

Modulation of ascorbate peroxidase activity by nitric oxide in soybean

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted to any university for a degree.

Signature.....

Date.....

Dedication

I dedicate this thesis to my loving parents, Elder & Mrs Egbichi

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List of Abbreviations

µg	Microgram
µl	Microliter
A600	Absorbance at 600 nm
A390	Absorbance at 340 nm
ABA	Abscisic acid
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA	Ascorbate
AtNOA1	Arabidopsis thaliana Nitric Oxide Associated 1
AtNOS1	Arabidopsis thaliana Nitric Oxide Synthase 1
CaCl ₂	Calcium Chloride
CAT	Catalase
cGMP	Guanosine 3, 5-cyclic monophosphate
Cl ⁻	Chloride ion
CNGCs	Cyclic nucleotide-gated ion channels
cNR	Cytosolic nitrate reductase
DETA	Diethylenetriamine
DETA/NO	2,2'-(hydroxynitrosohydrazono) bis-ethanimine
DHAsA	Dehydroascorbate

DHAR	Dehydroascorbate reductase
DNTB	5-(3-Carboxy-4-nitrophenyl) disulfanyl-2-nitrobenzoic acid
DTT	Dithiothretiol
EDTA	Ethylene diamine tetracetic acid
eNOS	Endothelial NOS
FW	Fresh weight
g	Gram
GDC	Glycine decarboxylase complex
GPOX	Guaiacol peroxidase
GPX	Glutathione peroxidases
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
GTP	Guanosine triphosphate
H ₂ O ₂	Hydrogen Peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
IAA	Indole acetic acid
iNOS	Inducible NOS
L-NAME	N ^G -nitro-L-arginine methyl ester
LNMAA	N ^G -monomethyl-L-arginine acetate
LNNA	N- ω -nitro-L-arginine
MAPKs	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDHA	Monodehydroascorbate

MDHAR	Monodehydroascorbate reductase
MeOOH	Methyl hydrogen peroxide
mg	Milligram
ml	Milliliter
Na ⁺	Sodium ion
NaCl	Sodium chloride
NaCN	Sodium cyanide
NaNO ₂	Sodium nitrite
NaNO ₃	Sodium trioxonitrate (V)
NBT	Nitroblue tetrazolium
NEM	N-ethylmaleimide
nNOS	Neuronal NOS
NO ⁺	Nitrosonium cation
NO ⁻	Nitroxyl anion
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
NO	Nitric oxide
NO-Lb	Nitrosyl-haemoglobin complex
NOS	Nitric oxide synthase
NR	Nitrite reductase
¹ O ₂	Singlet oxygen
O ₂ ⁻	Superoxide radical
OH	Hydroxyl radical
ONOO ⁻	Peroxynitrite
PAGE	Polyacrylamide gel electrophoresis

PCD	Programmed cell death
PM-NR	Plasma membrane-bound NR
PUFA	Poly-unsaturated fatty acids
PVP	Poly vinyl pyrrolidone
R	Reproductive stage
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SDP-PAGE	Sodium dodecyl sulfate- PAGE
sGC	Soluble guanylate cyclase
SNAP	S,N-acetyl penicillamine
SNP	Sodium-nitroprussiate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBE	Tris/borate electrophoresis
TCA	Trichloroacetic acid
TEMED	N,N,N',N'- tetramethylethylenediamine
Tris-HCl	Tris hydrochloride
U	Unit
V3	Third trifoliolate
V	Vegetative stage
v/v	Volume/Volume
w/v	Weight/volume

Summary

Salinity stress is one of the major environmental factors that lead to poor crop yield. This is due to overproduction of reactive oxygen species (ROS) which consequently lead to oxidative stress. Although these ROS may be required for normal physiological functions, their accumulation acts as a double edge sword, as they also cause oxidative damage to nucleic acids, lipids and proteins of plant cell membranes. Plants have evolved with an efficient antioxidant defensive system in order to protect and detoxify harmful effects of ROS. Ascorbate peroxidase (APX) is regarded as one of the major scavengers of H_2O_2 . Although some studies have described the role of nitric oxide (NO) in diverse physiological processes in plants, there is still much to know as regards to modulation of APX activity by nitric oxide in salinity-induced stressed plants. For the purposes of this study, the effect of salt and exogenously applied NO on APX, dehydroascorbate reductase and antioxidant metabolite content was determined. This study investigated the use of NO donor 2,2'-(hydroxynitrosohydrazono) bis-ethanimine (DETA/NO) and diethylenetriamine (DETA) on soybean.

The data obtained from this study shows that application of DETA/NO resulted in an increase of NO nodular content and also regulated APX activity. The NO-induced changes in APX enzymatic activity were coupled to altered nodule H_2O_2 content. Further analysis of APX enzymatic activity identified three APX isoforms for which augmented enzymatic activity

occurred in response to NO. By supplementing salinity-induced stress soybeans with NO, this study shows that tolerance to salt stress is improved. The underlying mechanism of the NO-mediated tolerance to salt is shown to be its role in modulating the plant antioxidant defense system thus maintaining redox status under salinity-induced stress. Here, although there was increased APX activity in salt stressed plant, supplementing the salinity-induced stressed plants with NO resulted to even higher APX activity which was sufficient to detoxify ROS. Furthermore, this study shows that the NO-mediated effect is not limited in antioxidant enzymes but also involves regulating antioxidant metabolite ratio through modulating the antioxidant enzymes that are involved in the ascorbate -glutathione cycle.

Chapter One

Introduction

Plants are constantly exposed to environmental stresses which eventually lead to changes in their physiology, morphology and development. Increasing evidence based on experiments in plants has shown a vital role of Nitric oxide (NO) in protecting against stress conditions (1). NO is a major signaling molecule and acts in several tissues to regulate a diverse range of physiological processes. This free reactive radical gas was initially considered just as a toxic gas. However this idea changed after the discovery of the signaling role of NO in regulating the cardiovascular system (2). In plants, the importance of in-depth studies on NO was prompted after the identification of the role of gaseous nitric oxide in senescence and plant defense against pathogens (3, 4). A vast range of processes related to growth and development which NO regulates in plants include induction of seed germination and reduction of seed dormancy (5,6), reduction of internodes length in stems (7,8), elongation of roots (7) and delay of senescence, promotion of stomata closure, stimulation of leaf expansion and inhibition of cell death in plant leaves (5).

Another major area directed on the study of NO is towards its involvement in coordinating several defense responses during both biotic and abiotic stress conditions in plants. The imposed level of stress on the plant can lead to the disruption of cellular redox homeostasis thus leading to conditions such as oxidative/nitrosative stress as a result of the generation of reactive oxygen species (ROS) (9). The ROS are by-products of electron transport reactions which are continuously produced during normal metabolic processes. Their role in plants can be complex. They are regarded as signaling molecules during cellular growth, control of stomata closing (10), stress responses (11, 12) and programmed cell death (13). However at

elevated levels ROS are lethal to the cell and this is usually accompanied by poor growth and low yield of cultivated crops.

Studies on adaptive mechanisms of plants have shown an increased basal level of NO in water and heat stressed plants, suggesting its importance in abating stress (14, 15). The protective mechanism of NO in plants during stress is linked to its ability to function as an antioxidant by directly scavenging the ROS, thus reducing cellular damage (16) and acting as a signaling molecule which eventually results in changes in gene expression (17).

Plants can also prevent or reduce the effect caused by the ROS by organizing a coordinated defense mechanism. This includes the scavenging of the ROS such as the superoxide radical and hydrogen peroxide by the use of antioxidants such as ascorbate (AsA), carotenoids and α -tocopherol, and by the use of an enzymatic antioxidant system. A list of these enzymes involved in the enzymatic antioxidant defense includes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), guaiacol peroxidase (GPOX) and glutathione reductase (GR) (18,19,20).

Diverse evidence support the involvement of NO in regulating plant responses to several environmental factors such as heavy metal toxicity, drought, extreme temperatures, salinity and oxidative stress (21-25). The data obtained from these studies involved the application of NO using a nitric oxide donor and usually with an NO scavenger. In this study, the modulation of the enzymatic activity of APX by NO in soybean root nodules is investigated. APX is regarded as the most important amongst the peroxidases in H_2O_2 detoxification and catalyzes the reduction of H_2O_2 to water by utilizing ascorbic acid as its electron donor (26, 27).

Nitric oxide

Since the last decade, NO has been recognized as a novel biological messenger in both plants and animals. Initially, plant researchers considered this readily diffusible gas as a toxic compound from industrial waste and exhaust gas. However, this concept changed later-on in the late 1980's after the NO signaling role in regulating cardiovascular system was discovered. Further discoveries on NO were on its involvement in signal transduction pathways controlling neurotransmission, cell proliferation, programmed cell death (PCD) and host response responses to infection (28). In plant biology, advancement towards further studies on NO increased after the discovery of its role of in senescence and plant defense against pathogens (3, 4).

Nitric oxide chemistry

NO is a colorless gaseous free radical molecule and has good solubility in water (29). The diffusion coefficient of NO in solutions closely resembles those of oxygen (O_2) and superoxide (O_2^-), nevertheless due to its small stroke radius and neutral charge, this molecule can afford an easy intra-membrane and trans-membrane diffusion (30). NO does not undergo dimerism and this property contributes to its ability to possess a longer biological half-life, as compared with other free radicals (28). NO possesses an electron structure which allows it to exist in three redox-related forms. This includes the uncharged free radical (NO^\cdot) with an unpaired electron, the nitrosonium cation (NO^+) and nitroxyl anion (NO^-).

NO^\cdot reacts readily with atmospheric O_2 to form several compounds which include NO_2^\cdot , N_2O_3 , and N_2O_4 . These compounds serve as an intermediate, by either reacting with cellular amines and thiols or undergoing hydroxylation to form nitrite (NO_2^-) and nitrate (NO_3^-) (8, 31). NO^\cdot also reacts with O_2^- and H_2O_2 to form peroxynitrite (ONOO^-), a highly reactive and destructive anion. NO^\cdot reacts with iron found in heme or iron cluster containing proteins to form iron nitrosyl complexes. This causes in changes in the structure and functioning of target proteins such as seen by the activation of soluble guanylate cyclase (GC) and the inhibition of aconitases. Some toxic effects of NO are attributed partly to its reaction with transition metal-containing proteins, oxygen and its ability to form adducts with amines and thiols of different stability (32). NO^+ is involved in nitrosation, an electrophilic attack on reactive sulfur, oxygen, nitrogen, and aromatic carbon centers in proteins, with thiols being the most reactive groups. Whereas not much has been documented on the physiological importance of NO^- , some studies suggest that this molecule could act as the stabilized form of NO (33, 34).

Nitric oxide generation in plants

One major important function of NO is to activate various signaling pathways. Hence, it is crucial that during this process, the effect exerted by this molecule at the specific site would be both rapid and efficient. As much as the production of NO could be due to chemical synthesis (35), there is evidence that NO production is also as a result of enzymatic activity.

In animal systems, NO is predominantly generated by nitric oxide synthases (NOS; EC 1.14.13.39). There are three isoforms which have different localizations and functioning. These include endothelial NOS (eNOS) and neuronal NOS (nNOS) which are present constitutively. They function in vasodilation and cell communication respectively, whereas the inducible isoform (iNOS) functions in immune defense against pathogens (36).

In plants there are two major proposed sources of NO namely NO produced from the utilization of arginine in a reaction catalyzed by NOS, using O₂ and NADPH and NO produced from nitrite either non-enzymatically or by a reaction catalyzed by nitrite reductase (37).

The L-arginine-dependent nitric oxide production system

In analogy to animals, plants seem to have NOS enzymatic activity which catalyses the conversion of L-arginine into L-citrulline with a simultaneous release of NO, through an intermediate, hydroxyl-arginine (8). This is an NADPH-dependent reaction and also requires other co-factors such as Ca²⁺ and calmodium (38).

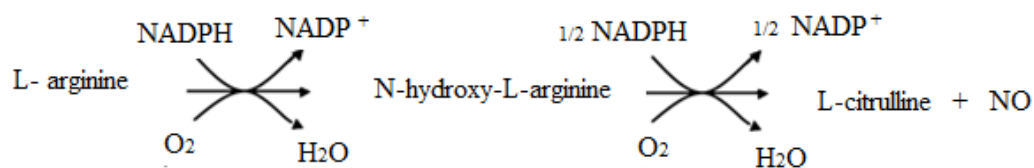


Figure 1-1. Reaction catalyzed by NOS : formation of citrulline and NO from L-arginine (38).

The schematic representation of the L-arginine-dependent NO production as shown in Figure 1-1, provides a convenient tool to investigate a possible similar NO production pathway in plants. The approach involves the use of compounds such as N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine acetate (LNMA), analogues of L-arginine which function as competitive inhibitors of animal NOS-mediated NO synthesis and thus treatment of plants with these inhibitors would imply the presence of NOS if it results in inhibition of NO synthesis. The presence of a gene encoding NOS in plants has been demonstrated previously but this gene was later shown not to be a NOS even though it influences NO production in plants (39, 40). Immune-gold labeling has been used to show that NOS-like enzymes were present in pea peroxisomes (41), however the cloning of a pea homologue of

NOS was not reported. Another immunological study (42) performed in maize roots and leaves using antibodies to mouse iNOS and rabbit nNOS indicated the presence of immune-reactive bands. A similar observation was recorded from a study (43) in pea leaves, where an antibody was raised against a synthetic peptide of the C-terminus of murine iNOS. There are other documented studies which have detected the NOS-like activity in roots and nodules of soybean (3), *lupines albus* (44) and in several other species such as tobacco (4). Nevertheless, efforts made to identify the genes encoding NOS proteins in higher plants have remained unsuccessful.

Two genes have been identified to have NOS-like activity in plants. The first enzyme was identified in tobacco as a virus infection-induced variant of the P protein of the mitochondrial glycine decarboxylase complex (GDC) and was designated as “plant iNOS”. The specific activity obtained from this study was however 30-times lower than obtainable in animals (45).

A seemingly breakthrough in plant NO research was the identification of a gene thought, albeit wrongly, to encode a nitric oxide synthase known as *Arabidopsis thaliana* Nitric Oxide Synthase 1 (AtNOS1) in *Arabidopsis* plants through sequence homology to NOS from the snail *Helix pomata* (46 , 47). This gene regulates growth and hormonal signaling and was thought to be the first *bona fide* NOS in plants. This gene encodes a 60 kDa protein and when expressed in *E. coli* caused an increase in NO synthesis in the *E. coli* cell extracts. When the corresponding *AtNOS1* was knocked out in *Arabidopsis*, the resulting mutant showed a low level of NO production in roots. Contrary to animal NOS (about 140 kDa), the much smaller AtNOS1 required no flavin or tetrahydrobiopterin, but only Ca^{2+} , CaM and NADPH. AtNOS1 seems constitutively expressed. Similarly to the variant of the P protein of the mitochondrial glycine decarboxylase complex, AtNOS1 does not have sequence similarities

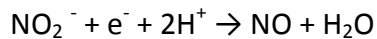
to any mammalian NOS (47). Progress towards identification of plant NOS had another setback after studies (48, 49) showed that AtNOS1 was a GTPase and not a NOS as initially suggested. This conclusion was drawn from the fact that the protein contains a GTP-binding domain and subsequently a GTPase activity without any NOS activity. There are suggestions that AtNOS1 interacts with other proteins to form a complex which can synthesize NO (50). Hence the protein was renamed *Arabidopsis thaliana* Nitric Oxide Associated 1 (AtNOA1). Irrespective of the intricate nature underlying the identification of the plant NOS, several studies are still ongoing in search of a true NOS in plants. One such study (51) characterized the sequence, protein structure, phylogeny, biochemistry, and expression of NOS from *Osterococcus tauri* (*O. tauri*). This is a unicellular species of green algae. The amino-acid sequence of *O. tauri* NOS identified from this study was shown to be comparatively similar to that of human NOS.

NOS-like activity localization in plants

Studies (52) using antibodies raised against animal NOS showed that a NOS-like protein was located in the cytoplasm of plant cells and subsequently translocated to the nucleus. These were the first documented occurrence of NOS-like immunoreactivity in plant cells. Plant NOS-like enzymatic activity was further investigated (53) and detected in the matrix of peroxisomes and in chloroplasts but not in the mitochondria of pea leaves. A more recent study also detected NOS-like enzymatic activity in peroxisomes from leaves and hypocotyls of sunflower (54). However, the findings obtained from these studies are in contrast with those of animals, where NOS activity had earlier been identified in the mitochondria (55, 56).

The nitrate/nitrite-dependent nitric oxide production

This is another enzymatic pathway for the generation of NO in plants by the use of nitrite as substrate.



The reaction shown above is catalyzed by nitrite reductase (NR) localized in various compartments of the cell such as the cytosolic nitrate reductase (cNR) (57) and a plasma membrane-bound NR (PM-NR) associated with a PM-nitrite: NO reductase that is root specific (58). In the reaction, nitrate is reduced to nitrite at the expense of NAD(P)H, and NR subsequently catalyzes a 1-electron transfer from NAD(P)H to nitrite, resulting in NO formation at an optimum pH 6.75 (59). Peroxynitrite is also produced simultaneously with NO by NR (57).

Evidence for NO production as a result of NR activity was first described in a study (60) which treated soybeans with herbicides. There are several recent studies (61-65) which confirm the involvement of NR in NO production. Work done on spinach and maize shows that NR-mediated NO production can be regulated by the phosphorylation status of the enzyme (66). A more recent study (67), described a diurnally opposite pattern to the wild type (low in day and high in night) of NO emission from plants constitutively expressing NR with a mutation where serine is replaced with aspartate (Asp). There is evidence on the involvement of the Ser residue in NR inactivation by phosphorylation. Replacement of Ser with Asp, which does not mimic phosphorylated Ser at the regulatory site in NR, was used in monitoring the regulation of NR by phosphorylation. Data obtained from this study indicate that the NR

activity in the mutant did not respond to changes in light/darkness that is otherwise observed in wild-type plants.

The NR-dependent NO generation, which occurs in the dark, is nitrite concentration-dependent and is possible only if the nitrite levels are higher than those of nitrates (68).

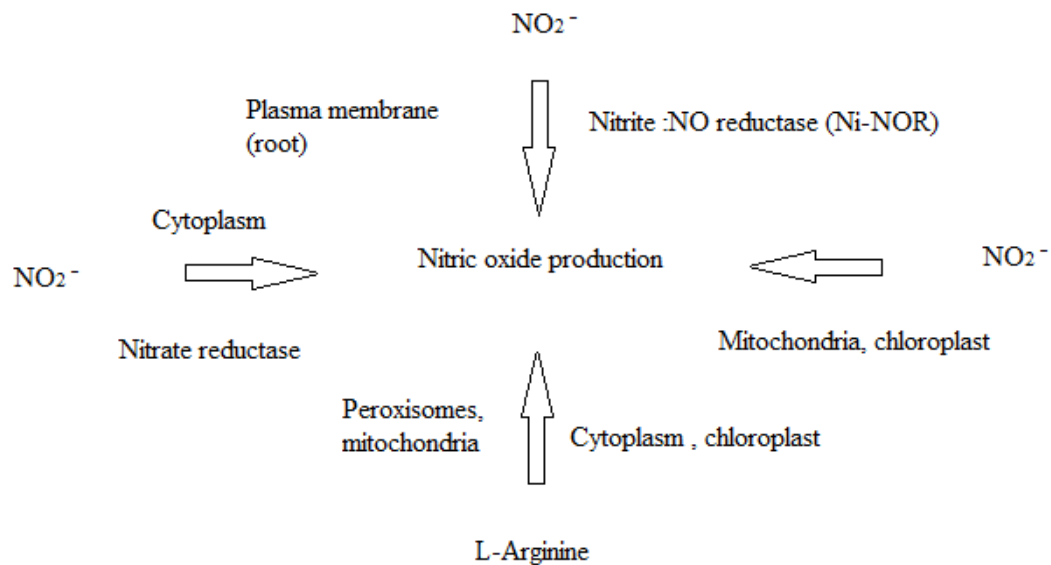


Figure 1- 2. Schematic representation of the various routes of NO production in plants cells (69).

Apart from enzyme-catalyzed NO production in plants, as shown in Figure 1-2, several non-enzymatic NO generation pathways have also been reported by several research groups. Tobacco mitochondria have been shown to reduce nitrite to NO (61) while ascorbic acid has been shown to reduce nitrite to NO and dehydroascorbic acid (DHAsA) (28). Soybean chloroplasts have also been shown to use either arginine or nitrite to produce NO (70), whereas carotenoids and light were reported to catalyze the production of NO from nitrites (71, 72). Furthermore, a reduction of nitrite to NO has also been shown to occur at low pH in the apoplast of barley aleurone cells (73).

Nitric oxide signaling in plants

In plants, NO is involved in several functions such as acting as a signaling molecule, mobilizing responses against stress and in defence against pathogens. Understanding the mechanism of action of NO has been a major interest of several researchers. The effect of NO is made possible through its direct and indirect interaction with several secondary messengers.

Direct effects of nitric oxide

NO can be directly involved in intracellular signaling, which eventually leads to some physiological changes that are mediated by events such as covalent post-translational protein modifications. Some of these modifications could also be as a result of a complex formed between NO and other reactive forms of nitrogen and oxygen. An example includes the reaction of NO with superoxide which leads to the formation of peroxynitrite (ONOO^-). This is a compound which can oxidize proteins at cysteine, methionine, or tryptophan residues or nitrate tyrosine residues to form nitrosyl tyrosine. The nitrosylation process is a reversible mechanism of direct NO effects on the cell (74, 75). Nitrosylation at cysteine residues is referred to as S-nitrosylation and that on glutathione is referred to as S-glutathionylation.

Indirect effects of nitric oxide

NO signaling in plant cells can be modulated indirectly when the effect is facilitated by its influence on other secondary messengers. The most commonly described pathways include the role in regulating the levels of guanosine 3, 5-cyclic monophosphate (cGMP), calcium ions levels, cADP ribose and MAPK kinase (76-78).

The presence of cGMP in plants has been validated by several mass spectrometry techniques (79, 80). In view of this, some studies have shown that cGMP is an NO signaling intermediate (81, 82). Further studies using exogenous application of NO, have shown an increase of cGMP levels both in tobacco and *Arabidopsis thaliana* (3, 83). The mechanism involves the activation of the sGC either by binding to the heme iron or by S-nitrosylating critical cysteine residues (84) which subsequently lead to the regulation of several cellular functions (61).

Another means of cGMP signaling is by binding and activating molecular targets. Although these targets have not been fully characterized, they are suggested to include cGMP-dependent protein kinases and cyclic nucleotide-gated ion channels (85). Some of the processes facilitated by cGMP include the induction of genes encoding chalcone synthase and ferredoxin NADP⁺ oxidoreductase and initiating anthocyanin biosynthesis in soybean (86).

NO also regulates signaling cascades by the mobilization of calcium ions (Ca²⁺). Ca²⁺ is an established and important intracellular secondary messenger in signaling cascades (87).

There are several documented studies on the inter-play between NO and Ca²⁺. NO has been shown to increase the level of free Ca²⁺ during osmotic stress in tobacco cells (88, 89). In a related study (90, 91) where NO donors were administered, an increase of intracellular Ca²⁺ was observed in *Vicia faba* and tobacco cells. A further study in tobacco indicates that the activation of defense genes by NO in tobacco is triggered by cGMP, and these genes are suggested to act through the action of cADPR which also regulates Ca²⁺ levels (92). Various data obtained from these studies (89-92) suggest that some effects of NO signaling are made possible via Ca²⁺-mediated pathways in plants.

NO can also act through cGMP-activated phosphates and protein kinases which include mitogen-activated protein kinases (MAPK's). Application of an NO donor has been shown to stimulate MAPK in both tobacco and *A. thaliana* leaves (93-95). Another study highlighting

MAPK as a target of NO action was demonstrated in cucumber. Here, the NO-dependent MAPK signaling cascade was shown to be activated during adventitious rooting induced by indole acetic acid (77). However, the mechanism underlying the activation of MAPK by NO has not been fully characterized. MAPK's have been shown to be involved in response to environmental and pathogens stress which results to signaling pathway leading to nuclear gene expression (96,97).

Physiological functions of nitric oxide in plants

The versatility of NO as a signaling molecule has prompted several investigations confirming its involvement in plant growth and development. There are several available commercial NO donors and they differ in their chemical structure, stability and factors promoting the release of NO such as temperature and pH level. This variation can lead to different biological effects and as such could be responsible for the variations obtained in results from studies using these NO donors. Another major point of consideration is the concentration of NO used in the various studies as the effect of NO on plant growth has been shown to be concentration-dependent (98). For instance, whereas exogenous application of high concentrations of NO donor inhibited growth in tomato, lettuce, and pea plants, application of low concentrations of NO stimulated growth (14).

Involvement of nitric oxide in plant growth and development

Studies utilizing treatment of either whole plants (99) or selected plant tissues such as roots (100) leaves (101) or shoot with NO donors have been used to demonstrate the role of NO in plant growth development. A low concentration of NO was able to increase the rate of leaf expansion in pea seedlings and similarly NO could also enhance the growth of tomato and

lettuce (102,103). Further studies (104) have also shown that NO possesses the ability to prolong the shelf life of some leaf fruits, vegetables and flowers. The underlying principle is thought to be the NO-dependent inhibition of ethylene accumulation. NO has also been shown to be involved in root development. This follows after studies (77,65) involving the use of NO donors such as sodium nitroprussiate (SNP) and S,N-acetyl penicillamine (SNAP) which induced the formation of adventitious and lateral roots in cucumber. This study further shows an increased endogenous NO level in plants after indole acetic acid (IAA) treatment (105).

Effect of nitric oxide on stomatal closure

The involvement of NO, apart from abscisic acid (ABA), as a regulator of stomatal closure has been documented (106). This role of NO is however linked with the presence of H₂O₂, a major component of ABA-induced stomatal closure (24,106). In another study (34), an increased endogenous level of NO was observed in peas and *Vicia faba* plants treated with abscisic acid. This increased level of NO is seen as a result of production from the NOS-like activity (48) that signals through protein S-nitrosylation (107), NR and Ca²⁺ sensitive ion channels (108) and is thought to influence the ABA-induced stomatal closure.

Effect of nitric oxide on chlorophyll content and photosynthesis

Chlorophyll is a porphyrin that constitutes the primary photoreceptor pigment for the process of photosynthesis in plants (109). It is produced in the chloroplast and is responsible for the green appearance of leaves, stems and green fruits before they ripen. NO donors such as SNP have been shown to increase the level of chlorophyll in potato, lettuce and

Arabidopsis (110). NO has also been shown to preserve chlorophyll in peas and potato (111). As such, the presence of NO ensures that the chlorophyll absorbs photons of light energy from a light source. However, the effect of NO directly on photosynthesis has not been fully elucidated, but several NO donors have been shown to differentially regulate the photosynthetic rate (110).

Effect of nitric oxide on seed dormancy

Under certain conditions, sown seeds are unable to germinate. This condition, referred to as dormancy, is as a result of a complex combination of factors including water, light, temperature, gas concentration, mechanical restriction, seed coat and hormone interactions. With the aid of nitrogen-containing compounds such as nitrate, nitrite, hydroxyl-amine and azide, the effect of dormancy can be reduced. The NO donor SNP has been shown to reduce seed dormancy in lettuce (4), Arabidopsis (112-114), and barley (110). These studies provide evidence that NO is involved in the regulation of seed germination.

Effect of nitric oxide on senescence

Plant senescence refers to a series of developmental events which are highly controlled and are characterized by several phenotypical changes such as loss of water, change in leaf colour, inhibition of flower formation and defoliation. Senescence is usually associated with ethylene production (115). Several studies have been carried out in order to investigate the anti-senescence property of NO. The results obtained from these studies shows that exogenous application of NO donor in pea leaves under senescence promoting conditions

decreased ethylene levels. A possible inference of this result is the inhibition of ethylene biosynthesis (14). A simple illustration suggesting the importance of NO in maintaining the post-harvest life of plant products relies on the observation that most unripe fruits contain high NO and low ethylene concentrations and the reverse is the case with ripening fruits (14).

Factors leading to stress in plants

Plant survival can be threatened and diminished as they are always bound to encounter stress conditions. Environmental stress could arise due to either biotic or abiotic factors. Biotic stress in plants includes stress conditions that arise due to infection, mechanical damage by herbivores or parasitism. On the other hand, abiotic stress results from negative impact exerted on the plant by a wide range of non-living factors such as water, radiation, temperature, and chemical stress.

Role of ROS and NO during abiotic stress in plants

The resulting effect exerted by the various abiotic stresses is molecular damage to plant macromolecules, ultimately perturbing metabolism and physiological functioning. This is often a result of the excessive production of ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl (OH) radical (116). The production of ROS is most commonly at the mitochondria, peroxisomes and chloroplast (117). There are several reactions proposed to account for the mechanism by which ROS levels could lead to the damage of essential plant biomolecules. ROS react with disulphide bonds in proteins. During this reaction, more radical moieties are generated, which leads to auto-oxidation of the protein. The oxygen radicals can also attack the deoxyribose moiety of DNA and,

subsequently, the sugar backbone of the DNA molecule is left with a non-coding gap and this leads to a strand break. ROS also react with poly-unsaturated fatty acids (PUFA) and form a carbonyl radical which initiates a chain reaction of lipid peroxidation. The resulting effect is membrane leakage, disintegrated membrane and eventual loss of membrane integrity (118). The formation of ROS is initiated when molecular oxygen accepts a single electron after which further reduction of the molecule to water occurs through a subsequent series of univalent electron transfers. The oxygen intermediates produced are the major cause of hazard to the cell (119). The first electron reduction reaction forms the O^{2-} molecule which interferes with metabolic processes due to its ability to reduce oxidized transition metal-ions present in protein. Apart from reducing transition metals, O^{2-} can also reduce unchelated bivalent cations. This leads to the formation of H_2O_2 , and can also be reduced by O^{2-} to the biologically dangerous hydroxyl radical (HO^\cdot).

Although ROS cause oxidative damage, some studies (120,121) have shown that basal level of ROS is required for normal plant physiological processes. Hence it is necessary that plants tightly control the concentration of ROS (122).

Several studies have shown that NO is induced by several abiotic factors and regulates plant response to abiotic stress (123). A few studies suggest NO as a stress inducing agent (124); this could however be as a result of the type or concentration of the NO donor used in the study, given that other studies have validated the protective role of NO against oxidative stress. The ability of NO to exist as a reactive free gaseous molecule enables it to scavenge other reactive intermediates. The protective property of NO against oxidative stress is thought to be based on its ability to directly or indirectly scavenge ROS. NO can react with lipid radicals and stop the propagation of lipid oxidation (125) and can also scavenge O^{2-} to

form ONOO^- . ONOO^- is a strong oxidant and is one of the major toxic reactive nitrogen species (32). It is extremely toxic to animal cells but not toxic in plant cells as its effect can be neutralized by ascorbate and glutathione (126, 127). Another mechanism by which NO protects the plant from oxidative stress is through its ability to act as a signaling molecule in a series of events which subsequently leads to changes in gene expression (128). Studies investigating the role and mechanism of NO in plant abiotic stress response using exogenous NO donor reported its ability to either neutralize the toxic effect of ROS generated by chemical stressors in potato and rice (129,130) or block ROS production in wheat seed (131). Further studies show that NO does not only reduce the oxidative stress by reacting directly and reducing the levels of ROS but can also change the activities of ROS-scavenging enzymes (132,133).

Effect of salinity on plants

Salinity is regarded as one of the major factors that affects worldwide agricultural yield. High saline soil could arise naturally as a result of poor irrigation management. Generally, plants could either be salt tolerant (halophytes) or sensitive (glycophytes); however the halophytes are relatively rare whereas most crops fall under glycophytes. Salt stress leads to the lowering of water potential, ion imbalance such as the toxicity of either Na^+ or Cl^- absorbed and interference with the uptake of essential nutrients (134,135). Other events such as membrane disintegration, cellular accumulation of ROS (a major cause of injury at cellular level during salinity stress) and inhibition of photosynthesis subsequently lead to plant death (136-139).

Legumes are considered sensitive or moderately sensitive to salt stress and as a result, there is a decline in legume yield under conditions of salinity. Several studies have shown the effect of salinity on legumes (140). These studies show that salinity reduces nitrogen fixation in legumes (141). High salt levels cause inhibition of root hair growth and decrease in the number of nodules per plant. Various studies have shown that salinity also reduces symbiosis, which results to low plant yield (142). Furthermore, both nitrogen fixation and nodule respiration are greatly reduced when legume plants are grown under saline conditions (143).

The morphologic effect exerted on plants arising from salinity is retarded growth due to inhibition of cell elongation (144) and a general reduction in growth parameters (145,146).

Influence of NO on salt stress

Several studies using the application of NO donors either on whole plants or cell cultures have demonstrated the involvement of NO in inducing tolerance against salinity. Application of SNP resulted in a decrease in the effect of salt stress in seedlings of rice, lupin and cucumber (147-149). In other similar studies, SNP under salinity stress was able to enhance seedling growth and increase the dry weight of maize and *Kosteletzkya virginica* seedlings (150,151,22). Although there is little known on the mechanism behind NO signaling network to induce tolerance against salinity, there is evidence from various studies that NO exerts its function by increasing the Na^+/K^+ ratio. This ratio is however dependent on the increased plasma membrane (PM) H^+ -ATPase as well as vacuolar H^+ -ATPase and H^+ -pyrophosphatase activities (22, 150, 152). This postulation is supported by studies which reported the induction of the expression of PM H^+ -ATPase in plants and to enhance salt tolerance of calluses under salinity in the presence of NO (153). The induction of salinity tolerance was

achieved through an increase in the K^+/Na^+ ratio—a process mediated by H_2O_2 and dependent on the increased plasma membrane H^+ -ATPase activity (154). Thus, it can be suggested that the NO-mediated regulation of Na^+ homeostasis and K^+ acquisition through increased expression of plasma membrane Na^+/H^+ antiporter and H^+ -ATPase-related genes plays a vital role in the salt tolerance mechanism in plants (155).

Plant protective response to abiotic stress

It is evident that plants are continuously exposed to environmental stress and thus are bound to face ROS. Apart from the NO mediating effect during abiotic stress, plant cells and organelles can also employ an antioxidant system. This includes a vast range of enzymatic and non-enzymatic antioxidants that functions in scavenging the reactive oxygen species (156-158) as shown in Figure 1-3.

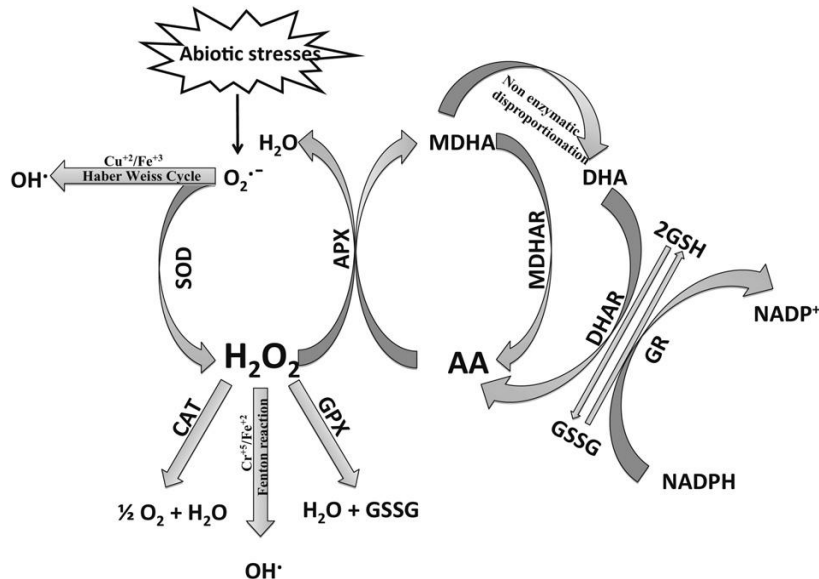


Figure 1-3. ROS and various antioxidant defense mechanisms (156).

Non-enzymatic antioxidants

The most abundant non-enzymatic antioxidant is ascorbic acid (AA), which has the ability to donate electrons to enzymatic and non-enzymatic reactions, making it one of the most powerful antioxidants (159,160). It can be found in all plant tissues but much higher in photosynthetic cells and meristems (19). Apart from its influence in protecting membranes by directly scavenging the O_2 and OH^- , AA is also important for the operation of the ascorbate-glutathione (AsA–GSH) pathway, regeneration of α -tocopherol and zeaxanthin and the pH-mediated modulation of PS II activity (161).

Another important metabolite found in plants that can directly detoxify ROS is the tri-peptide GSH. GSH mostly occurs in the reduced form in plant tissues and several cell compartments (162,163). It is also suggested to play a vital role in the detoxification of heavy metals (164). A study performed on leaves and chloroplast of *Phragmites australis*, reported a high antioxidant activity due to an accumulation of GSH which helped to protect photosynthetic enzymes against the thiophilic bursting caused by cadmium (165). Other reported functions of GSH include its role in growth and development, cell death and senescence, response to pathogens and enzymatic regulation in plants (166).

Plants also possess other compounds with antioxidant properties and these are involved in protecting membranes from oxidative damage caused by ROS. Tocopherols which are localized in the thylakoid membrane of chloroplasts are also regarded as antioxidants and they function in maintaining membrane stability and scavenging of singlet oxygen and lipid radicals (167). Flavonoids which are categorized into flavonols, flavones, isoflavones, and anthocyanins on the basis of their structure, are regarded as potent ROS scavengers. Under environmental stressful conditions flavonoids neutralize ROS before they cause oxidative

damage to cells (168). Certain pigments found in plants such as carotenoids also serve as antioxidants and helps to detoxify ROS in the plant (169).

Enzymatic antioxidants

In an effort to abate the damaging effect resulting from the accumulation of ROS during abiotic stress, plants mobilize a coordinated activity of several antioxidant enzymes. This antioxidant enzyme system includes superoxide dismutase (SOD), catalase (CAT), the components of the AsA-GSH cycle and glutathione peroxidases (GPX).

Superoxide dismutase (SOD)

SOD forms the first line of defense against ROS. This enzyme plays a pivotal role within the antioxidant network as it is solely responsible for the removal of O_2^- , the first ROS formed. It catalyzes the dismutation of O_2^- into H_2O_2 and O_2 (170). Based on the metal co-factor used by the enzyme, plant SODs are classified and identified into three classes which include Mn-SOD, Fe-SOD, and Cu/Zn-SODs (171). These enzymes are localized in different cellular compartments such as mitochondria, chloroplasts, glyoxysomes, peroxisomes, apoplast and the cytosol (172,173). Several studies have shown an increased SOD activity under salt stress in various plants such as mulberry (174) *CicerArietinum* (175) and *Lycopersicon esculentum* (176). Further studies on the effect of salinity and drought on *Glycyrrhiza uralensis* reported an increased SOD activity (177).

Catalase (CAT)

H₂O₂ formed from SOD activity, can be directly converted into H₂O and O₂ by the enzyme catalase. The activity of this tetrameric heme-containing antioxidant enzyme is crucial for ROS detoxification during stressed conditions (178). This enzyme is also able to react with and detoxify other hydroperoxides such as methyl hydroperoxide (179). Catalases (CATs) are mostly found in peroxisomes and glyoxysomes, although a specific isozyme, Cat3, is present in maize mitochondria (180).

The ascorbate-glutathione cycle

The enzymes in the ascorbate-glutathione cycle (181) include ascorbate peroxide (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). This system forms an efficient enzymatic defense system for the detoxification of ROS. Amongst the enzymes involved in this cycle, APX is thought to play a vital role as it scavenges H₂O₂ by utilizing AsA as its electron donor, thus protecting the cells from oxidative damage (182). In this cycle, AsA is converted to monodehydroascorbate (MDHA), which is spontaneously converted to dehydroascorbate (DHAsA), the final AsA oxidation product. MDHA can be reduced back to AsA by an NADH-dependent MDHA reductase (MDHAR). AsA can also be regenerated through a coupled reaction which involves dehydroascorbate reductase (DHAR) and an NADPH-dependent glutathione reductase. The last step of the cycle is when the oxidized glutathione (GSSG) is converted to its reduced form by NADPH-dependent glutathione reductase (GR). This step is essential in protection against oxidative stress, as it provides the reducing power into the antioxidant network (183). In this cycle, the ability of APX to remove H₂O₂ and the continuous maintenance of

cellular redox balance through regulation of the AsA and GSH pool is a major contributing factor to efficient ROS detoxification in plants. Some studies have reported a complete AsA-GSH cycle in chloroplast (184), peroxisomes, mitochondria (185) and cytosol (186-188).

Glutathione peroxidase

The glutathione peroxidases (GPXs) are large families of isozymes that also help prevent the damaging effect of excessive ROS during abiotic stress. These enzymes use GSH as a reductant to detoxify hydrogen peroxide, lipid hydroperoxides and alkyl hydroxyls and therefore protect plant cells against oxidative stress (189). A study (190) reported that salinity stress significantly increases GPX activity in *L. esculentum* Mill. cv “Perkoz” roots.

Importance of nodule redox balance in soybean physiology and growth

Leguminous plants such as soybeans are cultivated mostly for their seeds and as dairy substitute. Compatible rhizobia infect the root of this plant and lead to the development and formation of specialized root structures known as nodules (191). Nitrogen fixation in soybeans like other legumes occurs in these structures. Various processes that lead to ROS generation in nodules include oxidation of enzymes such as ferredoxin, autooxidation of leghemoglobin and electron carriers in mitochondria (192). The antioxidant enzymes and metabolites play a crucial role in the removal of ROS, symbiosis efficiency and promote nodule formation (193,194).

Structure and enzymatic properties of ascorbate peroxidase enzyme in soybeans nodules

Apart from higher plants, APX also occurs in algae (189), some cyanobacteria (190) and insects (195). Plant APXs are intracellular enzymes encoded in the nucleus and are abundant in root nodules of legumes, making up to 1% of the total protein content in the nodules (196). Soybean nodule APX has been the major subject of numerous biochemical studies (194). Its physiological role in scavenging ROS, with more affinity for H_2O_2 than catalase (197), makes it an important enzyme in plants during abiotic stress in consideration of the fact that abiotic stress causes elevated H_2O_2 levels in plant cells. The catalytic activity of this enzyme is as a result of the presence of two histidine (His) residues (198), namely His-42 and His-163 (Figure 1-4). His-42 is located on the distal side of the heme cavity whereas His-163 lies on the proximal end and forms the axial heme ligand connected to the heme iron.



Figure 1-4. Structure of the active site of APX with its substrate ascorbate. Amino acid residues forming hydrogen bonds with the substrate – green; residues responsible for binding of K^+ – blue; proximal and distal histidine – violet; residues near the distal histidine – orange; heme – yellow; ascorbate – light green (198).

Other residues around the distal histidine are Arg-38, Leu-39, Ala-40 and Trp-41 (198). One of the major distinguishing structural feature between APX and other plant peroxidases belonging to class III is the presence of a tryptophan residue at position 41 instead of phenylalanine (198,199). APX binds a single K^+ ion to the proximal domain and this ion is essential for its activity. APX activity can be lost in the absence of its electron donor. However it can be protected by other electron donors although their oxidation rates by the enzyme reaction are low (200). APX activity is inhibited by thiols and this inhibition is dependent on the presence of H_2O_2 (201).

Summary

During abiotic stress generated by various environmental factors, there is overproduction of ROS which consequently leads to oxidative stress. Although these ROS may be required for normal physiological functions, they acts as a double edge sword as their excessive level also causes oxidative damage to nucleic acids, lipids and proteins in plants. Plants have evolved an efficient antioxidant defensive system in order to protect and detoxify ROS. The antioxidant defense system includes a series of non- enzymatic metabolites and several antioxidant enzymes. Ascorbate peroxidase is regarded as one of the major scavengers of H_2O_2 . Its unique molecular properties and higher affinity for H_2O_2 makes it efficient and vital in the removal of this ROS by utilizing ascorbate as its electron donor. Several studies have described the role of Nitric oxide in diverse physiological processes in plants. However there is still scope for investigating the relationship between nitric oxide and APX as only limited data exists on this relationship.

Aims and objectives

Nitric oxide (NO) is a well-known signalling molecule that functions in several growth and physiological processes in plants. Although there are reports on the role of NO in enhancing antioxidant enzymatic activities, studies on its effect in regulating the activity of the various ascorbate peroxidase isoforms have not been reported. In view of the fact that there are vast areas of saline soil in South Africa and globally and there are a few studies describing the role of exogenous application of NO in ameliorating and improving tolerance to salt stress in soybean, this study aims at:

1. Determining the effect of exogenous application of a nitric oxide donor (2,2'-(hydroxynitrosohydrazono) bis-ethanimine) on
 - a. Nodule NO content
 - b. Ascorbate peroxidase activity
 - c. Nodule H₂O₂ content
2. Evaluating the effect of short-term salinity stress on soybean and if exogenous supply of NO could ameliorate the toxic effects of short-term salinity exposure, by analyzing its effect on inducing antioxidant enzyme activity and maintaining antioxidant metabolite ratios.
3. Determining the effect of exogenous supply of NO in improving tolerance to long-term salinity induced stress by evaluating its effect on ascorbate peroxidase activity and growth parameters.

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Chapter Two

Investigation of the effect of nitric oxide on the enzymatic activity of ascorbate peroxidase in soybean root nodules

Summary

Ascorbate peroxidase (APX) is one of the major enzymes that regulate the levels of H_2O_2 in plants and plays a crucial role in maintaining root nodule redox status. The aim of this study was to use mature and non-senescent nitrogen fixing root nodules from soybean plants to analyze the effect of exogenously applied nitric oxide (NO), generated from the NO donor DETA/NO, on the total enzymatic activity of soybean root nodule ascorbate peroxidase and the examine the effect of the exogenously applied NO on the enzymatic activity of distinct ascorbate peroxidase isoforms. Exogenously applied NO caused an increase in ascorbate peroxidase enzymatic activity. Further analysis of ascorbate peroxidase enzymatic activity identified three ascorbate peroxidase isoforms for which augmented enzymatic activity occurred in response to NO.

Proviso/Acknowledgement

The growth of plants, treatment of plants with DETA/NO (nitric oxide donor) or DETA (control for DETA/NO), protein extraction from root nodules, measurement of total ascorbate peroxidase enzymatic activity by spectrophotometry and the determination of effect of the various treatments (DETA/NO and the corresponding controls) on the enzymatic activity of ascorbate peroxidase isoforms by native in-gel electrophoresis/activity staining assays were done by me (Ifeanyi Moses Egbichi). However, Mr Alex Jacobs assisted

me in the feeding of the plants with nutrient solution and the application of DETA/NO to the plants. The measurement of nitric oxide content and the analysis of the pixel intensities on the native in-gel activity staining assays were done by Dr Marshall Keyster. The measurement of H₂O₂ content was done by Mr Ashwil Klein and all statistical analyses were done by both Dr Marshall Keyster and Mr Ashwil Klein. The work on NO content, H₂O₂ content and pixel intensity analysis of the native in-gel assay results is included in this thesis only because it is linked to the rest of the work that I did and is essential for sensible interpretation of the data from my work and brings appropriate perspective to the work done by me in this chapter.

Introduction

Nitric oxide (NO) was previously considered to be just a toxic gas but this concept has changed after several studies demonstrated the influence of NO in normal physiological processes in animals (1, 2). In plants, there is evidence validating the involvement of this gaseous signaling molecule in plant growth and development (3, 4), defense signal against pathogen infection (5, 6) and induction of programmed cell death (7,8). Unlike other free radicals, NO has a longer biological half-life. It has been suggested that the bioactivity of NO is concentration-dependent (9). Furthermore, NO can either be protective or toxic in different cell types, depending on its concentration (10).

The generation of NO in plants can be through enzymatic and non-enzymatic processes (11-14). The enzymes involved include nitric oxide synthase (NOS, EC 1.14.23.39) and nitrate reductase (NR, EC 1.6.6.1). The NOS enzyme is involved in the oxygen- and NADPH-dependent catalytic oxidation of L-Arginine to form L-citrulline and NO (15). This enzymatic reaction has been reported in extracts from peas (16), soybean and several other plants (17-

19). Genes previously thought to encode NOS in plants turned out not to be *bona fide* NOS (20-23). NR is another enzyme involved in the production of NO in plants (24) and depends on NAD(P)H for its electron source for the conversion of nitrite to NO (25). Studies utilizing different experimental conditions have validated involvement of NR in NO generation in plants (24, 26-28). The role of NO as a signaling molecule during abiotic stress conditions has been a subject to several studies. An increase in NO levels, caused by different stress conditions, suggests its role in maintaining plant redox homeostasis and alleviating oxidative stress (16, 23, 29, 30).

Accumulation of ROS such as the superoxide radical (O_2^-), hydroxyl radical (OH^\bullet) and hydrogen peroxide (H_2O_2) to levels in excess of the content that is required for protective cellular signaling can lead to cell death via a necrotic or programmed cell death (PCD) pathway in plant tissue (31-35). However, a role for ROS in nodule development and functioning has been suggested because basal level of ROS appear to be essential for initiating nodule developmental processes and maintaining nodule functioning (36, 37). It is thus reasonable to expect that a steady-state level of ROS would be crucial for the development of functional nodules and the maintenance of such functioning. However, any significant excess to this critical level could trigger oxidative stress and result in poor nodule functioning.

Plants are equipped with several enzymatic and non-enzymatic systems that help to neutralize and detoxify oxygen radicals and their intermediates in the cells. Ascorbate peroxidase (APX, EC 1.11.1.11) is a key enzyme regulating the level of H_2O_2 in plants through oxidation of ascorbate (AsA) to reduce H_2O_2 into H_2O (38-41). This activity of APX also leads to the formation of monodehydroascorbate (MDHA) which is spontaneously converted to ascorbate and dehydroascorbate (DHAsA). One of the characteristics that distinguish APX from other antioxidant enzymes is its high affinity for H_2O_2 - a factor which indicates its

crucial role in modulating the levels of H_2O_2 in cells (42). Various studies have shown that NO influences plant APX activity. However the result presented in these studies are contradictory in that when one of the studies shows inhibitory activity against APX in response to NO, exogenously applied in the form of various NO donors (43), the other shows that NO (accumulated in response to elevated H_2O_2) induces APX activity (44).

Although there are contradicting reports on the effect of NO on APX activity, studies on the effect of NO on various isoforms of APX have not been reported. This chapter aims at investigating the effect of exogenously applied NO on soybean (*Glycine max* L. merr. cv. PAN 626) root nodule NO content, APX total enzymatic activity and H_2O_2 content. Other aspect of interest examined includes the effect of exogenously applied NO on the enzymatic activity of APX isoforms in soybean root nodules using in-gel assays.

Materials and Methods

Materials

Glycine max L. merr. cv. PAN 626 seeds used for this work were kindly donated by Pannar (Greytown, South Africa) and all the chemicals were purchased from Sigma-Aldrich, unless otherwise stated. The Rhizobium inoculum *Bradyrhizobium japonicum* as the commercial peat-based HiStick 2 Soybean Inoculant was donated by Becker Underwood Ltd., West Sussex, United Kingdom. The filtered silica sand (98% SiO_2) was purchased from Rolfes® Silica (Pty) Ltd, Brits, North West, South Africa.

Methods

Plant Growth

Soybean 626 seeds were surface-sterilized in 0.35% v/v sodium hypochlorite for 10 minutes, followed by five washes with sterile distilled water. The seeds were imbibed in sterile distilled water for 1 hour and inoculated with *Bradyrhizobium japonicum* (commercial peat-based HiStick 2 Soybean inoculant). Seeds were sown in sand that had been pre-soaked in distilled water, in 15 cm diameter plastic pots (one plant per pot). The sand was kept moist by watering only with distilled water during germination.

The germinated seedlings were grown on a 25/19 °C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ during the day phase, in a randomized design. Once the plants reached the VC stage (when unifoliolate leaves are fully expanded), they were supplied with nitrogen-free nutrient solution containing 1 mM K_2SO_4 , 2 mM MgSO_4 , 3 mM CaCl_2 , 1 mM K_2HPO_4 buffer at pH 7.3, 25 μM H_3BO_3 , 2 μM MnSO_4 , 2 μM ZnSO_4 , 2 μM CuSO_4 , 2 μM Na_2MoO_4 , 0.1 μM CoSO_4 , 50 μM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3 at a three day intervals until they reached the V3 stage (third trifoliolate).

Treatment of Plants

Plants of the same phenological stage and similar height were selected for all experiments. The treatment was performed once the plants were at the V3 stage. The plants were chosen

randomly and divided into four groups. The first group, treated with nitrogen free nutrient solution only, served as the untreated. The second and third groups were treated with nitrogen free nutrient solution containing 0.5 μM and 10 μM 2,2'-(hydroxynitrosohydrazono) bis-ethanimine (DETA/NO) (NO donor) respectively. The fourth group was treated with nitrogen free nutrient solution containing 10 μM diethylenetriamine (DETA), which is chemically similar to DETA/NO but lack the NO moiety thus serves as a negative control. The treatment was performed for a time period of 24 hours and the root nodules were harvested immediately at the end of the treatment period.

Protein extraction from nodule tissue

Extracts were obtained from soybean root nodules by grinding the nodule tissue into a fine powder in liquid nitrogen and homogenizing 500 mg of the tissue with either 1 ml of homogenizing buffer [40 mM K_2HPO_4 , pH 7.4, 1 mM ethylene di-amine tetra-acetic acid (EDTA), 5% (w/v) poly vinyl pyrrolidone (PVP) molecular weight = 40,000] for determination of NO content and APX enzymatic activities or 10% trichloro acetic acid (TCA) for measurement of H_2O_2 content. The resulting homogenates were centrifuged at 12,000x g for 20 minutes and the supernatants were used for biochemical assays.

Measurement of NO content

The NO content was measured by using a haemoglobin-based assay as previously described by Murphy *et.al.* (45). Plants extracts were incubated with 100 Units of catalase and 100 U of superoxide dismutase for 10 minutes, followed by addition of oxyhaemoglobin to a final

concentration of 10 μM . The mixture was incubated for 2 minutes, followed by spectrophotometric measurement of NO content by following the conversion of oxyhaemoglobin to methemoglobin based on absorbance values read at 401 and 421 nm.

Determination of APX enzymatic activity

Plant APX activities were measured in nodule extracts by modifying a method previously described by Asada (46). The nodule extracts which were supplemented with ascorbate to a final concentration of 2 mM, were added to the assay buffer containing 50 mM K_2HPO_4 , pH 7.0, 0.1 mM EDTA and 5 mM ascorbate. The reaction was initiated by adding 1.2 mM H_2O_2 in a final reaction volume of 200 μl and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. For the determination of the response of *Glycine max* APX isoforms to exogenously applied NO, electrophoretic APX separation was carried out as previously described by Mittler *et al.*, (47) and non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed at 4°C in 7.5% polyacrylamide mini gels. Prior to loading extracts containing 50 μg of protein into the wells, gels were equilibrated with running buffer containing 2 mM ascorbate for 30 minutes at 4°C . After the electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 minutes and then transferred to a solution containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM ascorbate and 2 mM H_2O_2 for 20 minutes. The gels were washed in the buffer for a minute and submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N',N' tetramethylethylenediamine (TEMED) and 2.5 mM nitroblue tetrazolium (NBT) for 10-20 minutes with gentle agitation in the presence of light. The gel images were captured

and analyzed by densitometry using AlphaEase FC imaging software (Alpha Innotech Corporation).

Measurement of H₂O₂ content

H₂O₂ content was determined in the nodule extracts by modifying a previously described method by Velikova *et al.*, (48). *Glycine max* nodule tissue (100 mg) was ground to fine powder in liquid nitrogen and homogenized in 400 µl of ice-cold 6% (w/v) trichloroacetic acid (TCA). The extracts were centrifuged at 12,000x *g* for 30 minutes at 4°C and 50 µl of the supernatant were used to initiate the reaction in a mixture (total volume of 200 µl) containing 5 mM K₂HPO₄, pH 5.0 and 0.5M KI. The reaction was incubated at 25°C for 20 minutes and absorbance readings were recorded at 390 nm. H₂O₂ content was calculated using a standard curve based on the absorbance ($A_{390\text{ nm}}$) of H₂O₂ standards.

Determination of protein concentration

Protein concentrations for all assays were measured in the extracts as instructed for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories).

Statistical analysis

One-way analysis of variance (ANOVA) test was used for all data to evaluate statistical validity of the results and means were compared according to the Tukey-Kramer test at 5% level of significance, using Graph Pad Prism 5.03 software.

Results

Soybean root nodules were treated with varying concentration (5 μ M and 10 μ M) of NO donor DETA/NO for 24 hours at the V3 stage to assess the effect of the NO donor on the NO level in the root nodules and on APX catalytic activity. The effect of the exogenously supplied DETA/NO was compared against untreated samples as well as which were supplemented with DETA - an appropriate control for DETA/NO that is chemically and structurally similar to DETA/NO but lacks the NO moiety.

Effect of DETA/NO on NO content in soybean nodules

Apart from the outlined pathways of NO biosynthesis in plants, an elevation of NO can be achieved by pharmaceutical approaches through application of an NO donor. Exogenously applied NO donor would release NO which can diffuse readily through plant tissue. This study investigated if application of DETA/NO altered the level of nitric oxide in soybean root nodule tissue upon application of the donor to nodulated soybean.

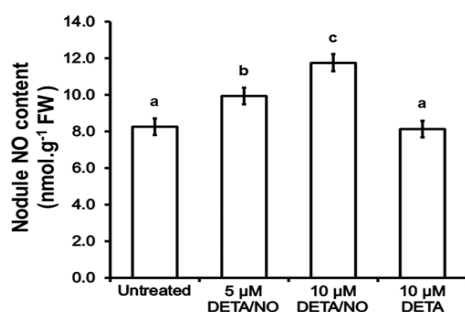


Figure 2-1. Nitric oxide content in soybean nodules as measured after treatment of nodulated soybean with the NO donor DETA/NO at final concentrations of 5 and 10 μ M or DETA (negative control for DETA/NO) at a final concentration of 10 μ M. Error bars represent the mean (\pm SE; $n = 3$) from data that are representative of three independent experiments.

As shown in Figure 2-1, application of DETA/NO caused an increase of NO content in soybean root nodules in a concentration-dependent manner. The DETA (10 μ M) treatment did not alter the nitric oxide content in the soybean root nodule and this is evident as there was no significant difference between the DETA treated plant nodule and the untreated plants. This indicates that the increase in NO level is as a result of the NO released by the NO donor DETA/NO.

Effect of DETA/NO on total APX enzymatic activity in soybean root nodules

Based on the observation that exogenous application of DETA/NO could increase NO content in soybean root nodules, it was hypothesized that this NO accumulation may lead to increased antioxidant enzyme activity. Therefore the effect of NO, which is released from the exogenous application of DETA/NO, on APX activity in soybean root nodules was determined although conflicting evidence show both inhibitory and stimulatory effects of nitric oxide donors on APX enzymatic activity (43, 44).

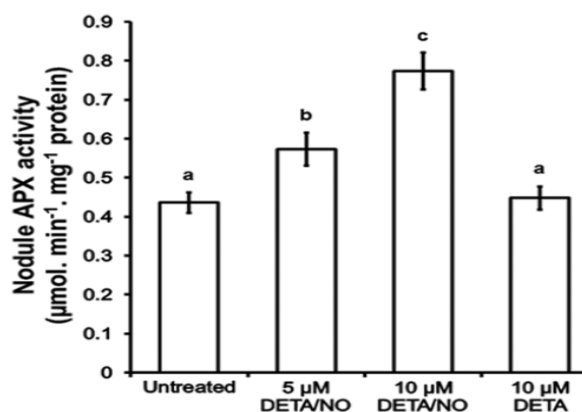


Figure 2-2. Nodule APX total enzymatic activity in response to treatment with various concentrations of DETA/NO or 10 μ M DETA, as measured by a spectrophotometric APX assay. Error bars represent the mean (\pm SE; n= 3) from data that are representative of three independent experiments.

DETA/NO was able to up-regulate total APX enzymatic activity in soybean root nodules as shown in Figure 2-2. However, no significant differences in APX activity were observed in response to treatment with 10 μ M DETA compared to the APX activity of untreated controls. Furthermore, the NO-induced increase in APX activity was 30% higher in response to 5 μ M DETA/NO than the controls, whereas it was approximately 75% higher in response to 10 μ M DETA/NO when compared to the controls.

Estimation of H₂O₂ content in soybean root nodules

It has been established that accumulation of H₂O₂ in plant cells leads to the occurrence of oxidative stress. Since exogenous application of NO (as DETA/NO) induced an increase in APX enzymatic activity, the relevance of this increased enzymatic activity in view of scavenging H₂O₂ was evaluated. Hence, the effect of application of DETA/NO on soybean root nodule H₂O₂ content after 24 hours was investigated.

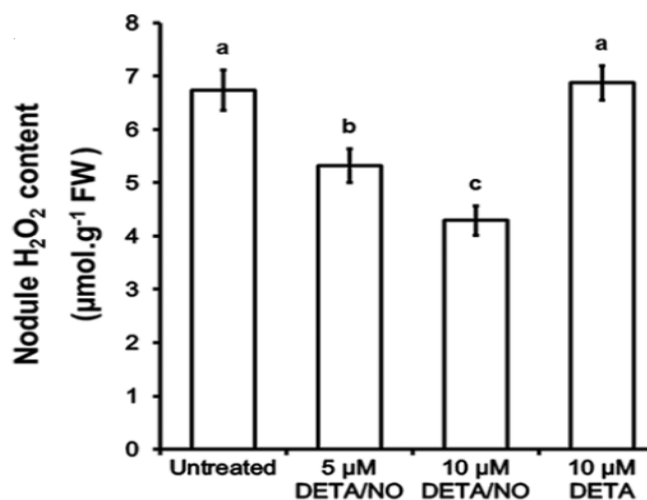


Figure 2-3. Effect of exogenously applied NO (as 5 and 10 μ M DETA/NO) or DETA (10 μ M) on soybean root nodule H₂O₂ content. Error bars represent the mean (\pm SE; n= 3) from data that are representative of three independent experiments.

Application of DETA/NO caused a reduction in H_2O_2 content of soybean root nodules as shown in Figure 2-3. H_2O_2 content was reduced by approximately 25% in response to 5 μM DETA/NO when compared to controls and was reduced by 40% in response to 10 μM DETA/NO when compared to untreated controls. Application of 10 μM DETA had no significant effect on the level of soybean root nodule H_2O_2 content when compared with untreated controls.

Determination of the effect of exogenously applied NO on APX isoforms

In the presence of H_2O_2 , APX prevents the formation of formazan resulting from H_2O_2 -dependent oxidation of ascorbate and then forming an achromatic band on a blue-purple background indicative of APX activity. In order to investigate the effect of NO on individual APX isoforms, native gel electrophoresis was carried out.

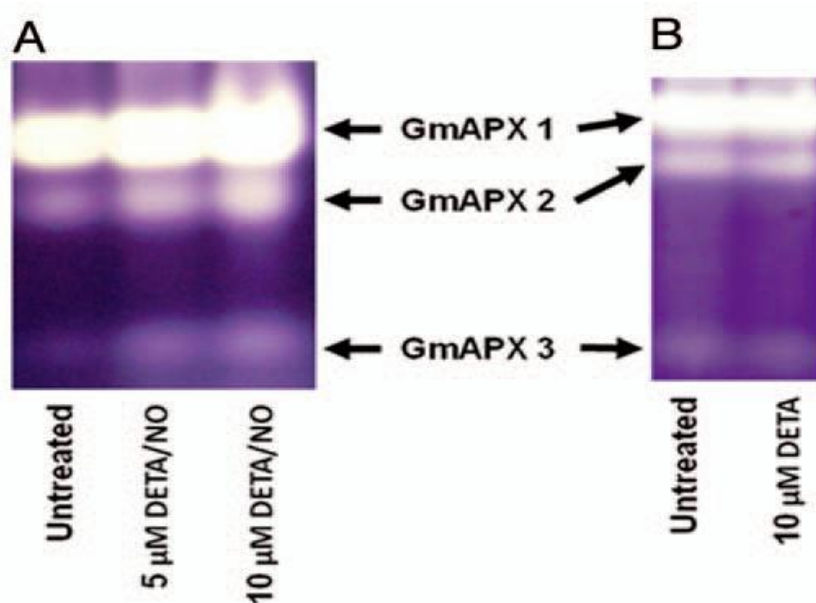


Figure 2-4. In-gel assay for nodule APX activity after treatment with 5 and 10 μM DETA/NO (A) or 10 μM DETA (B). The in-gel assay shows responses of different soybean root nodule APX isoforms to DETA/NO or DETA as indicated.

As shown in Figure 2-4A, NO up-regulated the enzymatic activities of three APX isoforms and this was observed on the basis of their different bands intensities. However the APX activity bands from samples treated with DETA appeared to have similar intensity as those from untreated samples (Figure 2-4B). The three APX isoforms were named as GmAPX 1, GmAPX 2 and GmAPX 3 based on their migrating distance. This result shows that application of NO as DETA/NO increases the activity of individual soybean APX isoforms.

Densitometric analyses of activity gels showed that the enzymatic activity of GmAPX 1 is upregulated by approximately 11% in response to 5 μ M DETA/NO and approximately 21% in response to 10 μ M DETA/NO (Figure 2-5A) when compared to GmAPX 1 enzymatic activity of untreated root nodule tissue.

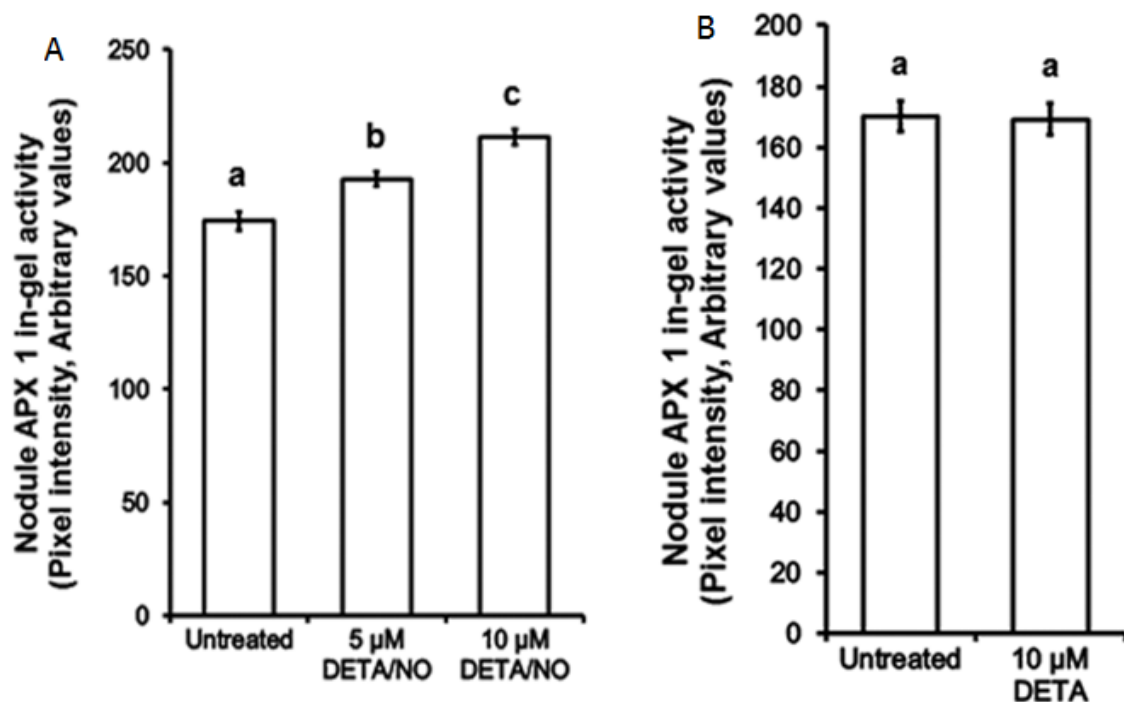


Figure 2-5. Effect of various concentrations of DETA/NO or DETA on the enzymatic activity of nodule GmAPX 1 isoform. Pixel intensities signifying the level of enzymatic activity of nodule GmAPX 1 isoform, derived from analysis of the intensity of the bands. (A) Response of GmAPX1 to treatment with 5 and 10 μ M DETA/NO. (B) Responses of GmAPX 1 isoform to 10 μ M DETA. Error bars represent the means (\pm SE; n = 3) of three independent experiments.

Treatment with 10 μ M DETA did not significantly alter the enzymatic activity of GmAPX 1 isoform, as revealed by analysis of the pixel intensities of the corresponding in-gel activity bands when compared to the untreated sample (Figure 2-5B).

The intensities of GmAPX 2 when treated with 5 μ M DETA/ NO and 10 μ M DETA/ NO (Figure 2-6A) increased by approximately 55% and 110% respectively when compared to GmAPX 2 enzymatic activity in the untreated control.

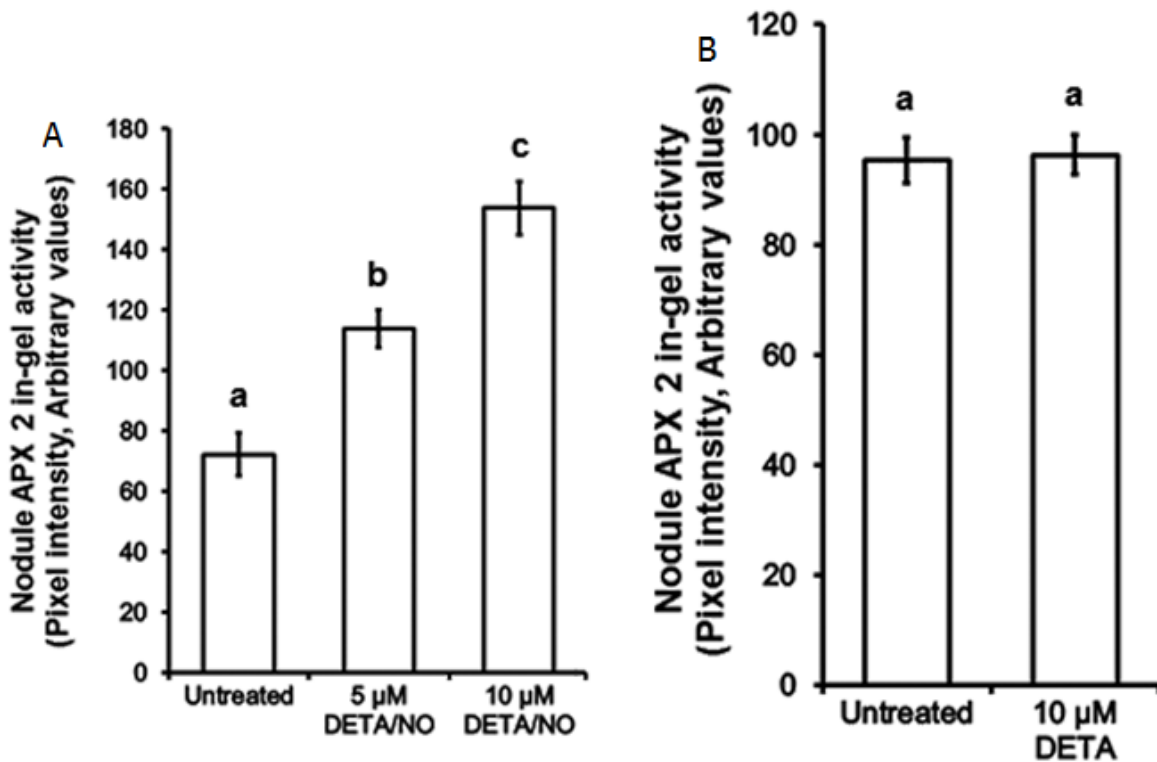


Figure 2-6. Effect of various concentrations of DETA/NO or DETA on the enzymatic activity of nodule GmAPX 2 isoform. Pixel intensities signifying the level of enzymatic activity of nodule GmAPX 2 isoform, derived from analysis of the intensity of the bands. (A) Response of GmAPX 2 to treatment with 5 and 10 μ M DETA/NO. (B) Responses of GmAPX 2 isoform to 10 μ M DETA. Error bars represent the means (\pm SE; n = 3) of three independent experiments.

However, the pixel intensity for GmAPX 2 on treatment with 10 μ M DETA was similar to the untreated sample (Figure 2-6B).

Furthermore, treatment of soybean root nodules with 5 μ M DETA/NO upregulated the GmAPX 3 enzymatic activity by approximately 228% compared to untreated controls (Figure 2-7A) while treatment with 10 μ M DETA/NO increased GmAPX 3 enzymatic activity by approximately 320% compared to untreated controls.

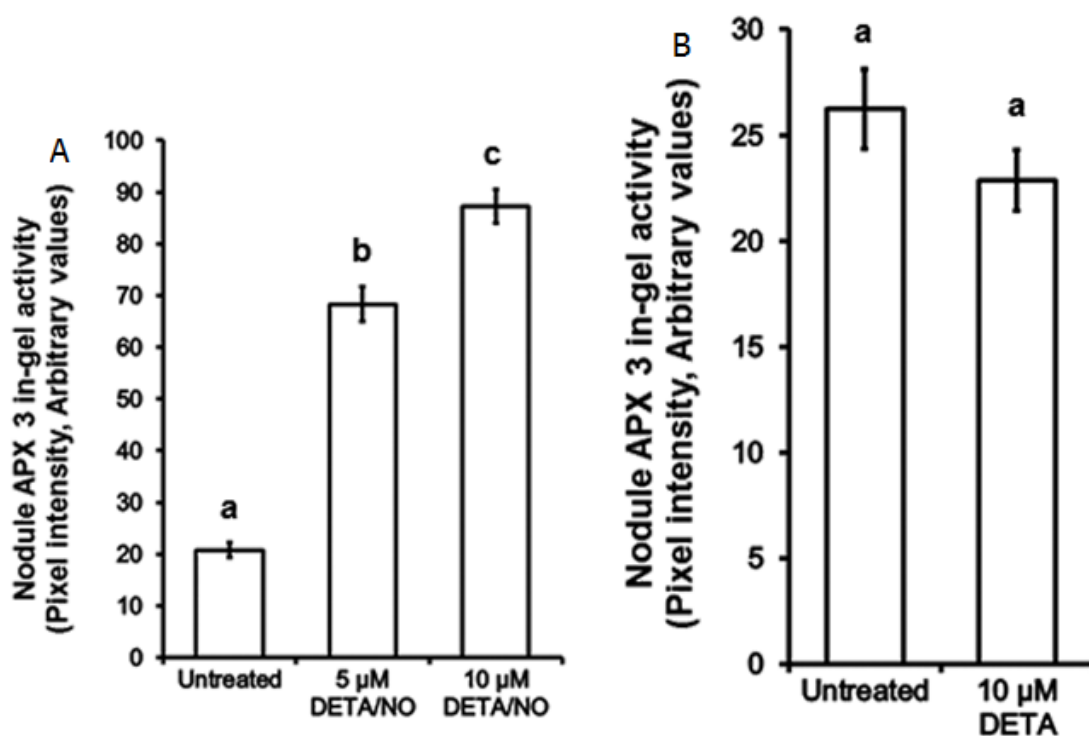


Figure 2-7. Effect of various concentrations of DETA/NO or DETA on the enzymatic activity of nodule GmAPX 3 isoform. Pixel intensities signifying the level of enzymatic activity of nodule GmAPX3 isoform, derived from analysis of the intensity of the bands. (A) Response of GmAPX 3 to treatment with 5 and 10 μ M DETA/NO. (B) Responses of GmAPX 3 isoform to 10 μ M DETA. Error bars represent the means (\pm SE; n = 3) of three independent experiments.

Similarly as observed with GmAPX 1 and GmAPX 2, treatment of soybean root nodules with 10 μ M DETA did not significantly alter the enzymatic activity of GmAPX 3 when compared to that of untreated sample (Figure 2-7B).

Discussion

In order to confirm that DETA/NO could release NO under the experimental condition in this study, it was necessary to measure the NO level in the treated soybean nodule samples and compare it to NO levels in the untreated and control samples. The result obtained from this study shows that application of the NO donor DETA/NO to mature soybean nodules increases the nitric oxide content in root nodule tissue in a concentration-dependent manner. The data from this study is in support of a study (49) which shows the influence of DETA/NO in reversing the effects of the NOS inhibitor N- ω -nitro-L-arginine (L NNA) on soybean nodule development and some nodule molecular/cellular activities.

It is evident that the NO pool in plants includes enzymatic sources such as nitrate reductase and NOS-like enzymes, and non-enzymatic sources (50). However, this study used the pharmacological application of NO via its donor DETA/NO to emulate NO production in soybean. DETA/NO was chosen in this study as an appropriate NO donor. Amongst the mechanism and kinetics of NO emission of this donor, the half-life of 56 hours of DETA/NO in solution, this NO donor releases NO in the uncharged (NO[•]) redox state believed to be the direct *bona fide* product of NOS enzymatic activity and it is NO[•] that has been demonstrated to be the redox form of NO that stimulates guanylate cyclase (GC, EC 4.6.1.2) activity (51, 52) to enhance the enzymatic rate of catalysis of the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) by soluble GC.

Furthermore, there is no toxic side effect recorded during the release of NO from DETA/NO. This is however not the case with some other NO donor such as sodium nitroprusside (SNP). Apart from NO, SNP also releases other several other products such as NaCN, NaNO₂, NaNO₃, ferrocyanide and ferricyanide (53). In fact, although addition of exogenous NO with

SNP can enhance antioxidant enzymes activities (53), there are also reports stating some negative effects of SNP on plant molecular and physiological processes (54, 55).

The fact that there was accumulation of NO in the DETA/NO-treated plants prompted further investigation on the H₂O₂ level in the soybean nodules. H₂O₂ acts in two different ways in plants based on its concentration. At a low concentration, it functions as a signaling molecule involved in signaling tolerance to biotic and abiotic stresses. However at high concentrations it leads to programmed cell death (PCD). It is likely that the concentration of H₂O₂ present in plants is dependent on the rate and extent of its production and on processes of its removal (56-58). The result presented in this study showed that the increase in NO content through application of DETA/NO has an influence in reducing the nodule H₂O₂ content. This result is in support of other studies which show the protective role of NO against H₂O₂ under water stress (59), heavy metal stress (60) and abscisic acid (ABA)-induced stress (61).

The investigation on the H₂O₂ level in mature soybean nodules shows that the presence of NO released from the NO donor DETA/NO leads to an effective reduction of H₂O₂. This suggests that in an event of extreme environmental condition, application of NO through DETA/NO might function in protecting the plant against oxidative damage. This is possible since NO can act as a signaling molecule which will activate, amongst the antioxidant enzymes, ascorbate peroxidase which plays an essential role in the detoxification of ROS. In order to confirm that NO has an effect on APX activity, total APX activity and in-gel activity were studied.

The result obtained from the spectrophotometric assay for total APX activity and in-gel analysis shows that application of NO, through DETA/NO, increased the APX enzymatic

activity in a dose-dependent manner. It is well established that the role of APX is to scavenge excess H_2O_2 formed in plant cells under normal and stress condition. There are suggestions that high doses of NO can be toxic to plants (62), and others have shown that high levels of NO can injure membranes, proteins, and nucleic acids in plant cells (63,64). As such, this study used low concentrations of (5 μM and 10 μM) DETA/NO which led to reduced nodule H_2O_2 content.

It is possible that APX can exist in more than one isoform depending on plant species. Therefore it was necessary to employ the in-gel studies since the spectrophotometric assay is unable to distinguish the different APX isoforms. In this study, we observed three isoforms in the untreated, DETA/NO treated and control soybean root nodules. However analysis of the in-gel enzymatic activities with regards to the responses of these isoforms to various concentrations of NO shows that they respond differently to varying concentrations of NO. Furthermore, the fact that GmAPX 1 does not respond to exogenous NO as markedly as GmAPX 2 and GmAPX 3 enzymatic activities, which were both up-regulated by 5 μM DETA/NO and 10 μM DETA/NO quite substantially, suggests that these two (GmAPX 2 and GmAPX 3) isoforms could be more important and could be suitable targets for genetic engineering for NO-mediated abiotic stress tolerance in plants.

In conclusion, this study suggests that the three APX isoforms identified from soybean root nodules may differ from each other in molecular and catalytic properties and belong to the ascorbate-specific class of plant peroxidases

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Chapter Three

The effect of exogenous application of nitric oxide on ascorbate peroxidase in salt stressed soybean root nodules

Summary

There are several abiotic factors which can cause molecular damage to plants either directly or through the accumulation of reactive oxygen species such as H_2O_2 . Whereas there are few publications suggesting NO as a stress inducing agent, this study supports literature suggesting a protective role of NO against abiotic stress. This is due to the fact that the NO donor 10 μ M DETA/NO used in this study was able to release NO which maintained the cellular redox homeostasis, regulated the level of H_2O_2 and prevented lipid peroxidation induced by short term NaCl stress. The ability of NO to show a protective function against NaCl induced oxidative stress was evident as it could increase the enzymatic activities of APX and DHAR and maintained the GSH/GSSG and ASC/ DHAsA ratio under salt stress conditions. Hence it functioned in ensuring a coordinated antioxidant defense system which is required for an effective scavenging of H_2O_2 from the cell. Furthermore the various findings obtained throughout this study showing the role of NO as a signaling molecule is evident given that no similar effect was obtained when DETA was applied to the plants.

Introduction

Abiotic stresses such as extreme temperatures, drought, salinity and chemical toxicity have been associated with pronounced decline in crop yield worldwide (1, 2). Amongst these stress factors, salinity is considered as one of the major factors that hinder plant growth and productivity (3, 4). Salinity imposes various effects on plants as a result of both ionic toxicity due to high ion concentration (Na^+ and Cl^-) and osmotic stress which consequently leads to the disruption of homeostasis, ion distribution and poor nutrient uptake.

One of the effects of salinity is oxidative damage at the cellular level due to increased production of reactive oxygen species (ROS) such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\text{HO}\cdot$) in plant cells (5). Accumulation of ROS causes cellular damage through oxidation of lipids, proteins and nucleic acids (6). Furthermore, there are several lines of evidence associating high salinity with changes in lipid metabolism. Amongst the various biomolecules, polyunsaturated fatty acids (PUFA) are the most susceptible targets to oxidative attacks mediated by ROS (7). Reaction of ROS with the PUFA leads to peroxidation which subsequently leads to degradation of biological membranes, rapid desiccation and cell death (8). ROS are produced by plants during normal processes such as photosynthesis, photorespiration and respiration. However high levels of ROS produced during salt stress are suggested to trigger stomatal closure which is associated with reduction of the CO_2/O_2 ratio in leaves (9), leading to a decrease in NADP^+ concentration and subsequent formation of more ROS (10). Amongst the different ROS, H_2O_2 is regarded as the most stable and at low concentration it functions as a signaling molecule

(11). But at high concentration, it becomes toxic, leading to programmed cell death (PCD), hence it is crucial for plants to regulate H_2O_2 intracellular concentrations (12).

Nitric oxide (NO) is now well recognized as an important signaling molecule in plants and is involved in several physiological processes such as promotion of seed germination or reduction of seed dormancy (13-16), regulation of plant development and senescence (17-19) and suppression of floral transition (20). There are other reports suggesting the role of NO in regulating the expression of genes involved in nodule development and nodule functioning in *M. truncatula* (21). More importantly, there are several studies showing an increase of NO production under unfavorable environmental conditions, hence suggesting the role of NO in mediating responses to abiotic stresses such as heat (22), drought, ultraviolet radiation (23), extreme temperature (24, 25) and heavy metals (26). Furthermore several studies using pharmacological approaches by the use of NO donors to treat plant under salinity stress have shown that NO plays a major role in the signaling network to enhance tolerance against salinity stress (27-30).

Plants are fully equipped with an array of antioxidant defenses aimed at protecting them from the oxidative effects exerted by ROS. The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) (31, 32), while the non-enzymatic antioxidants includes tocopherols, ascorbic acid (AA) and glutathione (GSH) (33-35). Amongst the anti-oxidant enzymes, APX is most crucial in regulating the level of H_2O_2 in plants and utilizes ascorbate (AsA) as its specific electron donor to reduce H_2O_2 into H_2O with the concomitant generation of monodehydroascorbate (MDHA) (36-38) and oxidized form of AsA -dehydroascorbate (DHAsA) (39). Amongst the antioxidant metabolites AsA, plays an essential role in the

removal of H_2O_2 by either reacting directly with H_2O_2 or via a reaction catalysed by APX. It is regenerated from MDHA and DHAsA in a reaction catalyzed by NAD(P)H-dependent monodehydroascorbate reductase (MDHAR, E.C. 1.6.5.4) and dehydroascorbate reductase (DHAR, E.C. 1.8.5.1). GSH is crucial in the regeneration of AsA and as such also functions in the regulation of H_2O_2 concentration and control of redox state in plants (40, 41).

There are numerous studies (42, 43) correlating, amongst other antioxidant enzymes, the rate and extent of increase of APX enzymatic activity under salt stress with plant tolerance to salinity. Furthermore, in view of the fact that increasing the level of NO by exogenous application of the NO donor 2, 2'-(hydroxynitrosohydrazono) bis-ethanimine (DETA/NO) resulted in an increase in maize biomass, scavenging of ROS and reduced extent of lipid peroxidation under salt stress (44), it is possible that application of DETA/NO could change the activity of APX in soybean (*Glycine max* L. merr. cv. PAN 626) root nodules under salt stress. In fact, in a recent related study (45), application of DETA/NO to nodulated soybean after 24 hours resulted in an increase in NO content and also increased the enzymatic activity of three soybean APX isoforms in a dose-dependent manner.

This chapter aimed to assess the degree of oxidative stress on nodulated soybean plants exposed to high NaCl concentrations for a period of 24 hours and whether an additional supply of NO (as DETA/NO) could alleviate the toxic effects of NaCl stress. Also, this chapter intends to elucidate and correlate the effect of NO on the APX enzymatic activity towards improving tolerance to salinity stress.

Materials and Method

Materials

Glycine max L. merr. cv. PAN 626 seeds used for this work were kindly provided by Pannar (Greytown, South Africa) and all the chemicals were purchased from Sigma-Aldrich, unless otherwise stated. The Rhizobium inoculum *Bradyrhizobium japonicum* as the commercial peat-based HiStick 2 Soybean inoculants was supplied by Becker Underwood Ltd., West Sussex, United Kingdom. The filtered silica sand (98% SiO₂) was purchased from Rolfes® Silica (Pty) Ltd, Brits, North West, South Africa.

Methods

Plant Growth

Soybean 626 seeds were surface-sterilized in 0.35% v/v sodium hypochlorite for 10 minutes, followed by five washes with sterile distilled water. The seeds were imbibed in sterile distilled water for 1 hour and inoculated with *Bradyrhizobium japonicum*. Seeds were sown in sand that had been pre-soaked in distilled water, in 15 cm diameter plastic pots (one plant per pot). The sand was kept moist by watering only with distilled water during germination. The germinated seedlings were grown on a 25/19°C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m⁻².s⁻¹ during the day phase, in a randomized design. Once the plants reached the

VC stage (when unifoliolate leaves are fully expanded), they were supplied with nitrogen free nutrient solution containing 1 mM K_2SO_4 , 2 mM $MgSO_4$, 3 mM $CaCl_2$, 1 mM K_2HPO_4 buffer at pH 7.3, 25 μM H_3BO_3 , 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 2 μM $CuSO_4$, 2 μM Na_2MoO_4 , 0.1 μM $CoSO_4$, 50 μM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3 at a three day intervals until they reached the V3 stage (third trifoliolate).

Treatment of Plants

Plants of the same phenological stage and similar height were selected for all experiments. The treatment was performed once the plants were at the V3 stage. The plants were chosen randomly and divided into six groups. The first group, treated with nitrogen-free nutrient solution only, served as the untreated. The second group was treated with nitrogen-free nutrient solution containing 10 μM DETA/NO (NO donor). The third group was treated with nitrogen-free nutrient solution containing 10 μM DETA (control). The fourth, fifth and sixth groups were treated with nitrogen free nutrient solution containing 150 mM NaCl, 150 mM NaCl plus 10 μM DETA and 150 mM NaCl plus 10 μM DETA/NO respectively. Plants were treated for a period of 24 hours and after which the soybean root nodules were harvested and placed on ice.

Protein extraction from nodule tissue

Extracts were obtained from soybean root nodules by grinding the nodule tissue into a fine powder in liquid nitrogen and homogenizing 500 mg of the tissue with either 1 ml of

homogenizing buffer [40 mM K_2HPO_4 , pH 7.4, 1 mM ethylene di-amine tetra-acetic acid (EDTA), 5% (w/v) poly vinyl pyrrolidone (PVP) molecular weight = 40,000] for determination of APX enzymatic activity, antioxidant metabolites and estimation of DHAR activity or 10% trichloroacetic acid (TCA) for H_2O_2 content and lipid peroxidation. The resulting homogenates were centrifuged at 12,000 x g for 20 minutes at 4°C and the supernatants were used for biochemical assays.

Measurement of H_2O_2 content

H_2O_2 content was determined in the nodule extracts by modifying a method previously described by Velikova *et al.*, (46). *Glycine max* nodule tissue (100 mg) was ground to fine powder in liquid nitrogen and homogenized in 400 μ l of cold 6% (w/v) TCA. The extracts were centrifuged at 12,000 x g for 30 minutes at 4°C and 50 μ l of the supernatant were used to initiate the reaction in a mixture (total volume of 200 μ l) containing 5 mM K_2HPO_4 , pH 5.0 and 0.5 M KI. The reaction was incubated at 25°C for 20 minutes and absorbance readings were recorded at 390 nm. H_2O_2 content was calculated using a standard curve based on the absorbance ($A_{390\text{ nm}}$) of H_2O_2 standards.

Lipid peroxidation

Lipid peroxidation was determined in soybean root nodules by measuring malondialdehyde (MDA) formation, using the thiobarbituric acid method (TBA) as previously described by Buege *et al.*, (47). Plant tissue (100 mg) was ground into a fine powder in liquid nitrogen. The tissue was homogenized in 400 μ l of cold 5% (w/v) trichloro acetic acid (TCA). The

homogenate was centrifuged at $12,000 \times g$ for 30 minutes at 4°C . Aliquots (100 μl) of the supernatant were mixed with 400 μl of 0.5% TBA (prepared in 20% TCA). The mixture was incubated at 95°C for 30 minutes and the reaction was stopped by placing the mixture on ice for 5 minutes. The mixture was further centrifuged at $12,000 \times g$ for 5 minutes at 4°C . The absorbance of the supernatant was measured at 532 nm and 600 nm. After subtracting the non-specific absorbance ($A_{600 \text{ nm}}$), the MDA concentration was determined by its extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as nmol g^{-1} of fresh weight.

Determination of APX enzymatic activity

Plant APX activities were measured in nodule extracts by modifying a method previously described by Asada (48). The nodule extracts which were supplemented with ascorbate to a final concentration of 2 mM, were added to the assay buffer containing 50 mM K_2HPO_4 , pH 7.0, 0.1 mM EDTA and 50 mM ascorbate. The reaction was initiated by adding 1.2 mM H_2O_2 in a final reaction volume of 200 μl and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. For the determination of the response of *Glycine max* APX isoforms to exogenously applied NO under salinity stress, electrophoretic APX separation was carried out as previously described by Mittler *et al.*, (49) and non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed at 4°C in 7.5% polyacrylamide mini gels. Prior to loading extracts containing 50 μg of protein into the wells, gels were equilibrated with running buffer containing 2 mM ascorbate for 30 minutes at 4°C . After the electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 minutes and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM

ascorbate and 2 mM H_2O_2 for 20 minutes. The gels were washed in the buffer for a minute and submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N',N'- tetra methyl ethylene di-amine (TEMED) and 2.5 mM nitroblue tetrazolium (NBT) for 10-20 minutes with gentle agitation in the presence of light. The gel images were captured and analyzed by densitometry using AlphaEase FC imaging software (Alpha Innotech Corporation).

AsA and DHAsA assay

AsA and DHAsA were determined in soybean root nodules by modifying a method previously described by Law *et al.*, (50). The following solutions were freshly prepared accordingly: 0.1 M standard solutions of AsA and DHAsA dissolved in 6% (w/v) TCA, 10 mM dithiothreitol (DTT) dissolved in 0.2 M potassium phosphate buffer (pH 7.4), 0.5% (w/v) N-ethylmaleimide (NEM) and 3% (w/v) FeCl_3 . The reaction was carried out in a 96 well-plate and to measure the total ascorbate, the reaction mixture consisted of 10 μl of plant extract, 10 μl of 10 mM DTT, 10 μl of 0.5% NEM and 20 μl of 0.2 M phosphate buffer (pH 7.4). For AsA content, the reaction mixture contained 10 μl of plant extract, 30 μl of 0.2 M phosphate buffer (pH 7.4) and 10 μl distilled H_2O . The rest of the steps were similar for both estimations. The following were added accordingly to each well of the plates; 50 μl of 10% TCA, 40 μl of 42% H_3PO_4 and 40 μl of 4% 2, 2' dipyridyl amide in 70% (v/v) ethanol. The total reaction mixture was made up to 200 μl by the addition of 20 μl of 3% iron (III) Chloride (FeCl_3). The solution was mixed and the plate was incubated at 42°C for 15 minutes. The absorbance was recorded at 525 nm and DHAsA was calculated as the difference between total ascorbate and AsA.

GSH and GSSG assay

Total (GSH+GSSG) and GSSG were determined in soybean root nodule by modifying a previously described method by Griffith (51). Nodule extract (1 ml) were neutralized with 360 μ l of 1M triethanolamine and 40 μ l of 2-vinyl pyridine was added for the measurement of GSSG to obtain GSH. The mixture was allowed to stand at room temp for 1 hour. The rest of the steps were similar for both estimations of total and oxidized glutathione. The following were added accordingly to each well of the plates; 190 μ l of reaction mixture consisting of 125 mM phosphate buffer (pH 7.5) containing 6.3 mM EDTA, 0.5 Units of glutathione reductase, 0.3 mM NADPH, 6 mM 5-(3-Carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid (DTNB) and 10 μ l of each of the above treated soybean root nodule extracts. Change in absorbance at 412 nm was recorded for 4 minutes. A reference curve was prepared with GSSG.

Determination of DHAR enzymatic activity

DHAR activity was measured in soybean root nodule extracts by modifying a method previously described by De Tullio *et al.*, (52). 10 μ l of the plant extracts (50 μ g of protein) was diluted in 40 μ l distilled H₂O and added into each well of a 96 well plate containing 80 μ l solution containing 2 mM GSH and 40 μ l 500 mM phosphate buffer(pH 7.0). For the blank, 80 μ l of distilled H₂O was added in place of GSH. The reaction was initiated by the addition of 30 μ l of 6.6 mM DHAsA. The assay measured the formation of AsA at 265 nm (ϵ = 14 mM⁻¹ cm⁻¹). Changes in absorbance at 265 nm were followed for a minute. The rate of enzymatic

DHAsA reduction was corrected by subtracting the values obtained in the absence of substrate GSH.

Determination of protein concentration

Protein concentrations for all assays were measured in the extracts as instructed for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories).

Statistical analysis

One-way analysis of variance (ANOVA) test was used for all data to evaluate statistical validity of the results and means were compared according to the Tukey-Kramer test at 5% level of significance, using Graph Pad Prism 5.03 software.

Results

Exogenously applied NO and 150 mM NaCl induced changes in APX activity and the redox state of soybean nodules.

H₂O₂ content in soybean root nodules

The level of H₂O₂ did not show any significant difference in the DETA (control) treated plants when compared with untreated controls (Figure 3-1).

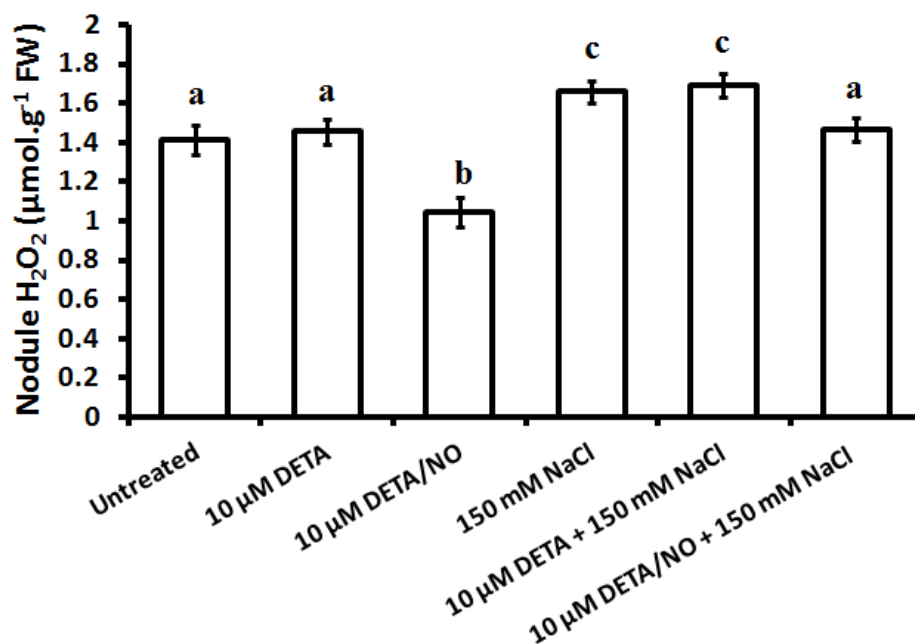


Figure 3-1. Effect of exogenously applied NO (10 μM DETA/NO) and salt stress (150 mM NaCl) on soybean root nodule H₂O₂ content. Error bars represent the mean (±SE; n= 3) from data that are representative of three independent experiments.

Application of DETA/NO resulted in approximately 25% decrease in H₂O₂ levels when compared to the untreated control. H₂O₂ content was increased by approximately 15% in response to 150 mM NaCl when compared to untreated controls. However there was no

significant difference in the level of H_2O_2 content between 150 mM NaCl and 150 mM NaCl combined with DETA. Application of 10 μM DETA/NO in combination with 150 mM NaCl in soybean root nodule resulted in approximately 12% reduction of H_2O_2 content.

Changes in lipid peroxidation

Salt stress induced oxidative damage to membrane lipids, as revealed by the amount of malondialdehyde produced in salt-treated nodules.

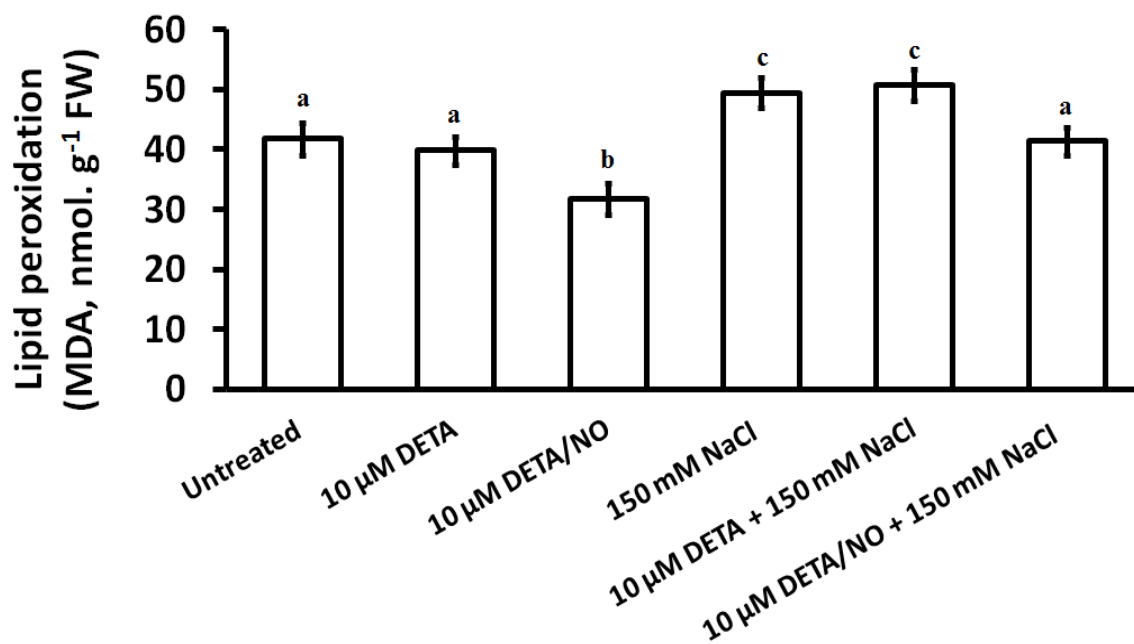


Figure 3-2. Effect of 150 mM NaCl and exogenously applied NO (10 μM DETA/NO) or DETA (10 μM) on lipid peroxidation in soybean root nodule. Error bars represent the mean ($\pm\text{SE}$; $n=3$) from data that are representative of three independent experiments.

Based on Figure 3-2, there was no marked difference in the lipid peroxidation level between the untreated control and DETA-treated soybean root nodules. Soybean root nodules treated with 10 μM DETA/NO exhibited low levels of lipid peroxidation. Lipid peroxidation was reduced approximately by 24% in response to 10 μM DETA/NO when compared to

untreated controls. The injury caused by salt to cellular membranes due to lipid peroxidation as reflected by the accumulation of the MDA levels was significantly increased by the addition of 150 mM NaCl. Nodules treated with 150 mM NaCl showed a 20% increase in lipid peroxidation when compared to the untreated control. A similar trend was also observed in soybean root nodules treated with 10 μ M DETA in combination with 150 mM NaCl. Interestingly, exogenous application of 10 μ M DETA/NO combined with 150 mM NaCl resulted in almost complete amelioration of the toxic effect of salt stress on lipid peroxidation. As shown in Figure 3-2, there was no marked significant difference on the level of lipid peroxidation in the 10 μ M DETA/NO combined with 150 mM NaCl treated soybean root nodules when compared to the untreated control.

Effect of exogenous application of DETA/NO on total APX enzymatic activity in salt-treated soybean root nodules

Based on observation that there was a marked decrease in the level of H_2O_2 and lipid peroxidation in 10 μ M DETA/NO treated plants as well as 10 μ M DETA/NO combined with 150 mM NaCl, further investigation was carried to determine the effect of exogenous application of DETA/NO on total APX enzymatic activity in salt-treated soybean root nodules. In a previous study (45), application of varying concentrations of DETA/NO resulted to accumulation of NO and moreover enhanced APX enzymatic activity in unstressed soybean root nodules.

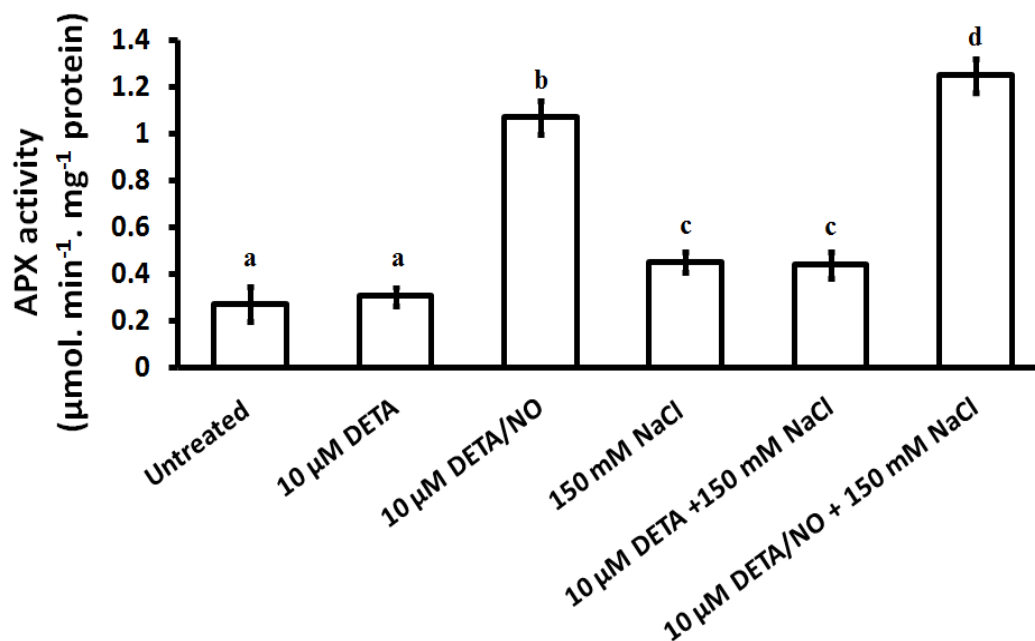


Figure 3-3. Effect of exogenously applied NO (10 μM DETA/NO or 10 μM DETA) and salt (150 mM or 10 μM DETA + 150 mM NaCl) on APX activity in soybean root nodule. Error bars represent the mean (\pm SE; $n=3$) from data that are representative of three independent experiments.

There was no marked difference in the total APX enzyme activity between the untreated control and DETA- (which lacks the NO moiety) treated soybean root nodules (Figure 3-3). However APX activities showed an increase in response to both salinity stress and the NO donor. The NO-induced increase in APX activity was 2.8-fold higher in response to 10 μM DETA/NO than the controls, whereas the APX activity was approximately 65% higher in response to 150 mM NaCl when compared to the untreated controls. There was also no significant difference in the level of APX activity in response to 150 mM NaCl when compared to 10 μM DETA + 150 mM NaCl. Interestingly, treatment of soybean root nodules with 10 μM DETA/NO + 150 mM NaCl resulted in the highest up-regulation of total APX enzymatic activity in root nodules. The increase in APX activity observed was 3.5-fold higher in response to 10 μM DETA/NO + 150 mM NaCl than the untreated control.

Effect of exogenously applied NO on three APX isoforms in NaCl treated soybean root nodules

Since the total APX activity was differentially up-regulated in response to various treatments as observed in the spectrophotometry assay, further investigation was carried out to determine the response of individual APX isoforms to NO in NaCl-stressed soybean root nodules using in-gel APX enzymatic assays coupled with pixel intensity analyses.

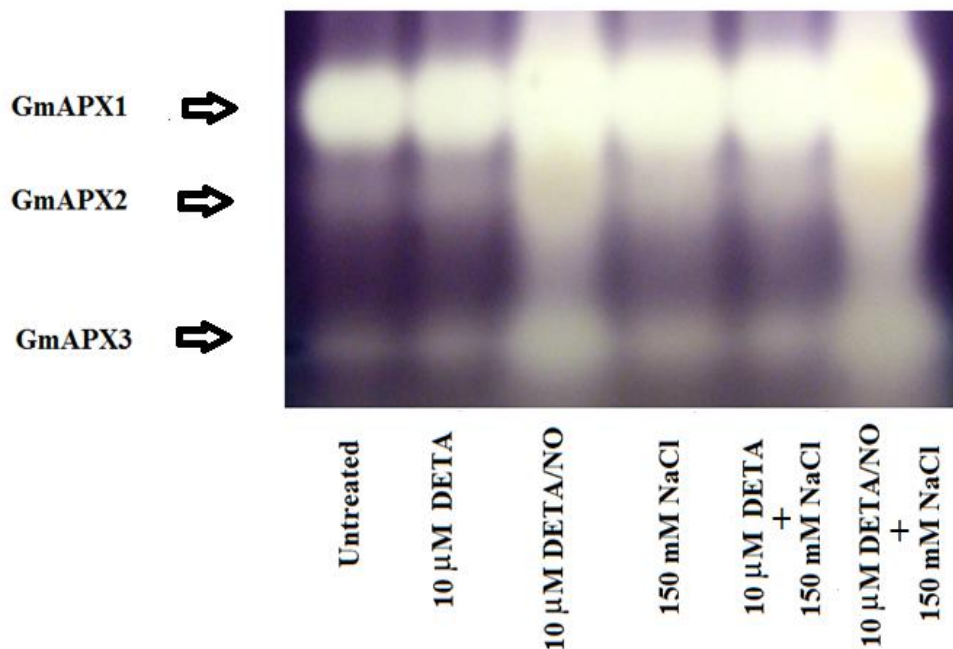


Figure 3-4. Effect of NO and NaCl on APX activity of *Glycine max* root. Lanes 1-6: Untreated, 10 μM DETA, 10 μM DETA/NO, 150 mM NaCl, 10 μM DETA + 150 mM NaCl and 10 μM DETA/NO + 150 mM NaCl respectively. The three isoforms are referred to as GmAPX1, GmAPX2 and GmAPX3 on the basis of their migration on the native PAGE gel.

Soybean root nodules treated with 150 mM NaCl or its combination with DETA; NO donor (10 μ M DETA/NO) or its combination with 150 mM NaCl showed higher increase in APX activity in an ascending order (Figure 3-4). This is visually evident from the varying intensities of the isoforms of the APXs in the native gel (Figure 3-4). However, further observation suggests that APX activity bands from the untreated and DETA treated samples have similar intensity.

Densitometry analyses of activity gels (Figure 3-5) showed that the enzymatic activity of GmAPX 1 was up-regulated by approximately 34% in response to 10 μ M DETA/NO when compared to GmAPX 1 enzymatic activity of untreated root nodule tissue.

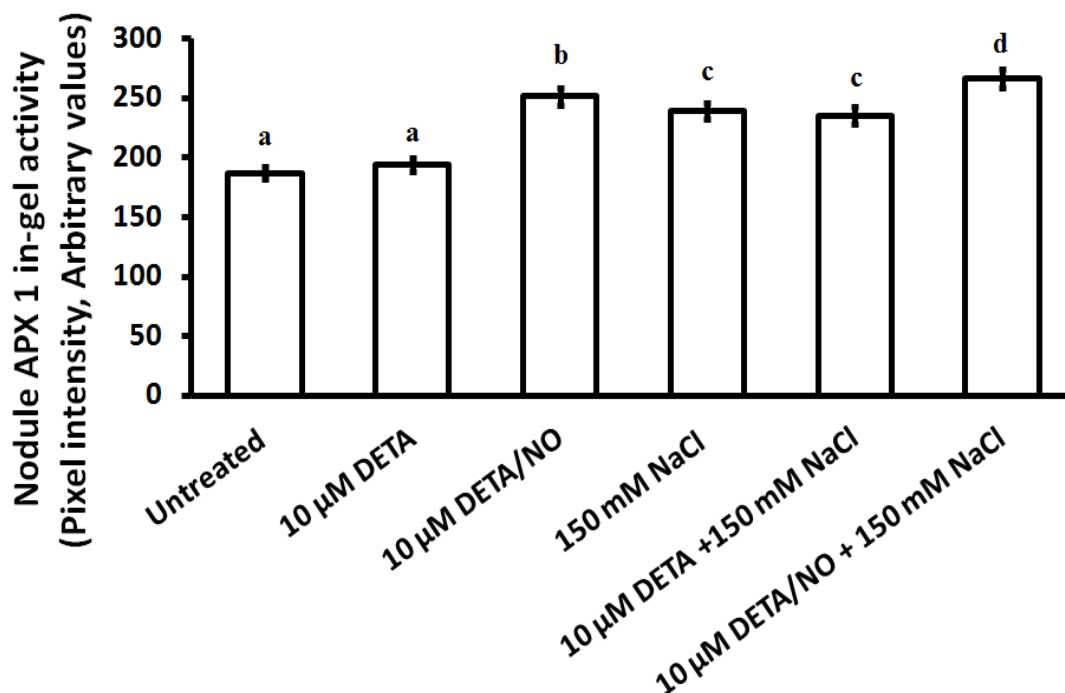


Figure 3-5. Pixel intensities signifying the level of enzymatic activity of nodule GmAPX 1 isoform, derived from analysis of the intensity of the bands. Response of GmAPX 1 to treatment with 10 μ M DETA, 10 μ M DETA/NO, 150 mM NaCl, 10 μ M DETA + 150 mM NaCl or 10 μ M DETA/NO + 150 mM NaCl. Error bars represent the means (\pm SE; n = 3) of three independent experiments.

Also, pixel intensities obtained indicates an up-regulation of enzyme activity by approximately 27% in response to 150 mM NaCl when compared to GmAPX 1 enzymatic activity of untreated root nodule tissue. A similar level of enzyme activity was observed in

response to treatment with the combination of 10 μ M DETA and 150 mM NaCl. Interestingly, pixel intensities obtained from the combined treatment of 150 mM NaCl and 10 μ M DETA/NO had the highest value. The enzymatic activity of GmAPX1 was up-regulated by approximately 42% in response to 10 μ M DETA/NO + 150 mM NaCl when compared with GmAPX 1 enzymatic activity of soybean untreated sample.

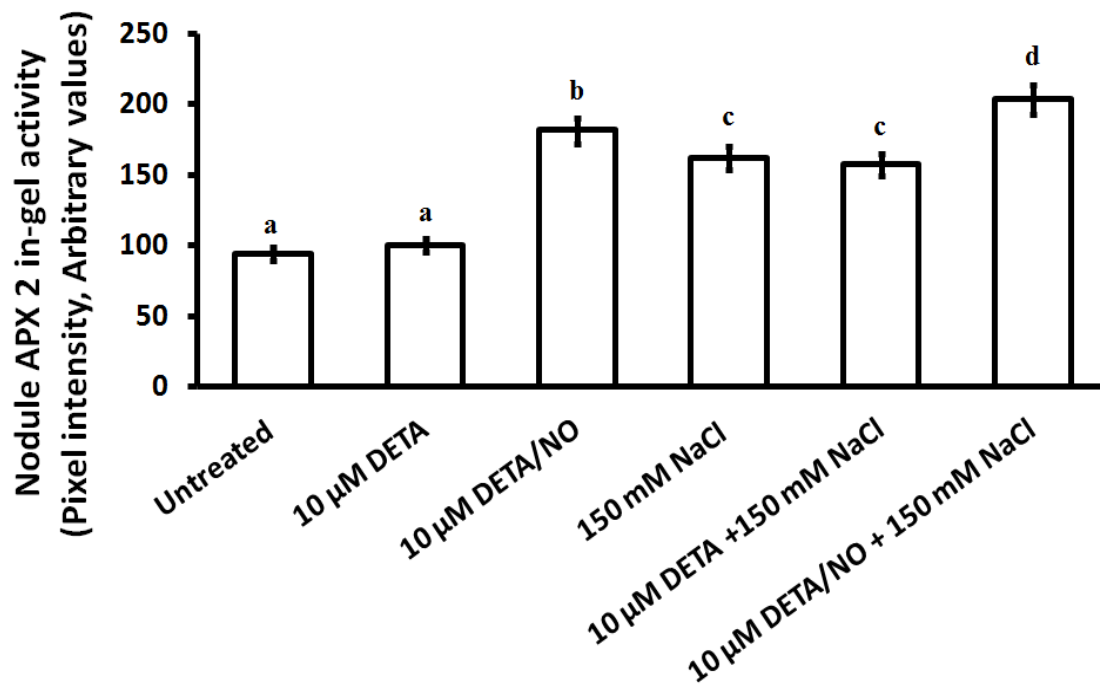


Figure 3-6. Pixel intensities signifying the level of enzymatic activity of nodule GmAPX 2 isoform, derived from analysis of the intensity of the bands. (A) Response of GmAPX 2 to treatment with 10 μ M DETA, 10 μ M DETA/NO, 150 mM NaCl, 10 μ M DETA + 150 mM NaCl or 10 μ M DETA/NO + 150 mM NaCl. Error bars represent the means (\pm SE; n = 3) of three independent experiments.

The enzymatic activity of GmAPX 2 was up-regulated by approximately 92% in response to 10 μ M DETA/NO when compared to the activity of GmAPX 2 in the untreated sample (Figure 3-6). The activity of GmAPX 2 in response to salt treatment or a combination of NaCl with DETA, which was also similar for these two treatments, was less than those of plants treated with the NO donor. The enzyme was up regulated by approximately 67% in response to the salt treatment, however the enzyme activity was more induced in the combined treatment

of NaCl and NO donor, with an up-regulation of approximately 115% in response to the 10 μ M DETA/NO + 150 mM NaCl treatment.

Densitometry analysis of the GmAPX 3 also shows a similar trend of enzyme induction by the various treatments as observed for GmAPX 2 (Figure 3-7).

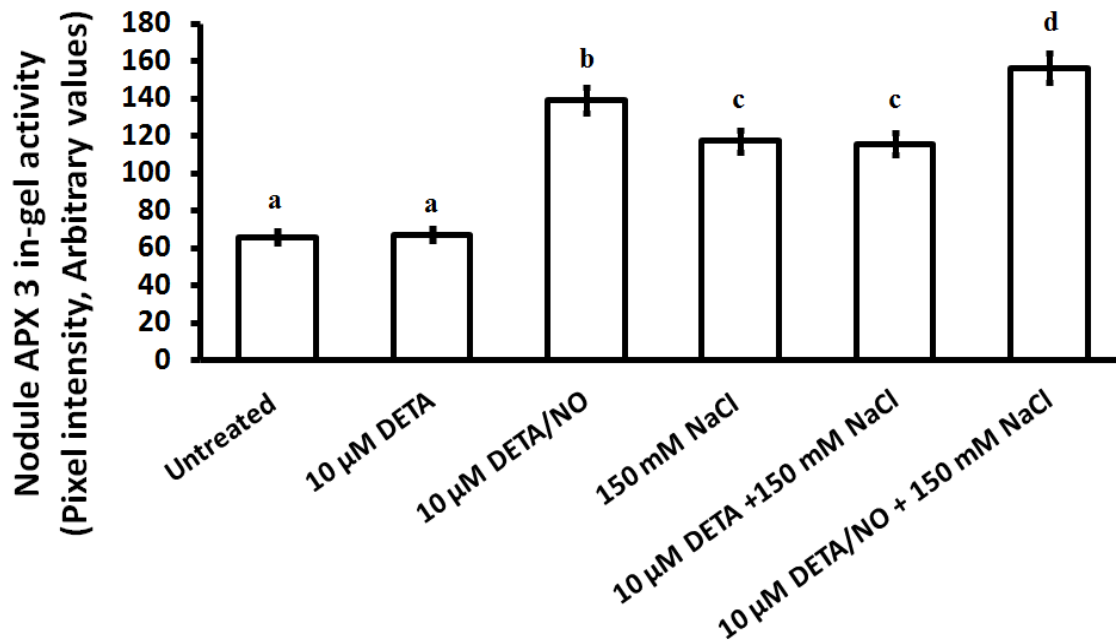


Figure 3-7. Pixel intensities signifying the level of enzymatic activity of nodule GmAPX 3 isoform, derived from analysis of the intensity of the bands. (A) Response of GmAPX 3 to treatment with 10 μ M DETA, 10 μ M DETA/NO, 150 mM NaCl, 10 μ M DETA + 150 mM NaCl or 10 μ M DETA/NO + 150 mM NaCl. Error bars represent the means (\pm SE; n = 3) of three independent experiments.

Whereas there was no significant difference for GmAPX 3 in response to the DETA treatment when compared with the untreated, GmAPX 3 was up-regulated by approximately 110% in response to 10 μ M DETA/NO, approximately 77% in response to 150 mM NaCl or its combination with 10 μ M DETA and induction of this isoform increased by approximately 137% in response to 10 μ M DETA/NO + 150 mM NaCl when compared to GmAPX 3 activity of the untreated root nodule tissue.

Levels of AsA and DHAsA

In view to the fact that the metabolism of H_2O_2 involving APX utilizes AsA as its electron donor, cellular levels of AsA and DHAsA were investigated in the variously treated soybean root nodules.

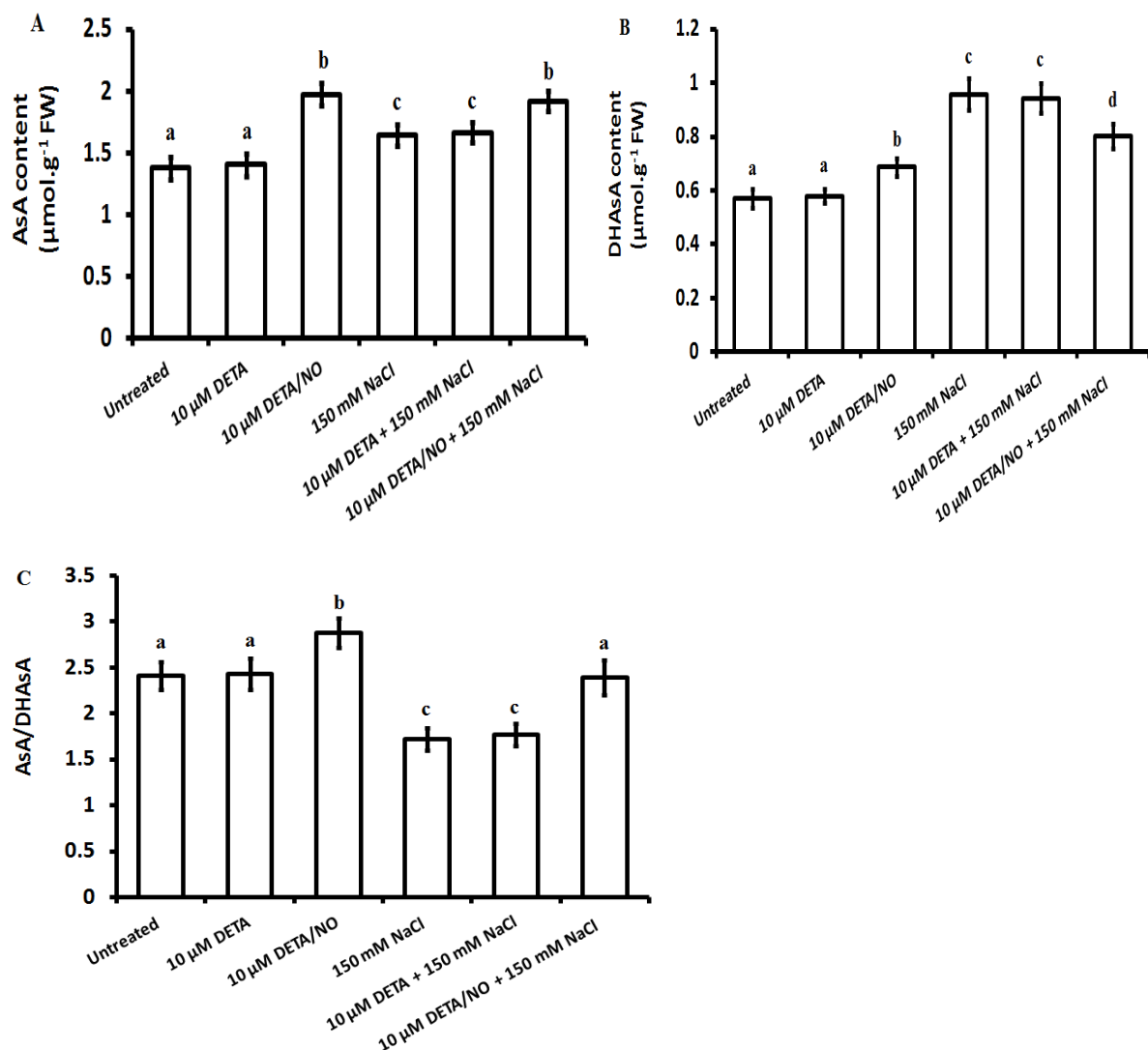


Figure 3-8. Effect of NaCl and DETA/NO treatments on ascorbate content (A), DHAsA content (B) and ascorbate redox ratio (C) in soybean root nodules. Treatments: Untreated, 10 μM DETA, 10 μM DETA/NO, 150 mM NaCl, 10 μM DETA + 150 mM NaCl and 10 μM DETA/NO + 150 mM NaCl. The data are mean values \pm SE (n=3).

As shown in figure 3-8A and B, there was no significant difference in AsA or DHAsA content of DETA treated soybean nodules when compared with the untreated samples. Whereas

there was a pronounced increase in AsA content in DETA/NO-treated soybean root nodules when compared to the untreated control, the corresponding increase in the level of its oxidized form (DHAsA) was moderate when compared to the untreated control. AsA was increased by approximately 43% and DHAsA by 20% in DETA/NO-treated soybean root nodules when compared to the level of the antioxidant metabolites in untreated levels. Treatment of soybean root nodules with 150 mM NaCl (either alone or in combination with 10 μ M DETA) resulted in a slight increase of AsA and a much higher increase in DHAsA in these treatments. The salt-treated soybean root nodules had an increase of 20% in their AsA content and whereas the increase in DHAsA was approximately 67% when compared to their respective untreated controls. However supplementing the salt treatment with an NO donor (10 μ M DETA/NO) restored AsA content considerably (even though not to the levels of the untreated control) and a similar restoration of DHAsA was observed for this treatment. Whereas there was no significant difference on the antioxidant ratio between the untreated and DETA-treated soybean root nodules, a decrease ($\sim 26\%$) in the AsA/DHAsA ratio was observed on the salt-treated soybean root nodules when compared with the untreated samples as shown in Figure 3-8C. However, DETA/NO treatments had a positive effect towards increasing ($\sim 19\%$) the ascorbate ratio. The ameliorative effect of NO under salinity stress was evident as the DETA/NO + NaCl treatment positively affected the reduced form of the antioxidant metabolite and thus maintaining the AsA/DHAsA ratio to that similar to the untreated control.

Levels of GSH and GSSG

Since GSH is a crucial antioxidant that participates in the AsA-GSH cycle for the scavenging of H_2O_2 , the effect of exogenously applied NO, as DETA/NO, on GSH content in salinity-induced oxidative stress soybean root nodules was investigated.

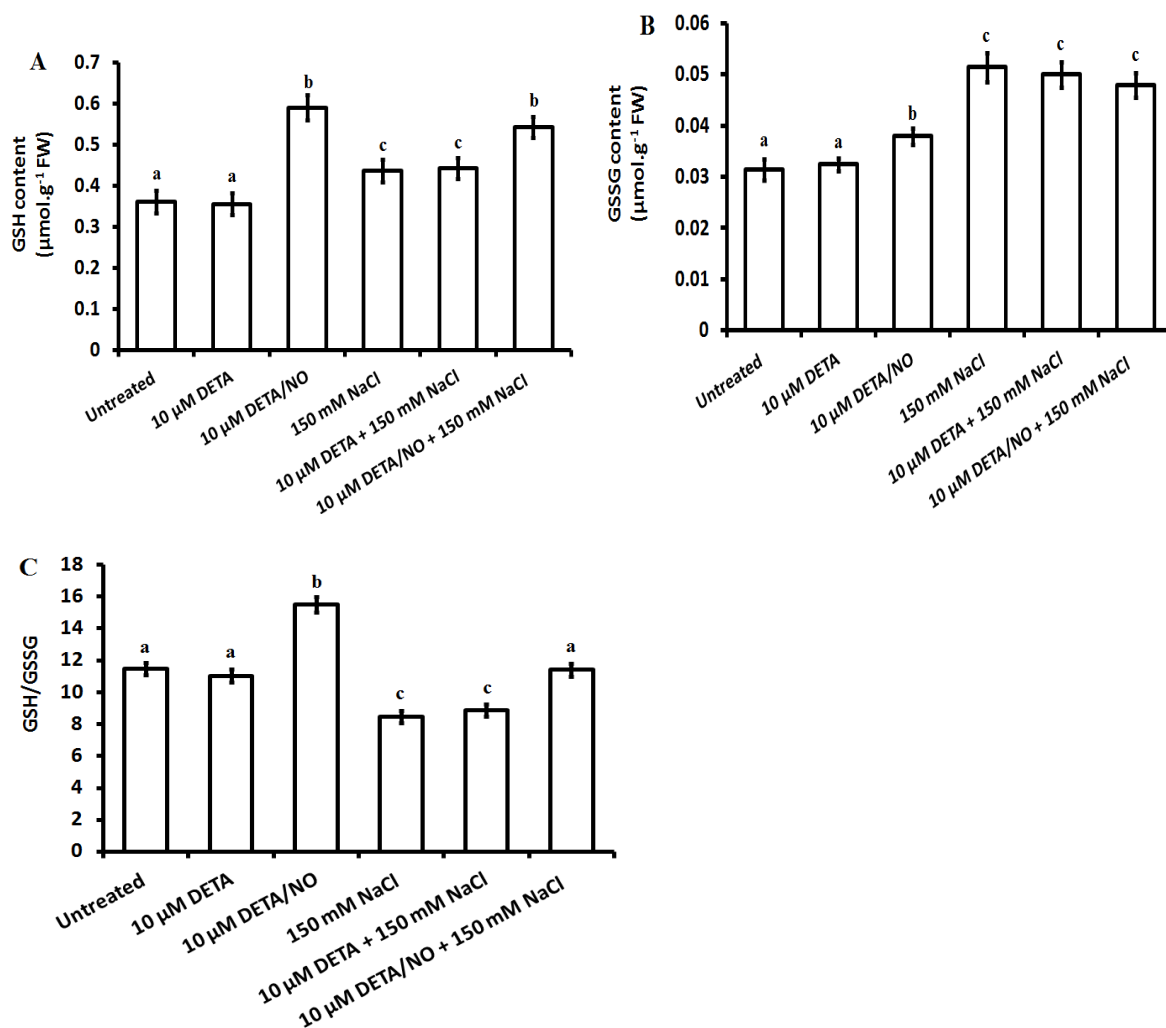


Figure 3-9. Effect of NaCl and DETA/NO treatments on glutathione content (A), GSSG content (B) and glutathione redox ratio (C) in soybean root nodules. Treatments: Untreated, 10 μM DETA, 10 μM DETA/NO, 150 mM NaCl, 10 μM DETA + 150 mM NaCl and 10 μM DETA/NO + 150 mM NaCl. The data are mean values \pm SE (n=3).

While there was no significant difference in the levels of GSH and GSSG in the 10 μM DETA-treated when compared to untreated values, a marked increase of GSH and a corresponding

marginal increase in GSSG in response to 10 μ M DETA/NO was observed (Figure 3-9). GSH level showed approximately 63% increase in response to 10 μ M DETA/NO whereas a 23% increase in the level of GSSG was observed in response to this treatment. On the other hand, a moderate increase of GSH with a high increase in GSSG levels was observed in response to 150 mM NaCl. This suggests an induction of oxidative stress by salt. GSH levels showed a 20% increase whereas the GSSG levels showed approximately 63% increase in nodules exposed to 150 mM NaCl. This trend was similar in the treatment where 150 mM NaCl was combined with 10 μ M DETA. However the combination of 10 μ M DETA/NO with 150 mM NaCl was effective in increasing GSH level by 50% when compared to the untreated plants but there was also an increase of GSSG by 52%. As shown in Figure 3-9C, application of the NO donor (10 μ M DETA/NO) was effective in increasing the GSH/GSSG ratio by 35% when compared to the untreated controls. The salt treatment resulted to a decrease of the GSH/GSSG ratio by approximately 26% when compared to the untreated samples. However, supplementation of 10 μ M DETA/NO to 150 mM NaCl was able to restore the shift of GSH from an oxidized state to the reduced state hence maintaining the GSH/GSSG ratio at a level similar to the level of nodules from untreated plants.

Effect of NO donor and salt on soybean root nodule DHAR activity

The enzymatic activity of DHAR is crucial, as it catalyses the reduction of DHAsA to AsA by utilizing reduced GSH. Since the product of this reaction is required by APX as an electron donor for scavenging of H₂O₂, further investigation was carried out to determine the effect of NO on DHAR activity in salinity-induced oxidative stress in soybean root nodules.

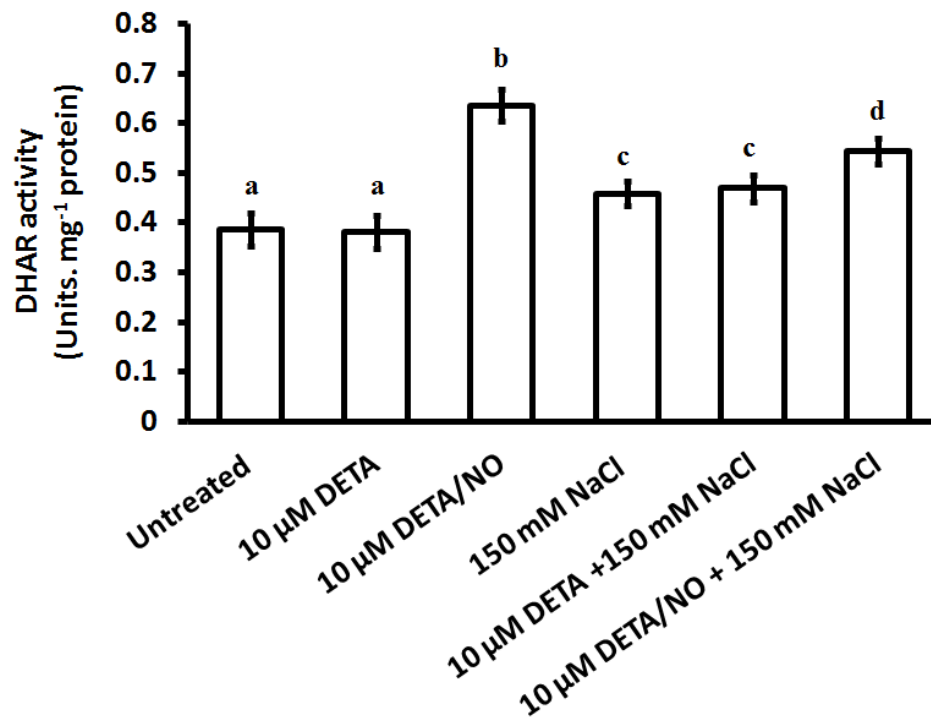


Figure 3-10. Effect of exogenously applied NO (10 µM DETA) and salt (150 mM NaCl) on DHAR activity in soybean root nodules. Error bars represent the mean (\pm SE; n= 3) from data that are representative of three independent experiments.

Compared with untreated control plants, treatment with 10 µM DETA exhibited no significant difference in DHAR activity (Figure 3-10). However treatment of soybean root nodules with the NO donor (10 µM DETA/NO) enhanced DHAR activity, as signified by approximately an increase of 64% in response to 10 µM DETA/NO. Although an increase in DHAR activity was observed in response to both 150 mM NaCl and 150 mM NaCl combined with 10 µM DETA, the increase was less pronounced (18% increase of DHAR activity) in these treatments when compared the increase caused by the NO donor. However, supplementation of the salt treatment with NO (150 mM NaCl + 10 µM DETA/NO treatment) resulted in elevation of DHAR activity by 40% when compared to the enzymatic activity of untreated soybean nodules.

Discussion

Application of 150 mM NaCl, either alone or combined with 10 μ M DETA, to the soybean root nodules caused lipid peroxidation. This was deduced from increased levels of MDA in these treatments when compared with the untreated samples, as was shown in Figure 3-2. This effect of salt stress on lipid peroxidation has previously been described (53, 54). However the data obtained from this study suggests that application of DETA/NO can protect plants from the effect of salt induced-membrane damage. This is evident not only from the ability of NO released from DETA/NO to maintain cellular membrane integrity as evidenced by the low level of lipid peroxidation of the soybean root nodules, but also by reducing the level of lipid peroxidation when supplemented on salt-treated plants. A similar protective effect of NO on membrane injury has been reported under salt stress (55). This protective role of NO against salt-induced membrane lipid peroxidation could be attributed to its ability to scavenge reactive intermediates (56). Furthermore, some evidence suggests that the reaction of NO with lipid peroxyl radicals reduces lipid peroxidation (57, 58).

Apart from extensive induction of lipid peroxidation, salinity stress is also associated with increased production of H_2O_2 (59). The accumulation of H_2O_2 during salinity stress can arise as a result of the imbalance in the rate of production and removal of ROS. As such, H_2O_2 content is often used as a marker that indicates the extent of oxidative stress in plant cells. As shown by the data obtained (Figure 3-1) from this study, samples treated with salt either alone or salt in combination with DETA recorded the highest level of H_2O_2 content. The result obtained from this study is similar, with reference to increased H_2O_2 content, to those observed in response to salt stress in various other studies using plant species such as rice (27), barley (53) and cucumber (54) and were also associated with oxidative stress.

Application of NO decreased the H₂O₂ content and reversed the salinity-induced stress. This effect of NO on H₂O₂ has been reported in a similar study (54) thus suggesting NO as a crucial molecule involved tolerance to salt stress.

Tolerance to oxidative stress is mostly associated with increased antioxidant activity. This suggests that the underlying mechanism for the protective role of NO against oxidative damage could depend on its role as a signaling molecule involved in the activation of plant antioxidant enzymes. In order to determine if the reduction of salt stress-induced oxidative damage as shown by decreased lipid peroxidation and H₂O₂ content could be due to the ability of NO to coordinate the induction of antioxidant defenses, its effect on APX and DHAR enzymatic activities and the antioxidant metabolites glutathione and AsA were investigated. The results from this study show that the total activities of APX (Figure 3-3) and DHAR (Figure 3-10) under salt stress were increased when compared to untreated controls, a mechanism often regarded as a defense response against salt stress (60). The marginal increase of APX enzymatic activity in response to salt treatment or the combination of salt with the DETA, and the corresponding increased lipid peroxidation and H₂O₂ content in soybean root nodules in these treatments point towards limited ability of the plant to induce its antioxidant system sufficiently to improve salt tolerance. Exogenously applied NO promoted APX and DHAR activities and this was maintained in treatments where NO was added in combination with salt, which was associated with efficient removal of H₂O₂ from the cell. The result obtained from this study is in contrast with a similar work on the response of APX activity to short term salinity exposure in *Phaseolus vulgaris* (61). In that study, application of salt decreased APX enzymatic activity in *P. vulgaris* and this may be a species-dependent or genotype-dependent response to salinity. However, the results reported in this dissertation are consistent with other studies (62) on the response of APX to

short-term salinity exposure in lentils. Application of salt resulted to an increased lentil root APX activity. This high APX enzyme activity accounted for the higher extent of protection from oxidative damage in the lentil root tissues. The contrasting responses of APX to these studies could also be a result of the varying concentrations of salt and duration of treatment amongst these studies. These findings are in agreement with other studies which reported an NO-induced increase in APX and DHAR under salt stress in cucumber roots (54), increased APX activity under salt treatments in wheat seedling leaves (63) and APX induction under salt stress in barley leaves (53). The inference made on the basis of the total APX activity can also be made based on the native gel staining of APX activity as shown in Figure 3-4 where NO increased the pixel intensities of the three APX isoforms, clearly showing NO-enhanced APX activity. Furthermore, analysis of the in-gel enzymatic activities also indicates that the effect of NO on the three APX isoforms was more pronounced when combined with 150 mM NaCl. It is evident that NO function as an antioxidant and results obtained from this study is supported by data in other literature (64, 65).

For effective conversion of H_2O_2 to H_2O , APX requires two molecules of AsA and also depends on DHAR for the reduction of DHAsA to AsA - a reaction that requires GSH as the reducing substrate (66-68). These antioxidants are important redox molecules that participate in the AsA/GSH cycle and their redox status directly affects the activity of APX. In fact, the ratios of these antioxidant metabolites are mostly used as markers of redox status.

The data obtained from this study shows that 150 mM NaCl directly affects the total AsA level, increasing the oxidation of AsA to DHAsA as shown in Figure 3-8. This resulted in decreased AsA/DHAsA ratio (Figure 3-8). Although the DETA treatment had no significant effect on the AsA/DHAsA ratio, application of 10 μM DETA/NO increased the ratio in the

absence of NaCl and effectively alleviated the effect of NaCl by maintaining the AsA/DHAsA ratios to a level similar to those of the untreated plants (Figure 3-8). The fact that NO maintained the AsA/DHAsA in the salt treatment is likely through increased DHAR activity and subsequent effective AsA regeneration. This is evident from the increased DHAR activity (Figure 3-10) in response to supplementation of 10 μ M DETA/NO to 150 mM NaCl-treated samples. The data obtained from 150 mM NaCl alone or in combination with DETA (which lacks the NO moiety) shows a slight increase of DHAR (Figure 3-10) activity, which is linked to the decreased AsA/DHAsA ratio. This study suggests that salinity-induced oxidative stress restricts the level of induction of DHAR activity while allowing for consumption of AsA by the elevated APX activity and the resulting effect is a poor AsA/DHAsA ratio.

The GSH content (Figure 3-9A and B) increased slightly under salt treatment (either alone or combined with DETA) whereas there was highly elevated GSSG content in these treatments. It is possible that the accumulation of GSSG in 150 mM NaCl could be due to the reaction of GSH with ROS generated by the high salt level. Hence as shown (Figure 3-9C), a decrease in the GSH/GSSG ratio was observed. Whereas DETA/NO treatment increased the ratios much above those in the untreated controls, NO was able to increase the GSH levels under salt stress in order to compensate for the high GSSG. As a result, the GSH/GSSG ratio was restored by exogenously applied NO in the salt treatment back to the level similar to the untreated control. It is possible that NO modulated GSH content in soybean root nodules by regulating its biosynthesis or via enhancement of glutathione reductase activity (69).

In conclusion the data obtained from this study, showing a higher level of reduced AsA and GSH pool or their recovery under salt stress respectively is consistent with the higher activities of APX and DHAR enzymes in 10 μ M DETA/NO treatments (either alone or in

combination with 150 mM NaCl). This further demonstrates the function of NO in promoting H_2O_2 scavenging through APX activity.

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Chapter Four

Effect of exogenous application of nitric oxide on salt stress responses of soybean

Summary

Salinity stress is one of the major factors that reduce annual agricultural products. This has led to numerous studies investigating on possible means to improve tolerance to salt stress. Nitric oxide is a gaseous signaling molecule involved in the regulation of diverse processes in plants such as growth, development and disease resistance. Apart from these aforementioned functions, there are multiple studies demonstrating the role of exogenous application of NO in mediating responses to abiotic stress. This chapter investigated the role of exogenously applied NO (DETA/NO) in ameliorating long term salinity stress on soybean. Long term salinity stress in the form of a final concentration of 80 mM NaCl over a 16 day period, drastically affected the plants as indicated by decreased biomass of shoots, roots and nodules of soybean plants. In contrast, supplementation with 10 μ M DETA/NO improved growth of soybean plants under NaCl as evidenced by increased shoot, root and nodule weights and nodule number. Further analysis showed that long-term salinity stress led to increased cellular H₂O₂ content and high levels of cell death in these plants. Treatments with NO, either as DETA/NO alone or in combination with NaCl, resulted in reversal of H₂O₂ to basal levels. The data obtained from this study indicates that application of DETA/NO resulted to increased enzymatic activity of APX. In view of these, the data obtained from this chapter suggests that the role of NO in increasing tolerance to salinity stress in soybean may result from its antioxidant capacity either by direct scavenging of H₂O₂ or its role in activating APX enzymatic activity crucial in scavenging H₂O₂.

Introduction

Crop plants are frequently exposed to various environmental stresses which generally results in decreased yield of agricultural produce. There have been several studies aimed at reducing the decline of agricultural crop produce resulting from these abiotic stress conditions. Amongst these environmental factors, soil salinity is regarded as one of the major factors that pose a great threat to agricultural yield. Most saline soils are observed in regions where there is limited rainfall, high temperature and inadequate soil management. In South Africa, saline soils have been documented (1). These are areas where cropping is predominantly performed under natural rainfall and includes regions where the soil is slightly saline and those where cultivation is practically impossible as a result of salinity and thus leading to low agricultural crop yield.

The deleterious effect of high salinity levels on plants can be attributed to several factors. This includes its capacity to disrupt the ability of roots to extract water hence inducing water stress, membrane disorganization, inhibition of several physiological and biochemical processes (2, 3). A combination of these factors thus results in reduced plant growth, development and survival. Furthermore, it is possible that plants which are exposed to long term salinity could experience ionic stress. The plants could subsequently undergo oxidative damage due to increased rate of production of reactive oxygen species (ROS) such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and singlet oxygen (1O_2) (4, 5). Excessive accumulation of ROS subsequently leads to inhibition of cell division, expansion and death (6).

Soybean (*Glycine max*) is one of the most important leguminous plants used as a source of vegetable protein and oil because of its high protein and oil content (7). Plants in general differ enormously in their ability to tolerate salinity. Soybean is mostly referred to as a moderately salt sensitive plant and hence can be severely affected by highly saline soils (8). Studies have shown the drastic effect of high salt levels in inhibiting soybean seed germination and seedling growth, reduction of nodulation and decrease in crop yield (9). There are other studies describing the effect of high salinity in reducing the number and weight of root nodules in soybean and other legumes (10-12). Some studies (13) have described reduced leaf area and lower root/shoot ratios as some of the effects of salinity on plants. Moreover, some studies investigated the ability of plants to tolerate high salinity levels by assessing the percentage biomass production in saline against control conditions after a long term exposure (14).

In order to minimize the effects of oxidative stress induced by salinity, plants have developed different mechanisms to remove or reduce the accumulation of ROS. This involves the mobilization of an array of antioxidant enzymes and several antioxidant metabolites that function in neutralizing and detoxifying ROS. Amongst the antioxidant enzymes, ascorbate peroxidase (APX) plays a major role in regulating the level of H_2O_2 , by catalyzing its conversion into H_2O through utilizing ascorbate as its electron donor (15-18). There are studies showing increased enzymatic activities of several antioxidant enzymes in plants under salt stress (19-21).

Nitric oxide (NO) is a signaling molecule that is involved in several physiological processes in plants. Apart from its role in promoting normal growth and development of plants at low concentrations (22), there are several lines of evidence supporting its role in alleviating the

oxidative damage of salinity in several plant species (23-25). Furthermore, data presented in the previous chapter of this thesis validates the protective role of NO against salt-induced oxidative stress. This was evident as application of the NO donor 2,2'-(hydroxynitrosohydrazono) bis-ethanimine (DETA/NO) alone and not DETA, increased the enzymatic activities of APX isoforms and maintained the GSH/GSSG and AsA/ DHAsA ratio of soybean under salt stress.

Although several studies showing the effect of exogenous application of NO in inducing salinity tolerance in plants have been reported, there are only a few studies showing this protective role over a long-term salinity exposure in soybean. Furthermore, given that the ability of plants to tolerate salinity can be estimated by evaluating their tissue and organ mass, an investigation on the role of NO to reduce salinity effect on soybean over long-term exposure to salt was prompted. Hence the main objective of this chapter was to analyze growth parameters (root nodule number and dry weight, shoot and root dry weights) of the plants grown under various treatments involving NO and NaCl. This chapter evaluates cell viability, H₂O₂ content and the enzymatic activity of APX isoforms in soybean root nodules in relation to the role of NO in alleviating oxidative stress induced by high salinity.

Materials and method

Materials

Glycine max L. merr. cv. PAN 626 seeds used for this work were kindly provided by Pannar (Pty) Ltd (Greytown, South Africa) and all the chemicals were purchased from Sigma-Aldrich, unless otherwise stated. The Rhizobium inoculum *Bradyrhizobium japonicum* as the commercial peat-based HiStick 2 Soybean Inoculant was supplied by Becker Underwood Ltd., West Sussex, United Kingdom. The filtered silica sand (98% SiO₂) was purchased from Rolfes[®] Silica (Pty) Ltd, Brits, North West, South Africa.

Methods

Plant growth

Soybean seeds were surface-sterilized in 0.35% v/v sodium hypochlorite for 10 minutes, followed by five washes with sterile distilled water. The seeds were imbibed in sterile distilled water for 1 hour and inoculated with *Bradyrhizobium japonicum*. Seeds were sown in filtered silica sand that had been pre-soaked in distilled water, in 15 cm diameter plastic pots (one plant per pot) and were grouped into six experimental test set: Untreated, 10 μ M DETA/NO, 10 μ M DETA, 80 mM NaCl, 10 μ M DETA/NO + 80 mM NaCl and 10 μ M DETA + 80 mM NaCl. Seeds designated for long-term salt treatment were gradually introduced to increasing NaCl concentrations. First, 10 mM NaCl was dissolved in nitrogen-free nutrient solution [1 mM K₂SO₄, 2 mM MgSO₄, 3 mM CaCl₂, 1 mM K₂HPO₄ buffer at pH 7.3, 25 μ M

H₃BO₃, 2 µM MnSO₄, 2 µM ZnSO₄, 2 µM CuSO₄, 2 µM Na₂MoO₄, 0.1 µM CoSO₄, 50 µM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3] and the solution was applied to the sand in the pot when the plants were at the V1 stage of growth. The gradual increase of NaCl concentration was essential to avoid large and sudden decrease in water potential. The germinated seedlings were grown on a 25/19 °C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m⁻².s⁻¹ during the day phase, in a randomized design.

Treatment of plants

Once the plants reached the VC stage (when unifoliolate leaves are fully expanded), they were supplied with nitrogen free nutrient solution containing 1 mM K₂SO₄, 2 mM MgSO₄, 3 mM CaCl₂, 1 mM K₂HPO₄ buffer at pH 7.3, 25 µM H₃BO₃, 2 µM MnSO₄, 2 µM ZnSO₄, 2 µM CuSO₄, 2 µM Na₂MoO₄, 0.1 µM CoSO₄, 50 µM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3 at three day intervals until they reached the V3 stage (third trifoliolate). For salt treatment, NaCl was supplied with the nitrogen-free nutrient solution at a final concentration of 10 mM initially when the plants were at the V1 stage, then the final concentration of the salt in the nutrient solution supplied to the plants was increased to 20 mM NaCl three days after the initial (10 mM) salt treatment. Three days later, the plants were supplied with nutrient solution containing a final concentration of 40 mM NaCl. Three days later, the plants were supplied with nutrient solution containing a final concentration of 80 mM NaCl and this application of 80 mM NaCl was repeated twice more at intervals of three days (so that the total number of days of plant

exposure to NaCl was 16 days). Plants were harvested one day (24 hours) after this final treatment.

A treatment regime similar to the one described above for NaCl was done with the NO donor DETA/NO at a final concentration maintained at 10 μ M throughout the treatment period either in the absence of NaCl or in combination with NaCl in increments similar to those described for the salt treatment. Plants were harvested one day (24 hours) after this final treatment. A similar control treatment was performed in which DETA (final concentration maintained at 10 μ M) was used in the place of DETA/NO either in the presence or absence of NaCl in a similar manner as in the increments described for the salt treatment. Plants were harvested one day (24 hours) after this final treatment.

The freshly harvested plants were used for measurement of growth parameters and nodule cell viability whereas the snap-frozen (in liquid nitrogen) nodules were used for all other assays (in which case the tissue was stored at -80°C until further use).

Measurement of growth parameters

Plants were carefully removed from the sand, avoiding any loss of roots or shoot during the process of harvesting. The root nodules from each set of the treatments were carefully detached from the roots and counted. The roots were cut off from the shoots and all fresh materials (roots, shoots and nodules) were weighed. The samples were oven-dried at -80°C for three days and the dry weights were recorded.

Evaluation of cell viability in soybean root nodules

In order to establish if application of NO (as 10 μ M DETA/NO) could maintain root nodule cell viability after long term salinity stress, evaluation of root nodule cell viability was carried out. This cell viability assay was estimated in soybean root nodules by modifying a method previously described by Sanevas *et al.*, (26).

The roots nodules (100 mg per treatment) from three different plants of each of the six treatments were harvested and stained with 0.25% (w/v) Evans Blue for 15 minutes at room temperature. The root nodules were then washed for 30 minutes in distilled water, followed by extraction of the Evans Blue stain (taken up by dead nodule cells) from root nodule tissue using 1% (w/v) Sodium Dodecyl Sulphate (SDS) after incubation for 1 hour at 55°C. Absorbance of the extract was measured at 600 nm to determine the level of Evans Blue taken up by the root nodule tissue.

Measurement of H₂O₂ content

H₂O₂ content was determined in the nodule extracts by modifying a previously described method by Velikova *et al.*, (27). *Glycine max* nodule tissue (100 mg) was ground to fine powder in liquid nitrogen and homogenized in 400 μ l of cold 6% (w/v) TCA. The extracts were centrifuged at 12,000 x *g* for 30 minutes at 4 °C and 50 μ l of the supernatant were used to initiate the reaction in a mixture (total volume of 200 μ l) containing 5 mM K₂HPO₄, pH 5.0 and 0.5 M KI. The reaction was incubated at 25 °C for 20 minutes and absorbance readings were recorded at 390 nm. H₂O₂ content was calculated using a standard curve based on the absorbance ($A_{390\text{ nm}}$) of H₂O₂ standards.

Determination of APX enzymatic activity

Plant APX activities were measured in nodule extracts by modifying a method previously described by Asada (28). The nodule extracts which were supplemented with ascorbate to a final concentration of 2 mM, were added to the assay buffer containing 50 mM K_2HPO_4 , pH 7.0, 0.1 mM EDTA and 50 mM ascorbate. The reaction was initiated by adding 1.2 mM H_2O_2 in a final reaction volume of 200 μ l and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$.

Determination of protein concentration

Protein concentrations for all assays were measured in the extracts as instructed for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories).

Statistical analysis

One-way analysis of variance (ANOVA) test was used for all data to evaluate statistical validity of the results and means were compared according to the Tukey-Kramer test at 5% level of significance, using Graph Pad Prism 5.03 software.

Results

After harvesting, the effect of salinity on growth parameters, cell viability, APX activity and H_2O_2 level were evaluated. Furthermore the effect of exogenously applied NO in alleviating salinity-induced oxidative stress over a long-term period (16 days) was also evaluated.

Plant growth parameters

As shown in Figure 4-1, plants treated with NaCl experienced a reduction in root dry weight compared to untreated plants. This reduction in root weight was however reversed by a combination treatment in which plants were exposed to NaCl combined with 10 μM DETA/NO.

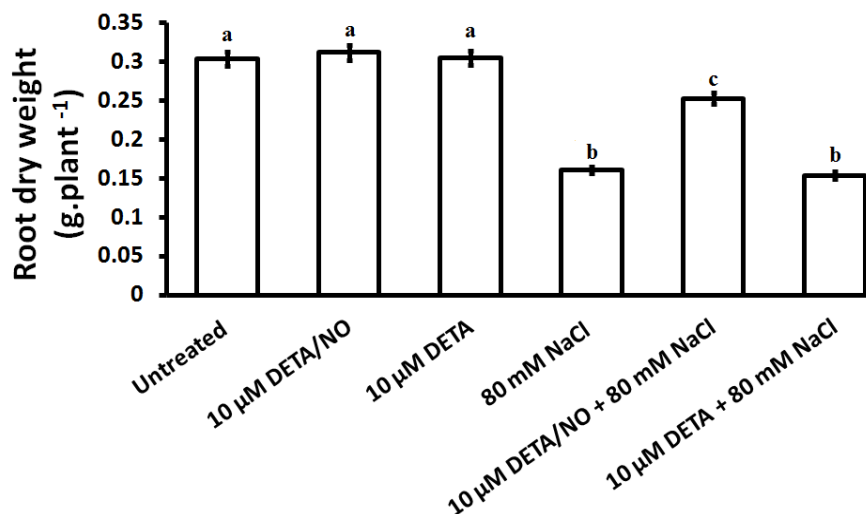


Figure 4-1. Effect of salinity-induced stress and application of exogenous NO on root biomass expressed as dry weight. Dry weights of soybean root were measured after 16 days of treatment with either nitrogen free nutrient solution only (Untreated), 10 μM DETA/NO, 10 μM DETA, a final concentration of salt at 80 mM NaCl, 10 μM DETA/NO + 80 mM NaCl final salt concentration or 10 μM DETA + 80 mM NaCl final salt concentration. Three plants were analyzed for each treatment. Data shown are the mean ($\pm\text{SE}$) of three independent experiments.

The improvement of root dry weight under salt stress was due to the presence of NO (released from the NO donor DETA/NO) and this is as such since there was no marked

improvement on shoot biomass treated with 80 mM NaCl combined with 10 μ M DETA (which does not release NO). There was no significant difference in root weight between untreated, DETA-treated plants and plants treated with 10 μ M DETA/NO. Exposure of soybean to salt resulted in a significant decrease of root weight (49% reduction) when compared to untreated controls whereas root weight in plants treated with NaCl combined with DETA/NO was only decreased by 16% when compared with untreated control.

Soybean shoot weights were similar amongst the untreated, 10 μ M DETA/NO and 10 μ M DETA-treated plants (Figure 4-2). However there was a drastic reduction of shoot weight as shown amongst NaCl-treated plants treated (Figure 4-2).

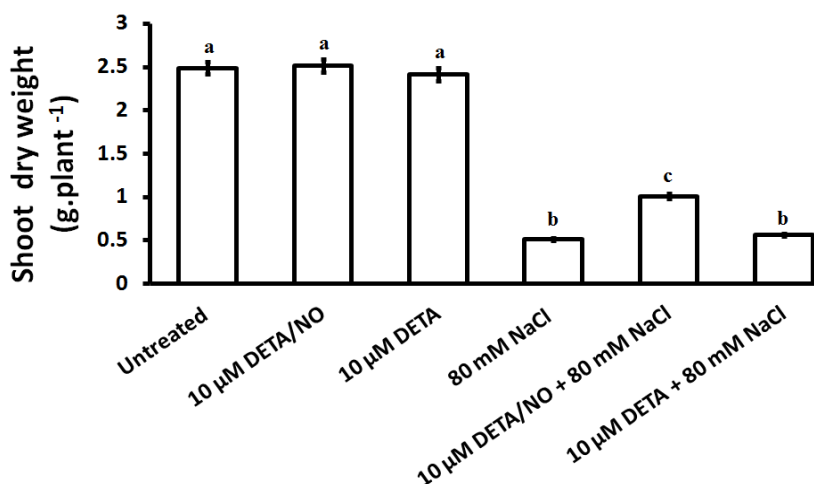


Figure 4-2. Effect of salinity-induced stress and application of exogenous NO on shoot biomass expressed as dry weight. Dry weights of soybean shoot were measured after 16 days of treatment with either nitrogen free nutrient solution only (Untreated), 10 μ M DETA/NO, 10 μ M DETA, salt (80 mM NaCl final concentration), 10 μ M DETA/NO + salt or 10 μ M DETA + salt. Three plants were analyzed for each treatment. Data shown are the mean (\pm SE) of three independent experiments.

Shoot dry weight was reduced significantly by approximately 79% in the NaCl-treated plants when compared to untreated controls (Figure 4-2). A similar reduction was observed when the plants were treated with NaCl in combination with 10 μ M DETA. Although there was a

decrease in shoot weight of plants treated with NaCl in combination with 10 μ M DETA/NO, this decrease (~ 52%) was less severe as there was an improvement on dry shoot weight although not entirely to the weight of untreated controls (Figure 4-2). This suggests that application of DETA/NO, and not DETA, has an effect in improving shoot biomass under salinity stress.

Similar to data obtained from the dry root and shoot weights, a drastic decrease in nodule dry weight was observed in plants treated with salt. Application of NaCl resulted in a significant decrease of dry nodule weight by 70% when compared to untreated control (Figure 4-3).

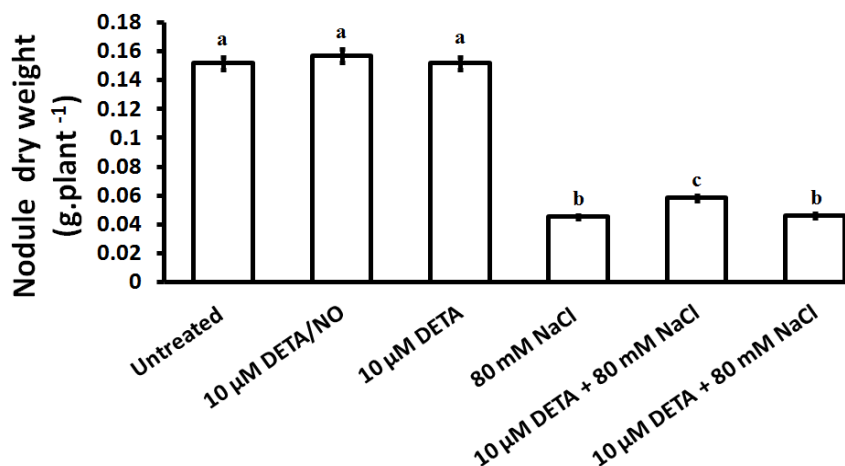


Figure 4-3. Effect of salinity-induced stress and application of exogenous NO on nodule biomass expressed as dry weight. Dry weights of soybean nodule were measured after 16 days of treatment with either nitrogen free nutrient solution only (Untreated), 10 μ M DETA/NO, 10 μ M DETA, NaCl (at a final concentration of 80 mM NaCl), 10 μ M DETA/NO + NaCl or 10 μ M DETA + NaCl. Three plants were analyzed for each treatment. Data shown are the mean (\pm SE) of three independent experiments.

As shown in Figure 4-3, reduction in nodule dry weight resulting from application of NaCl was considerably reversed when plants were treated with a combination of NaCl and DETA/NO. Nodule dry weight reduction of this set of plants was only 59% when compared to

untreated controls. Conversely application of NO via DETA/NO appears to reduce the harmful effect of salt stress on soybean root nodule biomass. This is evident since plants treated with NaCl in combination with 10 μ M DETA could not alleviate the salinity-induced reduction of root nodule weight.

Figure 4-4 shows varying nodule numbers of soybean root nodules exposed to different treatments. Nodule number was reduced significantly (60%) as a result of treatment of soybean with NaCl, moreover there was no significant difference in the number of nodules observed when NaCl was combined with 10 μ M DETA.

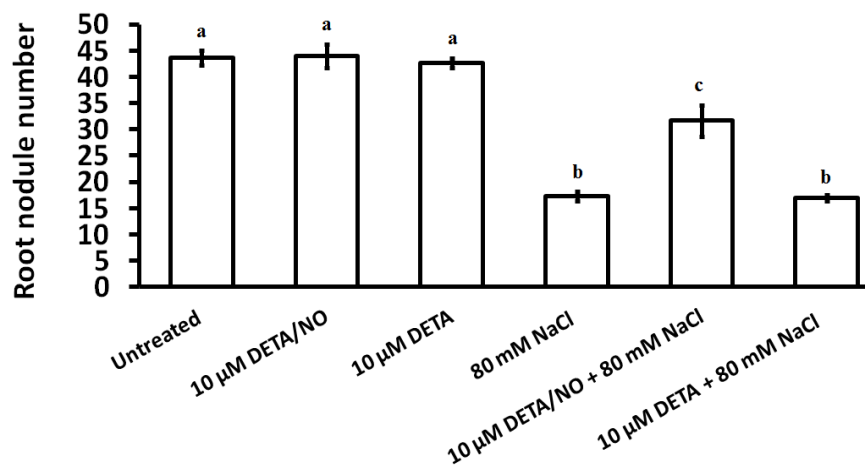


Figure 4-4. Effect of salinity-induced stress and application of exogenous NO on root nodule number. Numbers of root nodules were scored after 16 days of treatment with either nitrogen free nutrient solution only (Untreated), 10 μ M DETA/NO, 10 μ M DETA, salt at a final concentration of 80 mM NaCl, 10 μ M DETA/NO + salt or 10 μ M DETA + salt. Three plants were analyzed for each treatment. Data shown are the mean (\pm SE) of three independent experiments.

However nodule numbers in soybean plants treated with a combination of NaCl and 10 μ M DETA/NO was decreased by approximately 27% when compared to untreated control. This suggests that application of the NO donor could help in maintaining nodule number.

Nodule cell viability

Once it was established that exposure of plants to long-term salinity stress resulted in drastic loss plant growth, it was investigated if these plants could be experiencing more cell death than the untreated and DETA/NO-treated plants. Hence cell viability was carried out on the nodules of the variously treated plants.

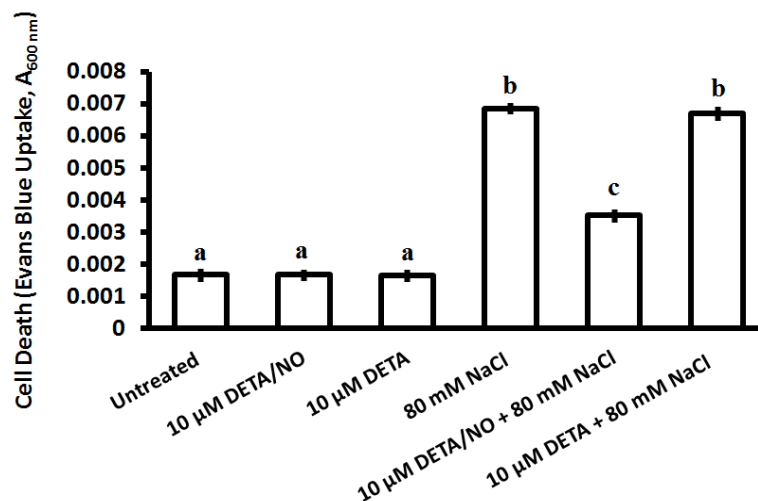


Figure 4-5. Changes in cell viability in soybean root nodules. The assay was performed after 16 days of treatment with either nitrogen free nutrient solution only (Untreated), 10 µM DETA/NO, 10 µM DETA, NaCl (final concentration of 80 mM NaCl), 10 µM DETA/NO + NaCl or 10 µM DETA + NaCl. Three plants were analyzed for each treatment. Data shown are the mean (\pm SE) of three independent experiments.

Treatment of plants with NaCl resulted to severe loss of nodule cell viability. This is indicated by the sharp increase of approximately 3-fold in Evans Blue uptake compared to untreated plants. The combination treatment of NaCl and DETA/NO on the plants resulted in a reversal of the reduction in nodule cell viability as shown in Figure 4-5. Improvement of cell viability was evident as there was a lesser increase (100%) of Evans Blue Uptake compared to untreated plants. On the other hand, a combination treatment with NaCl and DETA did not reverse the suppressive effect of long-term salt stress on nodule cell viability. This suggests that application of NO (as DETA/NO) is effective in maintaining nodule cell viability under salt stress.

H₂O₂ content in soybean root nodules

In view of the fact that cell death can result from excessive accumulation of ROS, data obtained from the cell viability assay prompted further investigation on the H₂O₂ level in soybean treated plants.

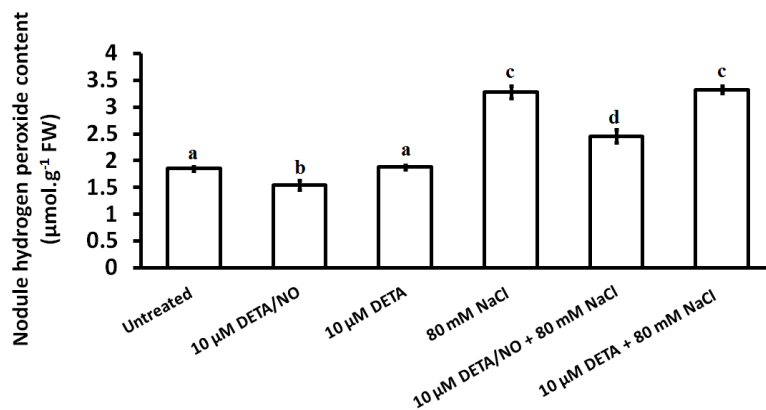


Figure 4-6. Effect of long-term salt treatment and application of exogenous NO on root nodule hydrogen peroxide content. H₂O₂ content was assayed after 16 days of treatment with either nitrogen-free nutrient solution only (Untreated), 10 μM DETA/NO, 10 μM DETA, NaCl (final concentration of 80 mM NaCl), 10 μM DETA/NO + NaCl or 10 μM DETA + NaCl. Three plants were analyzed for each treatment. Data shown are the mean (±SE) of three independent experiments.

Interestingly, whereas application of DETA had no effect in reducing H₂O₂ content, plants treated with 10 μM DETA/NO showed a significant decrease of H₂O₂ content by approximately 32% when compared with untreated control, as shown in Figure 4-6. Treatment of soybean plants with NaCl resulted in a significant increase of H₂O₂ content by approximately 78% when compared with untreated control. This increase in nodule H₂O₂ content was reversed by a combination treatment in which plants were exposed to NaCl combined with 10 μM DETA/NO. However such a reversal was not observed when plants were treated with a combination of to 10 μM DETA and NaCl. This suggests that long-term salinity exposure increases H₂O₂ content and NO acts to limit this effect by reducing the level of H₂O₂ in the nodules.

Total APX enzymatic activity

Data obtained from H_2O_2 analysis showing a reduced level of this ROS when plants were treated with either 10 μM DETA/NO or a combination of 10 μM DETA/NO with NaCl suggests that there could be induction of antioxidant enzyme activity that scavenges the H_2O_2 . In view of this, further investigation was carried out to determine the effect of exogenous application of DETA/NO on total APX enzymatic activity in soybean root nodules exposed to long-term salt stress.

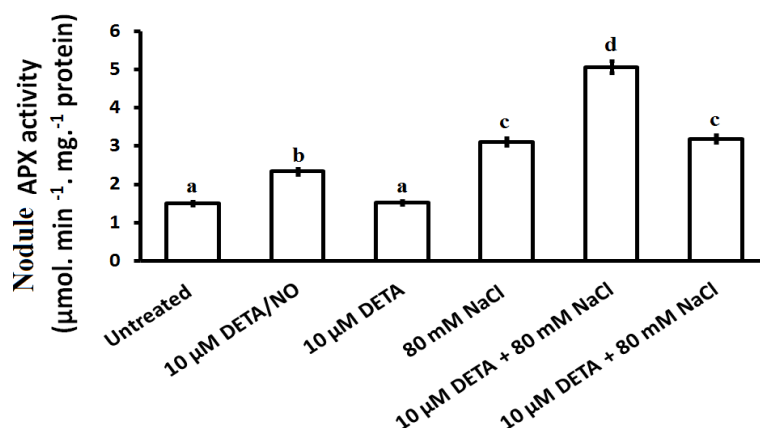


Figure 4-7. Effect of long-term salt treatment and application of exogenous NO on root nodule APX activity. APX activity was measured after 16 days of treatment with either nitrogen-free nutrient solution only (Untreated), 10 μM DETA/NO, 10 μM DETA, Salt (final concentration of 80 mM NaCl), 10 μM DETA/NO + Salt or 10 μM DETA + Salt. Three plants were analyzed for each treatment. Data shown are the mean (\pm SE) of three independent experiments.

As shown in Figure 4-7, whereas there was no marked difference in the total APX enzyme activity between the untreated and DETA-treated soybean root nodules, treatment of soybean plants with 10 μM DETA/NO resulted to a significant (~55%) increase of APX activity when compared to untreated samples. Also, there were marked increases of APX activity in response to long-term salt exposure. The salt-induced increase in APX activity was

approximately 107% higher in response to NaCl than the untreated control. No significant difference in the level of APX activity was observed in response to NaCl when compared to the combination treatment of 10 μ M DETA + NaCl on soybean root nodules. Interestingly, treatment of soybean root nodules with 10 μ M DETA/NO + NaCl resulted to the highest (~ 237) up-regulation of total APX enzymatic activity in root nodules when compared to the untreated control.

Discussion

Abiotic stress in the form of soil salinity has been a major focus of many research groups in plant science (3, 29). This stress factor imposes ionic toxicity and osmotic stress which subsequently results in nutrient deprivation and oxidative stress (30-32). Studies evaluating salt tolerance in different soybean cultivars assessed long-term salinity effects on the plants by measuring height, fresh and dry weights of the shoot (33).

There is vast amount of evidence validating the role of NO in conferring salt tolerance in several plant species by using pharmacological agents to manipulate endogenous NO levels (24, 34).

The results obtained from this study show that long-term salinity exposure caused a significant decrease in all soybean growth parameters. This reduction in the weight of the shoots, roots and nodules is indicative of the deleterious effect of salinity. Several studies suggest that plant growth reduction resulting from salinity could be associated with restrained water uptake by roots, leading to low plant survival (35). Furthermore, reduction in nodule dry weight and nodule number is a known occurrence in soybean in response to salinity stress (36-38). This is due to the fact that salt stress mitigates the aerobic respiration of nitrogen fixing bacteria and also reduces the leghemoglobin content in root nodules. The resulting consequence of this is a reduced energy level necessary for nitrogen fixation (39).

Findings from this study indicated that exogenous application of NO, as 10 μ M DETA/NO, had a protective effect against long-term salt stress-induced loss in soybean biomass. Furthermore, the observation that soybean plants exposed to long-term salinity stress in combination with 10 μ M DETA/NO treatment had improved biomass suggests that NO can act as growth regulator and thus limit the salinity-induced stress. The various results on

growth parameters obtained from this study are in agreement with other experiments which show that NO plays a role in the signaling network to induce tolerance against salinity (40). In a study on the role of NO (through the application of DETA/NO) in ameliorating salt stress, it was shown that NO acts by restricting lipid peroxidation induced by excessive O_2^- and H_2O_2 (41). In this study, application of DETA/NO to salt-stressed maize plants resulted in elevated NO content in maize tissue, which increased the capacity of the plant to tolerate the stress condition, resulting in improved biomass.

There are numerous studies which have shown amongst other ROS, increased levels of plant tissue H_2O_2 resulting from salinity stress (30). Although these ROS are essential in plant cells as they act as signaling molecules in mediating several physiological processes, their excessively elevated concentrations lead to damage of plant cellular structures and macromolecules such as lipids, enzymes and DNA. It is likely that the major underlying mechanism by which NO confers salt tolerance in plants is through restoration of redox balance. The reduction of H_2O_2 levels, when 10 μ M DETA/NO was supplemented to salt-stressed plants, highlights the significance of NO in facilitating the scavenging of H_2O_2 produced within the cell of the plant. This result is in agreement with studies which have shown the reduction of salt stress-induced elevation in ROS levels through exogenous application of NO (42). Further evidence suggesting that application of NO leads to salt tolerance is shown by the extent of cell death in the soybean plants. Long-term salinity stress led to severe cell death as deduced from the amount of Evans Blue uptake. Exogenous application of 10 μ M DETA/NO reversed this effect as cell death was considerably lowered. However this was not the case when salt stressed plants were treated in combination with 10 μ M DETA. This observation indicates the role of NO in maintaining plant cellular integrity under salt stress.

The observation that exogenous application of 10 μM DETA/NO to soybean plants led to reduced level of H_2O_2 within the soybean cell suggests that NO could act via induction of antioxidant enzymes during long-term salinity stress. In an effort to reduce the harmful effect of excess ROS arising due to salinity stress, plants mobilize an array of antioxidant enzymes to detoxify and control the balance of these ROS (43). Several studies have shown evidence of increased APX enzymatic activity under salt stress and this is associated with plant tolerance to salinity (44, 45). Furthermore it has been demonstrated in various studies using plant species subjected to different treatments that exogenous application of NO can stimulate antioxidant enzymes (24,46,47). In the present study, long-term salinity stress resulted in the increased total APX enzymatic activity when compared to untreated control. The result obtained from spectrophotometric analysis indicates that although application of 10 μM DETA/NO (alone) equally increased the APX enzymatic activity, long-term salinity stress (NaCl or NaCl in combination with 10 μM DETA) resulted in higher APX enzymatic activity. Nevertheless, the elevated APX activity under long-term salinity stress was not sufficient to reduce the harmful effect of salinity-induced stress as indicated in cell viability and H_2O_2 data shown in Figures 4-5 and 4-6, respectively. Soybean plants exposed to salt stress but simultaneously supplemented with 10 μM DETA/NO recorded the highest total APX activity. The relevance of this increased APX activity is shown by the corresponding decreased H_2O_2 content and improved cell viability. The inference drawn from this is that the highest level of APX enzymatic activity induced by exogenous application of 10 μM DETA/NO under salinity is an indication of the role of NO in stimulating the antioxidant enzyme, thus efficiently conferring some degree of tolerance to long-term salinity stress by enhancing the scavenging of H_2O_2 . The result obtained from this study is consistent with other studies (48) on the response of APX to long-term salinity exposure, where application of salt resulted in

increased root APX activity. This study supports the activation of antioxidant enzymes in the scavenging of ROS which can improve tolerance to salt stress

It is well established that in the plant cell, APX functions in the reduction of excess ROS into water and monodehydroascorbate (49). Overall, the results obtained from growth parameter analysis, H_2O_2 content, cell viability and APX enzymatic activity indicate that long-term salinity exposure leads to oxidative stress as evidenced by H_2O_2 levels and the accompanying cellular damage (inferred from reduction in nodule cell viability) in soybean plants. On the contrary, exogenous application of DETA/NO (an NO donor) improved salt tolerance in soybean plants due to the positive effects of NO on acting as an antioxidant in removing excess H_2O_2 , possibly via its ability to activate APX.

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Chapter Five

General Conclusion

The versatile role of nitric oxide (NO) in diverse physiological functions and coordination of an efficient defense response to biotic and abiotic stress in plants has made this signaling molecule an interesting subject in plant science. NO production in plants is categorized as either enzymatically or non-enzymatically. However, application of exogenous NO to plants through the use of various NO donors has been one of the major experimental approaches towards studying the involvement of NO in plants growth and development. The underlying key to a proficient dissemination of functions by NO lies in its chemistry and this includes its direct and indirect interaction with several secondary messengers. Although there are a few cases in which the mechanism of action and cell biological nature of NO response is still not conclusive.

It is evident that reactive oxygen species (ROS) are a major signal in various biological processes in plants. But excess level of ROS generated during abiotic stress is deleterious to the cells, resulting to oxidation of lipids, proteins and DNA. Thus it is necessary that the equilibrium between the production and removal of these molecules is consistently controlled. Plants possess an efficient anti-oxidant system which consists of antioxidant enzymes and non-enzymatic scavengers that acts in concert towards protecting the plant from the oxidative effects exerted from ROS. Amongst the antioxidant enzymes, ascorbate peroxidase enzyme is considered to play a crucial role in the removal of ROS and protecting

cells in plants. Hence this study was centered on nodular soybean APX activity and its response to abiotic stress in the form of salt stress and induction by exogenously applied NO. The first question addressed concerns the possible release of NO from the choice of NO donor used in this study, 2,2'-(hydroxynitrosohydrazono) bis-ethanimine (DETA/NO). This study showed that the NO donor could increase NO content in the treated soybean plants.

An interesting finding shown in this study is that exogenous application of NO via DETA/NO increases the enzymatic activity of three APX isoforms in soybean root nodules in a concentration dependent manner. Furthermore genetic manipulation of these isoforms could prove useful towards NO-mediated abiotic stress tolerance in plants.

Another subject addressed in this study is on the role of NO in ameliorating salinity induced oxidative stress in plants. This was investigated under short and long term salinity exposure in-order to evaluate molecular changes as well as plant growth parameters resulting from salinity-induced stress.

This study demonstrated that tolerance to salinity-induced stress in soybean is as a result of exogenously applied NO through DETA/NO which increased NO content and orchestrated the enzymatic defense system. Although there were increased APX activity level in response to salinity stress, the levels observed were not sufficient to induce tolerance to salinity stress as observed in this study, however supplementing with NO donor resulted in tolerance to salinity stress. Furthermore the role of exogenously applied NO in inducing tolerance to salinity is not limited to its induction of antioxidant enzymes but also in maintaining GSH/GSSG and ASC/ DHAsA ratio under salt stress conditions.

In conclusion, this study demonstrates the role of exogenous applied NO in mediating plant tolerance to salinity-induced stress. Furthermore, this study provides a background for future studies. It will be important to analyze the promoter regions of the three APX genes in-order to identify cis-acting elements that could be responsible for the NO-dependent regulation of the expression of the corresponding soybean nodule APX genes. This will establish if there is a relationship between the occurrence of such cis-acting elements and abiotic stress-dependent regulation of the expression of the APX genes.