerant (Attia et al, 2009; Koca et al, 2007). The data presented in this study agrees with the fact that NaCl increases the SOD activities above that of the controls. Although not measured in this experiment the increased SOD activity under salt condition is attributed to the induction of NO biosynthesis in response to NaCl (Neill et al, 2008). The SOD activities increased under salt and even increased further when nitric oxide donor was added to the incubation solution. The fact that this effect was reversed by addition of L-NAME suggests that nitric oxide is involved in modulating the activities of the SOD enzyme isoforms.

The SOD response pattern was rather different in the experiments involving the inhibition of endogenous nitric oxide biosynthesis in the cells (Figure 3-6). The cells rapidly lost the SOD activity with prolonged exposure to salt stress as compared to the other controls. This SOD inhibition may be due to the increased stress as more NO is scavenged from the medium and more NaCl added, which results in H₂O₂ accumulation as other antioxidants like glutathione may not be synthesized in adequate amounts (Innocenti et al, 2007)

From the above experiment, an increase in SOD activity is not only observed with the nitric oxide and NaCl treated samples. The untreated controls demonstrate a steady increase in SOD activity over time. Unlike the treated samples there is no observed inhibition of SOD at any point. This result exhibits a pattern similar to the one shown by the salt-only treatments. NaCl generates ROS that activate SOD activity (Neill et al, 2008). In the same manner, the non-treated samples exhibit some form of stress response. It should be noted that the nodules were harvested from the nodulated root sections that were incubated in various solutions in accordance with the experimental objectives and plan. Hence, the excised roots floating in solution could be experiencing some stress.

Identification of SOD isoforms in pea root nodules

There are two SOD isoforms that have been localized in the nodule tissue and the composition involves two Mn and two CuZn SOD isoforms (Becana et al, 1989). In this study the two Mn SOD isoforms and CuZn isoforms have also been identified on the native acrylamide gels (Figure 3-07). It is important to notice a contradiction between the leaf SOD and nodule SOD as evidenced by the existence of only one Mn SOD isoform in leaves compared to nodules (del Rio et al, 2003). The Mn SOD I in pea root nodules, was earlier identified through western blotting as a *Rhizobium* SOD, not a plant-based superoxide dismutase (Rubio et al, 2004). Hence, this variation observed in the SOD composition from the leaves and root nodules is justified.

Defferential regulation of SOD isoforms from the pea nodules by nitric oxide and salt

The Mn SOD appeared to be the most important in the pea nodules as compared to CuZn SOD as it is the one that is more stress-inducible. In other plant systems, studies reveal a dramatic increase in different SOD isoform activities in response to abiotic stress. For instance, the oxidative stress induced by NaCl induces the thylakoidal Fe SOD and CuZn SOD (Gomez et al, 2003). There are also reports that drought also induces the activity of CuZn SOD, Mn SOD and Fe SOD in leaves (Sharma et al, 2005). Furthermore, the dark-induced senescence causes increase in the activities of different SOD isoforms in perixosomes and mitochondria of pea leaves (del Rio et al, 2003). All isoforms increase in their activity directly proportional to senescence, where Mn SOD exhibits this increase even at transcript level (del Rio et al, 2003). These findings concur with the data from this work, in which 150 mM NaCl increases the activities of all the SOD isoforms as observed on the native gel except for the Mn SOD II.

Interestingly nitric oxide from nitric oxide donor SNP increased the activities Fe SOD and CuZn SOD but had no significant effect on the Mn SOD in leaf discs of mung bean subjected to heat shock (Yang et al, 2006). Hence our findings support the idea that nitric oxide increases enzymatic activities of all SOD isoforms in the pea nodule except for the Mn SOD II isoform.

Regulation of SOD isoform transcripts by nitric oxide

Superoxide dismutases activity is regulated at the transcriptional level. Salt stress has been reported to increase the transcription of the SOD isoforms. Kim et al, (2007)

reports that salt stress increased the Mn SOD and CuZn SOD but the Fe SOD transcripts remained unchanged. Also, in tobacco leaves, an increase in the SOD activities is associated with a transient increase in the transcripts levels of the SOD isoforms (Kurepa et al, 1997). A similar observation is noted in cucumber leaves where an increase in SOD activities is preceded by increases in SOD isoform transcripts (Deeparka et al, 2006). This data is similar to our findings that an increase in total SOD activity as obtained from the biochemical assays is regulated at the transcriptional level.

The regulation of SOD activity at transcript level is not fully understood. For instance, the CuZn SOD mRNA accumulates in cucumber leaves till 6 hours of exposure to metals then decreases drastically, while the corresponding protein activity escalates till 96 hours (Deeparka et al, 2006). Kurepa et al (1997) also reports higher transcript levels of SOD in younger leaves of tobacco compared to the older senescing leaves, but the enzymatic activity seems to be higher in the older leaves compared to the younger leaves. This differential regulation of SOD transcripts was suggested to be dependent on protein turn-over (Scandallios et al, 1993). Gao et al (2009) suggested the same reason for the decreased SOD mRNA transcripts in cucumber seedlings after 96 hours of metal treatment while the protein activities were escalating.

In this study, Cu-Zn SOD I transcript accumulation is negatively correlated with the corresponding protein activities, and it may as well be regulated by the protein turn-over. Different transgenic plants over-expressing the SOD isoforms have also been developed and conferred tolerance to different abiotic stress stimuli (van Camp et al, 1994; Gupta et al, 1993, Myougo et al, 2008 and Wang et al 2004). Such plants exhibited normal and healthy plant growth. Indeed SOD has direct physiological functions in plants; it functions in chloroplast development (Myougo et al, 2008).

3.5.0.0. Conclusion

This chapter aimed at studying the role played by nitric oxide in modulating superoxide dismutase and how SOD responds to salt stress. The main focus was to establish how NO affects enzyme activities of SOD isoforms from the pea nodule and to further study if the observed effect is controlled at the transcriptional level. From the data presented here it is concluded that nitric oxide modulates total SOD activity. The activities from the four individual SOD isoforms suggest that Mn SOD II, CuZn SOD I and CuZn SOD II contribute more to the total SOD activity. Mn SOD I seems to be negatively regulated by nitric oxide. Since it is a probably *Rhizobium*-derived SOD (Becana et al, 1989), these results could indicate the possible negative effect on the survival of bacteroids under a high level of nitric oxide concentration. However a study investigating the viability of the *Rhizobium* bacteroids in response to nitric oxide would be required to validate this point.

The manner in which the pea nodules respond to nitric oxide is similar to that in which they respond to NaCl. This might suggest that nitric oxide is a naturally occurring molecule that could be used in plant nodules to help with ROS defense. Thus application of biotechnology techniques involving up-regulation of nitric oxide synthesis under NaCl stress could possibly help increase the capacity of SOD to scavenge ROS under stress.

Regulation of superoxide dismutases is at transcriptional level but it cannot be ruled out that it could also be post-transcriptional as well. Mn SOD II and the two CuZn SOD isoform activities are regulated at the transcriptional level. Thus, the related coding

sequences can be over-expressed in plants or pea nodules to enhance the nodule tolerance to oxidative stress.

The approach with which this research was conducted has some limitations that would need to be considered in future research of this nature. First, it is important to study this kind of enzymatic response using intact plants rather than detached nodules as this would minimize any stress imposed by detachment on the nodules themselves. Although it is evident from our results that SOD is regulated at transcriptional level, the study involving the transient accumulation of SOD transcripts over a period of time would provide a much better picture of how the transcripts increase and decrease, and perhaps give clarity on whether the SOD protein turn-over regulates the SOD transcripts.

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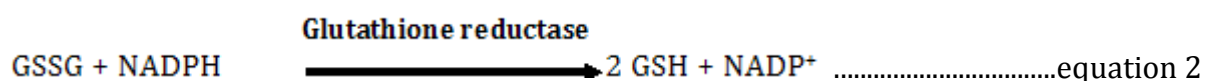
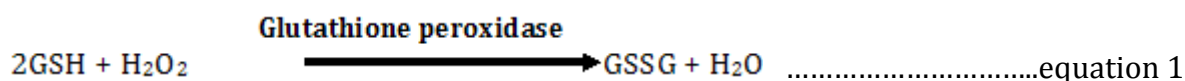
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Chapter 4 Regulation of glutathione peroxidase activity by nitric oxide

4.1.0.0. Introduction

Glutathione peroxidase (GPx) belongs to a large family of peroxidases (POX) found in a wide range of living organisms. It is an antioxidant enzyme and scavenges peroxides in a process that involves the use glutathione (GSH) (Ahmad et al, 2008) or thioredoxin (Mittler et al., 2002) as a reductant, yielding oxidized glutathione (GSSG) and water. The GSSG is reduced back to GSH by glutathione reductase (equation 1&2) in a process that consumes NADPH (Ahmad et al, 2008).



Glutathione peroxidase is perceived as one of the enzymes functioning in abiotic stress acclimatization during salt and heavy metal toxicity stress (Mittova et al, 2004). It also supports long term survival of yeast during growth under abiotic stress (Lee et al, 2007). Furthermore, abiotic stress tolerant plant genotypes have up-regulated gene expression of glutathione peroxidase that counteracts the effect of oxidative stress and this expression is observed after a prolonged exposure to abiotic stress (Csiszár et al, 2002). Glutathione peroxidase has often been used as a maker for oxidative stress tolerance

because of its role in oxidative stress tolerance (Caregnato et al, 2008 and Dazy et al, 2009).

Over-expression of GPx in plants induces a certain magnitude of tolerance to abiotic stress. This was indicated by the reduced cell death, low levels of monodehydroascorbate (MDA), and lower levels of lipid peroxidation in tobacco plants (Yoshimura et al, 2004). Transgenic tobacco plants over-expressing glutathione peroxidase in the cytosol and chloroplast had higher tolerance to 250 mM NaCl, chilling and ROS generated by methylviologen (Yoshimura et al, 2004). It is also worth noting that peroxidase (POX) activity is increased in the cells upon exposure to nitric oxide (Hung & Kao, 2004).

It is now agreed that the level of GPx activity in the cells determines the readiness of the antioxidant system to detoxify peroxides and determines the extent of abiotic stress tolerance in plants (Mittova et al 2003 and Lee et al, 2007). This chapter focuses on studying the regulation of the activity of glutathione peroxidase by salinity stress and how the activity is influenced by exogenous and endogenous nitric oxide.

4.2.0.0. Materials and methods

4.2.1.0. Plant material preparation

Medicago truncatula cv. Parabinga and *Pisum sativum* cv. Crusader (both kindly supplied by Agricol, South Africa) were inoculated with *Rhizobium meliloti* and *Rhizobium leguminosarum* respectively and grown on vermiculite. Plants were germinated on vermiculite pre-soaked in de-ionized water. The germinated seedlings were supplied

with the nitrogen-free nutrient solution consisting of 0.001 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 M potassium phosphate buffer, 0.00005 M FeEDTA, 0.001M CaCl_2 , 0.00005M KCl and the micronutrients in the proportions: 5 μM H_2BO_3 , 2 μM MnSO_4 , 2 μM ZnSO_4 , 2 μM $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$ and 5 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. The pH was adjusted to 6.2 with potassium hydroxide. Each pot containing a single plant was supplied with 150 ml of this nutrient solution after every three days. All the plants used for this experiment were grown under 16 hrs of light in the growth chamber for four weeks until nodule maturity.

4.2.2.0. Experimental conditions

Depending on the type of experiment, the four-week old plants were exposed to 1 hour, 3 hours, 6 hours and 24 hours of treatment with 100 μM sodium nitroferricyanide (SNP) or 50 μM Diethylenetriamine/Nitric oxide adduct (DETA/NO). SNP and DETA/NO are nitric oxide donors. For negative controls, plants were treated with only just nutrient solution. To determine if nitric oxide is required for GPx activity, some plants were treated with the nitric oxide synthase inhibitor N_ω -Nitro-L-arginine methyl ester (L-NAME). To evaluate the effect of salinity on GPx activity, the rest of the plants were treated with 150 mM NaCl. In the respective treatments, the relevant compound specific to that treatment was added as a supplement to the nitrogen-free nutrient solution. Potassium ferricyanide was used as a fixed control for SNP while DETA was used as a fixed control for DETA/NO.

4.2.3.0. Experimental design and data analysis

The experimental designs for all the experiment was a factorial design with a two-factor randomized complete block Design (RCBD) with three replicates. The treatment design

was a two-factor factorial design with 3 levels. ANOVA was used to detect the total variation amongst the treatments.

4.2.4.0 Preparation of enzyme extracts

Glutathione peroxidase enzyme was extracted by homogenizing the nodule tissue in extraction buffer (50 mM TRIS-HCl, pH 7.6, 0.15 mM NADPH) using an electric grinder. The homogenate was spun at 4 °C using a bench-top centrifuge at 20 000 *g* for 15 minutes. The supernatant was removed and desalted through a Sephadex G-25 column and used for GPx activity determination.

4.2.6.0. Determination of glutathione peroxidase enzyme activities

Glutathione peroxide activity was measured as per Mittova et al, (2004), with some modifications. In summary, the assay was carried out in GPx assay buffer (50 mM TRIS-HCl, pH 7.6, 0.15 mM NADPH, 15 mM GSH, 1 unit per ml glutathione reductase, 0.95 mM sodium azide. This reaction was initiated by addition of H₂O₂ to a final concentration of 0.075%. The glutathione peroxidase activity was then measured spectrophotometrically by following the decrease in absorbance at 340 nm. The extinction coefficient of NADPH (0.00373μM⁻¹) was used to determine the actual activity. The formula used for calculating GPx activity was as follows:

$$[(\Delta A_{340}/\text{min})/0.00373\mu\text{M}^{-1}] \times [0.19\text{ml}/0.02\text{ml}] \times [\text{dilution}] = \text{nmol}/\text{min}/\text{ml}$$

Where:

$$\Delta A_{340} = [A_{340} (\text{Time 2}) - A_{340} (\text{Time 1})] / [\text{time 1}(\text{min}) - \text{Time 2}(\text{min})]$$

The enzyme activity was expressed as nmol per milligram of protein.

4.2.7.0. Determination of protein concentration

All the protein contents in all the samples were measured as per Bradford et al, 1971.

4.3.0.0. Results

4.3.1.0. Effects of exogeneous nitric oxide on glutathione peroxidase activity in non-stressed legume nodules

After one hour of incubating the detached *M. truncatula* nodules in the three different solutions (SNP) in nutrient solution, nutrient solution only and the potassium ferricyanide in nutrient solution), the SNP treated nodules exhibited no significant difference in glutathione peroxidase (GPx) activity from the negative control. The overall GPx activity exhibited by the nodules under all the treatment conditions at this time point is the lowest compared to the rest of the incubation periods (Figure 4-1). GPx activities at 1 hour of treatment is 500% lower compared to the third hour treatment and 700% lower when compared to the sixth hour treatment (Figure 4-1).

The nodules show a substantial increase of GPx activity after 3 hours of incubation. At this time point SNP shows higher activity (about 10% compared to negative control) and slightly higher compared to the fixed control, potassium ferricyanide (FeCn). After 6 hours of incubation, the GPx activity of the SNP-treated nodules is about 20% higher than the activity from the negative control. At this time point both controls are way below the SNP treatment, thus indicating that nitric oxide from the SNP increases the activity of GPx in the root nodules of *Medicago truncatula* (Figure 4- 1).

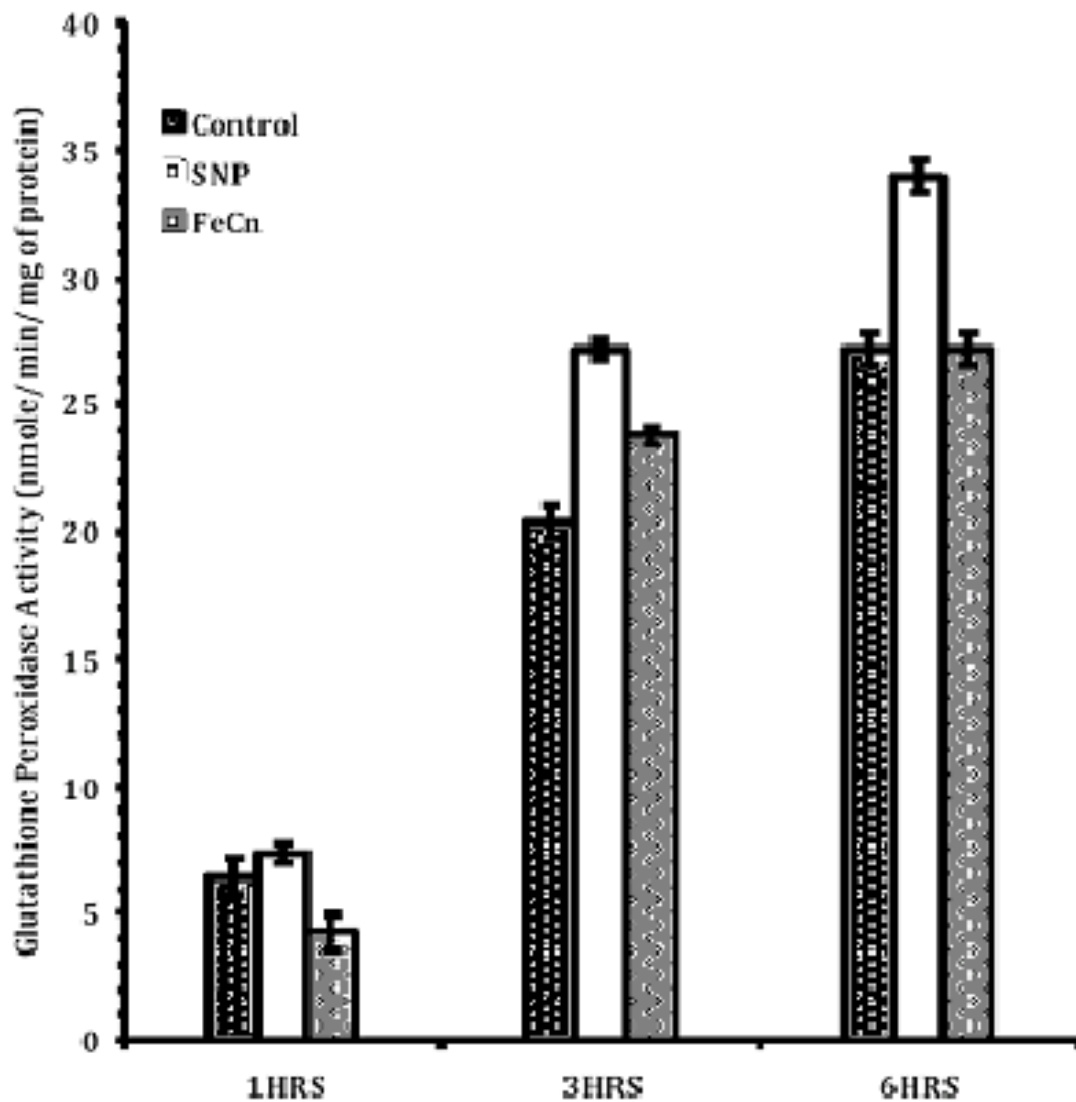


Figure 4- 1 The time course response of glutathione peroxidase activity in *M. truncatula* root nodules to nitric oxide treatments. SNP represents a nitric oxide donor treatment: sodium nitroferricyanide (FeCn) is a potassium ferricyanide treatment. The p value = (0.05), n ≥ 3. The bars represent the sample means and the error bars represent the standard errors of the means.

The SNP-treated nodules have significantly different GPx activities from the negative controls whereas the effect of FeCn is not significantly different from the negative controls. These observations indicate that the enzyme activity observed in SNP treatment is due to NO released from SNP and not the possible by-products of SNP break-down. It is thus appropriate to conclude that nitric oxide up-regulates glutathione peroxidase activity in *M. truncatula*. The GPX activities in all the treatments increase as the incubation period increases (Figure 4- 1). An increase in GPx activity in the nodules

that were exposed to NO treatment is initially observed after 3 hours where SNP treatment starts to exhibit the increased GPx activity above both controls.

The one hour treatment was excluded from the pea nodule experiments because, as observed in *Medicago* experiments (Figure 4- 1), a significant difference in glutathione peroxidase activity in response to nitric oxide is first observed at 3 hours of incubation. In the case of the experiments carried out on pea nodules, the nodulated roots were used for treatment and the nodules were harvested after snap-freezing in liquid nitrogen. In all the incubation periods the DETA/NO treatment exhibits higher GPx activity compared to the control (Figure 4-2). A different trend is observed after 24 hours of incubation where there is no further increase in GPx activity compared to the negative control (Figure 4- 2).

P. sativum nodules generally exhibited similar levels of GPx activity at all time periods as *Medicago* plants, and the activity trend becomes stable after 6 hours as there is no significant difference between the GPx activities for all the treatments at 6 and 24 hours of treatment (Figure 4- 2).

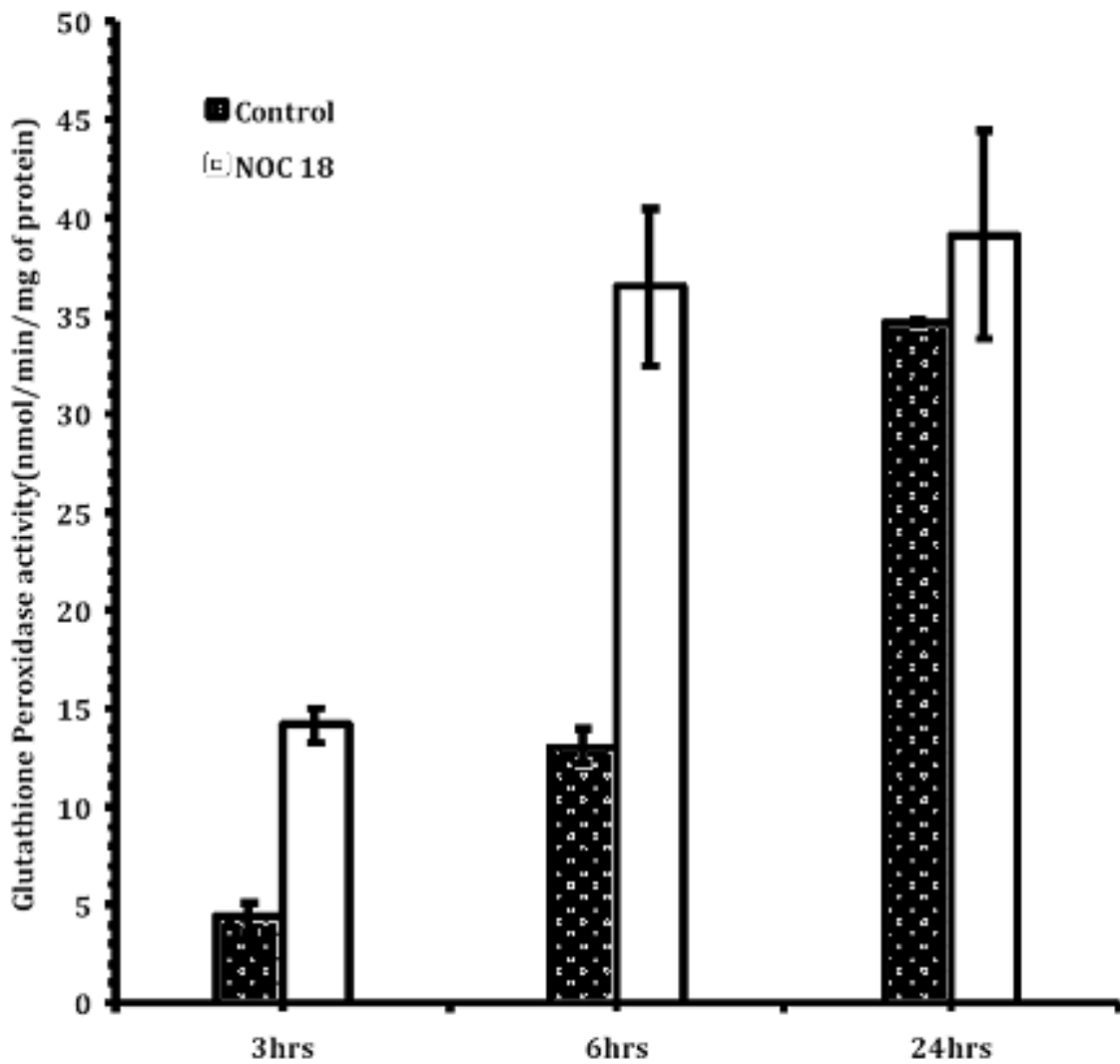


Figure 4- 2 The time course response of glutathione peroxidase activity in the *P. sativum* root nodules to nitric oxide treatments. NOC 18 represents a nitric oxide donor treatment with Diethylenetriamine/Nitric oxide adduct. The p value = 0.05, n ≥ 3. The bars represent the sample means and the error bars represent the standard errors of the means.

4.3.2.0. The effect of inhibition of nitric oxide synthase on GPx activity

L-NAME is a nitric oxide synthase (NOS) inhibitor and has been used in both animal and plant systems to inhibit NOS-dependent endogenous nitric oxide synthesis. In order to study the effect of NOS activity-derived nitric oxide on glutathione peroxidase activity, an experiment was set up that involved regulation of the endogenous synthesis of nitric oxide. In this experiment, the GPx activity in nodules incubated in nutrient solution was compared to the one from nodules in which the nitric oxide synthesis was inhibited by addition of 1mM L-NAME to the incubation solution. Further to this, a treatment was

also set up in which nodulated roots were incubated in the presence of 150 mM NaCl to induce salinity stress.

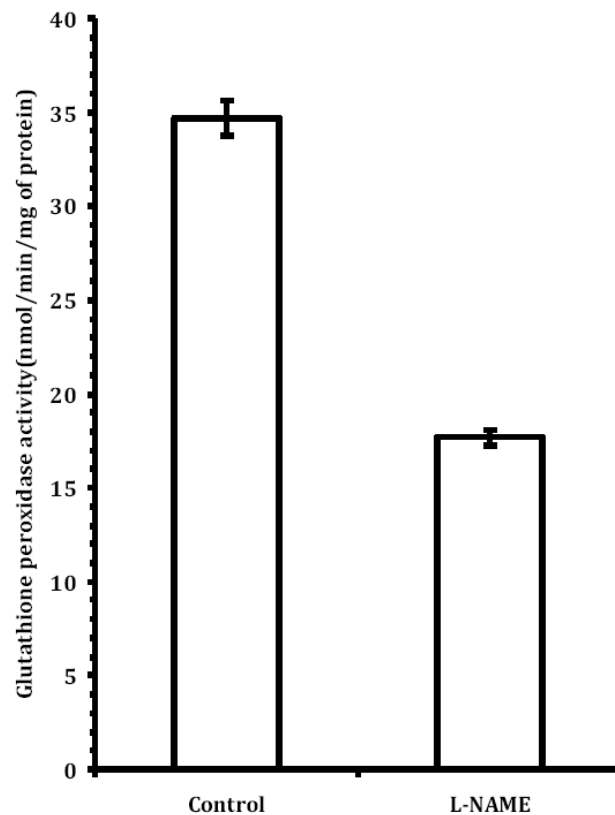


Figure 4- 3 The effect of L-NAME on *P. sativum* root nodule GPx activity under non-saline conditions after 3 hours of incubation in nutrient solution supplemented with 1 mM L-NAME. L-NAME is a nitric oxide synthase inhibitor. Control represents the negative control with nutrient solution at pH 6.2. The p value = (0.05), n ≥ 3. The bars represent the sample means and the error bars represent the standard errors of the means

Exposure to L-NAME treatment inhibited the activity of GPx under normal growing conditions, thus implicating the involvement of nitric oxide synthase-derived NO in activating the GPx activity (Figure 4- 3).

NaCl increases cellular nitric oxide concentrations and it is thought that this generated NO might trigger the activities of several antioxidants enzymes (Mittova et al, 2004). While NaCl increases the activity of the GPx way above the values observed for

untreated nodules, addition of L-NAME reverses the effect of the NaCl treatment (Figure 4- 4). This may suggest that an increase in GPx activity observed under salinity stress is triggered by nitric oxide.

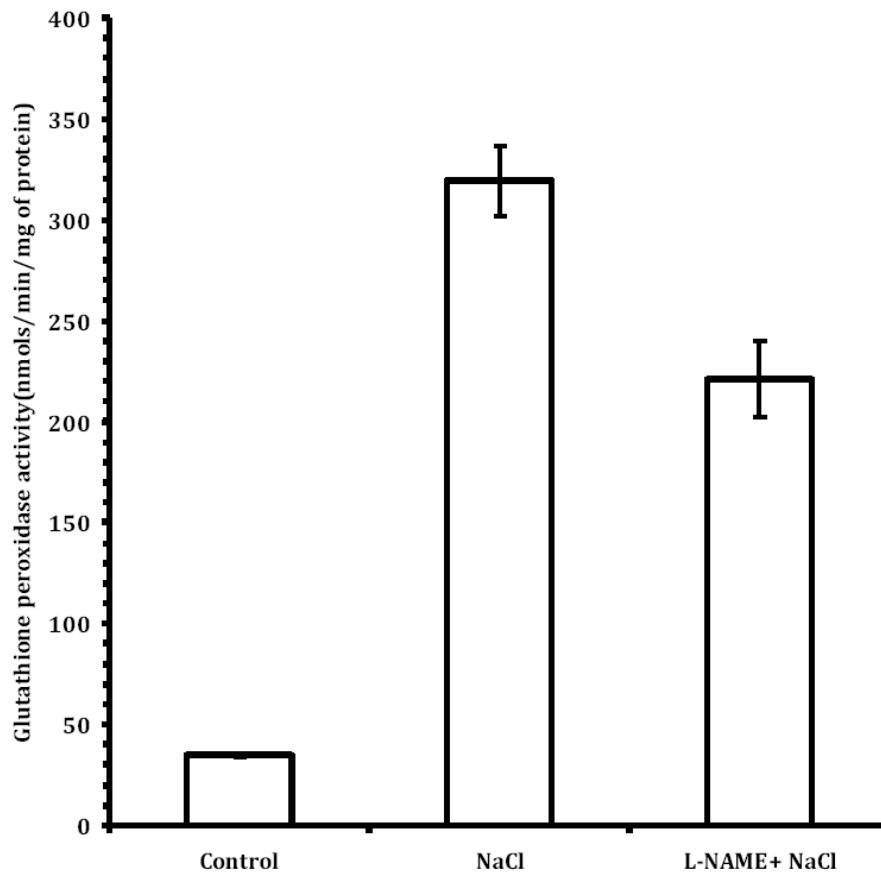


Figure 4- 4 The effect of L-NAME on GPx activity under saline conditions after 6 hours of incubation. L-NAME is a nitric oxide synthase inhibitor. Control represents the negative control with nutrient solution only at pH 6.2. The p value = 0.05, n ≥ 3. The bars represent the sample means and the error bars represent the standard errors of the means

4.3.3.0. Effect of exogenous nitric oxide on GPx activity in the nodules of legumes under salinity

M. truncatula and *P. sativum* root nodules exhibit high GPx activity under 150 mM NaCl than under the optimal conditions (an increase of about 500% in overall GPx activity is observed, Figure 4- 4 & 4- 5). The GPx activities from the negative controls are higher

under salt stress than under optimal conditions in both *Medicago* and pea. This effect is observed all across the treatments in both legumes (Figure 4- 4 & 4- 5).

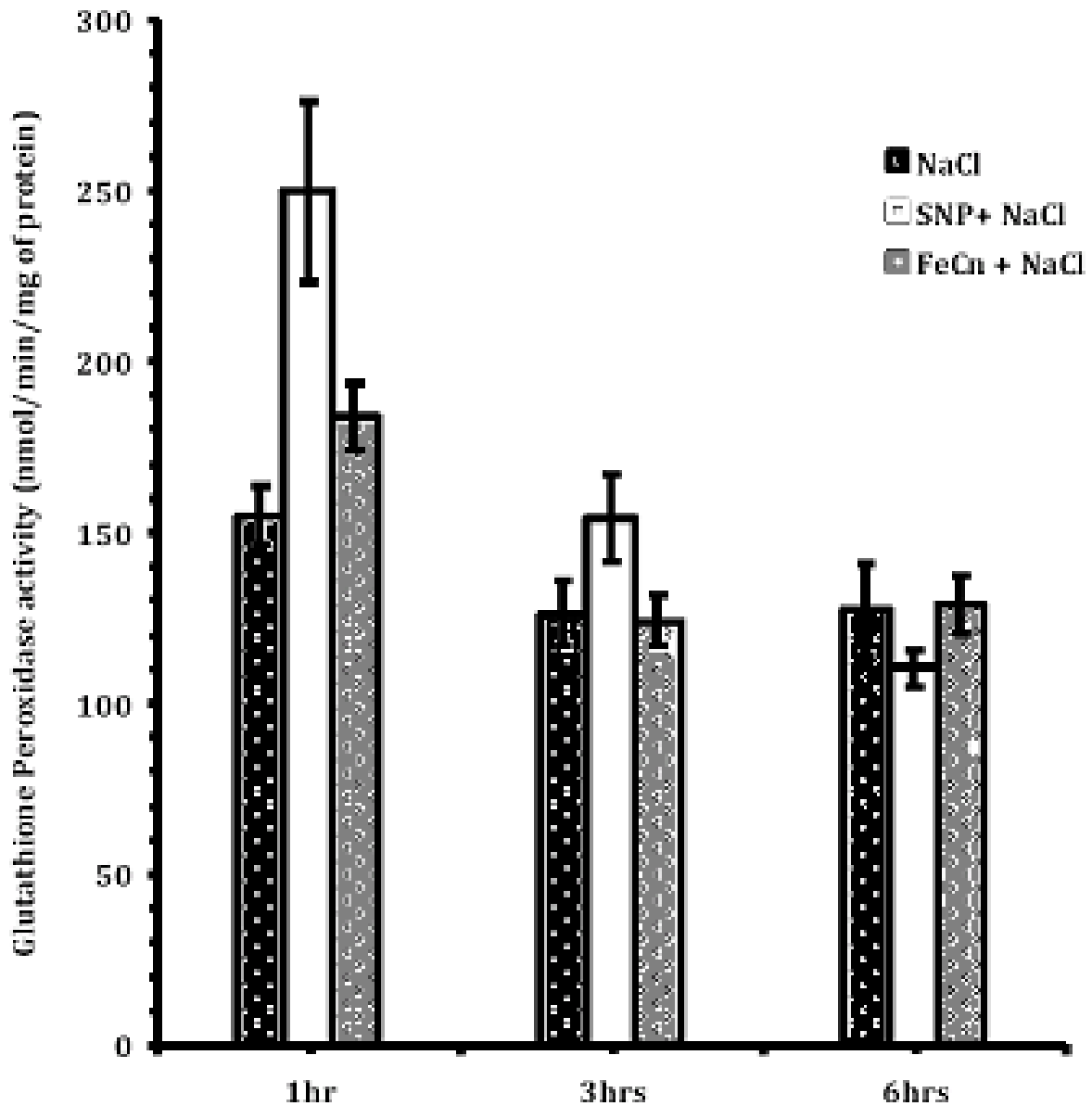


Figure 4- 5 The time course response of glutathione peroxidase activity in *Medicago truncatula* root nodules to endogenous nitric oxide treatments. SNP represents a nitric oxide donor treatment, FeCn is a potassium ferricyanide treatment. The p value = 0.05, n ≥ 3. The bars represent the sample means and the error bars represent the standard errors of the means.

In *Medicago* nodules treated with 150 mM NaCl, the GPx activity from SNP treatments is higher than both of the controls at 1 hour of incubation (Figure 4- 5). Although the SNP treatment exhibits the highest GPx activity, the overall GPx activity from all the treatments at this time point is the highest compared to the rest of the time periods.

After 3 hours of incubation the overall activity declines in all the treated nodules. The SNP-treated nodules exhibit an inhibition of GPx activity compared to the controls at 6 hours of incubation (Figure 4- 5). There is an early GPx response to SNP treatment followed by the decrease in activity as time approaches 3 hours and beyond.

In contrast to *Medicago* nodules, the pea nodules exhibited an increase in GPx activity that peaks at 6 hours of treatment then followed by the decrease at 24 hours. However, the GPx activity from the NO-treated pea nodules has remained higher than the controls at all time periods (Figure 4- 6). There are differences in the GPx responses to nitric oxide between these two legume species under NaCl stress.

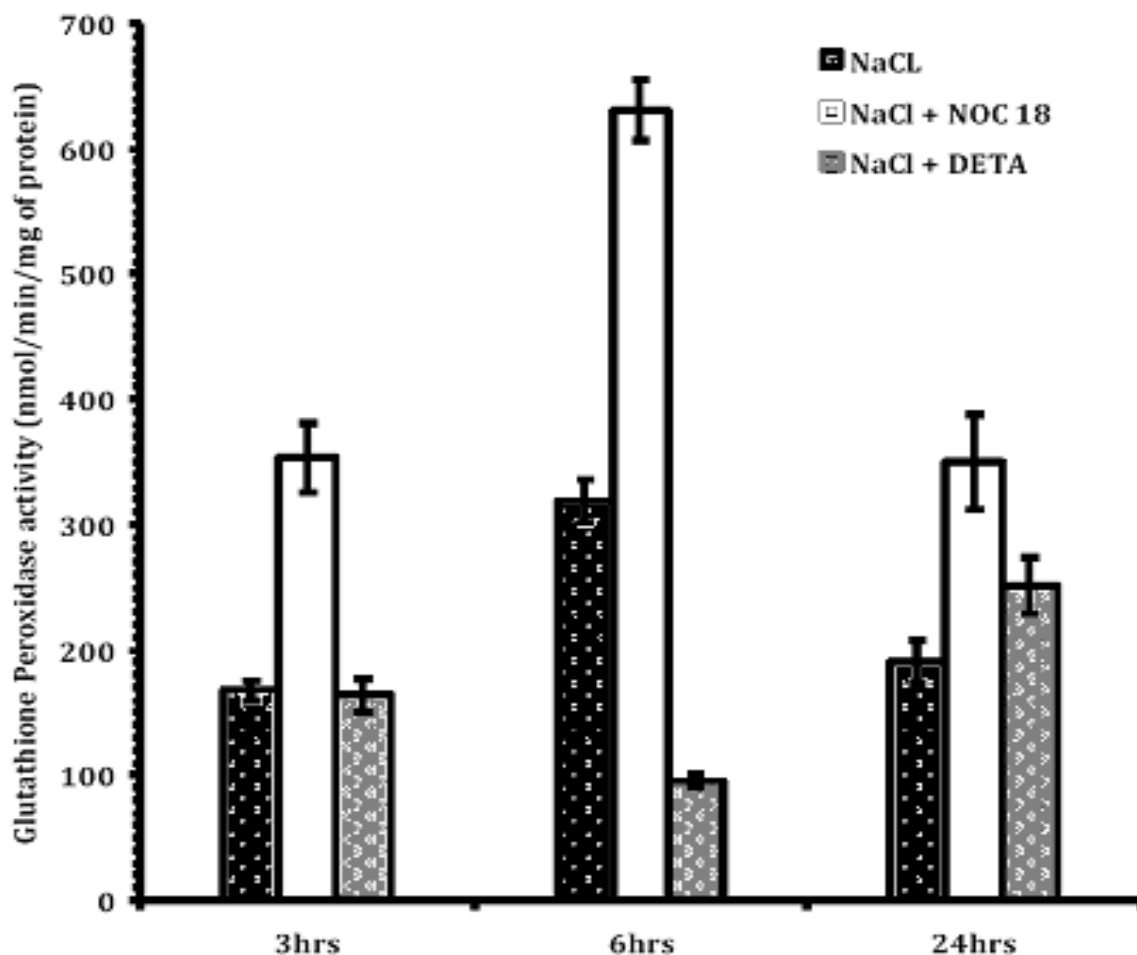


Figure 4- 6 Time course response of glutathione peroxidase activity in the *P. sativum* root nodules to endogenous nitric oxide treatments. NOC 18 represents a nitric oxide donor treatment with Diethyltriamine/Nitric oxide adduct. DETA is an analogue that does not release NO. The p value = 0.05, n ≥ 3. The bars represent the sample means and the error bars represent the standard errors of the means.

4.4.0.0. Discussion

Glutathione peroxidase belongs to a large family of peroxidase enzymes. These enzymes have a catalytic activity of scavenging peroxides in a chemical reaction that uses reduced antioxidant metabolites such as glutathione and thioredoxin.

This work focused on the glutathione-based antioxidant system, with specific attention paid to glutathione peroxidase. Glutathione peroxidase enzyme activity has been reported in plants and the genes encoding these enzymes have been isolated and characterized (Beeor-Tzahar et al, 1995, Mullineaux et al, 1998 and Roxas et al, 1997). Most importantly, glutathione peroxidase activity is increased under stress (Mittova et al, 2004).

Effects of nitric oxide on glutathione peroxidase activity under optimal conditions

In the late 20th century, it was established that NO generated from SNAP inhibits the activity of glutathione peroxidase from bovine serum by inactivating the cysteine residues (Asahi, 1995). This kind of inhibition was both time and NO concentration dependent. However with recent research findings in plant systems, NO protects maize plants from iron toxicity by up regulating POX and inhibiting GR activity (Sun et al, 2007). It is worthwhile to note that, with the higher levels of iron toxicity NO becomes less beneficial in increasing the POX activity (Sun et al, 2007). In agreement with findings on the role of NO in up-regulating POX activity is the observation from the work described in this chapter in which it is shown that nitric oxide from either SNP or DETA/NO in pea or *Medicago truncatula* increases the glutathione peroxidase activity.

Furthermore, the nitric oxide donors [N-*tert*-butyl-*a*-phenyl]nitron (PBN), sodium

nitroprusside and 3-morpholinopyridone] have been used and proved to protect leaves against oxidative stress-induced senescence caused by hydrogen peroxide (Hung et al, 2005).

Effects of nitric oxide on glutathione peroxidase activity under salinity

Higher peroxidase activities have been observed in rice seedlings exposed to several concentrations of NaCl (Koo et al, 2007). This chapter shows that salt-treated nodules exhibit higher glutathione peroxidase activity. It is interesting to note that with prolonged exposure to stress, both the nitric oxide-treated nodules and salt-stressed nodules exhibit reduced GPx activity. These findings are in agreement with the observations made on maize seedlings under iron toxicity (Sun et al, 2007). It is even more interesting to observe that despite an increase in general activity of glutathione peroxidase under salinity stress, the nitric oxide-treated nodules exhibit higher GPx activities at all time points than the other treatments in both *Medicago* and pea in this study. Hence, it is reasonable to speculate that nitric oxide from the nitric oxide donors and also the nitric oxide generated from nitric oxide synthase activity up-regulates GPx activity and possibly increases the capacity of the nodule tissue to detoxify peroxides under stress.

It should be noted that although nodules of the same type (morphologically, as both *Medicago* and pea form indeterminate nodules) were used for this study; different genotypes might have different responses to stress, nitric oxide or a combination of both. It should also be noted that over-expression of glutathione peroxidase, and thus increased corresponding GPx activity, increases plant tolerance to NaCl stress (Yoshimura et al, 2004).

Effect of inhibition of nitric oxide synthase-dependent nitric oxide synthesis on glutathione peroxidase activity.

From this study, it is observed that nitric oxide synthase-dependent production of nitric oxide is required for GPx activity under non-saline conditions. L-NAME is a nitric oxide synthase inhibitor often used to inhibit the synthesis of nitric oxide from nitric oxide synthase. It is thus appropriate to link the GPx activity in the cells to the effect of endogenous nitric oxide synthesized from nitric oxide synthase.

Exposure of maize seedlings to dehydration triggers the accumulation of intracellular nitric oxide (Zhang et al, 2006). Cotton calli exposed to NaCl also accumulate nitric oxide (Vital et al, 2008). It is thought that this accumulated nitric oxide may modulate antioxidant responses and processes that lead to stomatal closure (Niell et al, 2008). Nitric oxide accumulating during dehydration in maize seedlings is thought to be generated by nitric oxide synthase, as evidenced by increased activity of this enzyme under dehydration stress (Hao et al, 2007). It is therefore logical to relate the observed increase in activity of GPx under stress to the possible accumulation of nitric oxide in the nodules. In support of a role of nitric oxide synthase-dependent (and thus NO-dependent) modulation of GPx activity, 1 mM L-NAME reduces the GPx activity.

4.5.0.0. Conclusion

In conclusion, this study shows that exogenously applied nitric oxide increases the activity of GPx in the root nodules of the indeterminate type. This statement also holds the same for nitric oxide produced from nitric oxide synthase in these nodules.

However, future studies involving the application of NO donors/NOS inhibitors to intact nodulated plants instead of nodulated root systems would be more appropriate for studying temporal changes in GPx activity as this would minimize the likelihood of any influence of detachment of nodulated root systems on GPx activity. Despite the fact that detaching nodulated root systems from the plant might have some influence on GPx activity, it should be noted that the trend with which the NO and NaCl-mediated changes in GPx activity occur are likely to represent true responses to these compounds. Such postulation is made here because all the treatments (including controls) in this study were subjected to the same kind of detachment of nodulated root systems and so are appropriately compared.

There is a need to look at the effect of nitric oxide on the transcription of GPx. Such knowledge would help toward developing plants with increased GPx activity so as to counteract the effect of abiotic stress.

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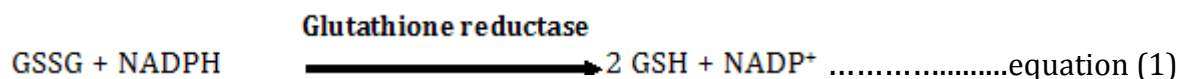
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Chapter 5 Regulation of glutathione reductase activity by nitric oxide

5.1.0.0. Introduction

Glutathione Reductase (GR. E.C. 1.6.4.2) is an important player in re-generation of glutathione in its reduced state, thus maintaining the homeostatically favoured redox status of the cell. This enzyme recycles oxidized glutathione (GSSG) back to its original reduced form (GSH) in a process that utilises NADPH as electron donor (Meister, 1988) (equation 1). GR is encoded by the nuclear genome and it has isoforms localized in the chloroplast, cytosol and mitochondria of the plant cell (Torres-Franklin et al, 2008).



Glutathione reductase plays an important role in plant stress tolerance as it has been reported that over-expression of chloroplastic glutathione reductase confers resistance to oxidative stress caused by chilling temperatures in maize (Koscy et al, 1996) and cotton (Kornyeyev et al, 2003). In emphasis of the significance of GR in stress tolerance, Foyer et al (1995) reported an enhanced antioxidant capacity in poplar as a consequence of chloroplastic GR over-expression. This chloroplastic GR resulted in an increase in the amount of reduced ascorbate and reduced glutathione accumulating in the cells, the two antioxidant metabolites that are indicative of abiotic stress tolerance. GR over-expressed in the chloroplasts of *Brassica* enhanced tolerance of these plants to cadmium (Cd) as demonstrated by increased chlorophyll contents and reduced oxidative

stress (Pilon-Smits et al, 2000). Indeed, recent studies also show that silencing of GR in tobacco plants exacerbate the effects of oxidative stress caused by methyl viologen (MV) (Ding et al, 2009). It is also interesting to observe that in rice seedlings, GR is one component of antioxidant system that responds to scavenge ROS under stress (Sharma & Dubey, 2005).

However, there are other reports emphasizing the existence of GR in plants but suggesting that this enzyme is not important in plant responses to oxidative stress. For instance, GR does not respond in any distinct pattern due to salt treatment (Mittova et al, 2004). Secondly, GR over-expression in cotton was not of any advantage to germinating seeds under temperature stress (Manah et al, 2009).

Despite the contrasting information regarding the response of glutathione reductase to stress, it should be noted that different plant species and tissues were used to establish the role of GR in stress responses. It is therefore difficult to correlate the published data and draw a general conclusion regarding the function of GR in oxidative stress responses. This study therefore intends to establish the response of this enzyme to abiotic stress and nitric oxide in the root nodules of legumes.

5.2.0.0. Materials and methods

5.2.1.0. Preparation of plant material

Seeds of *Medicago truncatula* cv. Parabinga and *Pussium sativum* cv. Crusader (both kindly supplied by Agricol, South Africa) were inoculated with *Rhizobium meliloti* and *Rhizobium leguminosarum* respectively. The seeds were germinated in vermiculite that

had been pre-soaked in de-ionized water and seedlings were propagated on this vermiculite. The germinated seedlings were supplied with nitrogen-free nutrient solution consisting of 0.001 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 M potassium phosphate buffer, 0.00005 M FeEDTA, 0.001M CaCl_2 , 0.00005M KCl and the micronutrients in the proportions: 5 μM H_2BO_3 , 2 μM MnSO_4 , 2 μM ZnSO_4 , 2 μM $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$ and 5 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. The pH was adjusted to 6.2 with potassium hydroxide. Each plant in a single pot was watered with 150 ml of this nutrient solution after every three days. Three weeks old nodules were used for experimental analysis.

5.2.2.0 Experimental conditions

Depending on the type of an experiment, four-week old plants were exposed to 1 hour, 3 hours, 6 hours and 24 hours of treatment with 100 μM sodium nitroferricyanide (SNP) or 50 μM Diethylenetriamine/Nitric oxide adduct (DETA/NO). SNP and DETA/NO are nitric oxide donors. For negative controls, plants were treated with only just nutrient solution. To determine if endogenously synthesized nitric oxide is required for GR activity, some plants were treated with the nitric oxide synthase inhibitor N_ω -Nitro-L-arginine methyl ester (L-NAME). To evaluate the effect of salinity on GR activity, the rest of the plants were treated with 150 mM NaCl. In the respective treatments, the relevant compound specific to that treatment was added as a supplement to the nitrogen-free nutrient solution.

5.2.3.0. Experimental design and data analysis

The experimental designs for all the experiments was a factorial design with a two-factor randomized complete block Design (RCBD) with three replicates. made up of 3 or

4 levels. Analysis of variance, full replicate model, was used to partition the experimental error and detect the variation amongst the treatments at 95% confidence intervals.

The nitric oxide concentration-dependency of glutathione reductase activity was determined by including a range of nitric oxide (5 μM , 20 μM and 50 μM) concentrations in which some were done in combination with 1 mM L-NAME. The nodulated roots were incubated in these treatments for 2 hours and the harvesting was done at 1 hour intervals.

5.2.4.0. Preparation of enzyme extracts

Glutathione reductase was extracted by homogenization in 10 times volume of extraction buffer (50 mM potassium phosphate pH 7.0, 1 mM EDTA, 0.1mM ascorbate and 0.1 mM glycerol. The homogenate was then centrifuged at 13 000 rpm on a bench top centrifuge at 4°C for 10 min. The supernatant was removed and desalted through a Sephadex G-25 column. The resultant eluate was used for GR activity determination.

5.2.5.0. Determination of glutathione reductase enzyme activity

The Glutathione Reductase activity was determined from the rate of oxidation of NADPH as measured by the decrease in the absorbance at 340 nm (using the extinction coefficient 0.00622 $\mu\text{M}/\text{cm}^{-1}$ for NADPH) as per Rao et al (1996). The reaction mixture composed of 100 mM potassium phosphate buffer, 0.2 mM NADPH, 0.5 mM GSSG and 20 μl of enzyme extract (Lee et al 2001 and Sgherri et al, 1994). The reaction was initiated by addition of 50 μl NADPH to all the wells. Absorbance was read at 340 nm, with

readings taken at 30°C every minute. The GR activity was measured as the rate of NADPH oxidation and was calculated using the following formulae:

$$[(\Delta A_{340}/\text{min})/0.00373\mu\text{M}^{-1}] \times [0.19\text{ml}/0.02\text{ml}] \times [\text{dilution factor}] = \text{nmol}/\text{min}/\text{ml}$$

Where:

$$\Delta A_{340} = [A_{340} (\text{Time 2}) - A_{340} (\text{Time 1})] / [\text{time 1}(\text{min}) - \text{Time 2}(\text{min})]$$

5.2.6.0. Determination of protein concentration

Protein contents in all the samples were measured as per Bradford et al, 1971.

5.3.0.0 Results

5.3.1.0. Effect of nitric oxide on glutathione reductase enzyme activities under non-saline conditions

In pea nodules the GR activities were generally much higher than in *M. truncatula* root nodules. Such differences may be a consequence of differing enzyme activities amongst different species. On the other hand, the nitric oxide donor used for treatment of pea nodulated roots, DETA/NO releases nitric oxide at higher concentration than SNP used in *M. truncatula* root nodules and has a longer half-life in solution (Hudson et al, 2001 and Keefer et al, 1996). Thirdly the nodules from Medicago and pea plant were exposed to chemicals at different states (in pea, the nodulated roots were incubated in treatment solutions while in Medicago the detached nodules from the roots were incubated in solutions), thus detaching nodules from the roots prior to incubation stage might have accelerated the natural senescence process due to wounding compared to the intact nodules.

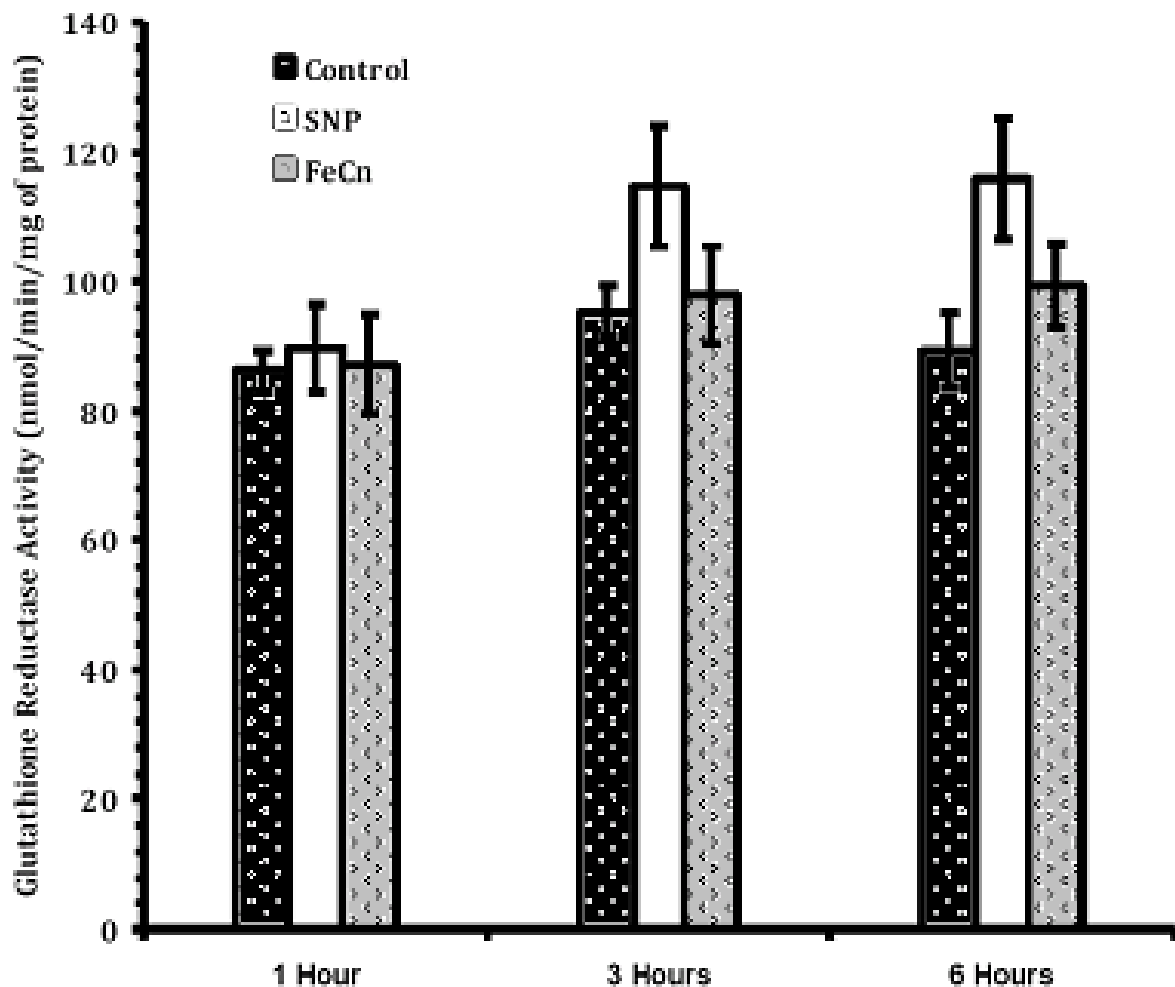


Figure 5- 1 Time course response of glutathione reductase enzyme activity to nitric oxide in *M. truncatula* root nodules. SNP represents a treatment with the nitric oxide donor 50 μ M sodium nitroprusside, FeCn is a treatment with 50 μ M potassium ferricyanide; and control represents a treatment with no chemical except for nutrient solution. The p-value = 0.05%, n \geq 3. The bars represent the sample means and the error bars represent the standard errors of the means.

In *M. truncatula*, there is no significant difference in GR activities between the nitric oxide treated nodules and their control counterparts initially. After 1 hour of incubation in the solutions containing nutrient solution, SNP and FeCn respectively, it is observed that NO released from SNP does not affect the activity of glutathione reductase. However, after 3 hours of incubation, a significant increase in GR activity is observed in SNP-treated *M. truncatula* root nodules followed by no change in activity in the successive period. While the other controls still have higher activities at these time

period compared to the 1 hour incubation, they are still lower than the activities observed in the SNP-treated samples (Figure 5- 1).

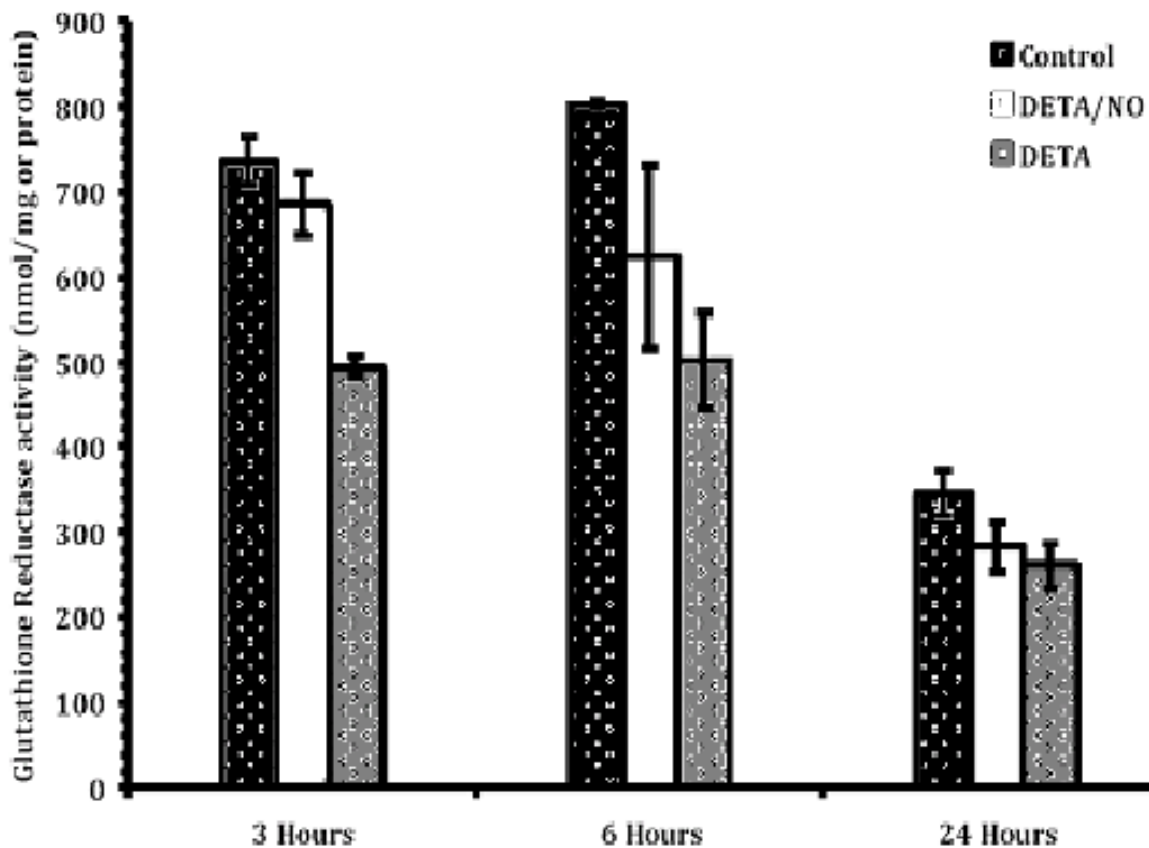


Figure 5- 2 Time course response of glutathione reductase activity in *Pisum sativum* root nodules to nitric oxide treatment under optimal nutrient conditions. DETA/NO (diethylenetriamine/nitric oxide adduct) represents a nitric oxide donor treatment, DETA is analogue of DETA/NO without nitric oxide. The p -value = 0.05%, $n \geq 3$. The bars represent the sample means and the error bars represent the standard errors of the means.

A pattern contradicting that observed in *Medicago* occurs in pea nodules. The activity of GR from the DETA/NO-treated samples at 3 hours in this case is significantly higher than in the DETA treatment and not significantly different from that of the negative control. This does not give a clear picture of the response of GR to DETA/NO; an attribute that was not observed in *M. truncatula* root nodules. However, the GR activity gets inhibited by an exposure of the nodules to DETA/NO treatment, as incubation period extends, suggesting the possibility of an involvement of NO in GR early responses (Figure 5- 2).

It is noteworthy to state that the GR activity from the untreated pea nodule has also declined substantially at 24 hours of incubation.

5.3.2.0. Effect of nitric oxide on glutathione reductase enzyme activity under saline conditions

Under saline conditions, GR activity is not affected by any of the treatments in *M. truncatula* root nodules. The fixed control (FeCn), negative controls and the SNP treatment exhibit statistically similar values. Based on these observations, it appears that GR enzyme activity is not responsive to nitric oxide generated from SNP under NaCl stress in *M. truncatula* root nodules (Figure 5- 3).

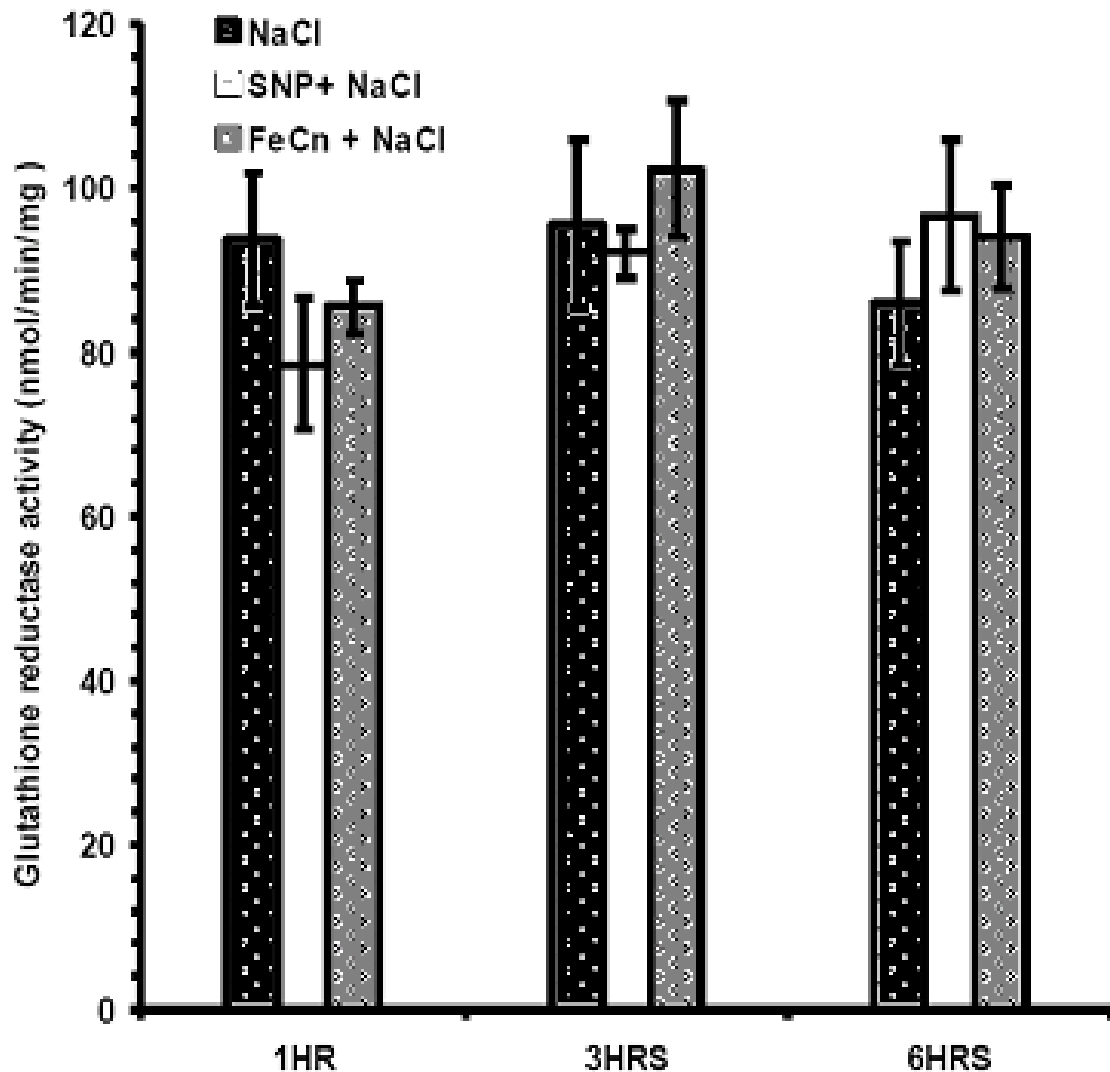


Figure 5- 3 Time course response of glutathione reductase activity in the *M. truncatula* root nodules to nitric oxide treatments under 150 Mm NaCl. SNP represents a treatment with the nitric oxide donor 50 μ M sodium nitroprusside, FeCn is a treatment with 50 μ M potassium ferricyanide; and Control represents a treatment with no chemical except for nutrient solution. The p-value = 0.05%, n \geq 3. The bars represent the sample means and the error bars represent the standard errors of the means.

5.3.3.0. Effect of inhibition of nitric oxide synthesis on glutathione reductase enzyme activities

When nitric oxide synthase-dependent biosynthesis of nitric oxide is inhibited in the nodules by exposure to 1 mM L-NAME, the GR activity decreases, and this decrease is

reversed by addition of diethylenetriamine/nitric oxide adduct to the incubation solution, resulting in activities higher than that of the control. This statement holds true only at early hours (within three hours) of incubation (Figure 5-4). Seemingly the lower inter cellular nitric oxide levels are required for enhanced GR expression, thus the response of GR to nitric oxide could be concentration dependent. For longer incubation periods (e.g. 6 hours), the pea nodules exhibit an inhibition of GR activity in response to NO treatment (Figure 5-4). The activities due to the fixed control are lower than the NO-treated nodules at least for the first two incubation periods.

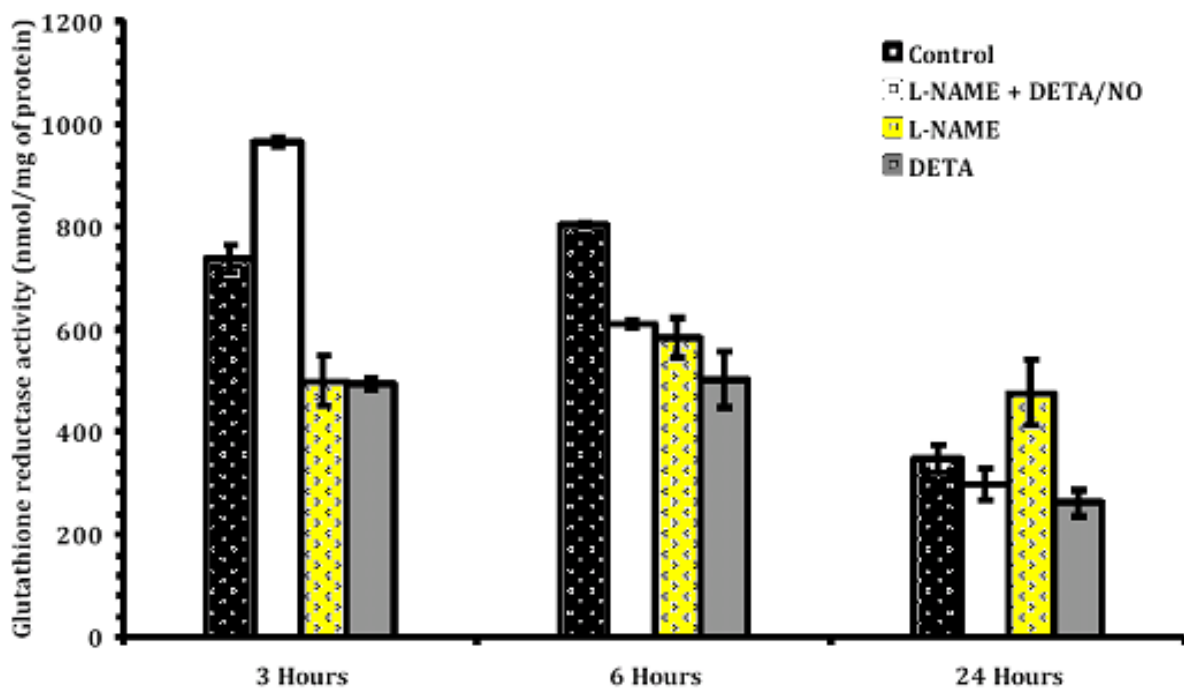


Figure 5- 4 The time course response of glutathione reductase activity in the *Pisum sativum* root nodules to endogenous nitric oxide under optimal nutrients conditions. DETA/NO (diethylenetriamine/nitric oxide adduct) represents a nitric oxide donor treatment, DETA (diethylenetriamine) is a fixed control treatment. L-NAME is the nitric oxide synthase inhibitor. The p -value = 0.05, $n \geq 3$. The bars represent the sample means and the error bars represent the standard errors of the means.

5.3.4.0. Effect of different concentrations of nitric oxide on glutathione reductase enzyme activities

In order to determine the NO concentration-dependency of GR activity, an experiment with a series of NO concentrations was set-up, the GR activities were measured in nodules for which DETA/NO was used as a nitric oxide donor in *P. sativum* root nodules (Figure 5- 5, 5- 6, 5- 7 & 5- 8). It is interesting to observe that GR in pea root nodules is inhibited by an increase in NO concentration (Figure 5- 5 & 5- 6). There is an observed decrease of about 30% after the first hour of incubation and 40% after two hours of incubation in the solutions containing nitric oxide donor (Figure 5-5).

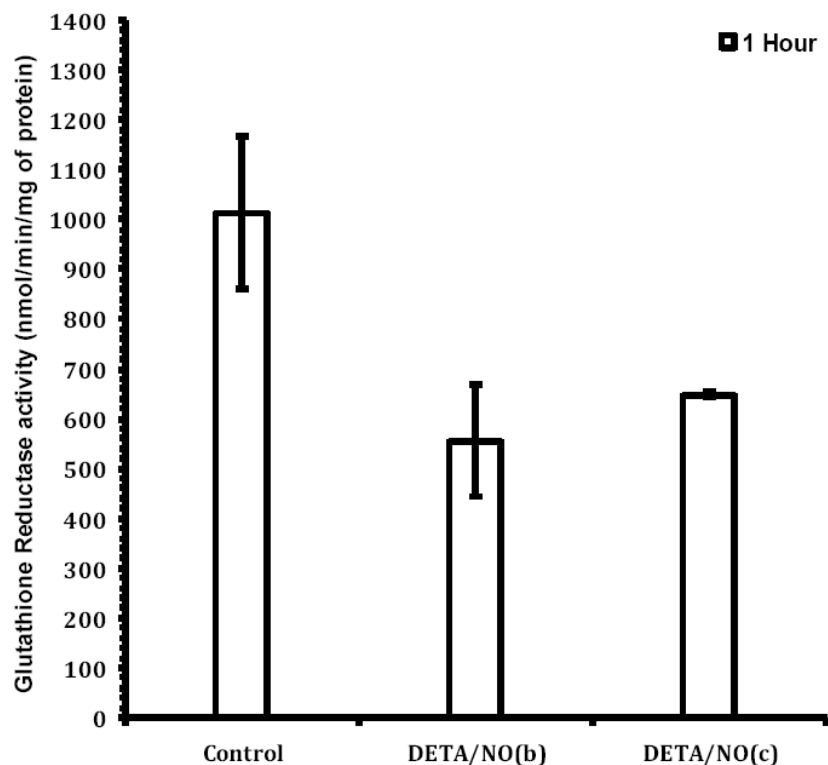


Figure 5- 5 Time course response of glutathione reductase activity in the *Pisum sativum* root nodules to different concentrations of exogenous nitric oxide treatments under optimal nutrient conditions. DETA/NO (diethylenetriamine/nitric oxide adduct) represents a nitric oxide donor treatment, DETA/NO (b) represents 20 μ M DETA/NO concentration in the incubation solution; DETA/NO (c) represents 50 μ M DETA/NO concentration in the incubation solution. The *p*-value = 0.05, *n* \geq 3. The bars represent the sample means and the error bars represent the standard error of the means.

After one hour of incubation, there is no observed difference in the GR activity between the two DETA/NO concentrations (Figure 5-5). However, their activities are lower than that of the control treatment. After two hours of incubation in the treatment solutions, there was a slight change in the activities of enzyme in response to nitric oxide (Figure 5-6).

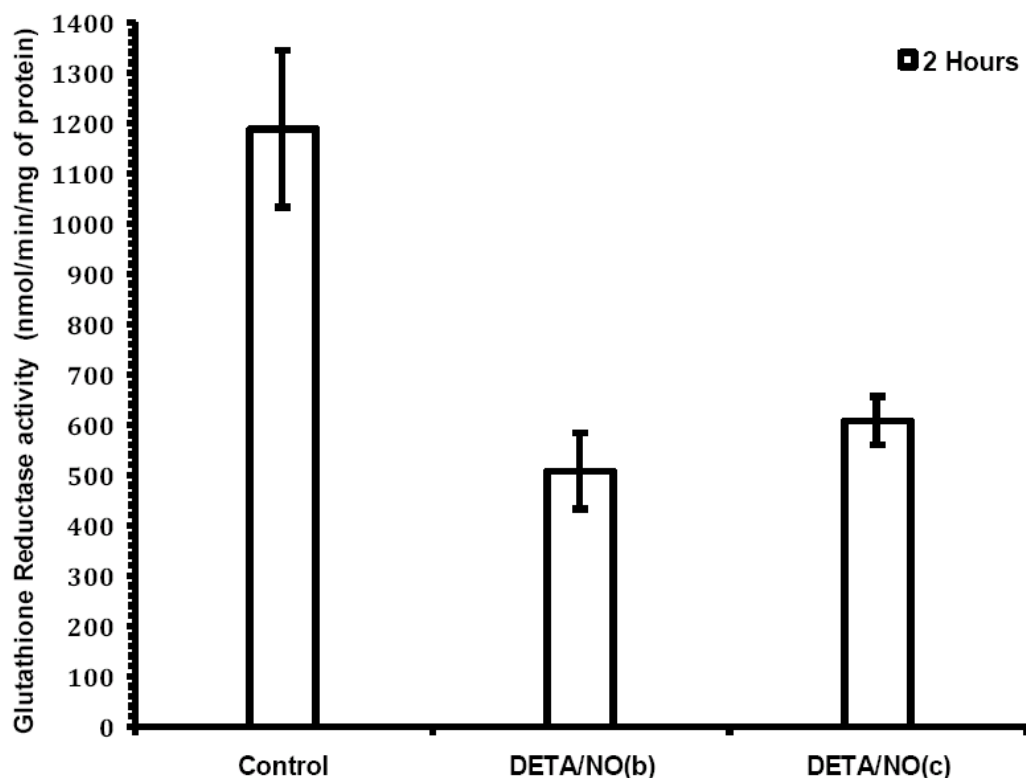


Figure 5-6 Time course response of glutathione reductase activity in the *Pisum sativum* root nodules to different concentrations of exogenous nitric oxide under optimal conditions. DETA/NO (diethylenetriamine/nitric oxide adduct) represents a nitric oxide donor treatment, DETA/NO (b) represents 20 μM DETA/NO concentration in the incubation solution; DETA/NO (c) represents 50 μM DETA/NO concentration in the incubation solution. The p -value = 0.05, $n \geq 3$. The bars represent the sample means and the error bars represent the standard errors of the means.

To further investigate the effect of NO concentration on GR activity, a series of concentrations of DETA/NO combined with LNAME were used to treat the nodules. Inhibiting the synthesis of NO endogenously with L-NAME, reverses the effects of

DETA/NO on GR activity and this effect is more explicit after 2 hours of incubation period than after 1 hour (Figure 5- 7 & 5- 8). GR activity is inhibited by 1 mM L-NAME while the 20 μ M and 50 μ M DETA/NO reverse the effect of L-NAME. The combination of 1 mM L-NAME and 20 μ M DETA/NO exhibits the highest GR activity after 2 hours of incubation in the root nodules of pea. Furthermore, even when synthesis of NO from NOS-like enzyme activity is inhibited with L-NAME treatment, the different concentrations of DETA/NO still show concentration dependency of GR activity on nitric oxide (Figure 5- 7).

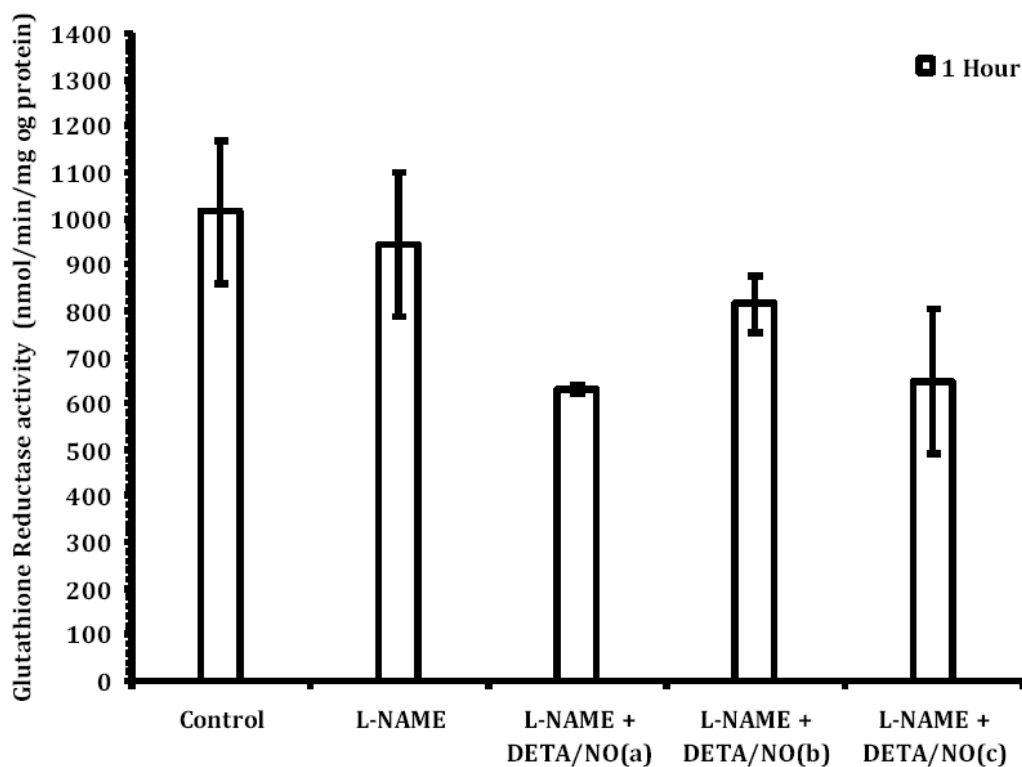


Figure 5- 7 Glutathione reductase activity response in the *Pisum sativum* root nodules to different concentrations of exogenous nitric oxide treatments combined with an inhibitor of nitric oxide synthase under optimal nutrients conditions for 1 hour. DETA/NO (diethylenetriamine/nitric oxide adduct) represents a nitric oxide donor treatment, DETA/NO (a) represents 5 μ M DETA/NO concentration in the incubation solution DETA/NO (b) represents 20 μ M DETA/NO concentration in the incubation solution; DETA/NO (c) represents 50 μ M DETA/NO concentration in the incubation solution. L-NAME is a nitric oxide synthase inhibitor. The *p*- value= 0.05, *n* \geq 3. The bars represent the sample means and the error bars represent the standard error of the means.

The control treatment after 1 and 2 hours of incubation exhibits higher GR activity in the root nodules of *P. sativum* compared to the rest of the treatments. It is interesting to see that L-NAME alone inhibits GR activity; this is more evident after two hours of incubation (Figure 5- 7 & 5- 8). The combination of L-NAME treatment with various concentrations of DETA/NO exhibits a distinct pattern. The GR activities are higher at 2 hours of incubation compared to the first hour. The lower NO concentration exhibited the lowest GR activity and 20 μM DETA/NO exhibited the highest GR activity in both time periods. Incubation in 50 μM DETA/NO does not result in the highest GR activity at the second hour although it exhibited statistically the same GR activity as in the first hour (Figure 5-7). The DETA/NO treatment reverses the effect of L-NAME after 2 hours of incubation.

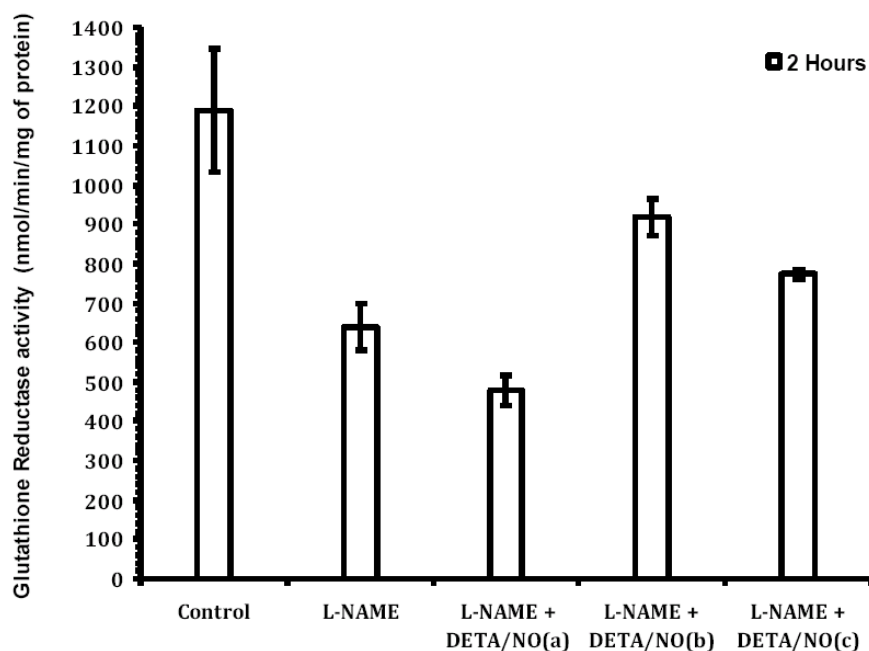


Figure 5- 8 Glutathione reductase activity in the *Pisum sativum* root nodules in response to different concentrations of exogenous nitric oxide treatments combined with inhibitors of nitric oxide synthase (L-NAME) under optimal conditions for 2 hours. DETA/NO (diethylenetriamine/nitric oxide adduct) represents a nitric oxide donor treatment, DETA/NO (a) represents 5 μM DETA/NO concentration in the incubation solution DETA/NO (b) represents 20 μM DETA/NO concentration in the incubation solution; DETA/NO (c) represents 50 μM DETA/NO concentration in the incubation solution. The p -value = 0.05, $n \geq 3$. The bars represent the sample means and the error bars represent the standard error of the means.

5.4.0.0. Discussion

Effects of nitric oxide on glutathione reductase activity under optimal growing conditions and NaCl stress

Glutathione reductase is an antioxidant enzyme that works to recycle oxidized glutathione back to its reduced form. It is a functional enzyme in plants and has been identified and isolated in the peroxisomes of pea leaves, shoots and roots of tomato (Jimenez, 1997 and Mittova, 2000).

The results from this work suggests that NO affects GR activity in a concentration-dependent manner. Indeed, NO induces an early increase in GR activity within 3 hours in *M. truncatula* but this increase is not observed beyond this time point. A similar trend is observed in plants subjected to NaCl stress. A similar pattern was also observed in tomato where salt treatment initially increased the GR activity followed by no further increase or decrease in GR activity in subsequent hours under salinity stress (Mittova et al, 2003).

There are several other studies demonstrating this time and/or concentration-dependent inhibition of glutathione reductase in response to various abiotic stresses, NO and heavy metal toxicity. In maize subjected to iron deficiency, nitric oxide from SNP rescued the plants from oxidative stress by up-regulating other antioxidant enzymes while GR activity was inhibited (Sun et al, 2007), hence an inhibition that is observed in peas at later stages of NaCl treatment is not an isolated case. Furthermore, Cd toxicity in sunflower affects some antioxidant enzymes but not GR, thus NO released from SNP does not cause any change in the response of the GR activity in those affected plants

(Laspina et al, 2005). Also, no changes in GR activities were observed in chickpea plants under salt stress (Sheokand et al, 2008). Based on results from this study, GR enzyme activity seems to be up-regulated by abiotic stress but this increase is followed by an inhibition of the GR activity at all the time points.

The glutathione reductase activity in *Pisum sativum* follows a different trend from that observed in *Medicago*. In *P. sativum* at 3 hours of incubation period and under low concentrations NO, there is an increase in the activity of GR but that activity drops with prolonged exposure to nitric oxide. GR functions to recycle GSSG back to GSH, hence the decrease in GR activity under stress could occur as a consequence of a decline in cellular GSH available for reduction under stress, or there is a possible alternative reductant being used by GR under such conditions. Over-expression of chloroplastic GR in poplar trees resulted in increased levels of the total glutathione pool and the GSH/GSSG ratios (Foyer et al, 1995). Glutathione levels and redox status form part of successive chapters within this thesis. The concentration-dependency experiments do not reveal a clear pattern in GR response to the concentration of NO. Although there could be a concentration-dependent response, it is not necessarily a linear response since different concentrations of DETA/NO do not show a linear response in GR activity.

5.5.0.0. Conclusion

Data from this study suggests that GR activity is modulated by nitric oxide in *Medicago* nodules. In pea nodules, nitric oxide causes an inhibition in GR activity that follows an early short-lived upsurge in this activity. For all their similarity in nodule morphology, the genotypes used here exhibit differences in their natural degree of tolerance to abiotic stress and this could account for their varying responses to nitric oxide exposure.

The inconsistency in the patterns of the plants antioxidant responses to increased GR expression has been recorded (Foyer et al, 1995; Mittova et al, 2003 and Ding et al, 2009). Also the non-responsiveness of GR to different types of stresses have been observed (Sheokand et al, 2008; Sun et al, 2007 and Laspina et al, 2005). Such differences do not follow a specific pattern, thus it is difficult to postulate any kind of a reason that brought about the differences in GR activities for this particular work.

The most important point is that, the *Medicago* and pea varieties used for this thesis are commercial varieties that have been bred for specific purposes. Thus, genetic rearrangements have occurred during several crosses made, and could have altered the original biochemistry of these genotypes, that tallies with their nodule morphology. However, there is no conclusive data to date indicating that nodules of the same phenotype must have similar biochemical response patterns with regards to antioxidant systems.

The application of knowledge on glutathione reductase in improving stress tolerance in plants is still in its infancy. There is a need for more research to establish the regulatory role of GR to or by other antioxidant enzymes. To make this point clearer, the study involving the regulation of temporal expression of this enzyme by both NO and stress in very early periods (minutes) would have improved our understanding of GR responses to NaCl and NO in this work.

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Chapter 6 Modulation of glutathione levels and glutathione redox status by nitric oxide

6.1.0.0. Introduction

Glutathione (GSH) is a low molecular weight tri-peptide that plays a major role in protecting plant cells against abiotic stress (Cossete et al, 1996). As an antioxidant it protects plant cells against oxidative stress, maintains the homeostatic redox status of the cell and thus supports the normal health of the cell (Ding et al, 2009). Elevation of GSH levels in the cells through over-expression of glutathione synthetase enhances plant tolerance to abiotic stress without any deleterious effect on plant growth (Zhu et al, 1999 and Xiang et al, 2001). The role of GSH in supporting normal growth under salt stress is also observed in yeast (Romero et al, 2001).

Exposure of cells to abiotic stress like salinity induces not only the accumulation of reactive oxygen species (ROS) but also other signalling molecules like nitric oxide (NO) in plant cells (Neill et al, 2008). Nitric oxide has been implicated in modulation of antioxidant metabolite levels/redox status and antioxidant enzyme responses under stress (Neill et al, 2008). GSH synthesis is also up-regulated upon plant exposure to stress, an indication of its vitality during oxidative stress. For instance, salinity induces the synthesis of glutathione in *Brassica* (Ruiz and Blumwald, 2002). GSH is also known to be responsive to nitric oxide; a molecule thought to be involved in abiotic stress signalling (Innocenti et al, 2007). NO affects the accumulation of GSH at the transcriptional level by up-regulating the transcription of glutathione synthetase and

consequently increasing cellular GSH levels in *Medicago truncatula* (Xu et al, 2009 and Innocenti et al, 2007).

The maintenance of higher ratios of reduced glutathione against the oxidized form is required for the maintenance of cellular redox homeostasis under stress as well (Ding et al, 2009). Reduced glutathione functions to scavenge peroxides in the ascorbate-glutathione cycle (Shao et al, 2008). The high levels of reduced glutathione protect the cells against oxidative stress (Cossett et al, 1996). This chapter evaluates the potential of nitric oxide to modulate the response of the glutathione pool in the nodules of legumes and the probable role in remedying the oxidative stress induced by salinity.

6.2.0.0. Materials and methods

6.2.1.0. Plant material preparation

Medicago truncatula cv. Parabinga and *Pisum sativum* cv. Crusader seeds (both kindly supplied by Agricol, South Africa) were inoculated with *Rhizobium meliloti* (for *Medicago*) and *Rhizobium leguminosarum* (for pea) and grown on vermiculite. The germinated seedlings were supplied with nitrogen-free nutrient solution consisting of 0.001 M MgSO₄·7H₂O, 0.001 M potassium phosphate buffer, 0.00005 M FeEDTA, 0.001 M CaCl₂, 0.00005 M KCl and the micronutrients in the proportions: 5µM H₂BO₃, 2 µM MnSO₄, 2 µM ZnSO₄, 2 µM CuSO₄·2H₂O and 5µM Na₂MoO₄·2H₂O. The pH was adjusted to 6.2 with potassium hydroxide. Each pot containing a single plant was supplied with 150 ml of this nutrient solution after every three days. All the plants used for this experiment were grown under 16hrs of light in the growth chamber for four weeks until nodule maturity.

6.2.2.0. Experimental conditions

Four-week old plants were exposed to either 1 hour, 3 hours, 6 hours or 24 hours of treatment with 100 μM sodium nitroferricyanide (SNP) or 50 μM Diethylenetriamine/Nitric oxide adduct (DETA/NO). For negative controls, plants were treated only with the nutrient solution. To determine if nitric oxide is required for regulation of glutathione levels, some plants were treated with the nitric oxide synthase inhibitor N_ω -Nitro-L-arginine methyl ester (L-NAME). To evaluate the effect of salinity on glutathione levels, the rest of the plants were treated with 150 mM NaCl. In the respective treatments, the relevant compound specific to that treatment was added as a supplement to the nitrogen-free nutrient solution. Potassium ferricyanide was used as a fixed control for SNP.

6.2.3.0. Experimental design and data analysis

The experimental designs for all the experiment was a factorial design with a two-factor randomized complete block Design (RCBD) with three replicates. Analysis of variance, full replicate model, was used to partition the experimental error and detect the variation amongst the treatments at 95% confidence intervals.

6.2.4.0. Preparation of enzyme extracts

Glutathione was extracted by grinding 10 mg of nodule tissue in of 5% (w/v) trichloroacetic acid (TCA), followed by centrifugation for 20 minutes at 20 000 *g* on a desktop centrifuge. The TCA was removed from the samples by extracting twice with two volumes of diethyl ether (Yanagida et al, 2004).

6.2.8.0. Measurement of glutathione pool content

The glutathione pool was determined as per Yanagida et al, 2004. Total glutathione (GSSG +GSH) was determined in a 200 μ L reaction mixture containing 0.25 mM NADPH, 10 mM potassium phosphate buffer (pH 7.5) and 0.6 mM DNTB (5,5'-Dithiobis-(2-Nitrobenzoic Acid) as per Pinto et al, 1999. The reaction contained 20 μ L of sample extract.

In order to assay for GSSG, 40 mM *N*-Maleimide (NEM) dissolved in 10 mM potassium phosphate buffer containing 5 mM EDTA was added to the supernatant to mask the GSH thiol group. The TCA and NEM were removed from the emulsion by extracting three times with two volumes of diethyl ether. The reaction for both assays was initiated by addition of 3 Units of GR in 1 ml of the reaction. The glutathione-reductase-DTNB recycling of total glutathione and oxidized glutathione was measured by monitoring absorbance of glutathione using a KC4 spectrophotometer from BIO-TEK (Version 2.5) at 412 nm for 1 minute every 2 seconds. The GSSG amounts were extrapolated from GSSG and GSH standard curves with known concentrations and the GSH quantity was calculated as the difference between the amount of glutathione pool and GSSG. The glutathione values were expressed in micromole per gram of tissue extracted (fresh weight).

6.3.0.0. Results

6.3.1.0. Effect of nitric oxide on total glutathione pool

Nitric oxide from SNP triggered an increase in total glutathione levels in the nodules compared to the controls. NO-treated *Medicago* nodules exhibited higher total glutathione level than the negative control (Figure 6-1).

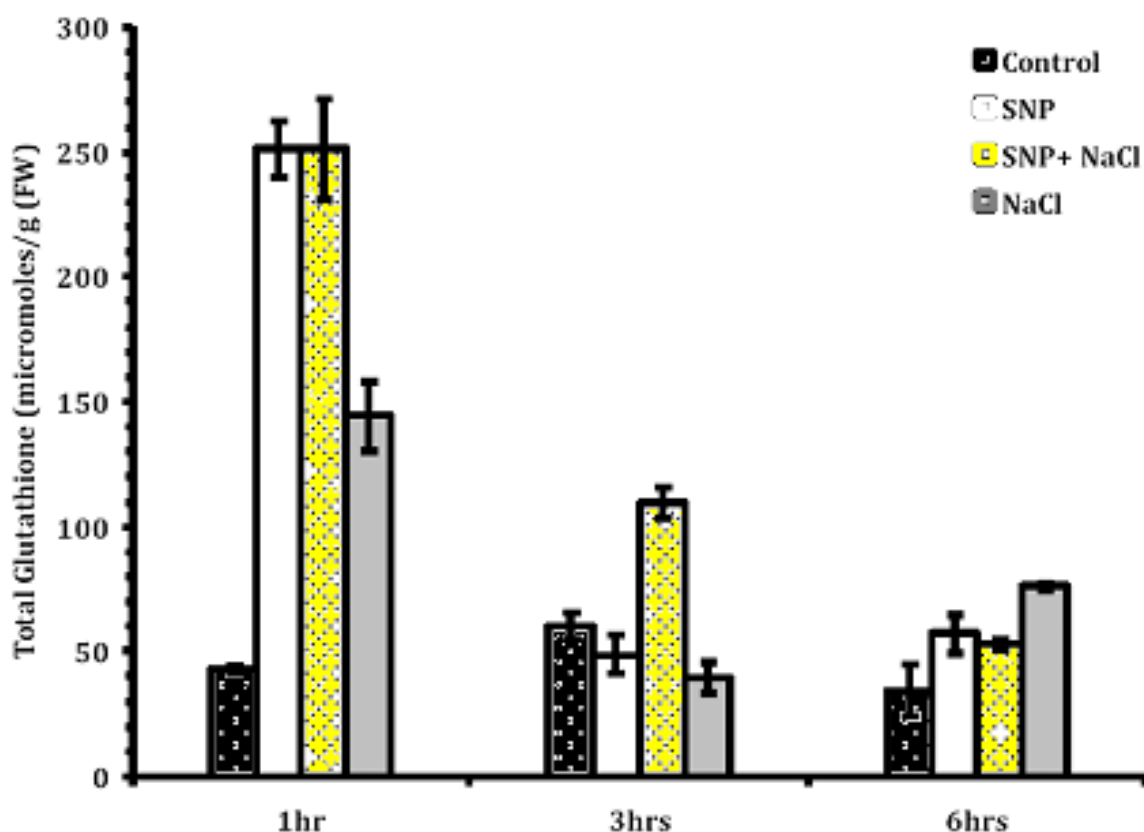


Figure 6- 2 Time course response of total glutathione in *M. truncatula* nodules to nitric oxide. Control represents the solution with only nutrient solution at optimal growing conditions; SNP is sodium nitroprusside, a nitric oxide donor; NaCl represents 150 mM sodium chloride. The total variation between the means was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the mean concentrations of total glutathione and the error bars represent the standard errors of the means.

Although there is an observed increase in glutathione levels in response to NaCl treatment as compared to the control treatments, addition of SNP (NO donor) to the

NaCl treatment increased the level further. The same pattern is observed in *P. sativum* root nodules; DETA/NO increased the glutathione levels in the nodules. This is observed throughout the time periods studied (Figure 6-2). However, there was a general decrease in total glutathione with an increase in incubation period regardless of the type of treatment the nodules have been subjected to. In *M. truncatula* there seems to be no difference between 3 hours and 6 hours of incubation in the levels of total glutathione (Figure 6-1).

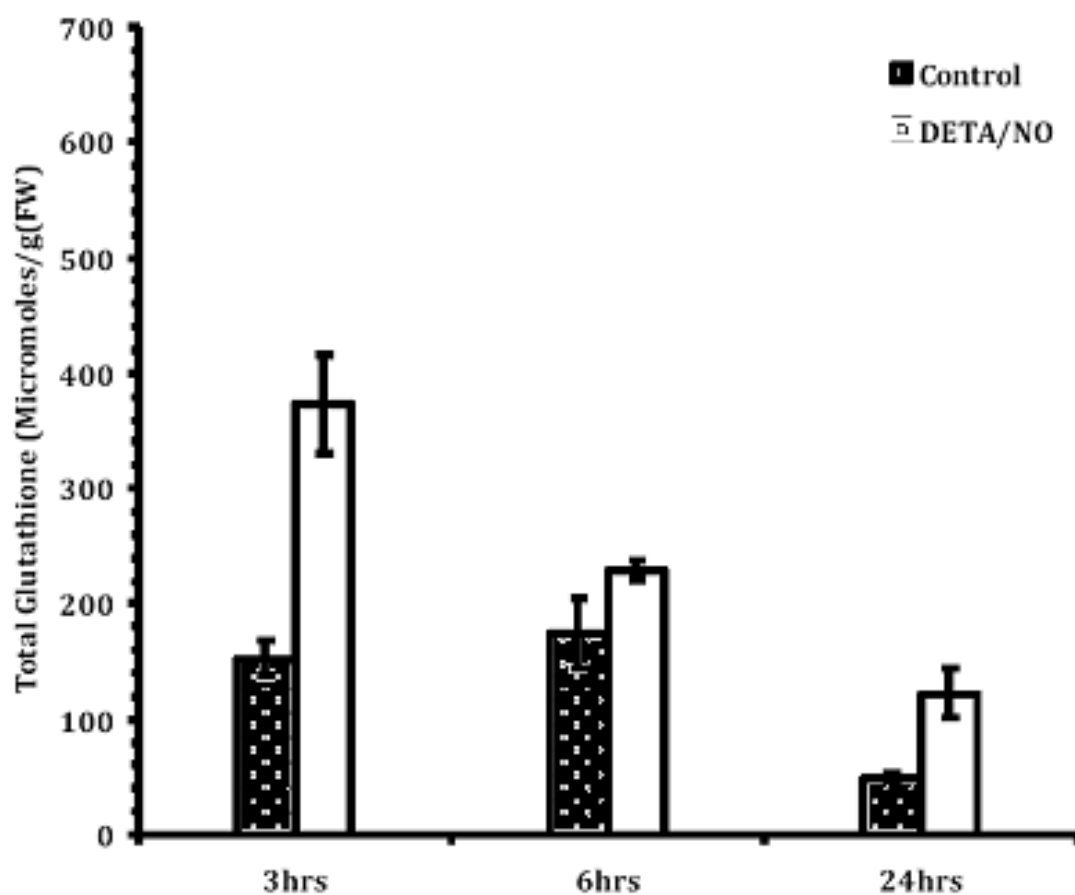


Figure 6- 3 Time course response of total glutathione of *P. sativum* nodules to nitric oxide. Control represents the negative control solution with only nutrient solution at optimal growing conditions; DETA/NO represents, a nitric oxide donor, 50 μ M Diethylenetriamine/Nitric oxide adduct. The total variation between the treatments was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the means of the total glutathione concentration per gram of fresh nodule tissue and the error bars represent the standard errors of the means.

Exposure of root nodules to nitric oxide leads to an increase in total glutathione levels but this effect is reversed by inhibition of nitric oxide synthase activity with L-NAME (Figure 6-3). There is an increase in total glutathione when the nodules are treated with DETA/NO following the inhibition of endogenous nitric oxide synthase activity. Treatment with DETA/NO reversed the inhibitory effect observed for L-NAME (Figure 6-3). The negative control treatments always exhibited lower glutathione levels than the DETA/NO-treated nodules.

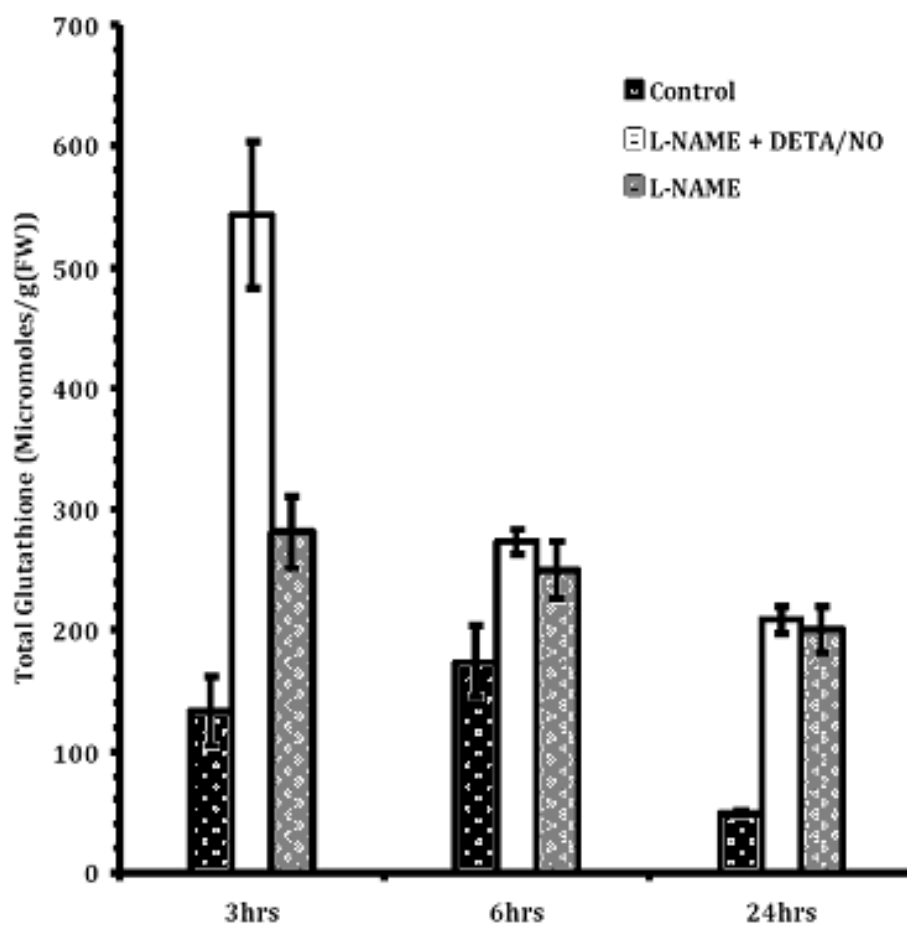


Figure 6- 4 Time course response of total glutathione to nitric oxide in *P. sativum* nodules. Control represents the negative control solution with only nutrient solution at optimal growing conditions; DETA/NO represents 50 μ M Diethylenetriamine/Nitric oxide adduct; L-NAME represents a nitric oxide synthase inhibitor. The total variation between the treatments was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the mean total glutathione concentration per gram of fresh nodule tissue and the error bars represent the standard errors of the means.

6.3.2.0. Effect of nitric oxide on reduced glutathione

Glutathione is synthesized in its reduced form and it is this form that is required to maintain the cellular redox homeostasis. Reduced glutathione is oxidized as it scavenges H_2O_2 in the reaction that is catalyzed by glutathione peroxidase. Hence, under oxidative stress more of reduced glutathione is consumed. From this work, the obtained results demonstrated that SNP and DETA/NO treatments increased the GSH content in the nodules (Figure 6- 4 & Figure 6- 5). A similar pattern is observed under 150 mM NaCl treatment, NaCl increases the GSH content of the nodules as the levels are higher than under negative control treatments. However, addition of SNP to NaCl incubation solution increases the GSH levels even more. In *Medicago*, this pattern is observed at early hours of incubation period and declines with the incubation period until the point of no significant difference between the treatments (Figure 6- 4).

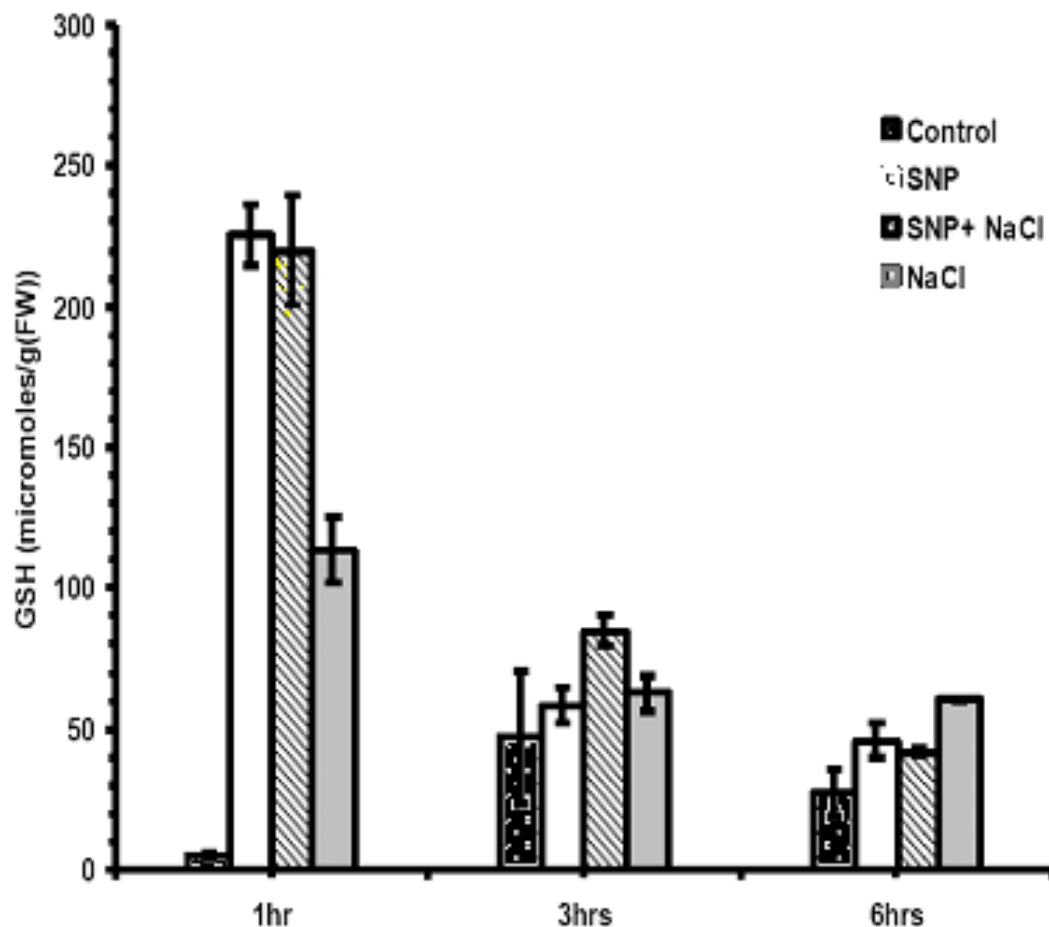


Figure 6- 5 Time course response of reduced form of glutathione per g of fresh weight of *M. truncatula* nodules. Control represents the negative control solution with only nutrient solution at optimal growing conditions; SNP represents sodium nitroprusside, a nitric oxide donor; NaCl represents 150 mM sodium chloride. The total variation between the means was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the mean concentrations of reduced glutathione and the error bars represent the standard errors of the means.

Endogenous nitric oxide has the same effect as the exogenous NO on the GSH levels. L-NAME reverses the effect of DETA/NO on GSH and restores its levels close to the negative controls levels. While L-NAME used in combination with DETA/NO explicitly increases the GSH levels in the nodules at 3 hours incubation, L-NAME lowers GSH levels and maintains these levels at the same point throughout the experimental period (Figure 6- 6). However, GSH levels from the control experiment decrease drastically after 24 hours incubation period.

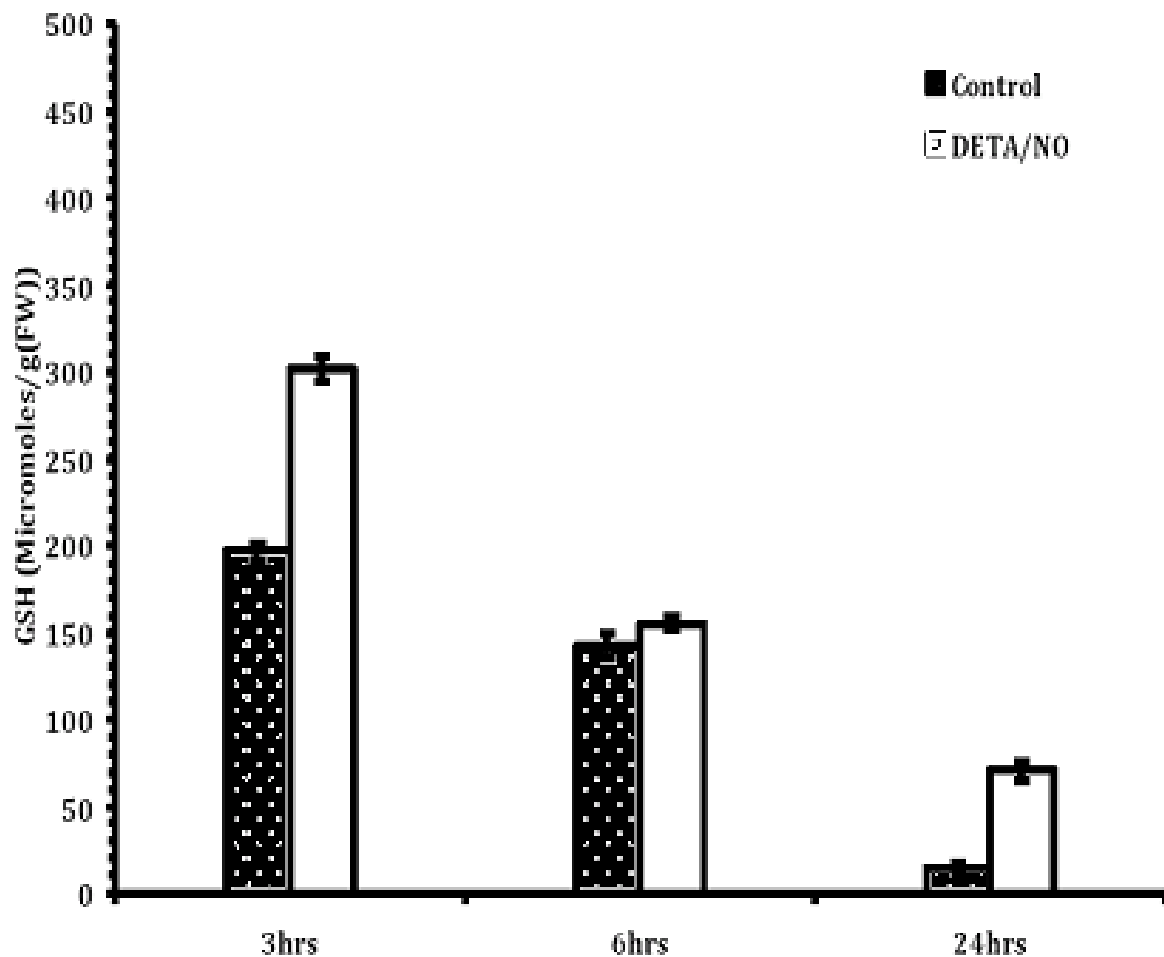


Figure 6- 6 Time course response of reduced form of glutathione to nitric oxide in *P. sativum* nodules. Control represents the negative control solution with only nutrient solution at optimal growing conditions; DETA/NO represents a nitric oxide donor (50 μ M Diethylenetriamine/Nitric oxide adduct). The total variation between the treatments was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the mean reduced form of glutathione concentration per gram of fresh nodule tissue and the error bars represent the standard errors of the means.

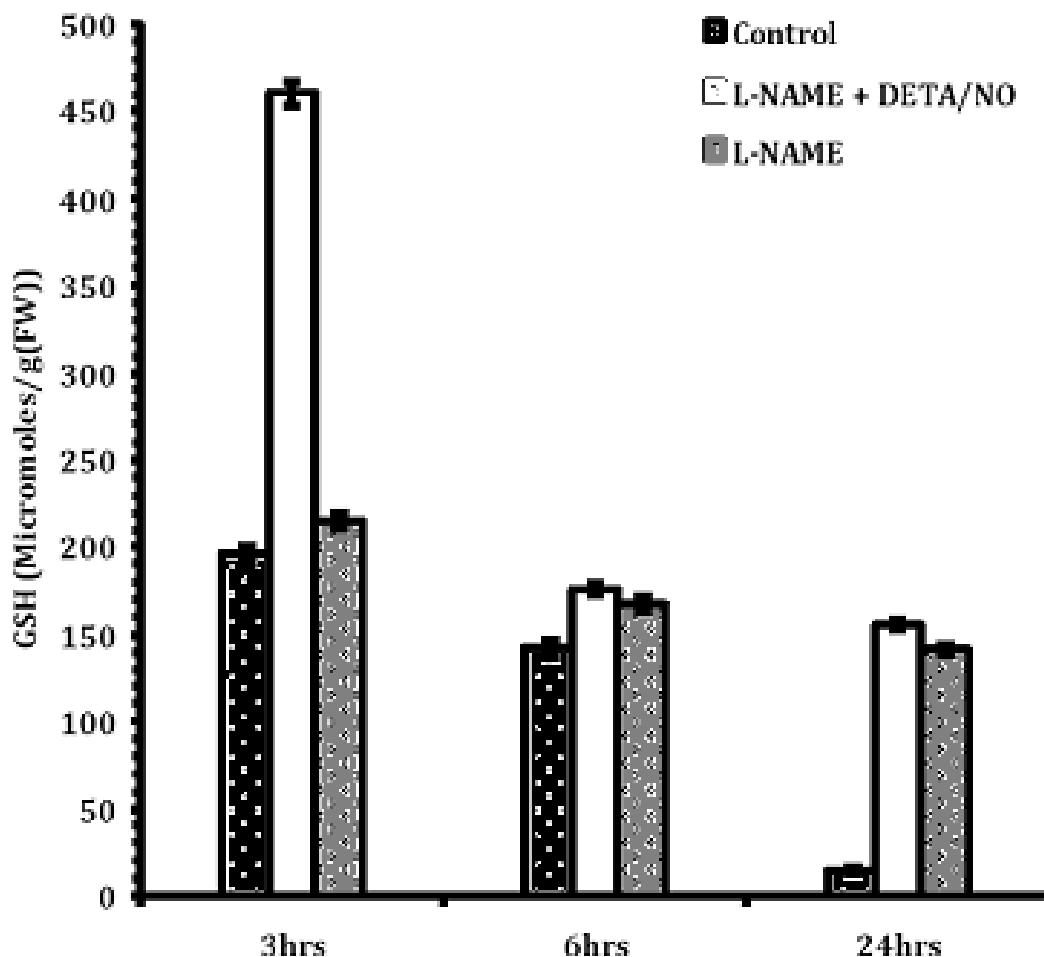


Figure 6- 7 Time course response of the reduced form of glutathione on *P. sativum* nodules to different treatments of nitric oxide. Control represents the negative control solution with only nutrient solution at optimal growing conditions; L-NAME represents a nitric oxide synthase inhibitor; DETA/NO represents 50 μ M Diethylenetriamine/Nitric oxide adduct (a nitric oxide donor). The total variation between the treatments was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the mean reduced form of glutathione concentration per gram of fresh nodule tissue and the error bars represent the standard errors of the means.

6.3.3.0. Effect of nitric oxide on glutathione redox status of the nodule

A high ratio of GSH/GSSG is required to maintain homeostatic redox state in plant cells. It is observed in this study that NO does not only increase total glutathione and GSH but also the GSH/GSSG ratios (Figure 6- 7). The data from *P. sativum* GSH/GSSG ratios show that DETA/NO-treated samples have higher ratios than the negative controls at all the time periods tested here (Figure 6- 7). This appears to be similar to the response

observed in *M. truncatula* root nodules after 1 hour and 3 hours of incubation in the treatment solutions, for *Medicago* nodules and pea nodules (Figure 6- 8).

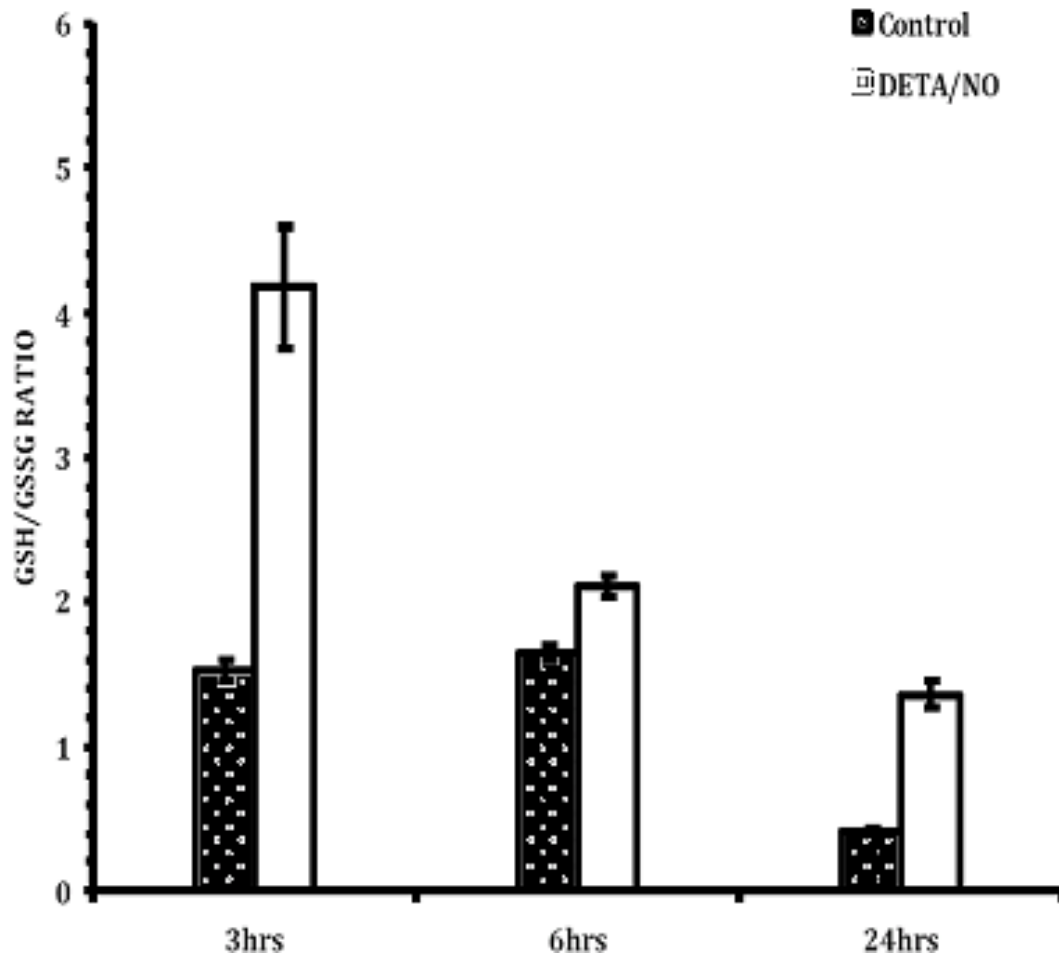


Figure 6- 7 Time course response of GSH/GSSG ratios of *P. sativum* nodules to different treatments of nitric oxide. Control represents the negative control solution with only nutrient solution at optimal growing conditions; DETA/NO represents a nitric oxide donor, 50 μ M Diethylenetriamine/Nitric oxide adduct. The total variation between the treatments was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the mean GSH/GSSG ratios and the error bars represent the standard errors of the means.

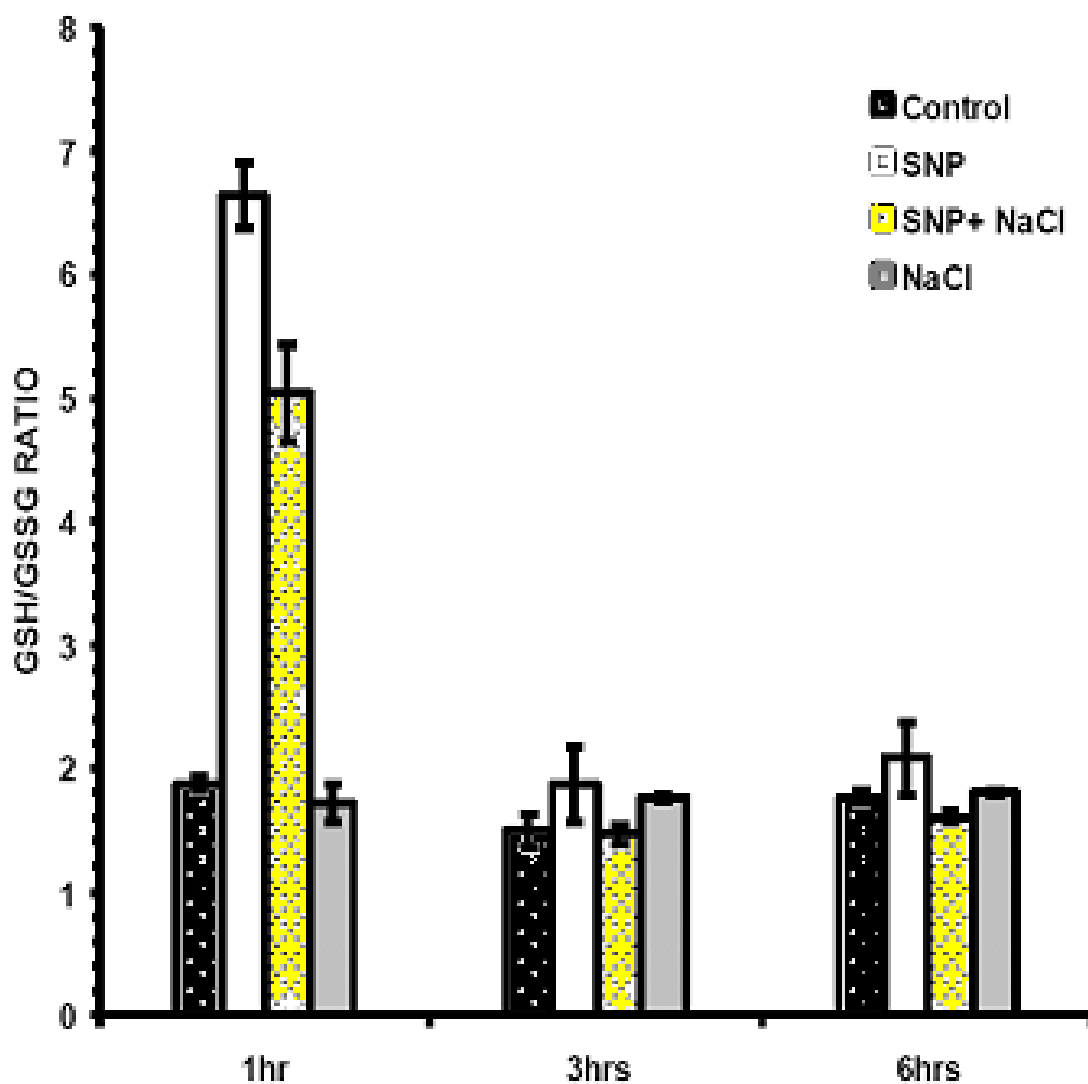


Figure 6- 8 Time course response of GSH/GSSG ratios of *M. truncatula* nodules to nitric oxide. Control represents the negative control solution with only nutrient solution at optimal growing conditions; SNP represents sodium nitroprusside, a nitric oxide donor; NaCl represents 150 mM sodium chloride. The total variation between the means was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the mean concentrations of glutathione ratios and the error bars represent the standard errors of the means.

Under salinity treatment, the nitric oxide donor SNP still increases the GSH/GSSG ratios high above the levels exhibited by NaCl treated samples. Although NaCl also increased both the total glutathione and the GSH content of the cells in the same way NO does, the GSH/GSSG ratios from the NO-treated samples are much higher than those from the NaCl-treated nodules at least at early periods of incubation (Figure 6- 8).

In order to prove that the higher ratios exhibited by NO donors are due to the activity of nitric oxide, an experiment in which nitric oxide synthase activity is inhibited was set up. The results obtained showed that DETA/NO is responsible for the increased GSH/GSSG ratios and this effect was reversed by treatment with L-NAME. A combined treatment in which L-NAME was used in simultaneous combination with DETA/NO still showed higher GSH/GSSG ratios than the negative controls. This effect is more evident in the first 3 hours of incubation (Figure 6- 9).

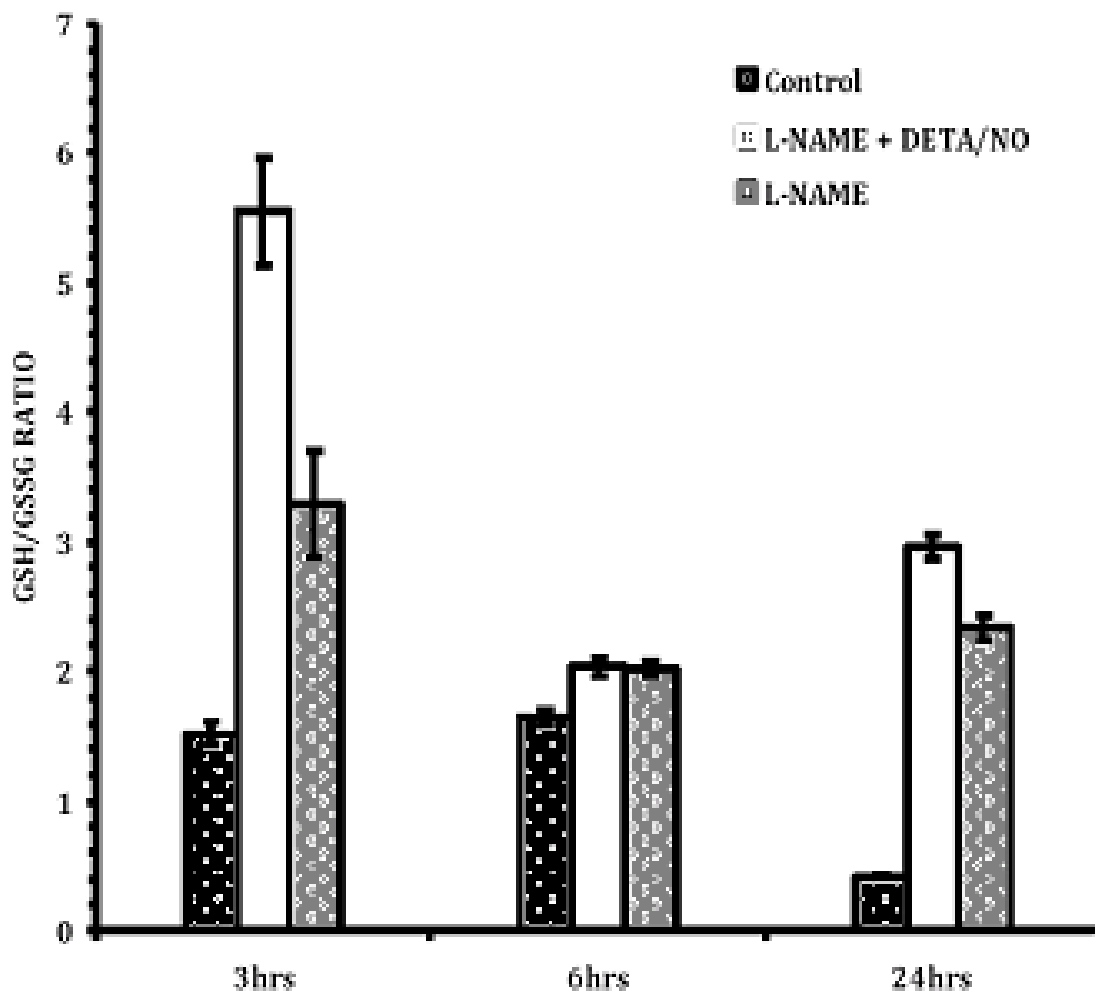


Figure 6- 9 Time course response of GSH/GSSG ratios on *P. sativum* nodules to nitric oxide. Control represents the negative control solution with only nutrient solution at optimal growing conditions; L-NAME represents a nitric oxide synthase inhibitor; DETA/NO represents, a nitric oxide donor, 50 μ M Diethylenetriamine/Nitric oxide adduct. The total variation between the treatments was tested using ANOVA, $p < 0.05$ ($n \geq 3$). The bars represent the mean GSH/GSSG ratios and the error bars represent the standard errors of the means.

6.4.0.0. Discussion

Glutathione is tri-peptide thiol whose function is to maintain redox homeostasis in the cell. Expression the enzyme/s synthesizing this molecule enhances plant tolerance to oxidative stress (Koscy et al, 2001). It is in fact more interesting to note that there is no toxicity of this molecule in the cell. Up-regulating glutathione levels in the cells has no reported cases of causing any defects on plant growth and development (Zhu et al, 1999; Xiang et al, 2001).

In this study, it is demonstrated that, in *M. truncatula*, glutathione content increases initially with salt stress. These findings are in accordance with the research findings from ground nut studies that salt induced high glutathione contents (Jain et al, 2002). In addition to this is the knowledge that more stress tolerant genotypes exhibit higher total glutathione under stress than the genotype with lower stress tolerance.

Under this study, a substantial difference in glutathione contents of salt-stressed nodules and non-stressed nodules is observed especially in the early hours of incubation. This increase in total glutathione and GSH levels indicates that NaCl triggers glutathione synthesis and secondly it might increase GR activity, hence the ratios are altered. Similar reports from cold stress also suggest an increase in total glutathione levels as a result of an increased synthesis rate (Koscy et al, 2001a). Furthermore, a similar trend is observed in wheat genotypes where tolerant varieties have higher total glutathione synthesis rate compared to the sensitive ones (Koscy et al, 200, 2004b, c). The cotton plant is another model validating the view that stress tolerance is mediated by glutathione synthesis (Gosset et al, 1996).

The changes in redox state of the cell have been studied and there are supporting lines of evidence to the findings of this study. A higher GSH/GSSG ratio is used as an indicator of a homeostatically favourable redox state. Hence freezing-tolerant wheat cultivars were found to have higher GSH/GSSG ratios compared to sensitive ones (Kocsy et al, 2001a) and these high ratios were further correlated with high heat tolerance (Kocsy et al, 2004b). Salt stress studied in tomato also revealed higher GSH/GSSG ratios in tolerant genotypes (Shalata et al, 2001). In this study, NO treatment exhibits higher ratios in both legume species, and under both the salt stress and optimal conditions.

Our study establishes that there are higher GSH/GSSG ratios at early hours of treatments in response to nitric oxide (regardless of the stress, nitric oxide-treated nodules exhibit the highest ratios at all the time points). This increase is followed by a decrease in the entire magnitude of the ratios in the successive hours indicating a change in the redox state of the cells regardless of the treatment. Similar changes in GSH/GSSG ratios have been observed in different plants under different abiotic stress conditions. It is reported that cold stress, drought stress and salt stress result in a steady decrease in the GSH/GSSG ratio as the stress progresses (Szalai et al, 2009). This kind of decrease in the GSH/GSSG ratio is attributed to mainly the scavenging of H_2O_2 by GSH to form glutathione conjugates or to further degradation of GSH. Supporting evidence observed from this study shows that the GSH/GSSG ratios decrease with time regardless of the treatment. The decrease in the GSH/GSSG ratios is accompanied by a decrease in both total glutathione and GSH content.

In this work it is shown that NO-treated nodules exhibit higher GSH content, total glutathione and GSH/GSSG ratios at early hours of the incubation period used in both legume species (Medicago and pea) for this study. An increase in these antioxidant

metabolites is more evident in pea nodules where DETA/NO (a nitric oxide donor) was used. It should be noted that although SNP is a nitric oxide donor it contributes to cell death at levels higher than those used for DETA/NO (Murgia et al, 2004). Hence, with extended exposure to SNP more oxidative stress might occur compared to DETA/NO. The other contributing factor in the difference in the levels of response between the nodules treated with different donors would be the way nodules from different genotypes have been treated with the various chemicals. In peas, the nodulated roots were incubated in the solutions while in *M. truncatula* only detached nodules were used. The nodules from the nodulated roots tend to survive and be actively fixing nitrogen for longer periods than the detached nodules; hence the detached nodules are expected to senesce earlier than the nodules that are still attached to roots. One cannot rule out the significance of the species/genotype-dependent responses.

Nitric oxide has been reported to enhance glutathione synthesis by up-regulating the expression of glutathione synthetase (Innocenti et al, 2007). Glutathione is synthesized in an ATP-dependent two-step enzymatic reaction that first involves production of glutamylcysteine from L-glutamic acid and L-cysteine in a reaction that is catalyzed by glutamylcysteine synthetase. Secondly, glutathione synthetase catalyses the formation of GSH by adding glycine to the C-terminal carboxyl group of glutamylcysteine. It has also been established that NaCl triggers an increase in NO cellular content and this NO is involved in oxidative stress signalling (Niell et al, 2008). Hence, it can be argued that an increase in total glutathione content in response to nitric oxide treatment and salt treatment compared to the controls is due to nitric oxide. It is therefore reasonable to speculate that NO can increase the GSH levels through increasing the synthesis rate (Innocenti et al, 2004) and also through increasing the reaction rate of glutathione reductase (Pilon-Smits et al, 2000). It has also been shown that increased GR activity

elevated the cellular glutathione levels without affecting glutathione synthesis (Foyer et al, 1995).

6.5.0.0. Conclusion

In this study, it is demonstrated that both exogenous nitric oxide and endogenous nitric oxide modulate the levels of the total glutathione pool; cause an increase in the levels of reduced glutathione and thus maintain high levels of the GSH/GSSG ratios. It is therefore concluded that nitric oxide improves nodule redox homeostasis.

The experimental system used in this work to a certain extent imposes limitations to explaining the presented data in full. Experiments based on whole plants in studying these responses would give a better perspective as to whether prolonged exposure of root nodules to nitric oxide would really protect plants against oxidative stress. However our findings show that NO protects nodules from oxidative stress induced by NaCl, through the maintenance of better homeostatic redox status.

Since it has been established that nitric oxide regulates glutathione biosynthesis at the level of transcription, the enzymes involved in glutathione biosynthesis can be targeted for genetic modification of plants with high glutathione content so that they are tolerant to abiotic stress.

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Chapter 7 Modulation of glutathione peroxidase activity by cGMP

7.1.0.0. Introduction

Cyclic guanosine monophosphate (cGMP) is a secondary signaling molecule and was identified in mammals decades ago. The cGMP pathway has been described as one of the means through which nitric oxide signaling is modulated. During cGMP synthesis from guanosine triphosphate (GTP), nitric oxide binds to the haeme domain of the soluble guanylyl cyclase (sGC); an enzyme that is responsible for synthesis of cGMP from other GTP. This accumulation of cGMP affects downstream biochemical processes that result in modification of proteins and their functions through phosphorylation (Mayer and Hemmens, 1997).

As a secondary messenger, cGMP is involved in plant cell signaling. The existence of cGMP in plants was demonstrated through mass spectroscopy in plants in the 20th century (Janistyn, 1983). Additionally, the existence of the proteins with guanylate cyclase activity in plants further proved the existence and functionality of the cGMP pathway in plants (Ludidi and Gehring, 2003). However, the identified plant proteins with guanylate cyclase activity were found to be independent of nitric oxide signalling (Ludidi and Gehring, 2003).

Even though there are no nitric oxide dependent proteins with guanylate cyclase activity in plants identified to date, several accounts in plant research link the nitric oxide signaling function to the cGMP pathway. For instance, the control of stomatal closure controlled by ABA and nitric oxide in the guard cells is dependent upon modulation by cGMP (Niell et al, 2003). The involvement of cGMP in osmotic stress is reinforced by findings showing that there is always an upsurge of intracellular cGMP upon stress exposure such as NaCl and heavy metal toxicity (Donaldson et al, 2004 and Palavan-Unsal & Arisan, 2009).

Apart from the osmotic responses, nitric oxide is implicated in modulating the activities related to plant growth and development through cGMP. The involvement of nitric oxide in root gravitropism in soybean and pollen polarity in *Ceratopteris richarddi*, are all modulated through cGMP (Xu et al, 2005 and Salami et al, 2007). Stress-induced generation of cGMP also seems to have an important function in regulation cation fluxes and thus regulating the absorption of Na⁺, Ca⁺ and K⁺ in the cells (Maathuis FM, 2006).

Nitric oxide synthesized during stress has an important role in protecting cells against oxidative stress. This important role was discussed (Niell et al, 2008) and furthermore the previous chapters of this thesis demonstrated it clearly by linking the protective role of NO during abiotic stress to antioxidant content and antioxidant enzyme activities. According to our knowledge to date there are no accounts that relate this modulation of antioxidants and enzyme activities by NO to the cGMP pathway. Glutathione peroxidase is an important antioxidant enzyme functioning in the acclimatization of cells to abiotic stress and its activity is modulated by nitric oxide. Hence in this work the involvement of the cGMP pathway in the cascade of events leading to GPx antioxidant enzyme activity was investigated.

7.2.0.0. Materials and methods

7.2.1.0 Preparation of plant material

Medicago truncatula cv. Parabinga and *Pisum sativum* cv. Crusader seeds (both kindly supplied by Agricol, South Africa) were inoculated with appropriate Rhizobia and were germinated on vermiculite pre-soaked in de-ionized water. The germinated seedlings were supplied with the nitrogen-free nutrient solution consisting of 0.001 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 M potassium phosphate buffer, 0.00005 M FeEDTA, 0.001 M CaCl_2 , 0.00005 M KCl and the micronutrients in the proportions: 5 μM H_2BO_3 , 2 μM MnSO_4 , 2 μM ZnSO_4 , 2 μM $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$ and 5 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. The pH was adjusted to 6.2 with potassium hydroxide. Each pot containing a single plant was supplied with 150 ml of this nutrient solution after every three days. All the plants used for this experiment were grown under 16 hours of light in the growth chamber for four weeks till nodule maturity.

7.2.2.0. Experimental conditions

Depending on the type of an experiment, four-week old plants were exposed to 1 hour, 2 hours and 3 hours of various concentrations of 8-pCPT-cGMP (a cell-permeable analogue of cGMP), L-NAME (a nitric oxide synthase inhibitor) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, an inhibitor of the soluble guanylyl cyclases).

The chemicals were dissolved in different solvents depending on their chemical stability. The stocks of 8-pCPT-cGMP and L-NAME were dissolved in water. ODQ was dissolved in absolute ethanol. Since L-NAME is a systemic nitric oxide synthase inhibitor, it was supplied to the nodulated roots 30 mins prior to other treatments.

7.2.3.0. Experimental design and data analysis

The randomized complete block Design (CRBD) with three replicates was adopted for these experiments. The Analysis of Variance (ANOVA), and was used to partition the experimental error and detect the variation amongst the treatments at 95% confidence intervals.

7.2.4.0. Preparation of enzyme extracts

Glutathione peroxidase enzyme was extracted by homogenizing nodule tissue in 10 volumes (v/w) extraction buffer (50 mM TRIS-HCl, pH 7.6, 0.15 mM NADPH, 15 mM GSH) using an electric grinder. The homogenate was spun at 4°C using a bench-top centrifuge at 20 000 *g* for 15 minutes. The supernatant was removed and desalted through a Sephadex G-25 column and used for GPX activity determination.

7.2.5.0. Determination of glutathione peroxidase enzyme activities

Glutathione peroxide activity was measured as per Mittova et al, 2004, with some modifications. In summary, the assay was carried out in GPX assay buffer (50 mM TRIS-HCl, pH 7.6, 0.15 mM NADPH, 15 mM GSH, 1 U/ml glutathione reductase, 0.95 mM sodium azide. This reaction was initiated by addition of H₂O₂ to a final concentration of 0.075%. The glutathione peroxidase activity was then measured by following the decrease in absorbance at 340 nm. The extinction coefficient of NADPH (0.00373μM⁻¹) was used. The formula used for calculating GPX activity was as follows:

$$[(\Delta A_{340}/\text{min})/0.00373\mu\text{M}^{-1}] \times [0.19\text{ml}/0.02\text{ml}] \times [\text{dilution factor}] = \text{nmol}/\text{min}/\text{ml}$$

Where:

$$\Delta A_{340} = [A_{340} (\text{Time 2}) - A_{340} (\text{Time 1})] / [\text{time 1}(\text{min}) - \text{Time 2}(\text{min})]$$

The enzyme activity was expressed as nmol per milligram of protein.

7.2.7.0. Determination of protein concentration

All the protein contents in all the samples were measured as per Bradford et al, 1971.

7.3.0.0. Results

7.3.1.0. Effect of different concentrations of cGMP on glutathione peroxidase activity

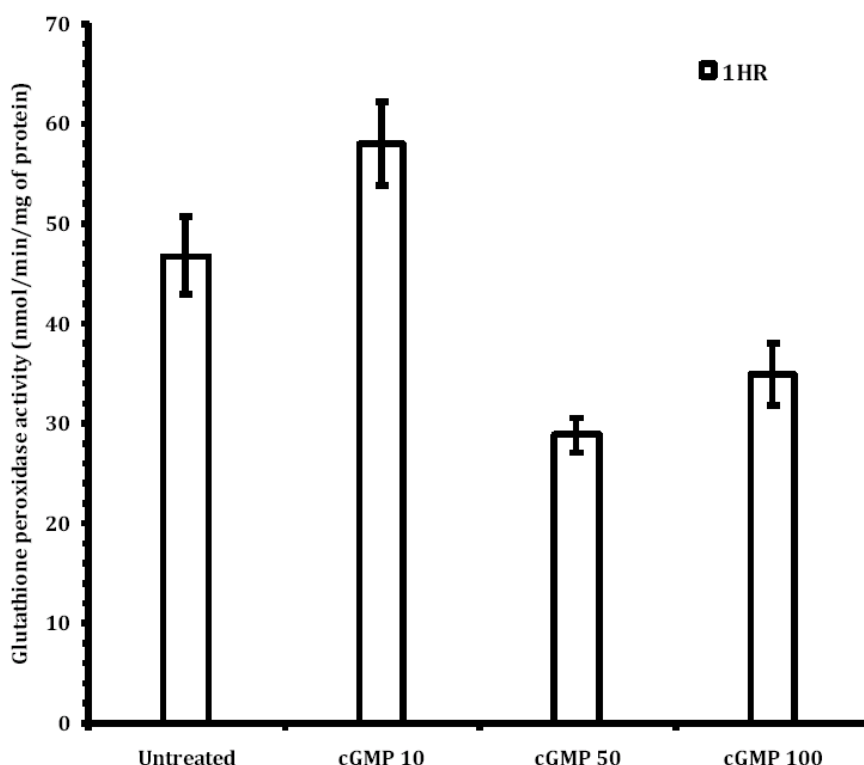


Figure 7- 1 Glutathione peroxidase activities in the pea nodules after incubation in nutrient solution, pH 7.2, supplemented with different concentrations of 8-pCPT-cGMP (cGMP) for 1 hour. cGMP 10 represents 10 μ M concentration of 8-pCPT-cGMP; cGMP 50 represents 50 μ M concentration of 8-pCPT-cGMP; cGMP 100 represents 100 μ M concentration of 8-pCPT-cGMP; while untreated represents just nutrient solution without 8-pCPT-cGMP.

Exogenously applied cGMP affects the activity of GPX in the nodules. From Figure 7- 1, it is observed that 10 μM cGMP causes an increase of about 30% in GPX activity when compared to the untreated sample. However, this increase is not sustained as the concentrations of cGMP increase; higher concentrations inhibit the activity; albeit that no significant difference is observed between 50 μM and 100 μM at 1 hour incubation period. It can thus be speculated that additional cGMP beyond the threshold levels does not necessarily increase the scavenging capacity of GPx.

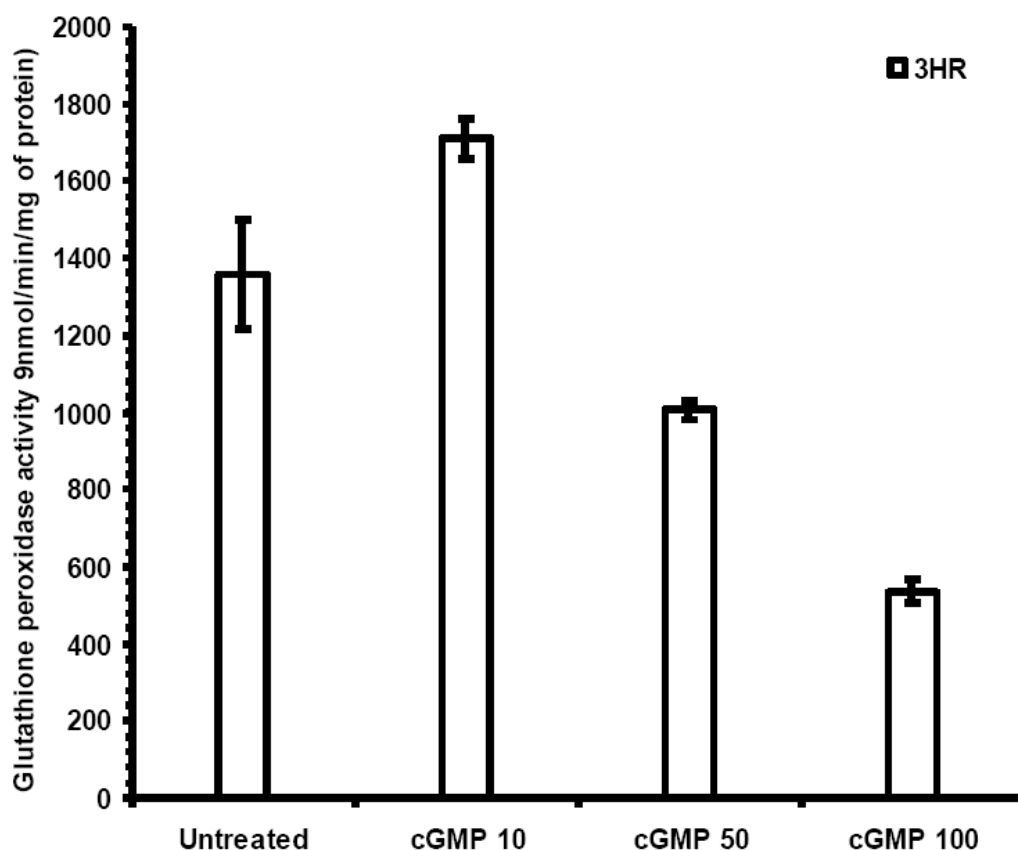


Figure 7- 2 Glutathione peroxidase activities in the pea nodules after incubation in nutrient solution, pH 7.2, supplemented with different concentrations of 8-pCPT-cGMP for 3 hours. cGMP 10 represents 10 μM concentration of 8-pCPT-cGMP; cGMP 50 represents 50 μM concentration of 8-pCPT-cGMP; cGMP 100 represents 100 μM concentration of 8-pCPT-cGMP; while untreated represents just nutrient solution without 8-pCPT-cGMP.

After 3 hours of incubation in similar solutions, the effect of different cGMP concentrations on nodular GPx activity at higher concentrations of cGMP is distinct from

that observed for the 1 hour treatment. There is an obvious difference between 50 μM and 10 μM cGMP. At 100 μM cGMP, the highest inhibition (about 50% compared to the untreated nodules) is observed. Thus the functionality of cGMP as a signaling molecule to trigger the activity of glutathione peroxidase is concentration-dependent (Figure 7-2).

7.3.2.0. Modulation of the Effect of cGMP on Glutathione Peroxidase activity By Nitric Oxide Synthase-Dependent Nitric Oxide.

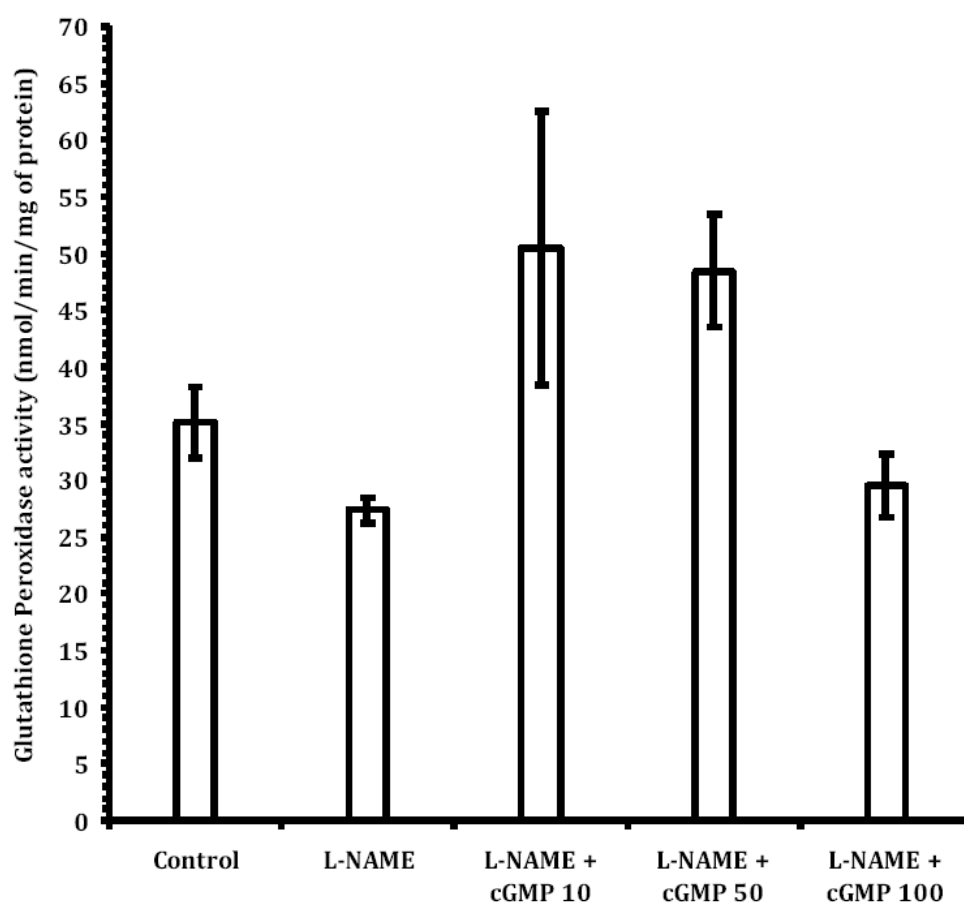


Figure 7- 3 Glutathione peroxidase activities in the pea nodules after incubation in nutrient solution, pH 7.2 supplemented with different concentrations of 8-pCPT-cGMP (cGMP) and 1 mM L-NAME for 3 hours. cGMP 10 represents 10 μM concentration of 8-pCPT-cGMP; cGMP 50 represents 50 μM concentration of 8-pCPT-cGMP and cGMP 100 represents 100 μM concentration of 8-pCPT-cGMP; while control represents just nutrient solution without any 8-pCPT-cGMP or L-NAME.

Figure 7- 3 above shows that inhibition of nitric oxide synthesis by 1 mM L-NAME inhibits the activity of GPx when compared to the control treatment. It is rather interesting to observe that addition of 10 μ M cGMP reverses the inhibition and increases the activity to about 25% higher than the control. However exposure of root nodules to 50 μ M cGMP does not cause a significant increase when compared to 10 μ M cGMP; they both at this time point increase the GPx activity to about the same magnitude. 100 μ M cGMP restores the GPX activity back to the level of the control.

7.3.3.0. Modulation of the effect of cGMP on glutathione peroxidase activity by soluble guanylyl cyclase.

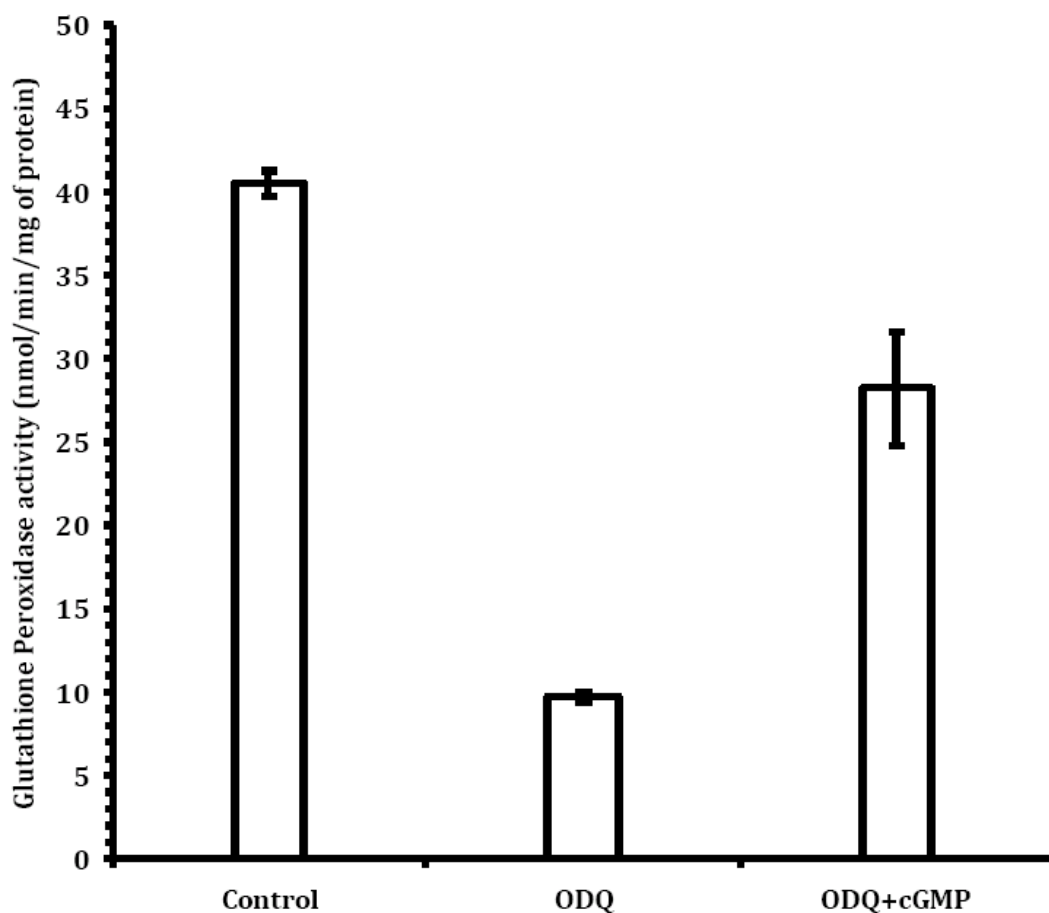


Figure 7- 4. Glutathione peroxidase activity in pea nodules after incubation in nutrient solution, pH 7.2, supplemented with 100 μ M 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 50 μ M 8-pCPT-cGMP (cGMP).

When incubating the nodules in the nutrient solution supplemented with 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), the glutathione peroxidase activity gets reduced compared to the control. There is a substantial decrease of about 73%. It is interesting however to note that the decrease caused by blocking the sGC is reversed by 50 μ M cGMP. All these observations can be summed up in one sentence: nitric oxide from nitric oxide synthase is required for GPx activity via interacting with sGC to stimulate cGMP synthesis in plant cells.

7.4.0.0. Discussion

The existence of a cGMP-mediated signalling pathway in plants has long been discovered and, though not well elucidated, there are pointers linking cGMP as a signaling molecule to the signaling effect of nitric oxide (Palavan-Unsal and Arisan, 2009). Cyclic GMP up-regulates the stress defense gene encoding phenylalanine ammonia lyase (PAL) in tobacco in the same manner as nitric oxide (Durner et al, 1998). Also, the nitric oxide produced via synthesis by rat NOS expressed in tobacco triggers the expression of defense genes in a pathway that involves functioning of cGMP down stream of nitric oxide (Duner, 1998). Indeed, in our study, it is observed that cGMP alone increases the activity of the antioxidant enzyme, glutathione peroxidase. In the previous chapter we established that NO also causes the upsurge of GPx activity both under salt stress and under optimal growth conditions.

It is further established that, just like nitric oxide (previous chapter), cGMP modulates the activity of GPx in a concentration-dependent manner. There seems to be a threshold cGMP concentration that would stimulate the activity of GPx and thus protect plants against oxidative stress. Higher concentrations of cGMP inhibit the activity of GPx (Figure 7- 2 & 7- 3). In mammals, the cGMP signalling pathway modifies proteins and their functions in a reversible phosphorylation process (Mayer and Hemmens, 1997). If this is true for plants, it is reasonable to conclude that the cellular response due to cGMP accumulation would be dependent upon the cGMP concentration. It should be noted that this is a first attempt to establish the relationship between the cGMP concentration and antioxidant responses in plants.

It is rather interesting though that cGMP reverses the inhibition of GPx activity by L-NAME, a nitric oxide synthase inhibitor. Incubation of nodules in L-NAME reduces GPx activity compared to the control experiments. However, addition of cGMP to the L-NAME-treated nodules reverses the effect of L-NAME. This change is also concentration-dependent. Thus, if substituting NO with cGMP exhibits a similar pattern as in NO treatment, it could suggest that cGMP and NO function in the same pathway to modulate the activity of glutathione peroxidase. Secondly, since inhibition of nitric oxide synthesis in the nodules causes a reduction in GPx activity, it can be concluded that NO is also required for GPx activity and its actions are transduced via NO-dependent stimulation of sGC to produce cGMP that is required for the GPx activity.

Nitric oxide interacts with sGC in the cGMP pathway (Feelisch et al, 1999). It is the activation of sGC that cause a rise in cGMP levels in the cell, which in turn activates protein kinases (Duner et al, 1998 and Wilson et al, 2008). In this study, it is observed that inhibiting the sGC also inhibits the GPx activity. However, this inhibition is reversed by addition of cGMP to the incubation medium. Since the chemicals used in this study are cell-permeable, and thus diffuse through the cell walls into the cell sap, it is reasonable to conclude that the activity of GPx in the nodules is dependent upon the concentration of cGMP. Indeed, the requirement of cGMP for plant signalling is not a new phenomenon, ODQ inhibits the transcription of stress response genes that are known to be up-regulated by nitric oxide and cGMP (Bowler et al, 1992).

It is noteworthy though to mention that abiotic stress triggers the accumulation of cGMP in the cells. For instance, NaCl stress in rice seedlings causes about 2-fold increase in cellular cGMP levels in the shoots and roots (Reggiani R, 1997). This is further evidenced by the findings in *Arabidopsis thaliana* where salt and osmotic stress causes a

rapid increase in cellular cGMP within 5 seconds of stress induction (Donaldson et al, 2004).

Cyclic GMP has a role in metabolic pathways leading to abiotic stress responses. It has been reported that exposure of *Arabidopsis* roots to 10 μ M cGMP modulated the influx and efflux monovalent cations in the cells thus regulating cation transport. Furthermore, cGMP has been found to regulate the uptake of sodium cations in *Arabidopsis* (Maathius et al, 2001; Rubio et al, 2003 and Maathius et al, 2006).

7.5.0.0. Conclusion

Form this study, it is concluded that cGMP is a signaling molecule involved in modulation of GPx activity and could be involved in the maintenance of redox homeostasis. Secondly, it is established that cGMP functions down stream of nitric oxide in the pathway that leads to regulation of glutathione peroxidase activity. Other signalling roles for cGMP were reported in *Ceratopteris* pollen polarity studies, where cGMP synthesized from sGC was found to work down stream in the pathway that leads to gravitational response of *Ceratopteris* pollen (Salmi et al, 2007). Similar findings in relation to cGMP were also made regarding root gravitropism in soybean (Hu et al, 2005). Cyclic GMP has also been placed downstream ABA and NO in the regulatory pathway that leads to closing of the stomata (Niell et al, 2003). Indeed, NO triggers the accumulation of cGMP in the cells (Hu et al, 2005; Durner et al, 1998).

Identification of the pathway that leads to the synthesis of cGMP in plants and the enzymes involved would open the doors for the possibilities of engineering plants with higher cGMP levels, potentially with more tolerance to abiotic stress.

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Chapter 8 Construction of a nodule specific promoter that is abiotic stress-responsive and the prospects of enhancing salinity stress tolerance in nodules

8.1.0.0 Introduction

Abiotic stress causes both physiological and biochemical defects that may lead to deterioration of plant yield/performance (Manchanda and Garg, 2008). In legumes, nodules serve as an integral source of environmentally safe, cheap and a natural soil nitrogen to the growing legume plant (Chrispeels and Sadava, 2003). This sustained supply of nitrogen from the nodules helps to meet the plant's nitrogen nutrient requirements, hence grain yield and crude grain protein content (Chrispeels and Sadava, 2003). Apart from natural senescence that occurs in nodules as they age (Puppo et al, 2005& Van de Velde et al, 2006), abiotic stress induces early senescence of legume root nodules (Puppo et al, 2005). Protection of nodule tissue from oxidative stress necessitates an urgent consideration in plant improvement programs (Puppo et al, 2005).

The significant role played by nitric oxide in modulating antioxidant responses in legume root nodules has been established in the previous chapters of this thesis. In summary, nitric oxide improves the antioxidant capacity of root nodules and this effect is transcriptionally regulated as observed for superoxide dismutase (chapter 3) and glutathione synthesis (Innocentie et al, 2004). Furthermore, this effect is modulated through cGMP (Chapter 6). Hence, regulation of either nitric oxide synthesis in the root

nodules under salinity stress, or up-regulating the synthesis/activity of superoxide dismutase and/or glutathione synthetase would be part of novel strategies in developing legume crop plants with better tolerance to salinity.

Constitutive expression of stress-responsive genes like dehydration response element binding protein (DREB) in plants has increased plant tolerance to abiotic stress, although sometimes it resulted in deleterious effects on other growth parameters (Miller J, 2007). For instance, drought-tolerant tomato plants were obtained by over-expressing *Arabidopsis* C repeat/dehydration-responsive element binding factor 1 (CBF1) under the control of cauliflower mosaic virus (CaMV35S) promoter (Hsieh et al, 2002). However, these tomato plants had stunted growth. On the other hand, when driving the expression of the CBF1 with abscisic acid inducible promoter; abscisic acid-response complex (ABRC1) in tomato, an increased stress tolerance was attained without any deleterious effect on the yield (Lee et al, 2003). The use of a non-constitutive seed-specific promoter (DS10) also resulted in higher levels of expression and also protected the seed from heat shock without affecting the growth parameters (Prieto-Dapena et al 2006). All these lines of evidence highlight the comparative advantage of inducible and tissue specific promoters in engineering plants for increased stress tolerance as opposed to constitutive expression of stress genes.

Root nodule development is facilitated by chemical dialogue between specific *Rhizobia* species and specific legume root hairs prior to the root hair infection (Schumpp et al, 2009). This dialogue, together with the physiological and molecular changes in the developing nodule tissues, results in accumulation of several polypeptides, which are encoded by a series of genes operating in a sequential, temporal manner, called nodulins (Verma et al, 1992). The nodulin genes have been isolated in the late 20th century

(Legocki and Verma, 1980) and their characterization revealed some conserved motifs in the promoter regions of such genes, demonstrating that they are responsive to environmental and chemical stimuli (Stougaard et al, 1990). Such motifs include consensus nodule sequences, enhancers, organ specific elements, strong positive elements (Stougaard et al, 1990).

Similarly, the plants respond to abiotic stresses such as salinity by synthesizing proteins that either protect plants from the immediate effects of stress (Manchanda and Garg, 2008) or are binding factors that will help trigger the transcription of stress adaptation genes (Gao et al, 2007). The abiotic stress-inducible genes are driven by promoters with specific regulatory elements (Yamaguchi-Shinozaki and Shinozaki, 2005 & Lee et al 1992). Such abiotic stress-responsive genes are classified into those that are abscisic acid dependent and abscisic independent (Yamaguchi-Shinozaki and Shinozaki, 2005).

In this work, we attempted to design and construct a nodule-specific promoter that is abiotic stress-responsive to drive the expression of genes that can combat the adverse effects of high NaCl concentration and dehydration in the root nodules.

8.2.0.0. Materials and methods

8.2.1.0. Construction of the chimeric promoter

8.2.1.1. Cloning of the ASREF/NSP Promoter

The Abiotic Stress Response Fragment (ASREF) and Nodule Specific Promoter (NSP) were isolated from the genomic DNA using primers that amplify the upstream (5' of the transcription start site) region of GmPM9 gene (Lee et al 1992) and N23 gene (Jorgensen

et al, 1991), respectively, using the primer sets; 5'ASREF: 5'-gaa aag ctt gtt tat gta agc ccc tat tgc-3' and 3'ASREF: 5'-cat tct aga tgc aca cgt gtc agt gca g-3' for the ASREF fragment and 5'NSP: 5'-gat tct aga gac att ttt aaa taa taa aat aaa gc-3' and 3'NSP: 5'-cat gga tcc taa tta ctt ggt tac tta gct ag-3' respectively. ProofStart™ DNA polymerase from Qiagen was used as per manufacture's protocol to amplify these fragments. ASREF and NSP primers were annealed at 56°C for 45 seconds and PCR yielded the DNA fragment of 175bp and 448bp respectively (Figure 8- 1).

The ASREF was first cloned as a HindIII/XbaI fragment into cloning vector pUC18 to produce a new plasmid pUC:AS. NSP promoter was then cloned into pUC:AS as Xba/BamHI fragment from pUC:AS/NSP. The ASREF/NSP fragment was then subcloned as a Hind III/ BamH I into the binary vector pBINPLUS.

8.2.1.2. Construction of plant expression vectors

The pBINPLUS binary vector has been modified in the lab to form a new vector with the β -glucuronidase (GUS) reporter gene fused to the nopaline synthase (nos) terminator, under the control of the cauliflower mosaic virus (CaMV 35S) promoter to form a new plasmid; pBPGUS. The ASREF/NSP fragment was sub-cloned following its sequencing into the pBPGUS as a BamHI/HindIII fragment, thus replacing the CaMV 35S promoter, to form the transformation vector pBAS/NSP with ASREF/NSP-GUS fusion. The pBPGUS plasmid was used as the control to study the functionality of the promoter (Figure 8- 1).

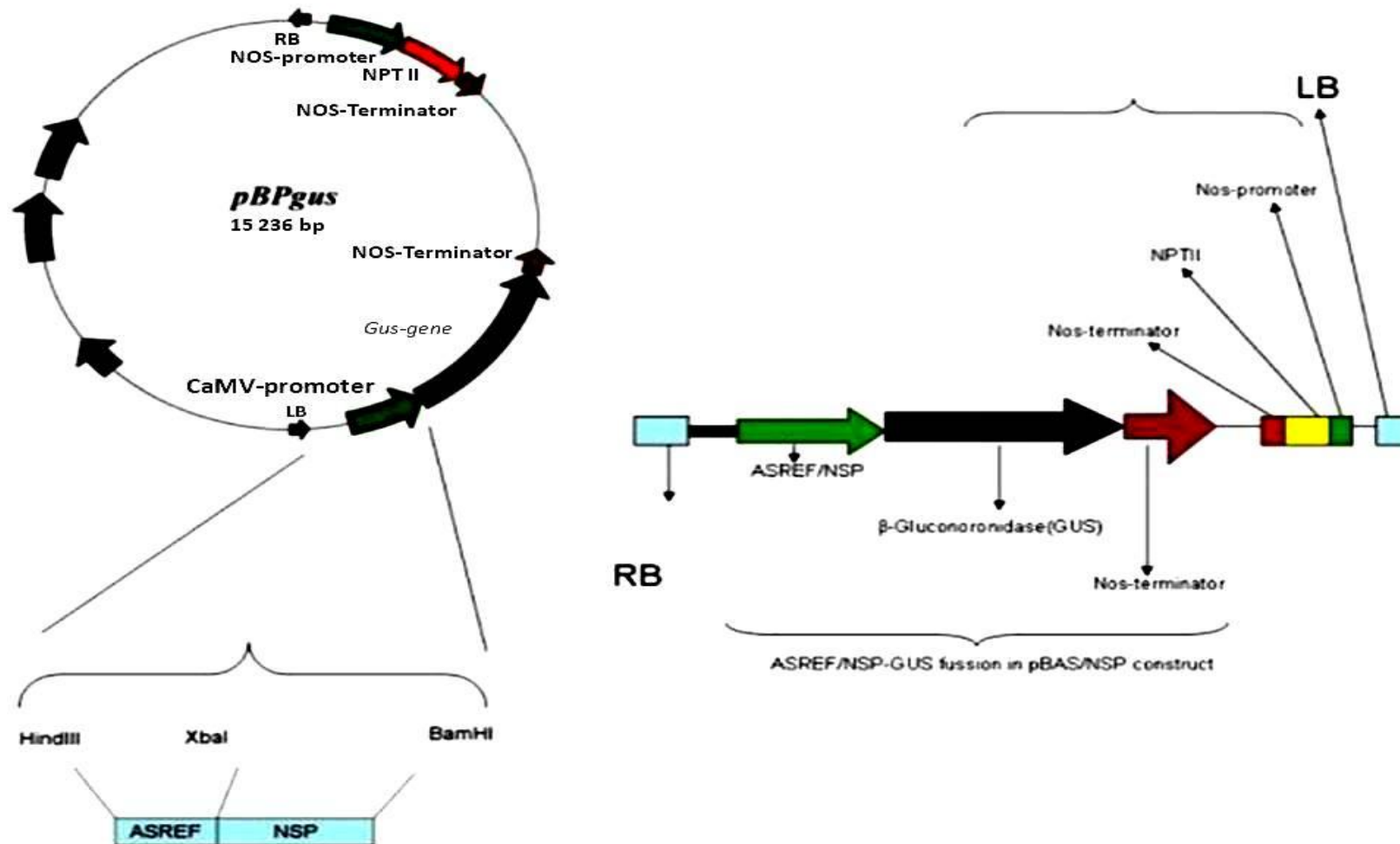


Figure 8- 1 Construction of pBAS/NSP plasmid. (Left side) original pBPGUS plasmid; (right side) The linearized pBAS/NSP plasmid after the insertion of ASREF/NSP chimeric promoter; (c) ASREF/NSP fragments with the resitriction sites flanking the two fragments ; ASREF and NSP. CaMV-promoter represents CaMV35s promoter.

8.2.2.0. Application of chimeric synthetic promoter technology

8.2.2.1. Identification of a nitric oxide synthesizing gene in *Medicago truncatula*.

The AtNOA1 has been identified and characterized in *Arabidopsis thaliana* as a nitric oxide associated gene (Guo et al, 2003). Although the actual direct involvement of this gene in nitric oxide biosynthesis has been disputed, it remains unchanged that its increased cellular expression result in enhanced nitric oxide generation (Guo et al, 2003; Moreau et al, 2007 and Uicker et al, 2007). For this reason the predicted amino acid sequence of the AtNOA1 (Accession number NP_190329) was used in protein homology searches of *Medicago truncatula* ESTs using tblastn from NCBI. The protein sequence with the highest homology based of the probability (e-value) was taken as the best match and its corresponding nucleotide sequence and cDNAs (MtNOA1) were used as the basis for gene isolation in *Medicago truncatula*. The primers for gene isolation and PCR were designed to amplify a full-length cDNA of the coding region of the identified gene (MtNOA1). The Primer3Input, version 0.4.0, (<http://www.frodo.wi.mit.edu>) program was used to design the primer set for amplification of a full length sequence of MtNOA1.

8.2.2.3. Analysis of the predicted MtNOA1 sequence based on web tools and the cloned sequence from *Medicago truncatula*

Both the predicted cDNAs and protein sequences were aligned with the putative nitric oxide associated protein from snail (Huang et al, 1997) and AtNOA1 using BLAST web

based tools (www.ncbi.nlm.nih.gov). Blastn was used to align the nucleotide sequences while the blastp was used to align the protein sequences. Following cloning of the MtNOA1 PCR product into the cloning vector, the isolated DNA fragment was sequenced and the resulting sequence was also analyzed to verify the accuracy of the PCR.

8.2.2.4. Medicago growth conditions

The *Medicago truncatula* cv. Parabinga was planted for total RNA extraction. Seeds were sterilized by washing the mechanically scarified seeds in 30% commercial bleach supplemented with 0.01% (V/V) tween 20 for 8 minutes. Then the seeds were rinsed 5 times with sterile water on the laminar flow bench. The seed were planted in tissue culture tubs on basal MS medium supplemented with 3% sucrose, under a light/dark cycle of 16/8 hours. Two weeks old seedlings were used for total RNA extraction.

8.2.2.5. Molecular cloning and characterization of MtNOA

The total RNA was isolated from *Medicago truncatula* using the RNeasy Plant Mini Kit (Qiagen) and the isolation procedure was as per manufacturer instructions. The full length cDNA of this MtNOA1 gene was synthesized from *Medicago truncatula* total RNA, using RT-PCR as per Superscript RT Kit (Invitrogen). First strand cDNA was synthesized using the MtNOA1 reverse primer. Then the second strand cDNA was amplified in PCR using the High Fidelity PCR Kit (Qiagen) according to the manufacturers' manual. The following primer set was used and annealed at 55°C; forward primer 5'- GTA GGA TCC ATG GCT ATC TTG TTC TCT ACA ATT G-3' and reverse primer 5'-GGA GTC GAC_TCA TGC AGA TAT CTC TAT ACT TGC-3', with BamHI and Sall restriction sites respectively.

The PCR product was ligated as a BamHI/SalI fragment in pUC18 cloning vector to form a pUC-MtNOA1 plasmid (Figure 8- 2). This plasmid was perpetuated in DH5α *E. coli* bacterial cells. The resulting clone was sequenced (Central Analytical Facility, Stellenbosch University) with M13 forward and reverse primers flanking the MCS region of the pUC18. The sequence from the cloned fragment was analyzed and aligned with the predicted MtNOA1 from the NCBI database.

In order to regulate the expression of NO in the nodules, MtNOA1 gene was sub-cloned from the pUC-MtNOA1 into pBAS/NSP-GUS plasmid, thus replacing the GUS gene, as a BamHI/SacI fragment (Figure8- 2).

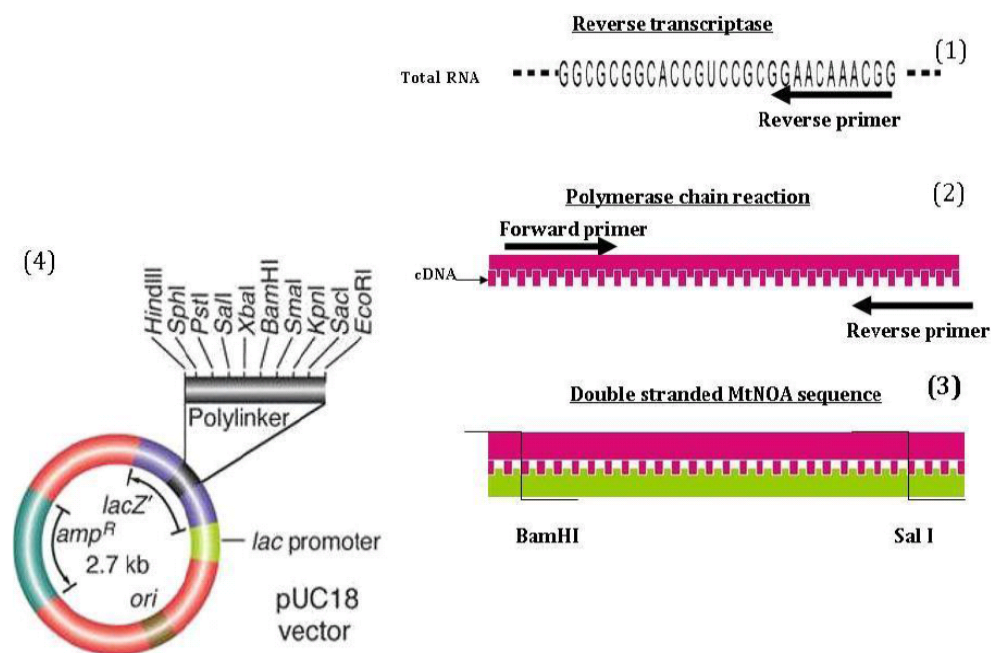


Figure 8- 2 Isolation of MtNOA1 from *Medicago truncatula* total RNA. (1) Reverse transcription with the reverse primer of full length sequence of MtNOA1; (2)PCR reaction on the single strand cDNA; (3) Double stranded MtNOA1 product with the restriction sites flanking the full length sequence and (4) pUC18 cloning vector with polylinker where MtNOA1 was ligated as a BamHI/SalI fragment.

8.2.3.0. Purification, manipulation and detection of DNA

DNA was stored in TE buffer at -20°C . While the total RNA extracted was resuspended in DNAase free DPEC water and stored at -80°C . Plasmid DNA was purified with the QIAprep® Spin Miniprep kit (Qiagen, Hilden), in which an overnight culture of 5ml was used. DNA concentration and purity was determined spectrophotometrically using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

Enzymatic modifications of DNA were done with commercially supplied enzymes and buffers (Fermentas). The incubation conditions were as suggested by the manufacturers. Endonucleolytic digestion was performed by different restriction

enzymes, hence incubation time was dependent on the amount of DNA and enzyme concentration. In general, 1 U enzyme was used to digest 1 μg DNA in an hour. Incubation temperatures were chosen as specified for each enzyme.

T4 DNA ligase (Promega) was used for ligating the DNA fragments. For cloning the ASREF, NSP and MtNOA1 in the vectors, a concentration of 100-200 ng DNA with a vector: insert ratio of 1:1 and 1 U ligase were used in the ligation reaction. The appropriate volume of buffer was used as per manufacturer instructions. The reaction was incubated at 16°C overnight.

The double stranded DNA fragments were separated electrophotically on 1% agarose gels. 1X TBE buffer was used for the electrophoresis of the DNA samples. Following staining with ethidium bromide, the separated fragments were visualized on an UV transilluminator and photographed with a high resolution camera.

DNA fragments were recovered and purified from aqueous PCR reactions with the QIAquick® PCR Purification Kit (Qiagen). It was extracted from the agarose gels using the QIAquick® Gel Extraction Kit (Qiagen, Hilden) as per manufacturer's manual.

8.2.4.0 Preparation of competent bacterial cells and DNA transformation

A overnight 2 ml culture was prepared by inoculating a 5 ml LB medium with a single colony of *E. coli* DH5 α . This was used to inoculate 50 ml of LB medium and the culture was grown overnight at 37°C with shaking at 200 rpm. The overnight culture was used to inoculate 300 ml LB and this new culture was grown at 37°C with shaking till the absorbance at 600 nm was 0.6. The bacterial culture was then transferred to sterile tubes and centrifuged at 2000 *g* at 4°C for 10 min. The excess LB was then removed from the tubes by decantation and the bacterial cells were then carefully resuspended and washed in 50 ml of ice-cold TFB-1 buffer and incubated on ice, then centrifuged as before. The TFB-1 buffer was removed as before and this time the cells were resuspended in 40 ml of ice-cold TBF-11 buffer. The resuspended cells were snap frozen in liquid nitrogen after preparing 300 μ l aliquots in 1.5 Eppendorf tubes. The frozen cells were stored at -80°C.

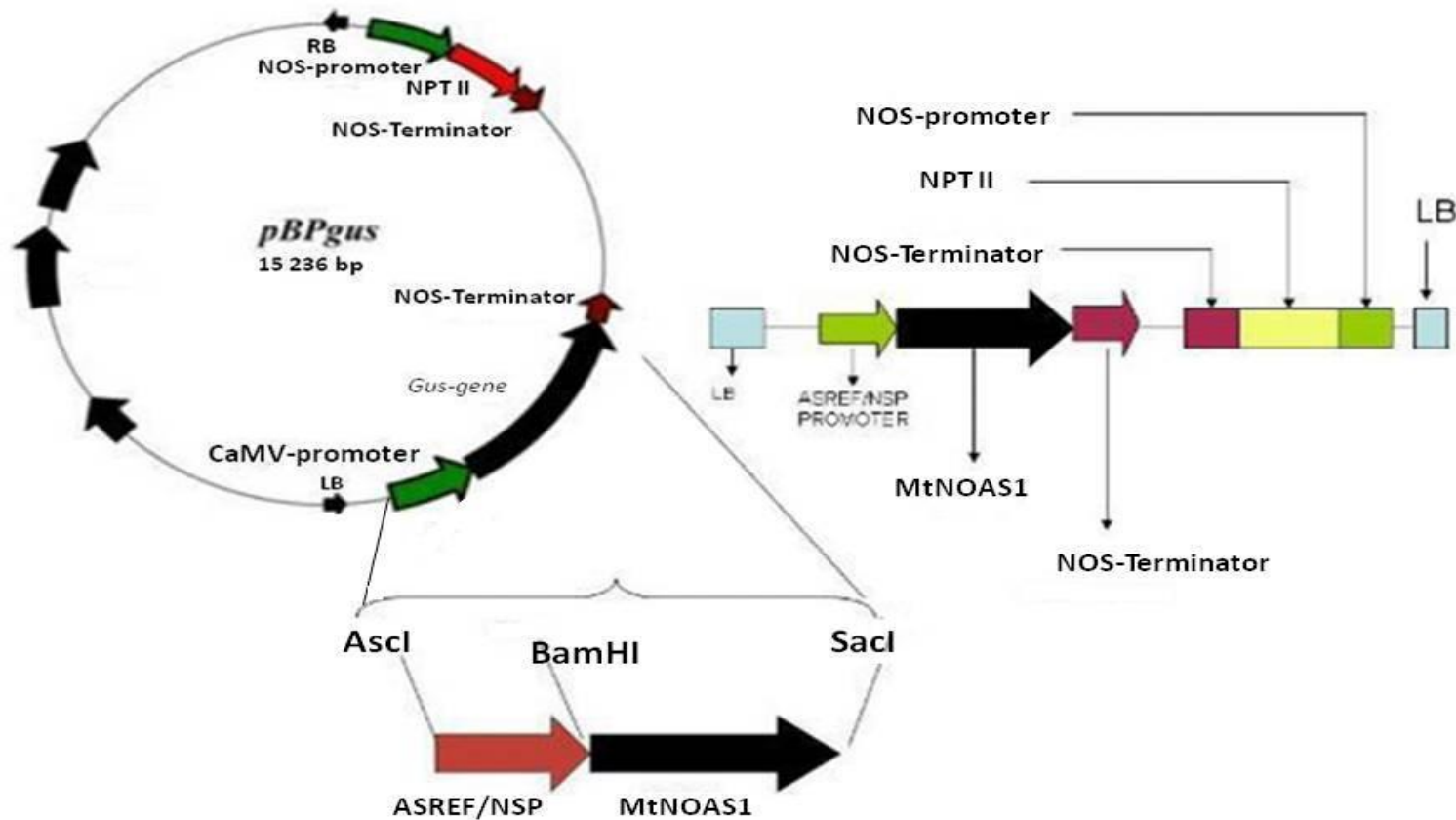


Figure 8- 3 Construction of pBASREF/NSP-MtNOA1 plasmid. (a) Original pBPgus plasmid before insertion of ASREF/NSP-MtNOA1 cassette; (b) Linearized pBASREF/NSP-MtNOA1 plasmid; (c) ASREF/NSP cassette. CaMV-promoter represents CaMV35s promoter.

8.2.5.0 Transformation with DNA

The ligated DNA fragments/plasmids were mobilized into the competent bacteria by the heat-shock method (Hanahan, 1983). The competent bacteria were thawed on ice and then mixed gently with 10 µl of the ligation reaction or plasmid DNA. This was incubated for 30 minutes on ice followed by incubation for 45 seconds at 42°C on a heating block. The suspension was then cooled immediately on ice for 15 minutes. About 0.5 ml of LB was then added to the suspension and the incubated at 37°C for 1 hour, then plated on LB solid medium supplemented with appropriate antibiotics. The cultures were grown overnight at 37°C in the dark.

For plant transformation, ARGUA 1 *Agrobacterium rhizogenes* strain was used to infect the plant tissue. This strain was cultured in YEP medium at 28°C overnight with shaking at 250rpm. The resulting culture was used and prepared for either transfection or for transformation by binary vector containing the sequences of interest. The competent cells for this agrobacterium were used for plasmid transformation (Christey & Braun, 2005). The heat shock method was used to mobilize the binary vector into the cells

8.2.6.0. Multiplex polymerase chain reaction

Multiplex PCR was carried with two primer pairs simultaneously so as to amplify the target fragments depending on the inserted fragment in the binary vector pBINPLUS. For the ASREF/GUS fragment, the primers 5'NSP, 3' GUS, 5' NPT II and 3' NPT II were used to amplify the NSP-GUS and NPT II gene fragments. While for AS/NSP-MtNOA1, 5'NSP, 3' MtNOA1, 5' NPT II and 3' NPT II were used to amplify NSP-MtNOA1 and NPT II gene fragments. Lastly, for the control plasmid pBPGUS, 5'GUS, 3' GUS, 5' NPT II and 3'

NPT II primers were used to amplify GUS and NPT II gene fragments. The primers were all annealed at 56 °C.

8.2.7.0. *Medicago truncatula* transformation

The *M. truncatula* cv. Jemalog seeds were scarified with concentrated sulphuric acid for 8 minutes, then surface sterilize in 30% commercial bleach for 10 minutes, then rinsed with sterile water 5 to six times. The seeds were imbibed in 10 µM BAP for 3 hours before placing on MS solid plates and allowed to germinate in the dark for three days. Hypocotyls of 3 days old seedlings were wounded several times with a needle, then co-cultivated for 15 minutes in co-cultivation medium (bacterial culture carrying the plasmid of interest supplemented with 0.154 g/ 25 ml DTT, 0.40 g/25 ml L-cystein and 100 µM acetosiringone), shaking in the dark. The seedlings were then blotted on a sterile filter paper and placed on MS medium supplemented with 100 µM acetosyringone and incubated in the dark for 3 days. The seedlings were then grown under 8/16 light/dark cycles. The three weeks old plants with well developed root systems were then transferred to sterile vermiculite where there have been inoculated with rhizobium. On the other hand some hairy root tips were cultured *in vitro* in MS solid medium without any exogenous phytohormones for further analysis and inoculation with rhizobium.

8.3.0.0. Results and discussion

8.3.1.0. Construction of the abiotic stress-inducible nodule-specific promoter

8.3.1.1. Cloning the abiotic stress-responsive and nodule-specific fragments

The PCR results revealed a fragment size of about for 163 base pairs for the abiotic stress-responsive (ASREF) and 439 for the nodule-specific (NSP) (Figure 8- 4). These fragments were subcloned into pUC-18 cloning vector to form a new vector: pUCAS/NSP. These fragments were sequenced with M13 primers in pUCAS/NSP, and the sequence analysis revealed the 602 base-pair fragment, homologous to the predicted ASREF/NSP from the data base (Figure 8- 5).

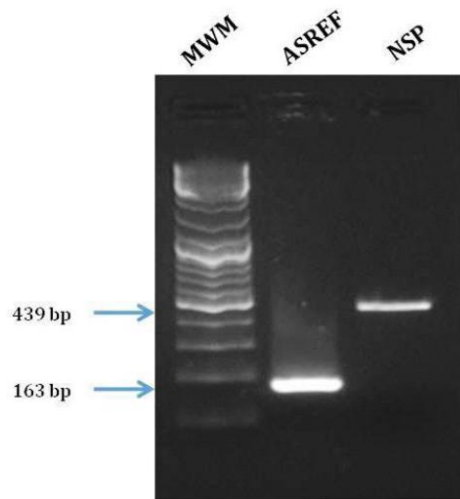


Figure 8- 4 PCR-amplified abiotic stress response element fragment from the soybean GmPM9 promoter (ASREF) and nodule-specific fragment with the core promoter from the soybean N23 gene promoter. Lane 1 is the 1kb DNA molecular weight maker (fermentas) MWM, lane 2 is the GmPM9 promoter fragment, ASREF and lane 3 is N23 promoter NSP.

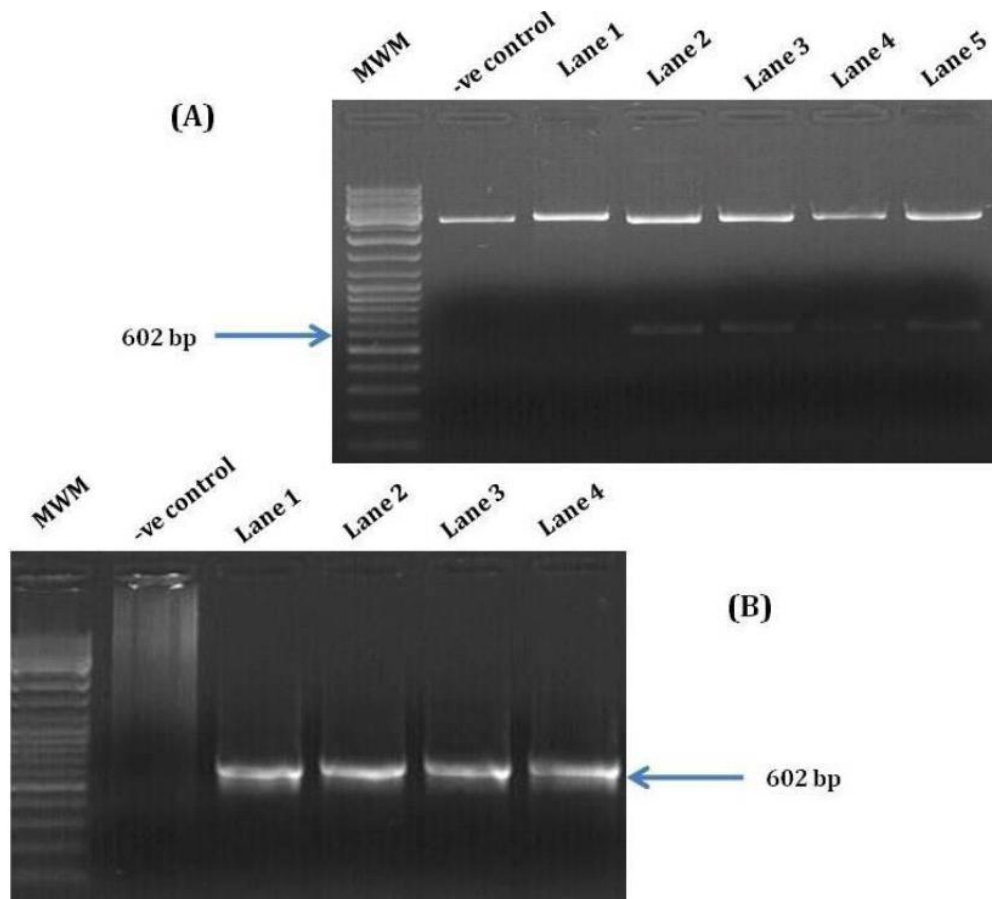


Figure 8- 5 Restriction digestion of pUC18 ligated to ASREF/NSP fragment, with BamHI and Sall (A) and PCR verification of the inserted fragment with the 5' end primer of ASREF fragment and 3' end primer of NSP fragment. The chimeric promoter has an approximate length of 600 bp based on its migration on agarose gels (B). Lanes represent either the PCR product or a restriction digest reaction performed on the plasmid DNA extracted from individual colonies. Lanes 2 to lane 5 carry the cloned fragment as verified by PCR (B). -ve controls in (A) and (B) represents the restriction digest reaction without the plasmid DNA. The molecular weight marker, MWM represents the 1kb DNA ladder (fermentas).

The sequence analysis of this clone reveals a very high homology to the fragments of GmPM9 and N23 promoter region. In general, the chimeric promoter has a core promoter with a transcription start site. The TATA box was identified and is located at about 55 bases from the transcription initiation site. There are nodule specific motifs concentrated 469 bases upstream of the TSS (Figure 8-7). Since the fragment was cloned from soybean as in Lee et al (2000), the arrangement of *cis*-acting motifs is similar to that in Lee et al (2000), as shown in Figure 8- 6.

WebSeq	AGCTTGTTTATGTAAGCCCTATTGCACGAGTGGTTGATTGCCACGTGTCCCTAACACTG	60
Full Clone	AGCTTGTTTATGTAAGCCCTATTGCACGAGTGGTTGATTGCCACGTGTCCCTAACACTG *****	60
WebSeq	TGTTGAAGCTCGTTGCAAAACAGACACGCGGCAATTACGTGTAAGACGATTAGTCCAATAA	120
Full Clone	CGTTGGAGCTCGTTGCAAAACAGACACGCGGCAATTACGTGTAAGACGATTAGTCCAATAA *****	120
WebSeq	TCCTCAGAAACTTGCCACGCGTACTGCACTGACACGTGTGCATCTAGAGACATTTTAAA	180
Full Clone	TCCTCAGAAACTTGCCACGCGTACTGCACTGACACGTGTGCATCTAGAGACATTTTAAA *****	180
WebSeq	TAATAAAATAAAGCAACTCTTAATTTTAAATGAAACATCCCTTTGTTAAACCGAATCTTCC	240
Full Clone	TAATAAAATAAAGCAACTCTTAATTTTAAATGAAACATCCCTTTGTTAAACCGAATCTTCC *****	240
WebSeq	ATAATGTAATAAATAAATGCTTGATGGAAGTTTTAAATTTGTTCTATCCAATACTCAAAGG	300
Full Clone	ATAATGTAATAAATAAATGCTTGATGGAAGTTTTAAATTTGTTCTATCCAATACCAAAGG *****	300
WebSeq	GTTGTAATAATTTTTTTTATCATTATATGTTGTAATATGAATGCAC TAGTAATAGTT	360
Full Clone	GTTGTAATAATTTTTTTTATCATTATATGTTGTAATATGAATGCAC TAGTAATAGTT *****	360
WebSeq	TAATGATAAAATATATTCTACAGATATATTTCTGTCTCTGGCAACTCGTGAGAATTGAA	420
Full Clone	TAATGATAAAATAYATTCTACAGATATATTTCTGTCTCTGGCAACTCGTGAGAATTGAA *****	420
WebSeq	TATATTATAAAGATGAAAGGTCGTTACAATTTTTTTTAGAATAAATATTATATACAATT	480
Full Clone	TATATTATAAAGATGAAAGGTCGTTACAATTTTTTTTAGAATAAATATTATATACAATT *****	480
WebSeq	C-TAGATTTTGTATAAAATTCACATATTGTATGAGTATAA-TACATGAGCACACACCAA	538
Full Clone	CCTAGATTTTGTATAAAATTCACATATTGTATGAGTATAARTACATGAGCACACACCAA * *****	540
WebSeq	ACTAGTCTCAAATTAAGTAAGGGGCTAATTATTAGCG-CTAGCTAAGTAAACCAAGTAATT	597
Full Clone	ACTAGTCTCAAATTAAGTAAGGTGCTAATTATTAGCGCTAGCTAAGTAAACCAAGTAATT *****	600
WebSeq	AG 599	
Full Clone	AG 602	

Figure 8- 6 Alignment of the predicted ASREF/NSP nucleotide sequence with the cloned sequences. WebSeq represents the predicted sequence based on the NCBI sequences and FullClone represents the nucleotide sequence of the isolated ligated fragments from GmPM9 and N23 genomic clones in pUC18.

The chimeric promoter has the abiotic stress-inducible *cis*-acting element and nodule specific *cis*-acting elements. The consensus motif for ABA inducibility, pyACGTGGC, is not found in this promoter hence the promoter might not respond to abscisic acid. A G-box is located at +43 and appears in repeats that are separated by three nucleotides. There are also five abiotic stress response motifs concentrated in the -560 to -410 region. Nodule-specific motifs appear from region -170 to -600. Just like in the N23 promoter, there are inverted repeats INVA and INVB that would act like enhancer

elements in the N23 promoter. The presence of an A/T-rich region that may serve as an enhancer region for the promoter activity is also present in this promoter. Such arrangements of the abiotic and nodules specific motifs in the promoter may warrant its activity in the nodules and under abiotic stress (Figure 8- 7).

```

-602 AGCITGTTTATGTAAGCCOCTATTGCAOGAGTGGTTGATTGCCCAAGTGTCCCTAACACTTGCGTTGGAGCTCGTTGCA
                                     (a)          (a)

AACAGACACGGGGCAATTACGTGTAAGACGATTAGTCCAATAATCCTCAGAACTTGCCACGCGTACTGCACTGACA
                                     (a)          (a)

CGTGTGCATCTAGAGACATTTTAAATAATAAAAATAAAGCAACTCTTAATTTTAAATGAAACATCCCTTTGTTAAACC
(c)          (d)          (e)

GAATCTCCATAATGTA AAAAATTAATGCTTGATGGAAGTTTTTAATTTGTTCTATCCAATACACAAAGGGTTGTA AA
                                     (e)

TATTTTTTTTATCATTTATATGTTGTAATATGAATGCACTAGTAAATAGTTTAAATGATAAAATAYATTTCTACAGAT

ATATTTCTGTCCTCTTGGCAACTCGTGAGAATTGAATATATTATAAAAGATGAAAGGTCGTTACATTTTTTTTTAGAAAT
                                     (d)          (f)

AAATATTTATATACAATTCTAGATTTGTTTATAAAATTCACATATTGTATGAGTATAARTACATGAGCACACACCA
                                     (g)          (h)          (l)

AACTAGTCTCAAATTAAGTAAGGTGCTAATTATTAGCGGCTAGCTAAGTAACCAAGTAATTAG -18

```

Figure 8- 7 Nucleotide sequence of the chimeric ASREF/NSP promoter containing the necessary *cis*-acting elements for nodule specificity and abiotic stress responsiveness. All the important motifs are underlined and labelled. The abiotic stress motifs are highlighted and underlined without labels. (a) represents enhancers, (b) represents (CA)_n elements, (c) represents a G-BOX(binding site for Inducer of CBF expression (ICE) binding site, (d) represents nodulin *cis* acting elements, (e) represents INVA and INVB, (f) represents a nodulin motif, (g) and (l)represent TATA box, (h) represents ROOTMOTIFTAPBOX1

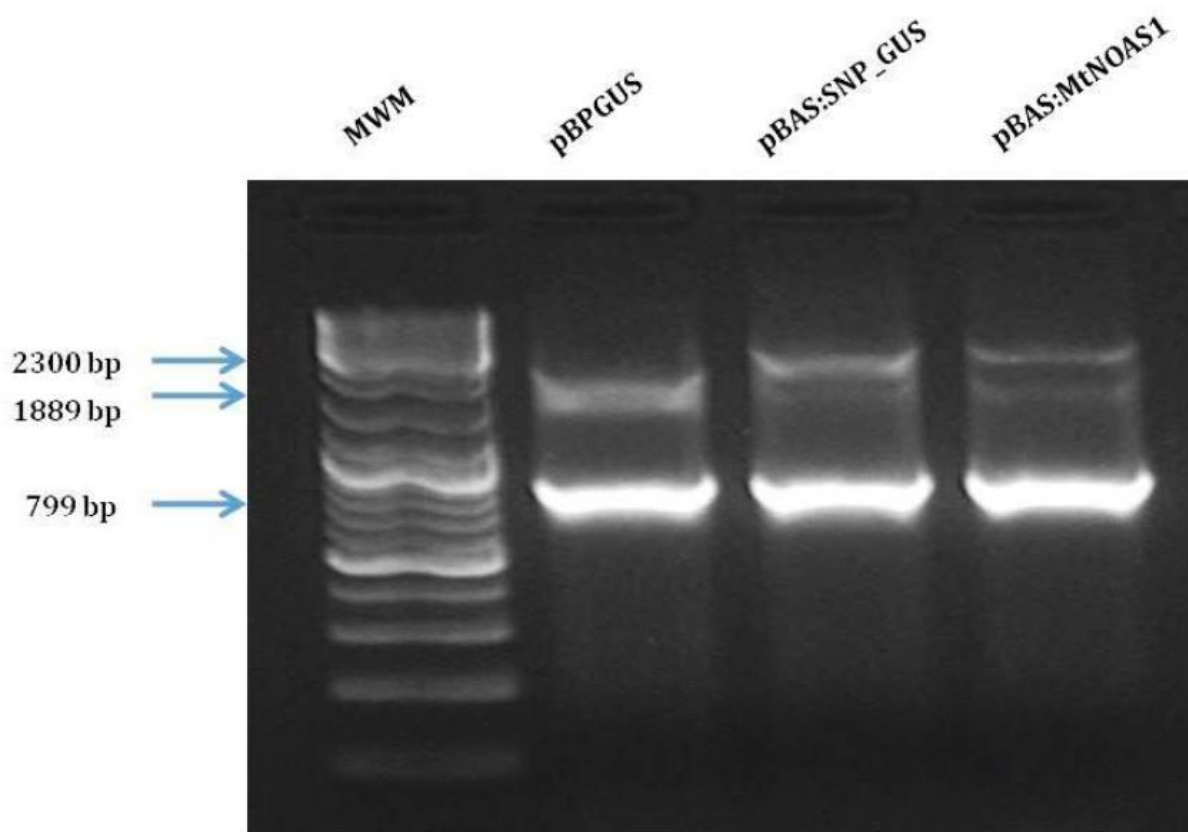


Figure 8- 8 Multiplex PCR of plasmid DNA from pBPGUS, pBAS:SNP-*GUS* and pBAS:MtNOA1. pBPGUS; the primers amplifying the full length sequence of NPT II and *GUS* gene were amplified. pBAS:SNP-*GUS*; the primer pairs amplifying the full length sequence of NPTII and nodule specific fragment (NSP) and *GUS* gene were used. pBAS:MtNOA; the primer pairs used were targeted at NPTII and secondly nodule-specific fragment (NSP) and MtNOA1.

8.3.2.0 Application of chimeric synthetic promoter technology and abiotic stress tolerance

Inducible promoters have been constructed and used to successfully drive the expression of transcription factors (Hsieh et al, 2002.). Unlike the constitutive promoters, the inducible promoters are more efficient in driving the expression of the genes and do not have deleterious effect on plant growth (Miller, 2007). In this project the homologue of AtNOA1 from *M. truncatula* has been isolated (Figure 8- 11), cloned into pUC18 and sequenced (Figure 8- 9 & 8- 10) and fused to ASREF/NSP promoter in the pBINPLUS binary vector. This was a suspected plant nitric oxide synthase (Guo et al,

2003) but was later ruled out to be a small GTPase and is involved indirectly in nitric oxide synthesis (Crawford et al 2006 and Zemojtel et al, 2006).

8.3.2.1 Identification and isolation of MtNOA1

Table 8- 1 the table showing the list of nucleotide sequences with the highest homology to AtNOA1 in *Medicago truncatula*. The Scores and E-value are stated.

Sequences producing significant alignments:	Score (Bits)	E- Value
gb ABD33306.1 Protein C4orf14 homolog, related [Medicago trunca	87.8	1e-18
gb ABN05857.1 Dynamin central region; Dynamin [Medicago truncat	26.9	3.1
gb ABN05954.1 Disease resistance protein [Medicago truncatula]	26.6	4.0
gb ABN06007.1 Homeodomain-related [Medicago truncatula]	26.2	4.2
gb ABN08114.1 Disease resistance protein; Calcium-binding EF...	26.2	4.4
gb ABD28508.2 Hydroxyacid dehydrogenase/reductase; 6-phospho...	26.2	4.6
gb AAT48629.1 putative auxin efflux carrier protein 9 [Medic...	26.2	5.2
gb ABN07977.1 Nicotianamine synthase [Medicago truncatula]	25.4	8.5
gb ABD28464.1 Ribosomal protein L30 [Medicago truncatula]	25.4	8.8

Following the web search of AtNOA1-homologous translated protein sequences from Medicago EST datasets using Blast, the protein with the highest homology appears to be Protein C4orf14 homolog with the accession number gb|ABD33306.1. This is a 616 amino acids long protein (Figure 8- 9). It appears to have high homology to the YqeH protein, a GTPase. Alignment of this retrieved sequence with AtNOA1 revealed a Score = 87.8 bits (216), and an E-value of 1e-18, with 28% identities and a 44% similarity. The cDNA corresponding to this sequence was used for primer design (cDNA accession number AC158502).

The designed primers were used to isolate this sequence from the high quality total RNA extracted from Medicago leaves. The reverse transcriptase reaction was completed and

the RT product was used to synthesize the second strand of the DNA fragment. RT-PCR was successful and the fragment size of about 2 kb was visualised on the agarose gels following illumination with UV light (Figure 8- 11).

```

Query 303 TKVDLLPSQVSEPTRIDRWVRHRASAGGAPKLSAVYLVSSRKDLGVRNVLSFVKDLAGPRG 362
          TK+DLLP      + WV          LS V+L SS+  GV V S ++   R
Sbjct 223 TKIDLLPKGTDMNCCIGDWVVEVTMRKRLNVLS-VHLTSSKSLDGVSGVASEIQEKKR- 280

Query 363 NVWVIGAQNAGKSTLINAFK-----KEGAKVTKLTEAPVPGTTLGILRIAGILSAK 414
          +V+++GA N GKS  INA K          A+ K ++ VPGTTLG ++I  + +
Sbjct 281 DVYILGAANVGKSAFINALLKTMAERDPVAAAQKYKPIQSAVPGTTLGPIQINAFVGGE 340

Query 415 AKMFDTPGILLHPYLLSMRLNREBQKMVEIRKELKPRSYRIKA-----GQA 459
          K++DTPG+  + + ++ ++  + + L+ +S+ I          G
Sbjct 341 -KLYDTPGVHLHHRQAADVHSDLLPALAPQNRLRGQSFDI STLPTQSSSSPKGESLNGYT 399

Query 460 IHVGGIARLDLIEASVQTMVYVTVWASPTVSLHMGKIENANEIWNHVGVRVLPPIGNDR 519
          GGI R+D+++A +T + T +  + +H  + A  + +GV L PP G ++
Sbjct 400 FFWGGIVRIDILKALPETCF-TFYGPKALEIHAVPTKTATAFYEAKLGVLTPPSGKNQM 458

Query 520 AE-LGTWKEREVKVSGSSWDVNCMDVSIAGLGWFSL 554
          E G  R +++ +          DV+I+GLGM S+
Sbjct 459 QEWKGLQSHRLLQIEINDAKRPASDVVISGLGWIISI 494

```

Figure 8- 9 Protein sequence alignment of AtNOA1 and MtNOA1. The query represents the predicted MtNOA1 sequence from the web and the sbjct represents web base AtNOA1 protein sequence. MtNOA1 (query) compared to AtNOA1 (subject) Score = 91.3 bits (225), E-value = 3e-16 Identities = 76/276 (27%), Positives = 128/276 (46%), Gaps = 28/276 (10%).

8.3.2.2. Sequence analysis of the predicted MtNOA and cloned sequence from *Medicago truncatula*.

The predicted nucleotide sequence of MtNOA, based on the web tools, is 1851bp long and encodes 606 amino acids (Figure 8-10). The accession number of the sequence is AC158502 and bears 44% homology to the AtNOA. Just like AtNOA, it has a short stretch of basic lysine residues (KKKKK) at the N-terminus (Moreau et al, 2008).

Query	1	MAILFSTIALPSTNVTSKLSILNNTSHSHALRHFSGNMTTKRPHKASSFI AFAVKNNPTIR	60
Sbjct	1	MAILFSTIALPSTNVTSKLSILNNTSHSHALRHFSGNMTTKRPHKASSFI AFAVKNNPTIR	60
Query	61	KTTPRRDSRNPLLSEGRDEDEALGPICPGCGIFMQDNDPNLPGFYQQKEVKIETFSEEDY	120
Sbjct	61	KTTPRRDSRNPLLSEGRDEDEALGPICPGCGIFMQDNDPNLPGFYQQKEVKIETFSEEDY	120
Query	121	ELDDEEDDGEEDNGSIDDES DWS EEL EAMLLGEENDDKVDLDGFTHAGVGYGNVTEEV	180
Sbjct	121	ELDDEEDDGEEDNGSIDDES DWS EEL EAMLLGEENDDKVDLDGFTHAGVGYGNVTEEV	180
Query	181	LERAKKKKVS KAEKKRMAREAEKVKEEVTVCARCHSLRNYGQVKNYMAENLIPDFDFDRL	240
Sbjct	181	LERAKKKKVS KAEKKRMAREAEKVKEEVTVCARCHSLRNYGQVKNYMAENLIPDFDFDRL	240
Query	241	ITTRLMNPAGSGSSTVVVMVVD CVD FDGSFPRTAVKSLFKALEGMQENTKKGKCLKLVL	300
Sbjct	241	ITTRLMNPAGSGSSTVVVMVVD CVD FDGSFPRTAVKSLFKALEGMQENTKKGKCLKLVL	300
Query	301	VATKVDLLPSQVSPTRLDRWVRHRASAGGAPKLSAVYLVSRKDLGVRNVLSFVKDLGAP	360
Sbjct	301	VATKVDLLPSQVSPTRLDRWVRHRASAGGAPKLSAVYLVSRKDLGVRNVLSFVKDLGAP	360
Query	361	RGNVWVIGAQNAGKSTLINAFAPKEGAKVTKLTEAPVPGTTLGILRIAGILSAKAKMFDT	420
Sbjct	361	RGNVWVIGAQNAGKSTLINAFAPKEGAKVTKLTEAPVPGTTLGILRIAGILSAKAKMFDT	420
Query	421	PGLLHPYLLSMRLNREEQKMVEIRKELKPRS YRIKAGQAIHVGGLARLDLIEASVQTMVY	480
Sbjct	421	PGLLHPYLLSMRLNREEQKM-----AGQAIHVGGLARLDLIEASVQTMVY	465
Query	481	TVWASPTVSLHMGKIE NANEIWNHVGVR LQPPIGNDRAAELGTWKEREVKVSGSSWDVN	540
Sbjct	466	TVWASP VSLHMGKIE NANEIWNHVGVR LQPPIGNDRAAELGTWKEREVKVSGSSWDVN	525
Query	541	CMDVSIAGLGFSLGIQGEATMKLWTNDGIEITLREPLVLDRAPSLEKPGFWLPKAISEV	600
Sbjct	526	CMDVSIAGLGFSLGIQGEATMKLWTNDGIEITLREPLVLDRAPSLEKPGFWLPKAISEV	585
Query	601	IGNQTKLEAQRK KLEDEDTEYMGASIEISA	631
Sbjct	586	IGNQTKLEAQRK KLEDEDTEYMGASIEISA	616

Figure 8- 10. Alignment of protein sequences of cloned MtNOA1 and the predicted MtNOA1 sequence. The query represents the amino acid chain predicted from the cloned nucleotide sequence from Parabinga RNA while the subject represents the predicted sequence from the the database.

The predicated amino acid sequence from the cloned nucleotide sequence has high homology to the predicted sequence with an insertion of 13 amino acids within the coding region of the gene (Figure 8-10). However, it is 632 amino acids in length. This slight difference may be brought about by the genetic difference between the cultivar

used in the study (Parabinga) and that used in the genome sequencing (Jemalong). This newly cloned sequence bears 46% homology with the AtNOA at amino acid level.

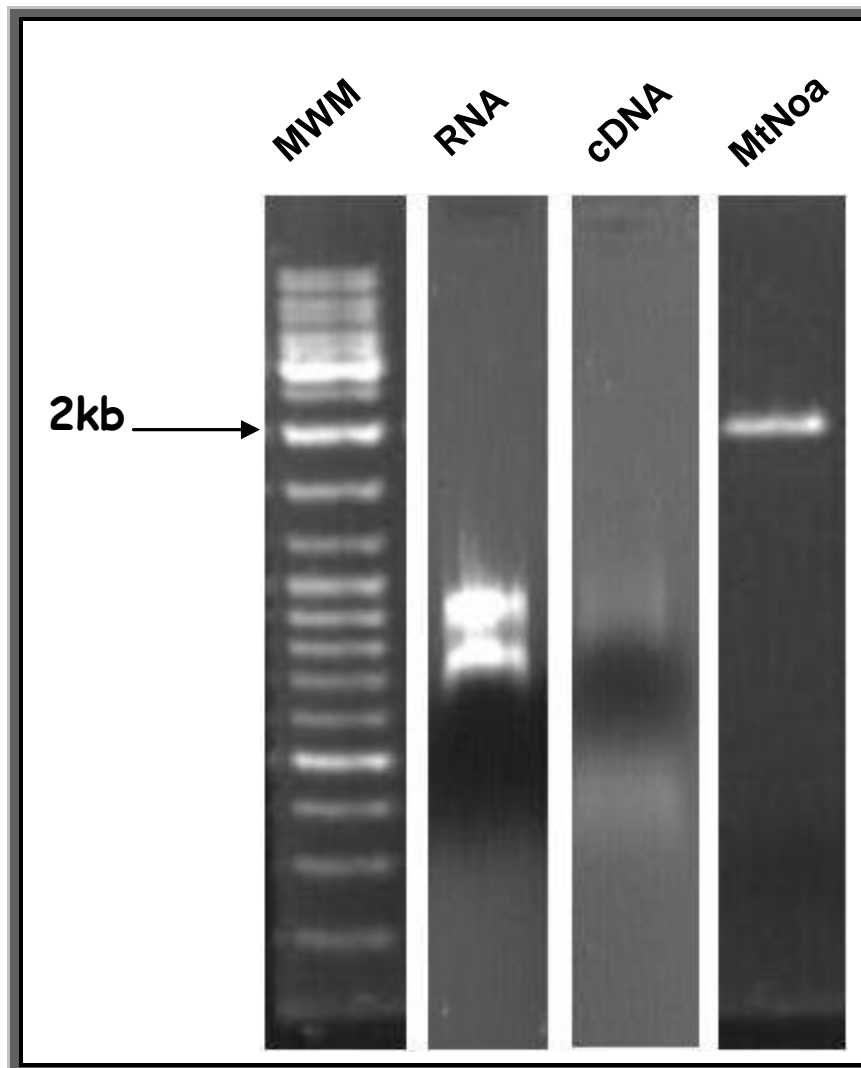


Figure 8- 10 the figure showing the isolation of MtNOA1 from total RNA extracted from *Medicago truncatula* leaves. The copy DNA was synthesized using the reverse primer. MtNOA1 has a fragment size of about 2kb. The first lane from the left is 1Kb DNA ladder (fermentas), the second lane is the total RNA extracted from the *Medicago truncatula* leaves, the third lane is the cDNA and fourth lane is the RT-PCR product synthesized using the MtNOA1 designed primers (forward and reverse primers).

8.3.3.0 *Medicago truncatula* transformation

Medicago truncatula cv. 2HA was successfully transformed with the rhizogens carrying the binary vector (Figure 8- 12). The 6 day old seedlings started to develop hairy roots from the infection site in tissue culture. These root tips were successfully propagated *in vitro* (Figure 8- 12).

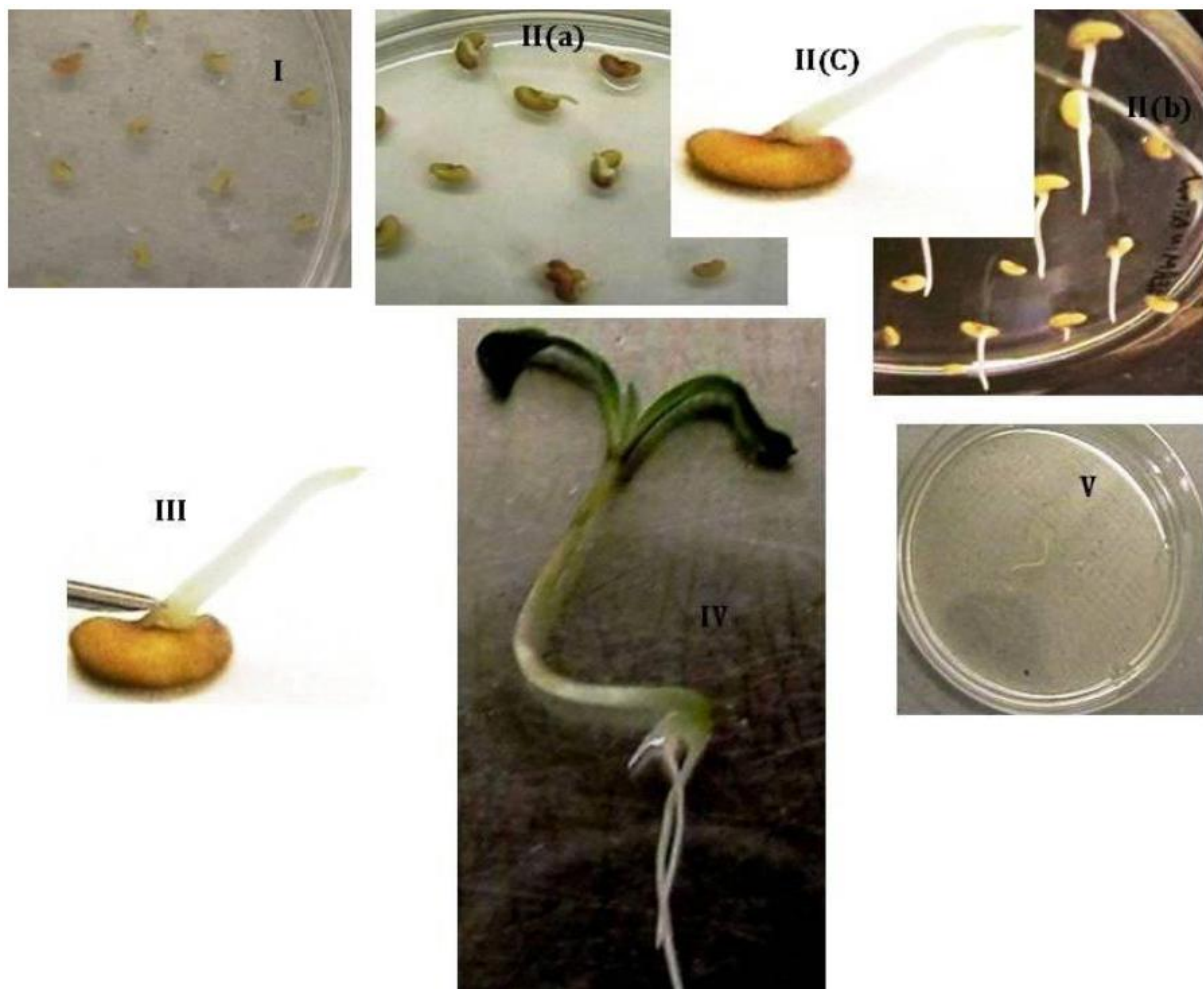


Figure 8- 11 illustration of the transformation process of *Medicago* seedlings with *A. rhizogens* ARGUA 1 strain carrying the binary vectors. I) Sterilized *Medicago truncatula* cv. 2HA seeds place on solid MS medium. II) Germinated *Medicago* seeds after 3 days of germination in the dark, plated placed up-side-down; (a), (b) & (c) are different views of the germinated seeds on solid MS plate. III) wounded region of the seedling prior to co-cultivation with *Agrobacterium rhizogens*. IV) Hairy roots development from the wounded region after 5 days of infection with rhizogens carrying the binary vector. V) Hairy root tip cut from the main plant and placed on solid MS medium for hairy root culture.

8.4.0.0 Discussion

Gene expression is regulated by *cis*-acting regulatory sequences found on the 5' UTR of the gene. This region has the *cis* acting element TATA box; generally called core promoter that allows the RNA polymerase to bind and initiate transcription of the downstream coding sequence. The core promoter works in collaboration with other conserved motifs that direct the temporal and sometimes tissue specific transcription of the gene. In addition to this, some promoters are chemically or environmentally activated. Gene expression is thus facilitated by the presence of the specific *cis*-acting sequences that bind specific proteins often called transcription factors and consequently drives the gene expression upon induction (Jorgensen et al, 1991). The promoter designed in this project has all the necessary *cis* acting sequences that are required for gene transcription.

There is a suggestion that nodulin genes are regulated at the transcriptional level (Verma et al, 1992). These nodule-specific genes are often driven by sequences 5' of the UTR of the promoter region, composed of the *cis*-acting elements that have been characterized through 'chimeric promoter- GUS fusion' experiments. Indeed the consensus nodule specific sequence AAAGAT, CTCTT, AATAA, and CACCC have been identified in several nodulin genes (Sandal, Bojsen and Marker, 1987; Stougaard et al, 1990, Szczyglowski et al 1994, Vieweg et al, 2004 Fehlbeg et al, 2005, Hohnjec N et al, 2000, Jorgensen J. E., 1987 and Jorgensen J.E, 1991). The motifs CTCTT and AAGAT are present in this chimeric promoter, together with the INVA and INVB that enhance the strength of the promoter in the nodules. The nodulin genes are classified into early (ENOD) and late nodulins.

Early nodulin gene promoter regions exhibit additional motifs that are not present in late nodulin genes. ENOD II genes are early nodulin genes that are involved in dialogue between the root hair and the bacteria prior and/or during infection (Andriankaja et al, 2007). The *in silico* analysis of the promoter region of this gene revealed a potential *cis*-acting element located at 5' UTR region of the promoter. The GC rich region termed the GCC box (5'-AGCCGCC-3' /5'-GCAGGCC-3') is identified as a binding site for NOD factor (NF) secreted by the compatible *Rhizobium* species in recognition of host plant roots. Working in conjunction with the GCC box is the potential CAAT box and HD-ZIP-like motif that is important for NF-induced gene expression (Andriankaja et al, 2007). Since the N23 gene is a late nodulin gene, these sequences are not present in its promoter region. Thus our synthetic promoter might be void of such *cis*-acting elements.

The late nodulin genes are also well characterized. The consensus motifs AATAA, AAAGAT, CTCTT and CACCC occur in the promoter region of the *glutamate synthase* gene that drives gene expression not only in the nodules but also in lateral root primordia, root nodule primordia and the fully mature nodules (Blanco et al 2008). Hence, nodulin gene expression is not only limited to the nodules but to other plant parts as well (Blanco et al, 2008). The A/T rich sequences found in promoters of nodulin genes are present in many plant genes and may not be responsible only for nodule specific expression of nodulins, hence they are denoted as organ specific elements (OSE). These A/T rich regions and -CTCTT motif are rather said to be OSE than nodule specific (Verma, 1992, Fehleberg et al, 2005). Based on this background, there is a possibility that the synthetic promoter may drive GUS expression even in the entire rooting system.

However, there are cases of nodule specific gene expression of a nodulin devoid of this A/T rich region (Hohnjec, 2000). The AAAGAT motif of one nodule specific promoter

has been found to direct the expression of the nodulin in the central part of the nodule (Syszyglowski et al, 1994). Other elements that function as enhancers of the expression level are important of efficient expression of the nodulin. One such sequence is a strong positive element (SPE) TTTAATATT (Fehlebrg et al, 2005).

The strength of a nodulin promoter is dependent upon the arrangement of the *cis*-acting elements on the 5' UTR of the gene in the promoter region and these elements work in an inter-dependent manner (Jorgensen et al, 1987). It has been found that the consensus sequences AAATAT and CTCTT plus the core promoter TATA box and CAAT box are not enough for efficient regulation of expression of a nodulin gene (Jorgensen et al, 1987). This suggests the presence of other important sequences that enhance efficient expression of a nodulin gene within the promoter region of the nodulin gene.

In the study of the N23 nodulin gene from soybean, several *cis*-acting elements have been identified and their functions characterized (Jorgensen et al, 1987). The functionality of this nodule-specific promoter is dependent on the presence of the positive element A and B (PE-AB) and it is denoted as a nodule specific element (NSP). The PE-A is composed of OSE, CTCTT and NAT binding element upstream of the INVA (a strong positive element). The study revealed the INVA as the core of the PE-A. The PE-B is found downstream PE-A and represents a weaker element; however it is required for driving efficient expression of nodulin genes (Jorgensen et al, 1991 and Stougaard et al, 1990).

On the other hand, abiotic stress-responsive promoters are classified into two main groups; those functioning through ABA-dependent pathways and those that function via ABA-independent processes and their main *cis*-acting elements are ABRE and DRE/CRT

respectively (Nakashima K and Yamaguchi-Shinozaki, 2006). ABRE requires activation by abscisic acid, hence ABRE regulated genes get expressed after accumulation of endogenous ABA. On the other hand, DRE motifs are induced by binding of proteins, such as ERDI (Nakashima & Yamaguchi-Shinozaki, 2006), that are synthesized upon abiotic stress exposure.

A single copy of ABRE element is not enough to drive the expression of the stress-inducible gene; additional sequences, called coupling elements (CE) are also required (Gómez-Porrás et al, 2007). However, additional copies of the ABRE motif of the DRE/CRT element do often serve as coupling elements (Gómez-Porrás et al, 2007). Unlike the ABBRE, DRE/CRT/LTRE element does not need multiple copies of the motif for efficient transcription of the gene. There are other motifs working in collaboration with these two major *cis*-acting elements to drive expression of the gene either in ABA-dependent and ABA-independent manner. Such *cis*-acting elements would be MYC, MYB, EDR1, NAC and 2F-HD protein (Nakashima & Yamaguchi-Shinozaki, 2006; Gutha L.R. and Reddy A.R, 2008). All these *cis*-acting regulatory sequences have been identified in many plant genes including late embryogenesis genes (LEA) (Yamaguchi-Shinozaki & Shinozaki, 2005, Goerge et al, 2008). In addition to this, the late embryogenesis gene from soybean (GmPM9) has been cloned and its promoter region characterized, hence the functional regions of the promoter for abiotic stress response induction are known (Lee et al 1992 & 2000). All these ABA independent *cis*-acting sequences on the GmPM9 promoter form part of the designed promoter for this project.

Nitric oxide synthase activity has been detected in plants (Cueto et al, 1996). However, the sequence of the gene encoding this protein has not been identified. The AtNOA1, thought to be a novel plant NOS, was later discovered to be a GTPase (Sudhamsu et al,

2008 and Moreau et al, 2007). However, it remains that AtNOA1 is involved in nitric oxide synthesis, though indirectly. Thus it can be used to study the effect of regulated synthesis of nitric oxide in the root nodule. The same gene has been used to study the role of nitric oxide in abiotic stress responses in *Arabidopsis thaliana* (Guo et al, 2003 and Zhao et al, 2007). MtNOA1 provides an opportunity for regulating plant nitric oxide synthesis in the root nodules so as to enhance antioxidant capacity of the root nodules of legumes under abiotic stress.

Over-expression of individual antioxidants in plant tissues has often protected plants against oxidative stress. Superoxide dismutase has been over-expressed in plants and enhanced plant tolerance to stress. This improved tolerance was also accompanied by an increase in activities of other antioxidant enzymes (Wang et al, 2004). Glutathione reductase was also over-expressed in poplar trees. This increase in GR transcripts also resulted in increased glutathione levels (Foyer et al, 1995). These points indicate that there is cross-talk as well between the antioxidants and antioxidant enzymes. Thus over-expression of one antioxidant actually increases the level of the others. Glutathione synthetase is an enzyme that is involved in synthesis of glutathione and it increases plant tolerance to stress (Zhu et al, 1999). Additionally, increasing the levels of glutathione in the plant cells does not cause any deleterious effect on plant growth. Thus increasing glutathione levels is one of alternatives to regulating antioxidants in the nodules upon stress induction.

In mammals, nitric oxide interacts with soluble guanylyl cyclase to catalyse synthesis of cGMP from GTP. A plant protein with guanylate cyclase activity has been isolated in plants (Ludidi & Gehring, 2003), but the protein identified has no nitric oxide-dependent

activity (Ludidi & Gehring, 2003). The limited knowledge on plant soluble GC again puts limitations in utilizing the cGMP pathway in modifying antioxidant responses.

The ASREF/NSP-gene fusion described in this chapter is one approach that can be used to regulate the synthesis of nitric oxide in the nodules. The antioxidant response to salinity in plants is mediated by nitric oxide; so is in the root nodules. The effect of ROS is counteracted by antioxidants and the associated enzymes, hence the encoding genes for the corresponding antioxidant enzymes and metabolites can be fused to ASREF/NSP promoter in the root nodules to develop plants with elevated antioxidant levels and better tolerance to salinity.

8.5.0.0 Conclusion

This chapter was aimed at designing and constructing an abiotic stress-inducible promoter that is nodule-specific and to explore the prospects of enhancing abiotic stress tolerance of the nodules. In order reach this goal the nucleotide sequences bearing the relevant *cis*-acting elements for both nodule specificity and abiotic stress induction had to be isolated from soybean genomic DNA and ligated together in a specific arrangement that would give a potentially functional promoter under the targeted environment. Secondly the objective was to build a transformation construct that would allow assessment of the functionality of the promoter in the nodules under abiotic stress. For the purposes of this chapter, the abiotic stress inducible- nodule-specific promoter was designed and constructed.

The abiotic stress-responsive fragment has been successfully isolated from the soybean GmPM9 gene, so is the nodule-specific fragment from the N23 promoter region. These

two fragments were fused together with the core promoter region from N23 to form a chimeric promoter. This was successfully sub-cloned into the binary vector pBINPLUS as a *GUS* fusion. The other construct, pBASMtNOA1 was also developed in which ASREF/NSP promoter was used to drive the expression of MtNOA1.

Although the promoter construct was successfully made and should theoretically be functional, there is a need to evaluate its functionality by delivering the vectors into the plant. The rhizogen transformation system has been initiated in *Medicago truncatula* cv. 2HA. The composite plants harbouring this ASREF/NSP-gene fusion are growing in tissue culture and await analysis. This rhizogen transformation is an efficient and fast method that can be used to study gene expression in the roots. However, in order to evaluate the effect of this transformation work, other approaches like somatic embryogenesis would be more relevant.

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Chapter 9 General conclusion

The vital role played by nitric oxide in oxidative stress responses in root nodules of legumes was established in this research project. Through the strategic study of the effect of both the endogeneously synthesized and exogenously supplied nitric oxide on the superoxides dismutase, together with the enzymes and metabolites of the glutathione peroxidase-glutathione antioxidant system, the role played by this molecule was partly established. The list of the selected candidate enzymes and metabolites for this study might not be enough to draw an overall solid conclusion on the behavior of the plant antioxidant system in response to nitric oxide. However, the study provides a clearer picture as to how legume nodules respond to abiotic stress and the possible interventions that utilize the scavenging capacity of nitric oxide as a coping mechanism, in plant abiotic stress responses, that can be employed in plant legume root nodule improvement programs. It should be noted that to our knowledge this is the first study targeting nodular antioxidant responses to NaCl and nitric oxide.

From this research project, it has been established that salt/abiotic stress tolerance in the legume nodules involves mediation by nitric oxide in a system that triggers an increase in antioxidant capacity of the nodule tissue. It is concluded that nitric oxide improves the redox homeostasis of glutathione pool. However this increase in antioxidant capacity is not linear; different antioxidants are up regulated at different times, indicating a possible signal transduction network amongst the antioxidants (Figure 9-1).

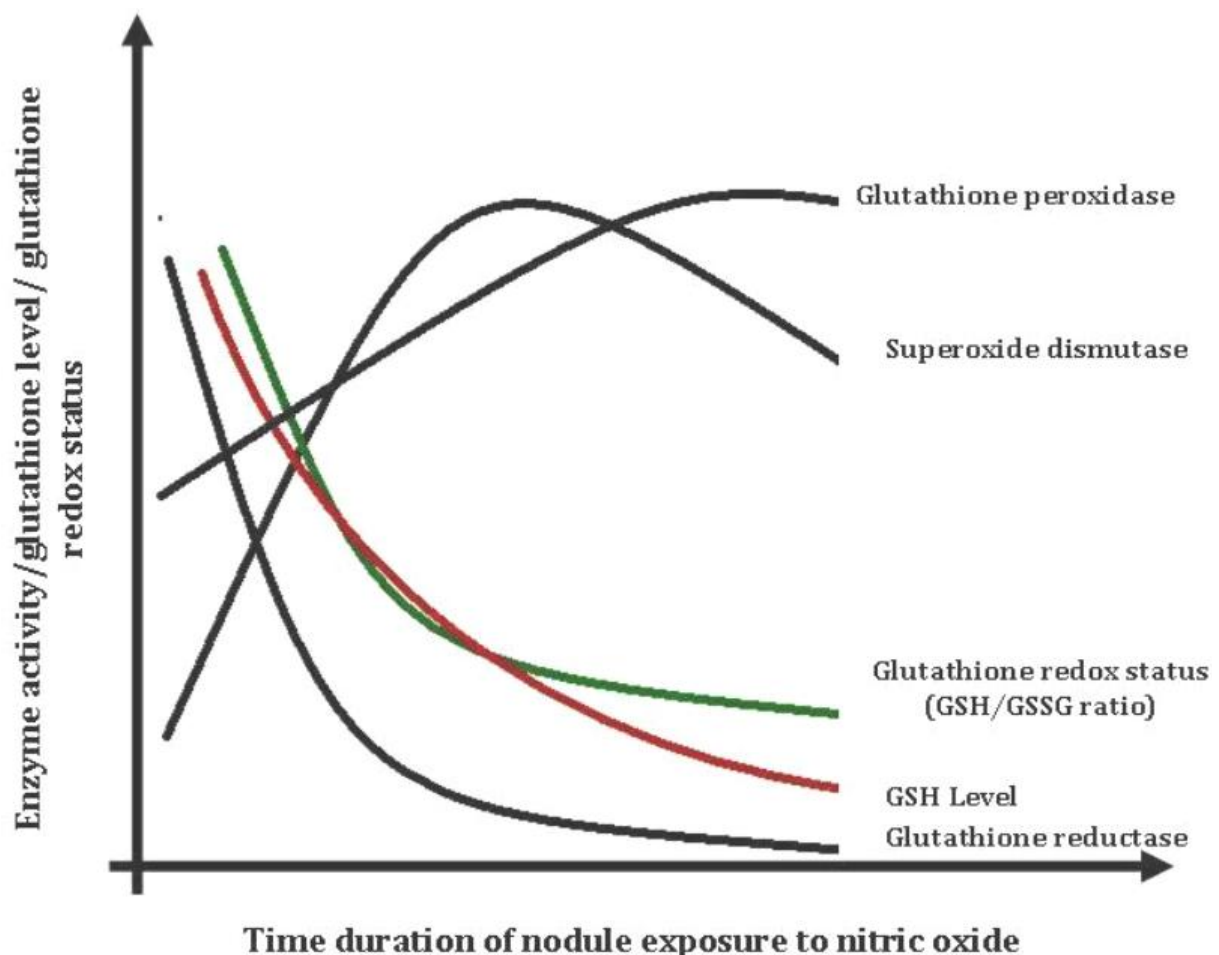


Figure 9- 1 Response of GPx, GR, SOD and glutathione pool in response to exposure to nitric-oxide. GSH represents the concentration of reduced glutathione in μmol .

Generally all the antioxidants investigated in this project are modulated by nitric oxide. Exposure of the nodules to nitric oxide results in an early increase in the activity of the antioxidant enzymes that is later either followed by a general decline in the overall level of the antioxidant or an increase depending on the enzyme. As illustrated in Figure 9- 1; GR and glutathione levels decline with time of exposure to nitric oxide, however the SOD and GPX levels increase. This pattern implicates possible availability of another reductant that might be involved in later stages of stress responses. However, this study did not go further into identifying some other plant reductants that have been implicated in the glutathione peroxidase system like thioredoxin. There is a possibility that the thioredoxin system takes over in scavenging ROS at later stages of nitric oxide exposure.

This research further establishes that some of the increases in enzyme activities are regulated at the transcriptional level. Superoxide dismutase activity in the nodules of peas is regulated at the transcripts level. The expression of different SOD isoforms is up-regulated in response to exposure to nitric oxide. The nitric oxide synthase gene has not been isolated in plants but such a gene can be targeted for genetically modifying legume nodules with increased salt tolerance.

One novel finding in this work is that the effect of nitric oxide on glutathione peroxidase in the pea nodules is modulated through the cGMP. Nitric oxide appears to be located upstream of cGMP in the nitric oxide/cGMP signalling network that leads to up-regulation of glutathione peroxidase activity. The GPx activity is inhibited by the absence of sGC activity. It is concluded that externally supplied cGMP increases the activity of GPx in the same manner as nitric oxide. However, endogenously synthesized cGMP in response to nitric oxide should have been quantified to verify that NO activates GPx through generation of cGMP.

The urgent need for enhancing abiotic stress tolerance in legumes, targeting specifically the nodule tissue, can be addressed via development of a stress-inducible nodule specific promoter driving expression of cytoprotective genes against the abiotic stress. By fusing the *cis*-acting elements from N23 late nodule gene promoter from soybean and stress responsive *cis*-acting elements from the GmPM9 gene promoter, a late embryo genesis gene, a 602 base pair long chimeric promoter has been designed and fused to the GUS gene in a binary vector pBINPLUS. This synthetic chimeric promoter was delivered into Medicago plants in a transformation process mediated by *Rhizogens*, to form composite plants, carrying the chimeric promoter in the roots. This promoter can be used to drive the expression of antioxidant genes in the nodules. For instance the

homolog of AtNOA1, which was thought to be a novel plant nitric oxide synthase, has been fused to the synthetic promoter. A similar system can be employed with other antioxidant genes or antioxidants regulatory coding sequences. Thus, this study provides the basis for enhancing legume nodule abiotic stress tolerance, with more emphasis on salt stress.

APPENDIX A

MEDIA FOR BACTERIAL GROWTH

LB (Luria-Bertani)

10g tryptone

5g yeast extract

5g NaCl

Adjust pH to 7.0 with 1N NaOH

TY MEDIUM

TFB-I:

15 % glycerol (v/v)

30 mM Calcium acetate

100 mM RbCl

10 mM CaCl₂

50 mM MnCl₂

Adjust pH to 5.8 with acetic acid

2 M CaCl₂ and MnCl₂ stock solutions were prepared, autoclaved and used for this buffer

TFB-II:

15 % glycerol (v/v)

10 mM MOPS

10 mM RbCl

75 mM CaCl₂

Adjust pH to 7.0 with NaOH

BUFFERS FOR AGAROSE GEL ELECTROPHORESIS

TE-buffer:

10 mM Tris·Cl, pH 7.6

1 mM EDTA

Autoclave, store at room temperature

10X TBE buffer stock

108g Tris

55g Boric acid

9.3g EDTA (pH 8.0)

Dilute to 0.5X before use.

1X TBE gives a working solution of 0.089M Tris-base/0.089M boric acid/0.002M EDTA

10X loading buffer for agarose electrophoresis

50mM NaOH

1mM EDTA

2.5% glycerol

0.025% bromophenol blue

SOLUTION AND BUFFERS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

Always wear latex gloves when working with acrylamide solutions.

PROTEIN GELS

SDS-PAGE (Laemmli-system)

Acrylamide stock (%T=30%, %C=2.7) purchased from sigma

Separating gel buffer stock

45.5g Tris

Dissolve in 250 ml ddH₂O, adjust the pH to 8.8 with HCl. Store at 4°C (stable for several months).

Stacking gel buffer stock

15.1g Tris

Dissolve in 250 ml of ddH₂O, adjust the pH to 6.8 with HCl. Store at 4°C (stable for several months). Check the pH of the buffer each time before use and adjust with HCl again if necessary.

2X sample treatment buffer for SDS-PAGE

4.6 % (w/v) SDS

10 % (V/V) 2-Mercaptoethanol

0.125 M Tris-HCl (pH6.8)

0.01 % (w/v) bromophenol blue

20% (v/v) glycerol

Tank buffer for SDS-PAGE

12% SDS-PAGE GELS

H₂O

30% acrylamide mix

1.5 M Tris (pH6.8)

10% SDS

10% ammonium persulfate

TEMED

10% SDS-PAGE GELS

H ₂ O	4.0ml
------------------	-------

30% acrylamide mix	3.3ml
--------------------	-------

1.5 M Tris (pH6.8)	2.5ml
--------------------	-------

10% SDS	0.1ml
---------	-------

10% ammonium persulfate	0.1ml
-------------------------	-------

TEMED	0.004ml
-------	---------

NATIVE ACRYLAMIDE GELS

2X sample treatment buffer for native acrylamide

10 % (V/V) 2-Mercaptoethanol

0.125 M Tris-HCl (pH6.8)

0.01 % (w/v) bromophenol blue

20% (v/v) glycerol

Tank buffer for native acrylamide

Tris base 3g (25 mM)

Glycine 14.4g (192 mM)

In 100 mL of H₂O

10% PAGE GELS (6ml/g)

30 % Acrylamide 2ml

Tris-HCl pH 8.8 1.5ml

dH₂O 2.5ml

10 % APS 30µl

TEMED 3µl

(No need for a staking gel)

Ribovalvin-NBT solution

Riboflavin 1mg

Nitro blue Tetrazolium (NBT) 2.5mg

APPENDIX B

AMINO ACID AND NUCLEOTIDE SEQUENCES

>NP_190329, AtNOA1

MALRTLSTFPSPRRHTTTRREPNTLTVIYRNPTTSIVCKSIANSEPPVSLSERDGFAAAAAPT
PGERFLENQRAHEAQKVVKKEIKKEKKEKKEEIIARKVVDTSVSCCYGCGAPLQTSVDVDS PG
FVDLVTYELKKKHHQLRTMICGRCQLLSHGHEMITAVGGNGGYPGGKQFVSADELREKLSHLR
HEKALIVKLVDIVDFNGSFLARVRDLVGANPIILVITKIDLLPKGTDMNCIGDWVVEVTMRK
KLNVLVSVHLTSSKSLDGVSGVASEIQKEKKGDRVYILGAANVGKSAFINALLKTMARDPVA
AAAQKYKPIQSAVPGTTLGPIQINAFVGGKELYDTPGVHLHHRQAAVVHSDDLPALAPQNRL
RGQSFDISTLPTQSSSSPKGESLNGYTFWGLVLRIDILKALPETCFTFYGPKALEIHAVPT
KTATAFYEAKLGVLLTTPPSGKNQMGEWKGLQSHRLQIEINDAKRPASDVAISGLGWISIEP
IRKTRGTEPRDLNEAEHEIHICVSVPKPVEVFLRPTLPIGTSGTEWYQYRELTDKEEEVPRK
WYF

>gi|87241448|gb|ABD33306.1| Protein C4orf14 homolog, related [Medicago truncatula]

MAILFSTIALPSTNVTSKLSILNNTSHSHALRHFSGNTTKRFHKASSFIAFAVKNNPTIRKT
TPRRDSRNPLLSEGRDEDEALGPICPGCGIFMQDNDPNLPGFYQQKEVKIETFSEEDYELDD
EEDDGEEDNGSIDDES DWDSEELEAMLLGEENDDKVDLDGFTHAGVGYGNVTEEVLERAKK
KKVSKAEKRMAREAEKVKEEVTVCARCHSLRNYGQVKNYMAENLIPDFDFRLITTRLMNP
AGSGSSTVVMVDCVDFDGSFPRTAVKSLFKALEGMQENTKKGKLPKLVLVATKVDLLPS
QVSPTRLDRWRHRASAGGAPKLSAVYLVSSRKDLGVRNVLSFVKDLGPRGNVWVIGAQNA
GKSTLINAFKKEGAKVTKLTEAPVPGTTLGILRIAGILSAKAKMFDTPGLLHPYLLSMRLN
REEQKMAGQAIHVGGGLARLDLIEASVQTMVTVWASPNVSLHMGKIEANAEIWNHVGVRLO
PPIGNDRAAELGTWKEREVKVSGSSWDVNCMDVSIAGLWFSGLGIQGEATMKLWTNDGIEIT
LREPLVLDRAPSLEKPGFWLPKAI SEVIGNQTKLEAQRRKKLEDEDETEYMGASIEISA

>(gi|62899132:c55332-54013, c53575-53408, c50792-50430) Medicago truncatula chromosome 7 clone mth2-73i16, complete sequence

ATGGCTATCTTGTCTCTACAATTGCACTTCCCTCCACAAACGTCACCTCCAAACTATCCAT
CTTAAACAACACTTCACATTCTCACGCACCTTCGCCATTTCTCAGGTAATACTACTAAACGCT
TTCATAAAGCTTCCTCCTTTATGCTTTTGCTGTGAAGAACAACCCACCATAAGAAAAACC
ACTCCAAGAAGAGATAGTAGAAACCCACTTTTAAGTGAAGGTAGAGATGAAGATGAAGCTCT
TGGACCCATTTGCCCTGGTTGTGGAATTTTCATGCAAGATAATGATCCAAATCTCCCTGGTT
TTTACCAACAAAAAGAGGTAAAAATTGAAACATTTTCTGAGGAGGATTATGAATTAGATGAT
GAAGAGGATGATGGTGAAGAAGAGGATAATGGGTCAATTGATGATGAGTCTGATTGGGATTC
TGAGGAATTGGAAGCTATGTTACTTGGTGAAGAAAATGATGATAAGGTTGATTTGGATGGGT
TTACACATGCAGGTGTTGGGTATGGTAATGTTACTGAGGAGGTTTTGGAGAGGGCTAAGAAG
AAGAAGGTTTCAAAGGCTGAGAAGAAGAGAATGGCTAGGGAAGCTGAGAAGGTGAAGGAGGA
GGTACTGTTTGTGCTAGGTGTCATTCCTTGAGAAATTATGGGCAGGTGAAGAATTATATGG
CGGAGAATTTGATACCGGATTTTGATTTGATAGGTTGATTACTACTAGGTTAATGAATCCT
GCTGGTAGTGGTAGTTCTACTGTTGTTGTTATGGTTGTGGATTGTGTTGATTTTGATGGTTC
TTTCCCGAGAACAGCTGTGAAGTCGTTGTTAAGGCATTGGAAGGTATGCAGGAGAATACAA
AGAAGGGTAAGAACTGCCAAAGCTTGTCTTGTGGCTACAAAGGTTGATCTCCTTCCGTCG
CAGGTTTCTCCGACGAGGTTGGATAGATGGGTTCCGGCACCGTGCAAGTGCTGGAGGAGCGCC
TAAATTAAGCGCGGTTTATTTGGTCAGTTCTCGAAAGGATTTAGGTGTGAGGAATGTGTTGT

CGTTTGTAAAGGATTTGGCTGGTCCTCGTGGGAATGTTTGGGTTATTGGGGCTCAAAATGCT
GGGAAGTCTACTCTGATCAATGCATTTGCGAAGAAAGAAGGAGCCAAAGTTACCAAGCTCAC
GGAAGCTCCAGTTCCTGGGACGACACTTGGGATCTTGAGGATTGCAGGAATTTTGTGAGCTA
AGGCTAAGATGTTTGATACTCCAGGGCTCTTGCATCCATATTTATTGTCGATGAGATTGAAT
CGGGAGGAACAAAAGATGGCTGGACAAGCCATACATGTTGGTGGCTTGGCAAGACTTGACCT
AATTGAAGCCTCTGTTCAAACAATGTATGTCACTGTTTGGGCATCACCAAATGTTTCTCTAC
ACATGGGAAAAATAGAAAATGCTAATGAGATTTGGAATAATCATGTTGGCGTCAGACTGCAG
CCTCCCATCGGTAATGACCGCGCAGCTGAACTAGGTACATGGAAAGAAAGGGAAAGTAAAAAGT
ATCTGGATCTAGTTGGGATGTCAACTGCATGGACGTATCAATAGCTGGCTTAGGTTGGTTTT
CTTTGGGTATCCAAGGTGAAGCAACCATGAAATTATGGACCAATGATGGAATTGAAATAACT
TTGAGAGAACCATTGGTACTTGACCGGGCCCCGTCCCTTGAAAAACCAGGTTTTTGGTTACC
AAAGGCTATATCTGAAGTTATTGGCAACCAACTAACTTGAAGCTCAAAGAAGGAAAAAAC
TTGAAGATGAAGATACAGAATACATGGGAGCAAGTATAGAGATATCTGCATGA

SUPEROXIDE DISMUTASE ISOFORM NUCLEOTIDE SEQUENCES

J04087.1 GI:169159 (Pea chloroplastic copper/zinc-superoxide dismutase mRNA, complete cds)

GCAACTAGCAATGGCTTCACAACTCTCGTCTCACCTTCACCTCTCTCTTCTCACTCTCTTC
TCCGAACATCTTTCTCCGGCGTCTCCGTCAAGCTCGCTCCCAATTCTCAACCCTTGCAACT
TCCAATTTCAAACCTCTCACCGTAGTTGCGGTGCCAAGAAAGCCGTCTCTGTCTTAAGGG
CACATCCGCCGTGAAGGTGTCGTCACCTCACTCAAGACGATGAAGGTCCAACAACAGTTA
ATGTTTCGTATCACTGGCCTTACTCCAGGGCTTCATGGTTTTTACCTACATGAGTATGGTGAT
ACCACAAATGGGTGTATCTCAACAGGACCACATTTTAATCCCAACAAGTTGACACATGGTGC
TCCTGAAGATGAAATCCGTGCATGCGGGTGACCTGGGAAACATAGTTGCTAATGCTGAAGGAG
TTGCAGAGGCGACAATCGTGGACAATCAGATAACCACTCACTGGCCCCAATTCAGTCGTTGGA
AGAGCCTTAGTGGTTCACGAGCTTCAAGATGACCTTGGAAGGGTGGACATGAACTTAGTTT
GAGCACTGGAAATGCTGGTGGAAAGATTAGCTTGTGGTGTGGTTGGCTTGACTCCAGTATAAA
TGCTTCAATGCTTTTGCACCAGCCTTGTTATTTTAACTGATGTTTGTATTTCTTTCATGTTATC
CTTGTTTTATGAAGCTTACTGTTATTTGTTTTCTTCAATTAGCAATGTAAAGATTTTAATG
TGTGAAAACATGACAGTCTCTACTAGATAGTTTCATCATGCAGTCAGGTGTGTTTCTTCCC
TAATAAAGTTTAATTTCTG

M63003.1 GI:169069 (Cu-Zn-superoxide dismutase Mrna, complete cds; cytosol)

GGATCACATTGAACAATGGTGAAGGCTGTGGCAGTTCTTAGTAACAGTAACGAAGTCTCGGG
TACTATTAACCTCAGTCAGGAGGGAAATGGTCCAACCACTGTAAGTGAAGTCTTGCTGGTC
TTAAGCCTGGCCTCCACGGCTTCCATATCCATGCCTTGGGAGACACCACAAACGGTTGCATT
TCAACTGGACCACATTTCAATCCTAATGGGAAGGAACATGGTGCCCCTGAGGATGAGACTAG
ACATGCTGGTGATCTTGAAATATCAATGTTGGTGTGATGATGGAAGTGAAGCTTCAACATTA
CTGACAACCATATCCCTCTCACTGGAACAACTCCATCATAGGAAGGGCTGTTGTTGTCCAT
GCCGATCCTGATGATCTTGGGAAAGGTGGTCACGAGCTTAGCAAACTACTGGAAATGCTGG
TGGCAGAGTAGCTTGTGGTATTATTGGGTTGCAAGGATAGATCACTACTCTCCACTGTGCGT
GCTGTTGAAGTTTTAGAAGAATAAATTGCACTCATCCCTCTTTGCTTGTTTAGGGTCTGAT
CTGTAAGTCCGGATAATGTGTTTTTGTGTTGTAATTGAAATCTCAATGGCTATATGACTGCACT
TGGTGTTTAATCAGTTACTTTCAGATGAAGTCTGTGGTGTGTTGTCATGCTTGTTTTTCAGTTT
CAGTATGATCTTAATTTAAGGAGTTGGGTTTTTAATAAAAAAAAAAAAAAAAAAAAA

X60170.1 GI:20901 (*Pisum sativum* mRNA for manganese superoxide dismutase)

GAATTCTAGAGTTTTTCCAGAATCTGCATTATCATCCTCTCTCTGTCTCTCTCCATGGCCGC
TCGAACCCTATTGTGCAGAAAACCCCTATCCTCCGTGCTCCGCAATGACGCAAACCAATCG
GAGCAGCCATAGCAGCCGCATCAACTCAATCCC GCGGTTTGCATGTCTTCACGCTCCCGGAT
CTCGCTTACGACTACGGAGCTTTGGAGCCTGTCATTAGCGGCGAAATCATGCAAATCCACCA
TCAGAAACACCACCAGACTTATATTACCAACTACAACAAAGCTCTCGAACAGCTTCACGATG
CCGTTGCTAAAGCTGATACATCTACCACCGTTAAGCTCCAGAATGCCATCAAATTC AACGGC
GGAGGTCATATCAACCATTCCATTTTCTGGAAAAATCTGGCTCCTGTTAGTGAAGGAGGTGG
TGAACCACCAAAGGAATCCCTGGGCTGGGCCATTGACACCAATTTTGGATCTTTGGAAGCAT
TGATACAAAAGATTAATGCCGAAGGTGCAGCTCTTCAGGCGTCTGGATGGGTGTGGCTTGGT
CTCGACAAAGACTTGAAGAGGCTTGTGGTTGAAACCACTGCAAACCAGGACCCACTGGTGAC
TAAAGGAGCAAGTTTGGTTCCATTGCTTTGGATAGATGTTTGGGAACATGCCTACTACTTAC
AGTACAAAATGTTAGACCAGACTATTTGAAGAACATTTGGAAAGTTATTA ACTGGAAACAT
GCCAGTGAAGTATATGAGAAAGAGAGCTCTTAATCTGAAGTGCTGAAGTGCTGCTTGGTGTG
GAACTTGGGACGACAGGTTTGCAGCTTGTGGCAATGGAATAAATGATGTCAAGTGATGTG
AAGTGATAGATAAAACCTTTCCTTTGATGTACTTAGACACTTAGAACTTGAGCAATCTGGCCG
AATAACCTTTAGAACCTTTTTTGGCTTAGTAGTACTCTTTTGTGAAAAA

Medicago MtNOA1 nucleotide and protein sequences as predicted

1 atg gct atc ttg ttc tct aca att gca ctt ccc tcc aca aac gtc
M A I L F S T I A L P S T N V
46 act tcc aaa cta tcc atc tta aac aac act tca cat tct cac gca
T S K L S I L N N T S H S H A
91 ctt cgc cat ttc tca ggt aat act act aaa cgc ttt cat aaa gct
L R H F S G N T T K R F H K A
136 tcc tcc ttt att gct ttt gct gtg aag aac aac ccc acc ata aga
S S F I A F A V K N N P T I R
181 aaa acc act cca aga aga gat agt aga aac cca ctt tta agt gaa
K T T P R R D S R N P L L S E
226 ggt aga gat gaa gat gaa gct ctt gga ccc att tgc cct ggt tgt
G R D E D E A L G P I C P G C
271 gga att ttc atg caa gat aat gat cca aat ctc cct ggt ttt tac
G I F M Q D N D P N L P G F Y
316 caa caa aaa gag gta aaa att gaa aca ttt tct gag gag gat tat
Q Q K E V K I E T F S E E D Y
361 gaa tta gat gat gaa gag gat gat ggt gaa gaa gag gat aat ggg
E L D D E E D D G E E E D N G
406 tca att gat gat gag tct gat tgg gat tct gag gaa ttg gaa gct
S I D D E S D W D S E E L E A
451 atg tta ctt ggt gaa gaa aat gat gat aag gtt gat ttg gat ggg
M L L G E E N D D K V D L D G
496 ttt aca cat gca ggt gtt ggg tat ggt aat gtt act gag gag gtt
F T H A G V G Y G N V T E E V
541 ttg gag agg gct aag aag aag aag gtt tca aag gct gag aag aag
L E R A K K K V S K A E K K
586 aga atg gct agg gaa gct gag aag gtg aag gag gag gtt act gtt
R M A R E A E K V K E E V T V
631 tgt gct agg tgt cat tcc ttg aga aat tat ggg cag gtg aag aat
C A R C H S L R N Y G Q V K N
676 tat atg gcg gag aat ttg ata ccg gat ttt gat ttc gat agg ttg
Y M A E N L I P D F D F D R L
721 att act act agg tta atg aat cct gct ggt agt ggt agt tct act
I T T R L M N P A G S G S S T
766 gtt gtt gtt atg gtt gtg gat tgt gtt gat ttt gat ggt tct ttc
V V V M V V D C V D F D G S F
811 ccg aga aca gct gtg aag tcg ttg ttt aag gca ttg gaa ggt atg
P R T A V K S L F K A L E G M

856 cag gag aat aca aag aag ggt aag aaa ctg cca aag ctt gtt ctt
 Q E N T K K G K K L P K L V L
 901 gtg gct aca aag gtt gat ctc ctt ccg tcg cag gtt tct ccg acg
 V A T K V D L L P S Q V S P T
 946 agg ttg gat aga tgg gtt cgg cac cgt gca agt gct gga gga gcg
 R L D R W V R H R A S A G G A
 991 cct aaa tta agc gcg gtt tat ttg gtc agt tct cga aag gat tta
 P K L S A V Y L V S S R K D L
 1036 ggt gtg agg aat gtg ttg tcg ttt gta aag gat ttg gct ggt cct
 G V R N V L S F V K D L A G P
 1081 cgt ggg aat gtt tgg gtt att ggg gct caa aat gct ggg aag tct
 R G N V W V I G A Q N A G K S
 1126 act ctg atc aat gca ttt gcg aag aaa gaa gga gcc aaa gtt acc
 T L I N A F A K K E G A K V T
 1171 aag ctc acg gaa gct cca gtt cct ggg acg aca ctt ggg atc ttg
 K L T E A P V P G T T L G I L
 1216 agg att gca gga att ttg tca gct aag gct aag atg ttt gat act
 R I A G I L S A K A K M F D T
 1261 cca ggg ctc ttg cat cca tat tta ttg tcg atg aga ttg aat ccg
 P G L L H P Y L L S M R L N R
 1306 gag gaa caa aag atg gtt gag ata cgg aag gaa ctt aaa cct cgt
 E E Q K M V E I R K E L K P R
 1351 tca tat aga att aag gct gga caa gcc ata cat gtt ggt ggc ttg
 S Y R I K A G Q A I H V G G L
 1396 gca aga ctt gac cta att gaa gcc tct gtt caa aca atg tat gtc
 A R L D L I E A S V Q T M Y V
 1441 act gtt tgg gca tca cca act gtt tct cta cac atg gga aaa ata
 T V W A S P T V S L H M G K I
 1486 gaa aat gct aat gag att tgg aat aat cat gtt ggc gtc aga ctg
 E N A N E I W N N H V G V R L
 1531 cag cct ccc atc ggt aat gac cgc gca gct gaa cta ggt aca tgg
 Q P P I G N D R A A E L G T W
 1576 aaa gaa agg gaa gta aaa gta tct gga tct agt tgg gat gtc aac
 K E R E V K V S G S S W D V N
 1621 tgc atg gac gta tca ata gct ggc tta ggt tgg ttt tct ttg ggt
 C M D V S I A G L G W F S L G
 1666 atc caa ggt gaa gca acc atg aaa tta tgg acc aat gat gga att
 I Q G E A T M K L W T N D G I
 1711 gaa ata act ttg aga gaa cca ttg gta ctt gac cgg gcc ccg tcc
 E I T L R E P L V L D R A P S
 1756 ctt gaa aaa cca ggt ttt tgg tta cca aag gct ata tct gaa gtt
 L E K P G F W L P K A I S E V
 1801 att ggc aac caa act aaa ctt gaa gct caa aga agg aaa aaa ctt
 I G N Q T K L E A Q R R K K L
 1846 gaa gat gaa gat aca gaa tac atg gga gca agt ata gag ata tct
 E D E D T E Y M G A S I E I S
 1891 gca tga 1896
 A *