Nitric oxide signaling and cysteine protease activity in the modulation of abiotic stress responses in soybean and maize

By

Mawethu Pascoe Bilibana

Supervisor: Dr NN Ludidi

Abstract

Nitric Oxide (NO) is an essential bioregulatory molecule in plant growth, development, and tolerance against biotic and abiotic stresses. In legume root nodules, abiotic stresses impose restraint on metabolic capacity of bacteria and cause oxidative damage to cellular macromolecules, leading to inhibition of nitrogenase activity. In this study, the primary aim was to determine the influence of NO signaling on cysteine protease activity in soybean (*Glycine. max* [L] Merr) root nodules. Intact plants were treated with a NO donor, diethylenetriamine/nitric oxide adjunct (DETA/NO), 8-(4-chlorophenylthio)-[CPT]-cGMP, sorbitol and sodium chloride (NaCl). The root nodule cysteine protease activity was measured using the chromogenic substrate N-benzoil-L-p-nitroanilide (L-BAPNA). The results demonstrated NO as acting both as a protection against programmed cell death (PCD) at low exogenously applied NO concentrations, or as inducing PCD through regulating the cysteine proteases activity in root nodules when NO is applied at elevated concentrations. In the root nodules, the activity of cysteine protease is regulated either through cyclic guanosine monophosphate (cGMP)-dependent during abiotic stress or cGMP-independent pathways during normal root nodule development. The purpose of this research was to highlight the importance of NO in cell signaling and cysteine protease activity in legume root nodules. We also focused on the effect of abiotic stress on two maize genotypes as well as the influence of abiotic stress on cysteine protease activity in the abiotic stress-sensitive maize genotype than the tolerant genotype. The study suggests that cysteine protease activity can be used as early screen to identify abiotic stress-sensitive/tolerant maize genotype upon exposure to abiotic stress.

Declaration

I hereby declare that "**Nitric oxide signaling and cysteine protease activity in the modulation of abiotic stress responses in soybean and maize**" is my own work that has not been submitted for any degree or examination at any other university and that all the source I have used or quoted have been identified and acknowledged by complete references.

Mawethu Pascoe Bilibana   September 2010

Signed………………………

Thesis presented in partial fulfillment of the requirements for the degree of Magister Scientiae at the institute for Plant Biotechnology, Stellenbosch University, South Africa.

I 'm dedicating this thesis to my late grandmother Mrs Ethel Siphika, I will always miss her encouragement and endless love.

Acknowledgment

Table of Contents

1.1 Grain Legumes

1.1.1 Maize as a crop plant

1.2 Legume symbiosis

1.2.1 Root nodule development

1.2.2 Root nodules

1.2.3 Nitrogen fixation in root nodules

1.3 Nitric oxide in plants

1.3.1 Nitric oxide signalling during nodule development

1.3.2 The source of Nitric oxide in plants

1.3.3 Modulation of physiological processes by nitric oxide

1.3.4 Nitric oxide as a biological messenger

1.5 Programmed cell death in plants

1.5.1 Role of NO in programmed cell death in plants

1.5.2 Modulation of protease activity in plants

1.5.2.1 Classification of proteases

1.5.2.2 Proteolytic machinery

1.5.2.3 The main catalytic classes of proteases

1.5.2.4 Cysteine Proteases

1.5.2.4.1 Cysteine Families (Clans)

1.5.2.4.2 Papain-like enzymes (Clan CA)

1.5.2.4.3 Catalytic Mechanism of papain-like proteases

1.5.2.4.3.1 Structure and function of papain-like proteases

1.5.2.4.4 Caspases (cysteinyl-aspartic-acid-proteases)

1.5.2.5 Inhibition of protease catalytic activity

1.5.2.6 Role of cysteine proteases in plant responses to abiotic stress

1.5.2.6.1 Cysteine proteases in root nodules in response to abiotic stress

List of figures and tables

Abbreviations

BSA	Bovine serum albumin
E-64	Trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane
cm	Centimeter
DETA/NO	Diethylenetriamine/Nitric Oxide adjunct
DTT	Dithiothreitol
g	Gram
k	Kilo
L	Litre
L-BAPNA	N-benzoil-L-p-nitroanilide
M	Molar
mM	Milimolar
ml	Millitre
min	Minute
NO	Nitric Oxide
NOS	Nitric Oxide synthase
nM	Nanomolar
$O.D._{600}$	Optical density (at 600nm)
PMSF	Phenylmethylsulphonyl fluoride
μg	Microgram
μl	Microgram
μM	Micromolar
v/v	Volume per volume
w/v	Weight per volume
%	Percentage

Chapter 1

1.Literature review

1.1 Grain Legumes

Grain legumes (e.g. soybeans, peas and beans) provide a good source of high quality protein for human and animal nutrition as well as being used in diverse industrial applications such as production of biofuels and pharmaceuticals. In general, abiotic and biotic stresses decrease plant growth and crop yield, resulting in negative impact on world food supply (Linsenmair *et al*., 2001; Walley *et al*., 2007; Jagadeeswaran *et al*., 2009). Indeed, legumes are constantly exposed to a plethora of external environmental factors during their growth including ozone exposure, wounding, water/nutrition deficiency, salinity, pathogen and pest attack; all of which are major causes of legume crop yield reduction. However, numerous studies show that legumes have an ability to adapt to variations in environmental conditions due to various adaptation and detoxification mechanisms (Evans 1976; Crockford *et al.,* 1996**;** Linsenmair *et al*., 2001; Piechalaka *et al*., 2002; Walley *et al*., 2007). Hence, legumes have evolved a nitrogen-fixing mechanism by forming a symbiosis with bacteria of the genus *Rhizobium*, *Sinorhizobium* and *Azorhizobium*, which leads to the formation of a new organ, the root nodule (figure 1.1).
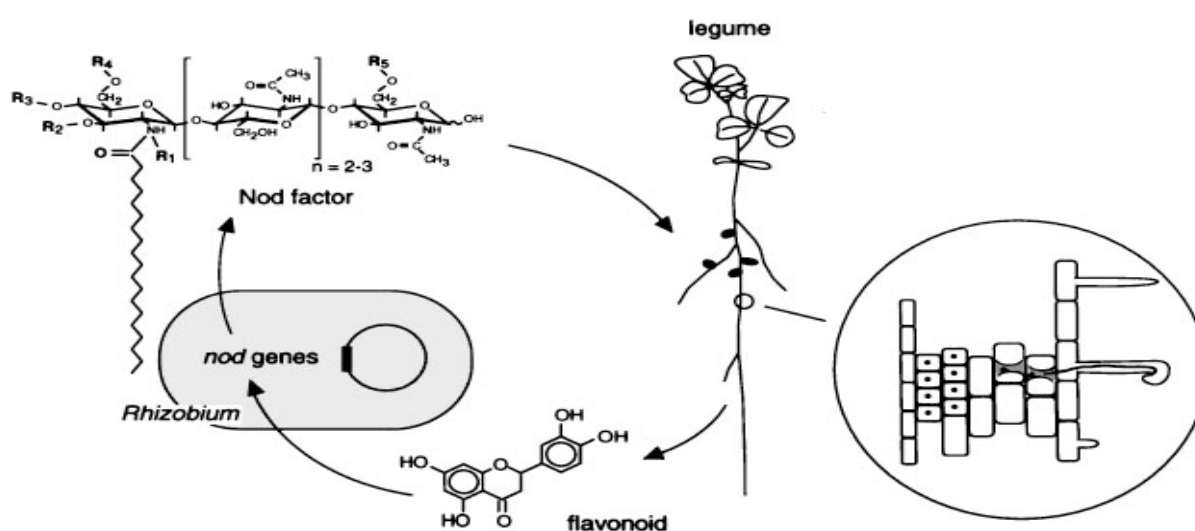
Figure 1.1. Signal exchange in the *Rhizobium*-legume symbiosis. The insert illustrates an infection thread passing through the root cortex toward a cluster of dividing cells that will become a nodule primordium (Schultze and Kondorosi**,** 1998).

The symbiosis between leguminous plants and rhizobia is of agronomic importance, reducing the need for use of nitrogen fertilizer for soil enrichment to enhance yield in agriculturally important plants. Hence, the presence of root nodules allows legumes to fix atmospheric nitrogen and export it to other parts of the plant in return for carbohydrates necessary for the energy-consuming process of nitrogen fixation figure 1.2 (Schultze and Kondorosi, 1998). Studies at both the physiological and molecular level have provided some basic understanding of several steps that occur during the establishment of the symbiosis, controlled by molecular signal exchanges between the plant and its rhizobial symbiotic partner (Genoud *et al.,* 2001).
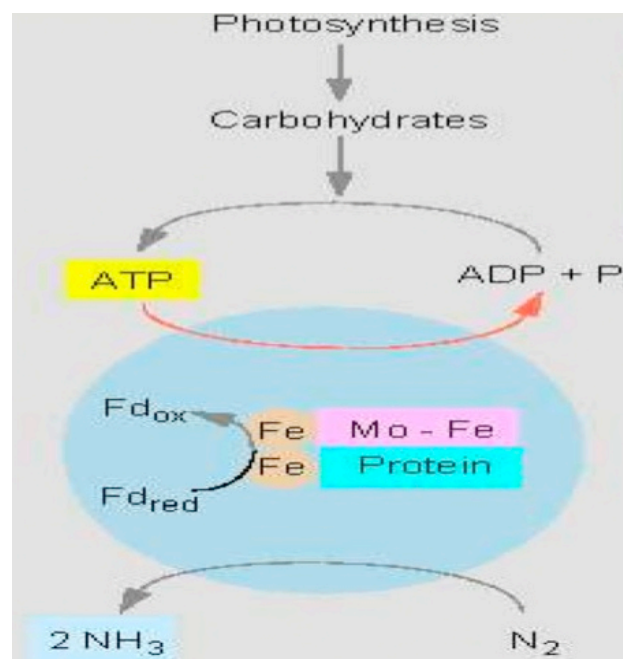
Figure 1.2. Schematic of metabolic interactions in a root nodule cell containing N_2-fixing bacteroids.

1.1.1 Maize as a crop plant

Maize (*Zea mays* L.) is extensively grown as a cereal crop throughout the world, serving as human and animal food source in sub-Saharan Africa, Asia, and Latin America also use as livestock feed and in ethanol industry in the United States and Asia. Maize is a tall, warm-weather annual grass with a shallow root system, hence the requirement for planting in adequately rain-fed soils (Vollbrecht and Sigmon, 2005). Maize uses C4 carbon fixation, which is the attribute for maize as a water-efficient crop than C3 plants such as legumes (e.g. soybeans). However it regarded as drought sensitive during silk emergence (Vollbrecht and Sigmon, 2005.). In terms of production yields, maize is ranked first for most important cereal crop in the world seconded by rice and then wheat.

Several studies report enormous losses of agricultural yields which are attributed either to susceptibility of maize to abiotic stress such as drought or lack of tolerance against nutrient-deficient soils (Mmbaga and Lyamchai 2001; Mkhabela et al., 2005).

1.2 Legume symbiosis

Nitrogen gas (N_2) constitutes about 80% of atmospheric gases but cannot be assimilated by most living organisms (Ebbing and Gammon, 1999; Prescott et al., 2005). Living organisms use the ammonia (NH_3) and nitrates for metabolism of nitrogen-containing biomolecules necessary for various biochemical processes essential for growth (Ridge, 1992; Schultze and Kondorosi, 1998; Soto et al., 2006). Hence, most organisms would suffer from nitrogen deficiency despite the presence of atmospheric N_2 and this is due to the lack of nitrogen-fixing abilities in such organisms whereas legume are able fix nitrogen due present of bacterium symbiosis in organ called root nodules (Ridge, 1992; Schultze and Kondorosi, 1998; Soto et al., 2006).

1.2.1 Root nodule development

The development of root nodules is mediated by lipochitooligosaccharide signals (LCOs) commonly known as nodule-inducing (Nod) factors. Nod factors are secreted by rhizobia and act as signals that induce cells within the root cortex to divide and develop into nodule primodia when legumes are grown in nitrogen-deficient conditions (Ridge, 1992; Schultze and Kondorosi, 1998; Soto et al., 2006). The rhizobia attach to root hairs and penetrate the root cortex through an infection thread where nodule primodia develop (Soto et al., 2006). Upon successful entry into the cortex the rhizobia become surrounded by a host-derived membrane and differentiate into nitrogen-fixing bacteroids (figure 1.1, Soto et al., 2006). The expression of rhizobial genes encoding the Nod factors is initiated when plants secrete flavonoids upon sensing nitrogen deficiency (figure 1.1, Ridge, 1992; Schultze and Kondorosi, 1998; Soto et al., 2006).

1.2.2 Root nodules

The morphology of root nodules on several perennial legumes such as alfalfa and clover are fingerlike whereas the mature nodules may be similar to a hand with center mass and protruding portions. Nodules with such morphology are named indeterminate nodules (Table1.1, Strozycki and Legocki, 1995; Gualtier and Bisseling, 2000; Downie, 2005).

Additionally, nodule morphology on annual legumes, such as beans and soybeans, are round and can reach the size of a large pea and are commonly known as determine nodules (Strozycki and Legocki, 1995**;** Gualtier and Bisseling, 2000; Downie, 2005). The crucial role of root nodules is to enable plants to extract nitrogen from the air and turn it into soluble molecules (figure 1.3 A, Strozycki and Legocki, 1995**;** Gualtier and Bisseling, 2000; Downie, 2005). Nodules usually turn green, white or grey when they are no longer fixing nitrogen and may eventually be discarded by the plant in such cases, yet healthy nodules are pinkish or reddish in colour due to accumulation of leghemoglobin (figure 1.3 B, Strozycki and Legocki, 1995**;** Downie, 2005). The role of leghemoglobin in root nodules is to controls oxygen flux to prevent the damaging effect of oxygen on nitrogenase activity while ensuring delivery of oxygen to rhizobia for sustenance of their metabolic activity (Arredondo-Peter *et al.,* 1998). Leghemoglobin has a similar role as mammalian haemoglobin as it regulates the concentration of free oxygen inside nodules. In addition limited or lack nitrogen fixation occurs as a consequence of the use of an inefficient *Rhizobium* strain, deprived plant nutrition or abiotic stress (Strozycki and Legocki, 1995**;** Gualtier and Bisseling, 2000; Downie, 2005).

Table 1.1: Tabulation of some of the characteristics of indeterminate and determinate nodules (Marije Scholte, PhD thesis, November 2002)

	Indeterminate nodule	Determinate nodule
Site of initial cell divisions	Inner cortex	Outer cortex
Meristem	Persistent meristem	No persistent meristem
Nodule form	Elongated	Round
Geographic region of plant origin	Temperate regions	Subtropical and tropical
Example of symbiotic association	Medicago-Sinorhizobium	Glycine-Bradyrhizobium

A)

B)
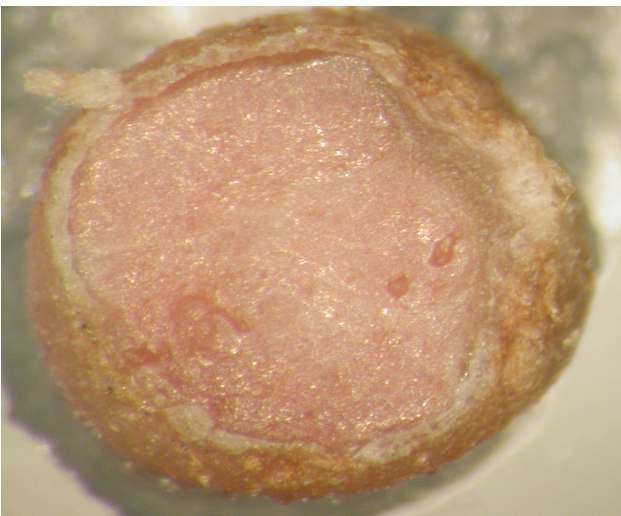
Figure 1. 3. A) Root nodules of soybean under optimal nitrogen fixation. B) Phenotype (pink or reddish color) of haemoglobin-containing root nodules (Photo courtesy of Dr. Ndiko Ludidi, SU)

1.2.3 Nitrogen fixation in root nodules

The nitrogenase enzyme complex catalyzes nitrogen fixation. Nitrogenase is highly conserved and widely distributed amongst rhizobia. Its uses Fe-S and Mo-Fe as co-factors that serve in reduction and transfer of electrons from ferredoxin to N_2 (figure 1.2, Shlessman *et al.,* 1998). Recent reports demonstrate a decline in nitrogenase activity that could be caused by increased diffusion of O_2 in the nitrogen fixation root nodule

(Downie, 2005; Garrett and Grisham, 2005). It is thought that the Fe-S cofactors are irreversibly inhibited by oxygen and thus the plant oxygen-binding protein leghemoglobin is important for regulating oxygen levels to prevent its inhibitory effects on nitrogenase activity in the root nodules (Lee *et al.,* 1995).

The structural mechanism of nitrogenase enzyme is comprised of two components of metalloproteins. The iron-sulphur protein cluster (Fe-protein) or nitrogenase reductase is a homodimeric protein, which forms one redox active 4Fe-4S center (P-cluster) that symmetrically bridges two identical protein subunits. The molybdenum-iron protein cluster (MoFe-protein) in nitrogenase is organized as an a_2b_2 tetramer containing two 4Fe:4S clusters and an FeMo cofactor. Each of the P-clusters in nitrogenase contains 8Fe-7S that is bridged by 6 cysteines while FeMo cofactor contains two 7Fe-1Mo-9S-1homocitrate clusters in each a subunit.

The role of homocitrate is believed to be to supply the Mo atom with two oxo ligands (Leigh, 1995). The Fe-protein–MoFe-protein complex reduction reactions require electron transfer from the iron-sulphur protein component to the molybdenum-iron (MoFe) protein component in a reaction that is coupled to the hydrolysis of MgATP. During this process the MgATP binds to nitrogen reductase and is hydrolysed two ATPs per electron transfer (eq.1, Schlessman *et al.,* 1998).

The flow of electrons proceeds from a [4Fe-4S] cluster in the 4Fe: 4S protein to a P-cluster or [8Fe-7S] in the MoFe protein and finally to an iron-molybdenum-sulfurhomocitrate cofactor (FeMoco) that acts as the site of substrate binding and reduction. This reduction of N_2 to $2NH_3$ by nitrogenase requires the hydrolysis of 16 ATP molecules (figure 1.2).

$$N_2 + 8H^+ + 8e^- + 16\ MgATP \longrightarrow 2NH_3 + H_2 + 16\ MgADP + 16\ Pi \quad (Eq.\ 1)$$

1.3 Nitric oxide in plants

Nitric oxide (NO) in plants acts as a cellular signaling molecule involved in variety physiological processes including plant growth and development, stomatal closing, ripening of fruit and senescence of organs as well as plant responses to abiotic and biotic stresses (Besson-Bard*, et al.,* 2008). NO is an inorganic short-lived free radical gas with high reactivity due to the presence of an unpaired electron.

In animals, it has important roles in key physiological processes such as neurotransmission, immunological and inflammatory responses, Programmed Cell Death

(PCD) and smooth muscle relaxation (Mur *et al.,* 2005**).** NO is readily diffusible through the cell membrane and can react with a variety of intracellular and extracellular targets. In some cases, these interactions are cytotoxic and result in cell death (Beligni and Lamattina, 2000; Clarke *et al.,* 2000; Arasimowicz and Floryszak-Wieczorek, 2007).

In plants, it plays a crucial role in disease resistance and the induction of hypersensitive cell death by H_2O_2 in soybean (*Glycine max*) suspension cultures (Wendehenne *et al.,* 2001**)**.

In tobacco plants, NO activity mediates the function of SA in the systemic acquired resistance (SAR) signaling pathway (Song and Goodman, 2001).

1.3.1 Nitric oxide signalling during nodule development

Nitric oxide (NO) is a key molecule in inter- and intracellular signalling in plants, implicated in host defense (Neill *et al.,* 2003). A recent study showed that the phytohormone auxin (indole-3-acetic acid, IAA) and NO are required for indeterminate nodule formation (Pii *et al.,* 2007).

An IAA-overproducing mutant produces NO, where auxin-induced adventitious root formation and NO acts as a second messenger and operates downstream of IAA to control nodule number in indeterminate legumes (Pii *et al.,* 2007). Moreover, Naya and co-workers (2007) revealed that defense responses in alfalfa nodules during drought and oxidative stress impose restraint on metabolic capacity of bacteroids and oxidative damage to cellular macromolecules, leading to inhibition of nitrogenase activity. Thus N_2-fixation declines when ROS and antioxidants interact with signals mediated by hormones such as abscisic acid (ABA) during establishment of symbiosis and senescence in legume nodules (Puppo *et al.,* 2005).

1.3.2 The source of Nitric oxide in plants

The production of NO in mammalian systems is primarily accomplished by three different isoforms of nitric oxide synthase (NOS) enzymes, which catalyze a two-step oxidation reaction of L-arginine and NADPH; in the presence of O_2; to N-hydroxyarginine, NO, L-citrulline and $NADP^+$(Figure 1.4, Alderton *et al*. 2001). The NOS isoforms are fundamental heme flavoproteins (130 to 160 kDa), classified as constitutive, neuronal (nNOS), endothelial (eNOS) and inducible (iNOS).

The eNOS and nNOS isoforms produce NO at much lower rates than iNOS and thus serve primarily to generate NO for signaling during the steady-state conditions of the cell.

On the other hand, iNOS makes large amounts of NO as a cytotoxic agent in immune responses. In plants, it appears that NO is not only produced from nitrite but also from L-arginine (L-Arg), a substrate for NO synthesis in mammalian systems.

The generation of NO in plants is from three different enzymatic pathways and at least one non-enzymatic reaction. The non-enzymatic reaction involves ascorbic acid (AA) at acidic conditions (pH below 4). Enzymatic sources of plant-derived NO include a nitrate/nitrite reductase-dependent pathway and a L-Arg (NOS-like)-dependent pathway (Delledonne *et al.,* 2001; figure 1.4). The nitrate/nitrite pathway involves cytosolic nitrate reductase (cNR) and a root-specific plasma membrane nitrite-NO reductase (Ni-NOR). Yamasaki *et al*. (1999) demonstrated NO production by cNR in a reaction catalyzed by a purified cNR through one-electron reduction of nitrite via the use of NADH as an electron donor. In addition, nitrite (NI-NOR) uses reduced cytochrome c as an electron donor instead of NADH *in vitro*. Hence, NO biosynthesis is thought to be from reduction of nitrate to nitrite in the root apoplast using succinate as an electron donor (Meyer and Stohr, 2002).

1.3.3 Modulation of physiological processes by nitric oxide

A fair body of literature demonstrates the role of NO in the modulation of a broad range of biological processes such as plant growth, development, and resistance to biotic and abiotic stresses (Crawford and Guo, 2005; Delledonne, 2005). Accumulating evidence suggests that Programmed Cell Death (PCD) is affected by the level of NO in plants and animals. In particular, the ratio of NO to H_2O_2 is crucial for determining whether cells will or will not undergo PCD (Crawford and Guo, 2005; Delledonne, 2005; Zago *et al.,* 2006). Comparative effects of various NO donors on PCD and reactive oxygen species (ROS)-scavenging systems in plant cells have been investigated (figure 1.5; Murgia *et al.,* 2004), in which it was shown that high NO concentrations induce cell death and suppression of ROS scavenging systems whereas no negative effects are observed for low NO concentrations. Moreover, cytokinin induces PCD through the increase of NO levels during the progression of senescence (Carimi *et al.,* 2005). PCD is well documented in plants during developmental processes and abiotic stress. For instance, PCD is reported during plant-pathogen interactions in which the NO and ROS react to produce highly toxic peroxynitrite molecules that kill both cells of the pathogen and host (Planchet *et al.,* 2006; Van Breusegem and Dat, 2006). On the other hand Pagnussat *et al,* 2003, postulated NO as mediator of auxin-induced adventitious root formation in cucumber. Furthermore, gravitropic bending in soybean (*Glycine max*) is mediated by NO

(Hu *et al.,* 2005).

A)
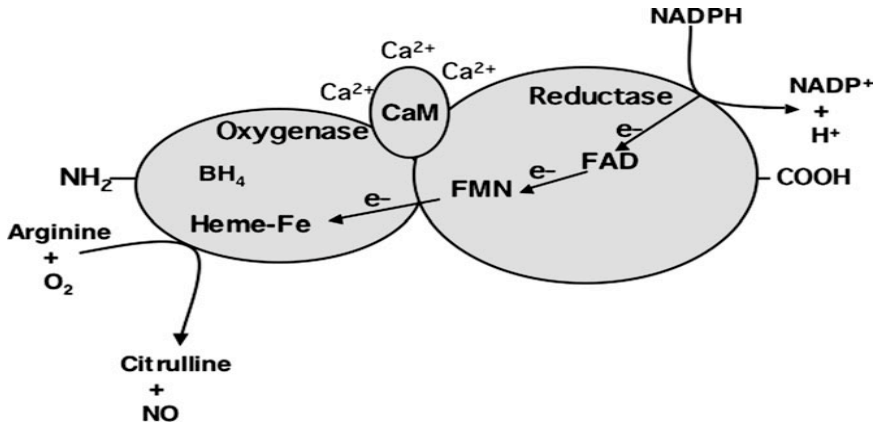
B)
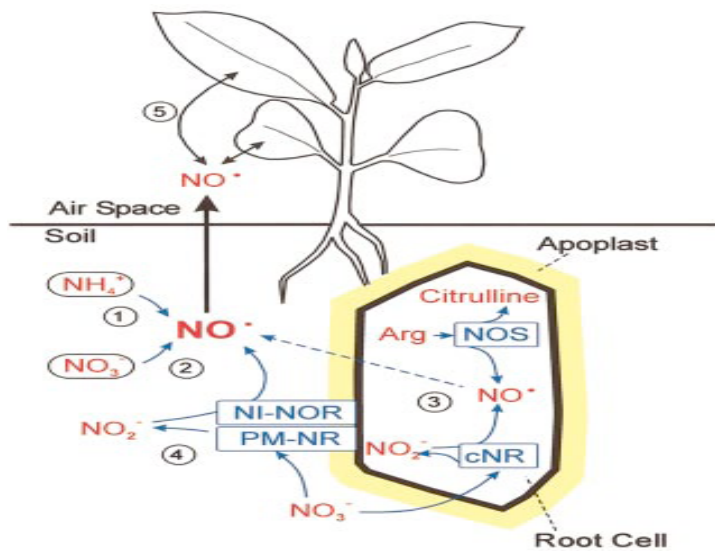
Figure 1.4. Mammalian and plant NOS structure. A). Illustration of mammalian NOS structure with two domains separated by a calmodulin-binding site and indication of flow of electrons from NADPH to haem-Fe (Crawford, 2006). B) Proposed root and rhizosphere synthesis and circulation of NO in higher plants. (1) Bacterial nitrification; (2) bacterial denitrification; (3) In root cells NO is synthesized by cNR and/or NOS; (4) PM-NR in the root apoplast synthesized NO from NI-NOR; (5) exchange of NO (probably mainly released from the soil) between the atmosphere and aerial plant organs (Stohr and Ullrich, 2002).
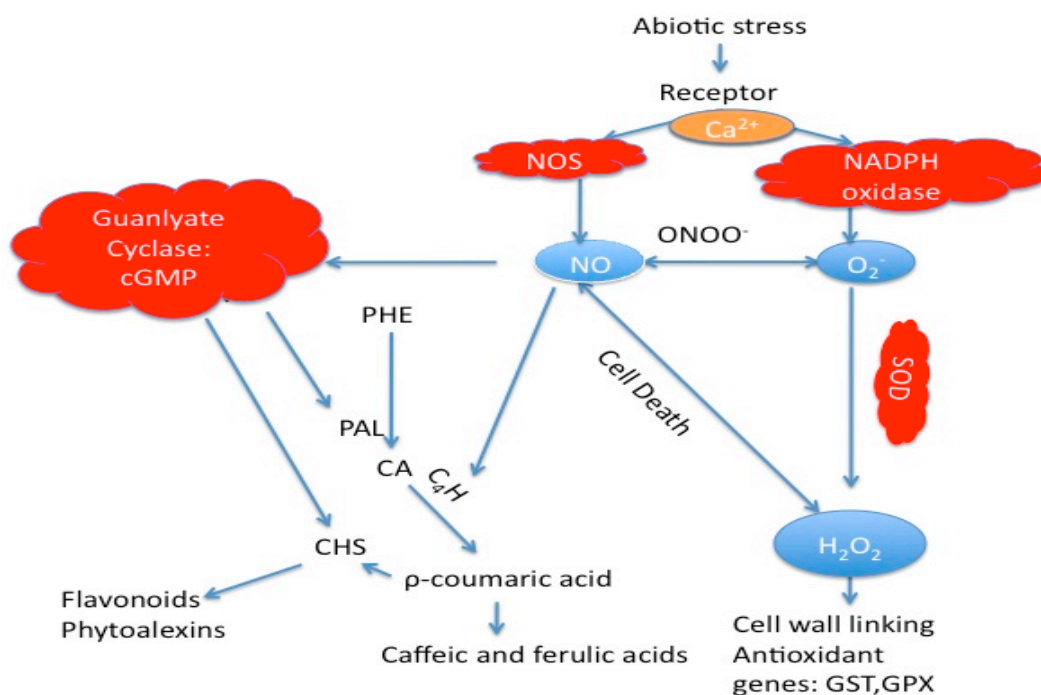
Figure 1.5. Depiction of NO and H_2O_2-mediated plant abiotic stress responses. CHS, 3chalcone synthase; C_4H, cinnamic acid-4-hydroxylase; CA, cinnamic acid; Ca^{2+}, calcium; cGMP, cyclic GMP; GPX, glutathione peroxidase; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; PAL, phenylalanine ammonia lyase; PHE, phenylalanine; SOD, superoxide dismutase.

1.3.4 Nitric oxide as a biological messenger

The physiological function of NO in plants predominantly involves the induction of distinctive genes, including the expression of defence-related genes against pathogens and apoptosis/PCD, maturation and senescence, stomatal closure, seed germination, root development and the induction of ethylene production (Beligni and Lamattina, 2000). Beligni et al (2002) showed that endogenous NO modulates antioxidant activity and delays PCD in barley aleurone layer.

1.5 Programmed cell death in plants

Plants activate PCD, a genetically programmed process of cellular suicide that requires active gene expression under a variety developmental events (Woltering, *et al*., 2002; Rupinder *et al*., 2007). Generally PCD in plant cells is perceived as a fundamental genetically controlled route that functions in the regulation of development and responses to biotic and abiotic stresses (Woltering, *et al*., 2002). During the hypersensitive response, PCD facilitates the elimination of specific cell types that are redundant, damaged, or infected (del Pozo and Lam, 1998; Woltering, *et al.,* 2002). PCD is characterized by loss of plasma membrane integrity and production of characteristic DNA

"ladders" resulting from the inter-nucleosomal cleavage of nuclear DNA into short 180-bp fragments (Woltering, *et al*., 2002, Rupinder *et al*., 2007). The most and consistently observed feature of PCD is the induction of a series of cytosolic proteases known as caspases. Caspases, Bax and Bcl-2 family members (pro- and anti-apoptotic) are some of the molecular and biochemical events that regulate the execution of PCD (Swidzinski *et al.,* 2004).

1.5.1 Role of NO in programmed cell death in plants

Senescence and the hypersensitive response in plants are characterized by an increase in reactive oxygen species (ROS) and NO production, which are signal molecules involved in cell death (Greenberg and Yao, 2004; Van Breusegem and Dat, 2006). High concentrations of NO donor treatment in plant tissue initiate chromatin condensation and DNA fragmentation (Arasimowicz and Floryszak-Wieczorek, 2007). Rapid increases in NO levels directly precede H_2O_2 generation and occur before the appearance of visible HR-type cell death symptoms (Arasimowicz and Floryszak-Wieczorek, 2007). However, NO can inhibit catalase and peroxidase and this may potentially regulate ROS levels in the cell, e.g. during PCD in xylem formation (Leshem *et al.,* 1998; Navarre et al, 2000; Clark *et al.,* 2000; Lamattina *et al.,* 2003; Murgia *et al.,* 2004). Li *et al* 1997 reported that NO is an endogenous regulator of caspase activity in rat hepatocytes. Moreover, NO interacts with cysteine and tyrosine found in proteins and thiol groups of other molecules. In fact, NO reversibly inhibits seven members of the caspase family by S-Nitrosylation (Dimmeler, S *et al.,* 1997; Romero-Puertas *et al.,* 2007).

S-nitrosylation is a post-translational modification mechanism that induced through rich redox and reactive nature of NO, which can modulate signaling pathways in plants (Romero-Puertas *et al.,* 2007). Owing to unique chemical properties of NO in forming metal-nitrosyl complexes in the presence of transition metals such as Fe, Cu, or Zn, NO can have regulatory effects on certain enzymes (Stamler *et al.,* 2001). Hence, chemical modification through the NO moiety on a sulfur atom in Cys to form an S-NO bond is known as S-nitrosylation (Stamler *et al.,* 2001; Lindermayr *et al.,* 2006). However, reducing agents (e.g. dithiothreitol, DTT) prevent inhibition of cysteine protease and restore enzyme activity. Indeed, S-nitrosylated proteins can be easily denitrosylated, allowing cells to flexibly and precisely adapt protein function in response to environmental signals (Rusterucci *et al.,* 2007). Interestingly, there is a report demonstrating that NO-donors (e.g. *S*-nitrosoacetyl-penicillamine, SNAP) inhibit falcipain, the *Plasmodium*

falciparum trophozoite cysteine protease (Venturini *et al.,* 2000). Furthermore, NO has been reported to inhibit caspases by S-nitrosylation of cysteine residues at the active site (Lindermayr *et al.,* 2006).

Protein S-nitrosylation of the Cys catalytic residue in cysteine proteases is assisted by adjacent basic and acidic amino-acid residues (e.g. Cys–His catalytic dyad,) (Colasanti *et al.,* 2001; Belenghi *et al.,* 2007). Perhaps the role of NO's inhibitory actictivity on caspases is to provide a mechanism for the cell to block undue apoptosis, which makes them tremendously important in determining the fate of the cell. It is suggested that S-nitrosylation might play an important role in plant disease resistance (Rusterucci *et al.,* 2007). Delledonne *et al.* (1998) have shown that inhibitors of NO accumulation blocked the localized cell death, known as hypersensitive response (HR), induced in plant-pathogen interactions and this blockage lead to the spread of disease and bacterial growth in *Arabidopsis thaliana*.

1.5.2 Modulation of protease activity in plants

Proteolytic activity in plant systems is a requirement in many aspects of cellular regulation including seed storage proteins in germination, efficient recycling of amino acids in senescence and PCD, activation of zymogens, removal of aberrant proteins, degradation of proteins as a part of the homeostatic cycle (Rawlings and Barrett, 1994; Wiederanders, 2003; Antão and Malcata, 2005). The activity of proteases has been correlated with the initiation of 'cellular suicide' induced by either biotic or abiotic stress and it has been shown that the activation of cysteine protease is a prominent event in the control of developmental and pathogen-activated PCD in plant cells (Kirschke *et al*., 1995; Lam *et al.,* 1999). A cysteine protease in tomato was found to accumulate in the leaves of drought-stressed plants (Rawlings and Barrett, 1994). The vacuolar processing enzyme VPE (Cysteine protease with substrate specificity for aspartic acid) is induced during wounding and also exhibits a caspase-1 activity involved in tobacco mosaic virus–induced hypersensitive cell death (Rawlings and Barrett, 1994).

1.5.2.1 Classification of proteases

Proteolytic enzymes (peptidase or protease) catalyse the hydrolytic cleavage of peptide bonds in their target proteins to remove non-functional proteins and release amino acids for recycling (Mottram *et al*., 2003; Asp *et al*., 2004; Fedorova and Brown, 2007).

Proteases are practically found all plants, animals and microorganisms. They can be classified either as exopeptidases that act only on termini of polypeptide chains at a free N-terminus, and carboxypeptidases, which are distinguished according to their substrate specificity (Antão and Malcata, 2005). Those which act inside the peptide chains are classified as endopeptidases and are divided into six mechanistic classes based on the kind of active site residue (cysteine, serine, aspartic, threonine, metalloprotease classes of protease Figure 1.6 (Powers *et al.,* 2002; Antão and Malcata, 2005). In general, the mechanism of action of protease activity is to polarize the carbonyl group of the substrate's peptide bond (Antão and Malcata, 2005; van der Hoorn, 2008).

Rawlings & Barrett (1993) have grouped the protease enzymes into families and clans. The classes are subdivided based on the kind of active site (catalytic type) residue used to cleave a peptide bond. Enzymes belonging to subclass S (serine proteases) have a Ser catalytic activity; C (cysteine proteases) contain a Cys active site; A (aspartic proteases) have an Asp active site residue instead; M (metalloproteases) depend on a metal ion (i.e. Zn^{2+}); and T (Threonine protease) have a Thr active site (Wiederanders 2003; Antão and Malcata, 2005). Within a protease class there are several clans of proteases, which are recognized by a letter following the catalytic class (e.g. clan CA within class C). Moreover, within each clan, proteases are comprised of families based on evolutionary relationships, which can be recognized by a number following the catalytic type (i.e. family C1 within clan CA in figure 1.6; Wiederanders, 2003; Antão and Malcata, 2005).
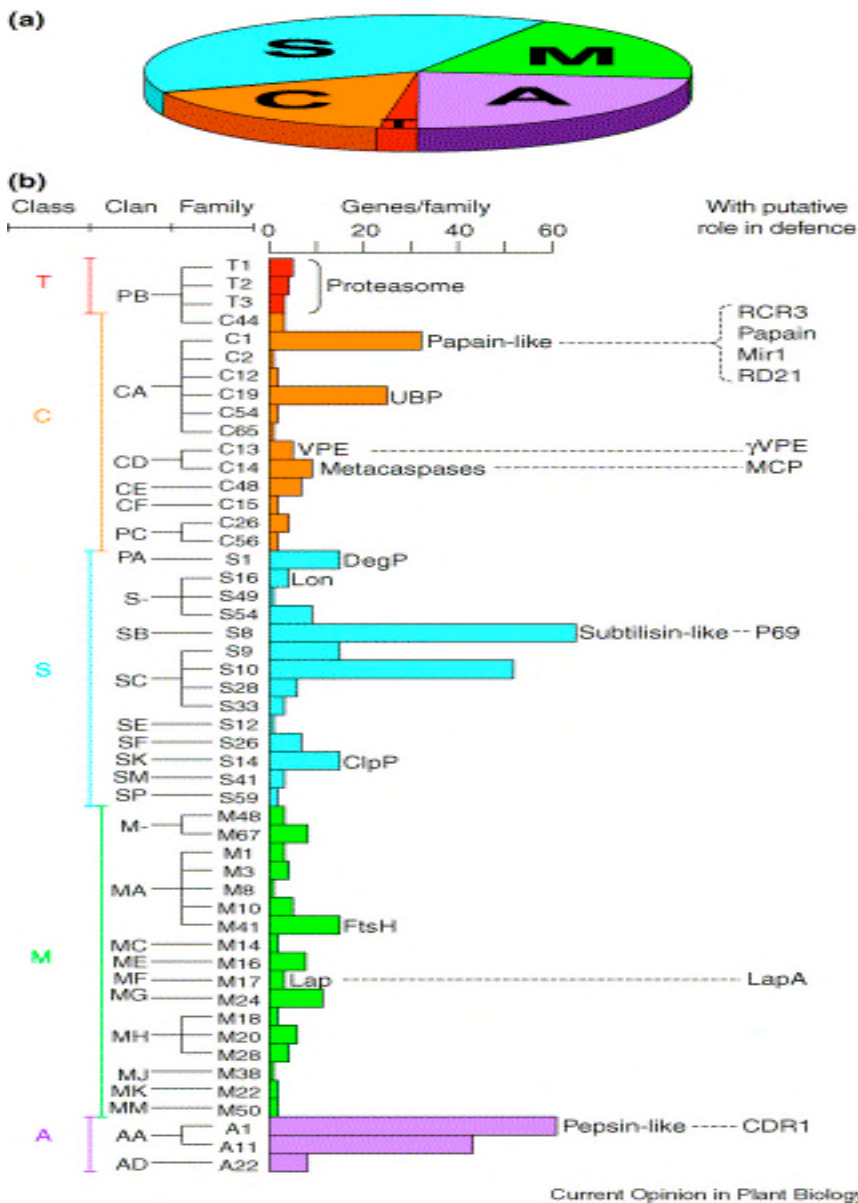
Figure. 1.6. Each protease class consists of several clans of proteases, which are identified by a letter following the catalytic class (e.g. clan CA within class C). Members of a single clan have a common evolutionary origin on the basis of their conserved tertiary structure and order and spacing of catalytic residues (van der Hoorn and Jones, 2004).

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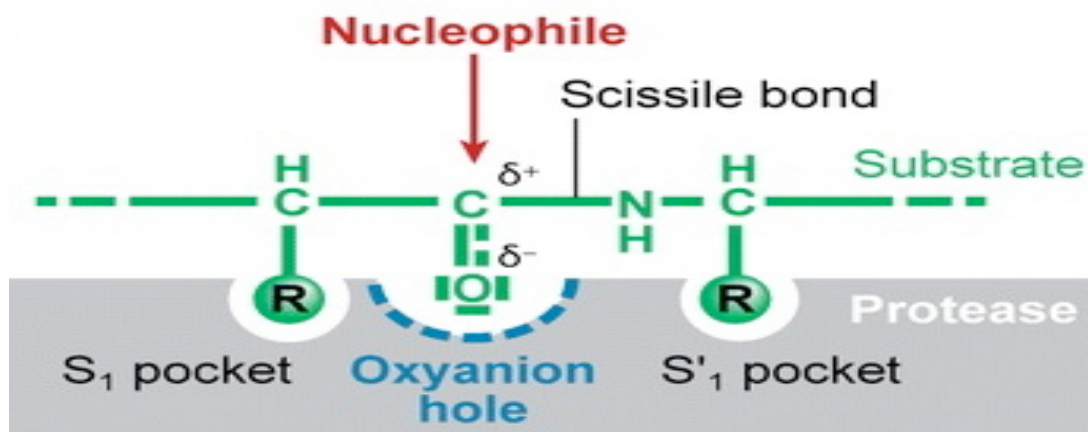
Figure 1.7. The illustrating the cysteine proteases active site cleft. The substrate protein (green) binds by means of amino acid residues (R) to the substrate-binding site of the protease (gray) by interacting with substrate (S) pockets of the enzyme. The scissile peptide bond is adjacent to a carbonyl group, which is polarized by the enzyme by stabilizing the oxyanion hole (blue); this makes the carbonyl carbon susceptible to nucleophilic attack (van der Hoorn, 2008).

1.5.2.2 Proteolytic machinery

The enzymatic mechanism of serine and cysteine proteases is through covalent enzyme/substrate complexes, where they perform a strong nucleophilic attack on the side-chain of the carbonyl group of the peptide bond in their catalytic site (figure 1.7) (Rawlings and Barrett, 1994; Wiederanders, 2003; Antão and Malcata, 2005). However, the proteolytic machinery of aspartic proteases and metalloproteases is heavily depended on general acid/base catalysis (Rawlings and Barrett, 1994; Wiederanders, 2003; Antão and Malcata, 2005).

1.5.2.3 The main catalytic classes of proteases

The major differences between the catalytic classes are on the basis of the nucleophilic and oxyanion stabilizing properties of each class (Table 1.2). Two residues in the backbone predominantly stabilize the oxyanion hole in Cys and Ser protease. On the other hand, water molecules are employed as a nucleophilic molecule in metalloproteases and aspartic proteases; resulting in electrostatic interactions with the metal ion (Me^{2+}) or aspartate (Asp), respectively (Dunn, 2001; van der Hoorn 2008). In addition Me^{2+} and Asp stabilize the oxyanion of metalloproteases and aspartic proteases, respectively. It is thus commonly thought that the main distinction between the catalytic classes is the nature of the nucleophile and oxyanion stabilizer (figure 1.7; Dunn, 2001; van der Hoorn 2008).

Table:1. 2: The four major catalytic classes of proteases

Catalytic class	Nucleophile	Oxyanion stabilizer
Cysteine proteases	H_2O -Me^{2+}	-NH-(2x)
Serine proteases	H_2O-Asp	-NH-(2x)
Metalloproteases	Cys-His	Me^{2+}
Aspartic proteases	Ser-His	H^{+}-Asp

1.5.2.4 Cysteine Proteases

Cysteine proteases (endopeptidases); also known as thiol proteases due to a nucleophilic group of sulfhydryl of cysteine residue; play important roles in plant cells, including the catabolism of protein reserves in seeds, leaf and flower senescence, responses to environmental stress conditions, oxidative stress and nodulation (Grudkowska and Zagdańska, 2004; van der Hoorn and Jones, 2004; Zhang *et al*., 2008). Cysteine proteases are fundamental for the survival of all kinds of organisms, and constitute nearly 2% of an organism's genes (Grudkowska and Zagdańska, 2004). Barrett and Rawlings (1996) argue that cysteine proteases evolved from at least seven different origins, each of which has produced a group of cysteine peptidases with distinctive structures and properties. Hence cysteine proteases comprise a group of enzymes, which share extensive sequence homology and are characterized by a catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic dyad of conserved Cys and His residues (Barrett *et al.,* 1998; van der Hoorn and Jones, 2004). The phylogenetic analysis of the peptidases in each evolutionary line can be seen not only in their 3D structures, but regularly also in the 2-D structures that are represented by a set of one or more modern-day families, termed a clan (Barrett *et al.,* 1998; van der Hoorn and Jones, 2004).

Cysteine proteases are well known to operate via nucleophilic attack on the sulfhydryl group of cysteine, with catalytic mechanisms similar of the serine type proteases, in

which a nucleophile and proton donor/general base are required site (Rawlings and Barrett, 1994; Wiederanders, 2003; Antão and Malcata, 2005). Histidine in cysteines protease catalytic activity is used as a proton donor. Most cysteine protease families contain a third residue to orientate the imidazolium ring of His, which serves the same purpose (proton donor) as aspartate, Asp102 in the serine protease site (Rawlings and Barrett, 1994; Wiederanders, 2003; Antão and Malcata, 2005). Furthermore the catalytic dyad of Cys, His, and Asn in order of sequence and the fourth residue Gln19 preceding the catalytic cysteine help to form the 'oxyanion hole'. The oxyanion hole is an electrophillic centre which forms a stable tetrahedral intermediate by orientating imidazolium ring in the catalytic His site (Rawlings and Barrett, 1994; Wiederanders, 2003; Antão and Malcata, 2005).

1.5.2.4.1 Cysteine Families (Clans)

The cysteine protease superfamily consists of about 20 families which are comprises six major families: Clan CA contains the families of papain (C1), calpain (C2), streptopain (C10), ubiquitin specific peptidases (C12, C19), and families C3, C4, C5, C6, C7, C8, C9, C16, C18 and C21 of viral cysteine proteases (Barrett *et al.,* 1998, van der Hoorn and Jones, 2004). Clan CD contains the families of clostripain (C11), gingipain R (C25), legumain (C13), caspase-1 (C14) and separin (C50). These enzymes are specifically governed by the interactions of the S1 subsite, wherein binding at the P1 site of the substrate is a critical mechanical characteristic. Contrary to other clans, clan CD enzymes are unaffected by the traditional cysteine peptidase inhibitor E-64 (van der Hoorn, 2008). Clan CE contains the families of adenain (C5) from adenoviruses, the eukaryotic Ulp1 proteases (C48) and the bacterial YopJ proteases (C55). Clan CF contains only pyroglutamyl peptidase I (C15). The picornains (C3) in clan PA form the majority of enzymes in this clan and have probably evolved from serine peptidases. The cysteine peptidase activities in clans PB and CH are autolytic only. Worth noting is that some of the clans are of mixed type and contain families with two catalytic types or more catalytic types (Barrett *et al.,* 1998; van der Hoorn and Jones, 2004; van der Hoorn, 2008). Furthermore the order of cysteine and histidine residues (Cys/His or His/Cys) in the linear sequence differs between families (figure 1.6).

1.5.2.4.2 Papain-like enzymes (Clan CA)

Cysteine proteases of the papain super family are widely distributed in nature (Grudkowska and Zagdańska, 2004; van der Hoorn and Jones, 2004). Clan CA is generally the largest and belongs to the C1 family that is subdivided into subfamily C1A (extracytoplasmic PLCPs) that contain proteases with disulfide bridges and are abundant in vesicles, the vacuole and the apoplast (Grudkowska and Zagdańska, 2004; van der Hoorn and Jones, 2004). The second family consists of proteases that lack disulfide bridges found in the cytoplasmic PLCPs (family C1B). Surprisingly, plants comprise of only C1A subfamily; with proteolytic activity implicated in pathogen perception, disease resistance signaling, defense against insects and also in senescence (Grudkowska and Zagdańska, 2004; van der Hoorn and Jones, 2004).

1.5.2.4.3 Catalytic Mechanism of papain-like proteases

Papain was the first cysteine protease to have its amino acid sequenced and 3-D structure determined through X-ray crystallography (Grudkowska and Zagdańska, 2004; van der Hoorn and Jones, 2004). Papain is a 212 amino acid cysteine protease from the latex of unripe fruit of *Carica* papaya. This enzyme is considered to be the archetype (model) of cysteine peptidases (Barrett *et al.,* 1998; Grudkowska and Zagdańska, 2004; van der Hoorn and Jones)*.* The Molecular weight of papain is 23.4 kDa and its pH optimum is 5.5 - 7.0, having high stability at elevated temperatures (Barrett *et al.,* 1998; Grudkowska and Zagdańska, 2004). The catalytic site of papain contains the catalytic triad Cys25–His159–Asn175 at which the thiolate anion in Cys25 serves as the nucleophile that attacks the carbonyl group of the substrate to give an acyl enzyme intermediate and a free amine (Barrett *et al.,* 1998, Grudkowska and Zagdańska, 2004; van der Hoorn and Jones, 2004; van der Hoorn, 2008). A water molecule then reacts with the intermediate to give a carboxylic acid and the free enzyme. Traditionally papain catalytic mechanisms specifically prefer bulky residues at the S_2 subsite and small hydrophobic residues at the S_1 subsite (figure 1.7) (Grudkowska and Zagdańska, 2004; van der Hoorn and Jones, 2004; van der Hoorn, 2008).

1.5.2.4.3.1 Structure and function of papain-like proteases

All papain-like cysteine proteases share similar sequences and have similar 3-D structures (Rawlings and Barrett, 1994; Grudkowska and Zagdańska, 2004). Proteases

in clan CA; which include papain, cruzain, and cathepsin; have subunits of a and b proteins composed mainly of antiparallel b-sheets with segregated a and b regions. Papain contains a 'V' like shaped active site made of two catalytic residues, namely Cys25 and His159, each from N- and C terminal domains respectively (Barrett and Rawlings, 1996; Grudkowska and Zagdańska, 2004; van der Hoorn and Jones, 2004). The active site of papain contains seven sub-sites each capable of accommodating a single amino acid residue of a peptide substrate (Barrett and Rawlings, 1996). The subsites are located on both sides of the catalytic site, four on the N-terminal side and three on the C-terminal side (Barrett and Rawlings, 1996; van der Hoorn and Jones, 2004).

1.5.2.4.4 Caspases (cysteinyl-aspartic-acid-proteases)

Clan CD enzymes differ fundamentally from those in clan CA, which allows them to be readily distinguished experimentally in several ways (Barrett and Rawlings 1998; Grudkowska and Zagdańska, 2004). Firstly the clan CD peptidases are unaffected by the conventional cysteine peptidase inhibitor E-64 (Mottram *et al.,* 2003; van der Hoorn and Jones, 2004). Peptidases within a family can have some sequence similarity, but very different biological mechanisms and functions (Hilley *et al.,* 2000; Lillico *et al.,* 2003; Mottram *et al.,* 2003). Caspases belong to clan CD and are members of the C14 family of cysteine proteases that cleave substrates after aspartate residues (hence their name) and play a role in apoptosis (Mottram *et al.,* 2003; van der Hoorn and Jones, 2004). Asparaginyl-specific cysteine endopeptidase family cleaves peptide bonds with Asn or Asp (less efficiently) in the P1 positions at the C-terminal only at acidic pH (Hilley *et al.,* 2000; Lillico *et al.,* 2003; Mottram *et al.,* 2003; Grudkowska and Zagdańska, 2004). Vacuolar processing enzymes (VPEs), belonging to family C13, are known as legumains and evolved independently of papain-like proteases (Mottram *et al.,* 2003; Grudkowska and Zagdańska, 2004). VPEs have been determined in various species including *Ricinus communis* seeds, *Glycine max* cotyledons, germinating *Vicia sativa* seeds and in different organs of *A. thaliana* and are implicated in mediating the processing of seed storage proteins (Muntz, 2002; Mottram *et al.,* 2003). One of these VPE genes (γVPE) is induced upon wounding (Muntz, 2002; Mottram *et al.,* 2003). VPEs are also present in the cell wall and their function is not restricted to precursor protein processing but also includes protein breakdown in the vacuole or cell wall (Muntz, 2002; Mottram *et al.,* 2003). Legumains can be divided in two subfamilies: those specific for seeds (βVPE) and

others (γVPE and αVPE) specific for vegetative organs (Muntz, 2002; Mottram *et al.,* 2003). An immunocytochemical analysis confirmed the specific localization of bVPE in the protein storage vacuoles and γVPE in the lytic vacuoles (Muntz, 2002; Mottram *et al.,* 2003).

1.5.2.5 Inhibition of protease catalytic activity

Proteolytic activity is essential for maintenance of homeostasis and depends heavily upon correct sorting and trafficking of proteases within cells, so their activities need to be kept strictly under control (Bobek and Levine, 1992; Rawlings *et al*., 2004). Proteolytic processing by cysteine proteinases has also been shown to regulate the assembly of many viral proteins including HIV-1 (Hellen and Wimmer, 1995; Chavan *et al.,* 2001). It doesn't come as a surprise that the mode of interaction of protein inhibitors with enzymes contributed to novel approaches in the design of synthetic inhibitors for use as drugs (Hellen and Wimmer, 1995; Chavan *et al.,* 2001). Furthermore, some research groups in the field of plant biotechnology have genetically modified crop plants expressing inhibitors of the digestive enzymes of their insect pests and increasing tolerance to abiotic stress (Rzychon *et al*., 2004; Habib and Fazili, 2007; Rawlings *et al*., 2008). Naturally, plants are equipped with a large arsenal of protease inhibitor genes that participate in the control of proteolysis in defense against herbivores (Habib, and Fazili, 2007; van der Hoorn, 2008).

Inhibitors of cysteine proteases are widely distributed among plants, animals and microorganisms (Dubey *et al.,* 2004; Habib, and Fazili, 2007; Rawlings *et al*., 2008). They are subdivided into three families (stefin, cystatin and kininogen) based on their sequence homology, the presence and position of intrachain disulfide bonds, and the molecular mass of the protein (Dubey *et al.,* 2004; Rawlings *et al*., 2004; Rzychon *et al*., 2004; Habib, and Fazili, 2007; Rawlings *et al*., 2008; van der Hoorn, 2008). The plant serpins have a molecular mass of 39 - 43 kDa and inhibit caspases and papain-like cysteine proteases and are thought to function in protection of plants against pathogens (Dubey *et al.,* 2004; Rzychon *et al*., 2004; Habib and Fazili, 2007; van der Hoorn, 2008). The superfamily of cystatins consists of tightly and reversibly binding inhibitors against cysteine proteases. This family contains proteins of about 120–126 amino acids with a molecular mass about 13.4–14.4 kDa (Rawlings *et al*., 2004; Rawlings *et al*., 2008). Recent sequence analysis shows that similarities between stefins and cystatins but

Phytocystatins do not contain free Cys residues (Fernandes *et al*., 1993; Zhao *et al*., 1996). The mode of the inhibitory activity of cystatins is frequently limited to specific organs or to particular phases during plant growth environmental cues (Dubey *et al.,* 2004; Rzychon *et al*., 2004; Habib, and Fazili, 2007; van der Hoorn, 2008).

1.5.2.6 Role of cysteine proteases in plant responses to abiotic stress

Abiotic and biotic stresses such as wounding, low or high temperatures, drought, high salinity and pathogens have been demonstrated in various plants to regulate the expression of cysteine protease genes (Groten *et al.,* 2006). Fan *et al.,* 2009 had elucidated the expression of a senescence-associated *CsCP* cysteine protease gene during the development of navel orange (*Citrus sinensis* L. Osbeck). The expression of *CsCP*, a papain-like transcript, is induced by hypoxia (3% O_2) during postharvest peel pitting (Fan *et al.,* 2009). Furthermore, drought stress increases levels of azocaseinolytic activity in winter wheat (*Triticum aestivum* L.) while recovery reverses the effect of drought on the cysteine protease activity (Simova-Stoilova *et al.,* 2006*)*. Also Vance and colleagues (1979) showed that senescent nodules have elevated levels of protease activity, which is also reflected by a decrease in nodule soluble protein concentration and is accompanied by a decrease in leghemoglobin and the loss of bacteroids in nodule cortical cells; which are characteristic features of nodule senescence (Groten *et al.,* 2006). In general, intracellular cysteine proteases are involved in stress-induced protein degradation and the re-organization of metabolism (Zagdañska and Winievski, 1996). Simova-Stoilova *et al* argue that high cysteine protease activity in drought-stressed winter wheat might be due to drought sensitivity rather than drought tolerance. Furthermore, plants respond to osmotic stress by regulating gene expression including genes responsive to other dehydration-related stresses, such as salinity and cold temperature. *TDI-65*, a drought-induced cysteine protease that was isolated and characterized in tomato (*Lycopersicon esculentum*) leaves during water stress has a function related to drought-induced senescence and programmed cell death (Harrak *et al.,* 2001). Furthermore, PsCYP15A (belonging to the C1 family of clan CA), a cysteine protease from pea (*Pisum sativum* L.) and is up-regulated at transcript level in shoots and root nodules under water deficit (Vincent and Brewin, 2000).

1.5.2.6.1 Cysteine proteases in root nodules in response to abiotic stress

AsNODf32, a gene encoding a nodule-specific cysteine protease, has been isolated and characterized in Chinese milk vetch (*Astragalus sinicus,* Naito *et al.,* 2000). The AsNODf32 gene is expressed predominately in the nodule senescence zone, interzone between the infection and the nitrogen fixing zone and is also believed to have endoplasmic reticulum-retention signals due to the presence of either of the C-terminal tetrapeptides, HDEL and KDEL (Naito *et al.,* 2000). A number of the cysteine protease genes in root nodules show a highly specific temporal and spatial expression during nodule senescence, which in turn is not only induced by development towards nodule maturity and ageing, but abiotic and biotic stresses contribute to the induction of nodule senescence (Asp *et al.,* 2004). The *Pisum sativum* PsCYP15a is a putative drought-inducible wilting-associated cysteine protease, which is also expressed in the meristematic region and in the central nitrogen-fixing region and is associated with the adaptation to changes in cell turgor as well as protein turnover in the symbiosome. Cysteine proteases are essential for plant signalling in soybean nodule development and senescence (Alesandrini *et al.,* 2003).

1.6 Objectives of the study

This study aims to examine the role of nitric oxide and abiotic stress on cysteine protease activity in root nodules of soybean and to evaluate the effect of abiotic stress on two maize genotypes having varying tolerance to the abiotic stress.

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Chapter 2

2. Cysteine protease activity (CPA) of soybean root nodules in response to nitric oxide (NO).

2.1 Introduction

Cysteine proteases in legume root nodules regulate a number of processes including development, defense responses to root invasion by microorganisms, adaptation of host cells to physiological stresses and control of nodule senescence (Sheokand and Brew, 2003). Alesandrini *et al*. (2003) have shown that both a cysteine protease and H_2O_2 are involved in senescence of determinate nodules of soybean (*Glycine max*) and this process is thought to occur via programmed cell death (Matamoros *et al*., 2003). The characteristic features of nodule senescence include a decline in nitrogen fixation, increased rate of formation of reactive oxygen species, reduction of antioxidant metabolites and the triggering proteolytic activities that cause larges scale protein degradation (Pfeiffer, 1983; Pladys and Vance 1993). Abiotic stress induces premature senescence in root nodules, which reduces ammonia-assimilating enzyme activities and is characterized by high levels of cysteine protease activity (CPA) (Pladys and Vance, 1993). To date, there is no documentation of the influence of nitric oxide on CPA in root nodules. In this study we determined the role of NO in root nodules by assaying the activity of papain-like proteins.

2.2 Material and Methods

2.2.1 Chemicals and reagents

All chemicals were purchased from Sigma Aldrich Chemical Company and Bio-Rad. Soybean seeds were kindly donated by Pannar Seeds, Greytown, South Africa.

2.2.2 Seeds sterilization

Seeds of soybean (*Glycine max* (L.) Merr.) cv. 626 (kindly donated by Pannar, Greytown, South Africa) were surface-sterilized in 0.35% sodium hypochlorite and 0.005% Tween-20 for 10 minutes, followed by five washes with sterilized demineralized water. The seeds were allowed to imbibe in water for 1 hour by leaving them in the beaker with sufficient water (just enough to cover the seeds) to allow for easy seed coat breakage.

2.2.2.1 Growing Soybean plants in the greenhouse

The seeds were sown 3 cm into damp vermiculite (pre-soaked in distilled water) in 15 cm diameter pots. The seeds were allowed to germinate on a 25/19°C day/night temperature cycle and 16/8 hours light/dark regime with a photon flux density of 300 μmol photons.m^{-2}.s^{-1} during the day (light) period. Additionally, the bases of the pots (pot tray) were watered with distilled water to ensure that the vermiculate is kept moist during germination until the VC stage (when the unifoliolate leaves are completely unrolled, are opposite each other and the first node is visible). When seedlings reached the VC stage, the vermiculate was watered with nitrogen-free nutrient solution [1 mM K_2SO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.2, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $CuSO_4$, 5 μM Na_2MoO_4, 5 μM $CoSO_4$, 50 μM Fe-NaEDTA and 10 mM 4-(2-hydroxethyl)-1-piperazineethanesulfonicacid (HEPES) at pH 7.2. At this stage the plants were romoved from vermiculate and the plant root systems were inoculated by immersion in a suspension of *Bradyrhizobium japonicum* (as the commercial inoculant 'Nodulator Peat-based Soybean HiStick 2'(Becker Underwood Ltd, Little Hampton, West Sussex) made up of 5 grams of inoculant mixed with 500 ml of demineralized water, then returned to the vermiculate in the same pot. From then onwards, plants were watered with 250 ml of nitrogen-free nutrient solution (every two days on the base of the tray. Plants were treated and analyzed at the beginning of the V3 stage (when there are two fully expanded trifoliolate leaves and the third trifoliate leaves start to emerge). The interior of the nodules at this stage was reddish pink in colour (indication of presence of optimal levels of leghemoglobin and active nitrogen fixation). Intact plants were treated with a nitric oxide donor, cGMP, NaCl and sorbitol, followed by measurement of cysteine protease activity in the root nodules.

2.2.3 Treatment of intact plants

2.2.3.1 Application of exogenous NO·

For 'Untreated' samples, intact plants growing in vermiculate were supplied with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.2 and used for CPA assay at 0 and 6 hours of treatment. For 'NO' samples, intact plants growing in vermiculate were supplied with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.4 containing either 10 μM, 50 μM or 100 μM of the nitric oxide donor 'diethylenetriamine/nitric oxide adjunct' (DETA/NO) and used for Cysteine protease activity assays at 0 and 6 hours of treatment. A control treatment

('DETA') with a chemical similar to DETA/NO, except that it lacks the NO molecule, was set up by supplying intact plants growing in vermiculate with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.4 containing 50 μM Diethylenetriamine (DETA) and used for cysteine protease activity assays at 0 and 6 hours of treatment. CPA was determined only from the nodules of the nodulated roots as previously described (Zhang *et al*., 2008).

2.2.4 Sample preparation

Ten milligrams of treated root nodules with (DETA/NO, DETA) and untreated root nodules, were homogenized in 200 μl of protein extraction buffer (10 mM HEPES, pH 7.4, 450 mM Mannitol, 7 mM Na_2EDTA, 7 mM $CaCl_2$, 5 mM $MgCl_2$, 20 mM ascorbic acid and 10 mM DTT), followed by centrifugation at 10 000 *g* for 10 minutes at 4°C to sediment the debris. The supernatants were transferred into clean tubes and stored at - 80°C for later use. Protein concentrations were estimated by the Bradford method using Bovine serum albumin (BSA) as the standard for generation of a standard curve (Bradford, 1976)**.**

2.2.5 Spectrophotometry

Spectrophotometric analyses were carried out using an µQuant (Bio-Tek Instruments) or FLUOstar OPTIMA (BMG LABTECH) spectrophotometer operated via WinUV software (Varian Ltd) or OPTIMA Software respectively.

2.2.5.1 Analysis of CPA

CPA of nodule crude extracts was determined by measuring the proteolytic cleavage of the chromogenic substrate N-benzoil-L-p-nitroanilide (L-BAPNA) (Sigma) according to Zhang *et al*., 2008, with slight modifications. Briefly, crude nodule extracts containing 50 μg of total protein were incubated in 170μl of Phenylmethylsulphonyl fluoride (PMSF) buffer (100 mM phosphate buffer, 10 mM DTT, 1 mM PMSF) for 1hour at 37°C with L-BAPNA at a final concentration of 0.6 mM in a final volume of 200 μl. CPA was measured in a spectrophotometer at 405 nm. Enzymatic activity was expressed in $nM.mg^{-1}.min^{-1}$ (specific activity) and determined by calculating nanomolar p-nitroaniline formed per milligram of protein per minute at 37°C, using a molar extinction coefficient of 9600 for p-nitroaniline at 405 nm [i.e., specific activity = optical density x 1000/(milligrams of protein in lysate x time of reaction (minutes) x 9.6) for L-BAPNA. Each assay was repeated three

times. PMSF is a protease inhibitor for both serine and cysteine protease, however its effect is reversible in cysteine protease in the presence of 10 mM DTT.

The data are presented as mean and standard deviation and analysed using online Student's t-test. A P-value less than 0.05 present will similar alphabet letter considered as level of significance for all tests.

2.3 Results

2.3.1 Exogenously applied NO modulates CPA in a concentration-dependent manner

CPA in root nodule crude extracts were assayed following treatment of soybean with various concentrations (10 μM, 50 μM and 100 μM) of (DETA/NO, figure 2.1). The concentration dependency of the effect of exogenously applied NO donors on CPA show significant differences amongst the various concentrations used ($P < 005$). CPA was reduced by 10 μM (DETA/NO) while an induction of the CPA was observed for treatments with 100 μM of (DETA/NO) after 6 hours of treatment. Exogenously applied NO showed no significant changes in nodule CPA at 0 hours in the 'Untreated' and 'DETA/NO' samples (figure 2.1). After 6 hours of treatment with 10 μM DETA/NO, nodule CPA decreased by 39% compared to the of the cysteine activity obtained for the 'Untreated' samples (figure 2.1). Augmentation of CPA increases with an increase in NO concentration (figure 2.1). It is noteworthy that a 50 μM of DETA/NO results in CPA similar to the 'Untreated' samples. To establish if the effect of DETA/NO on CPA is a result of NO and not the by-product (DETA) of the donor, root nodules were incubated with 10 μM (DETA/NO) or DETA as control (figure 2. 2). CPA in samples treated with 10 μM DETA was similar to the activity observed in the 'Untreated' samples at 6 hours of incubation.
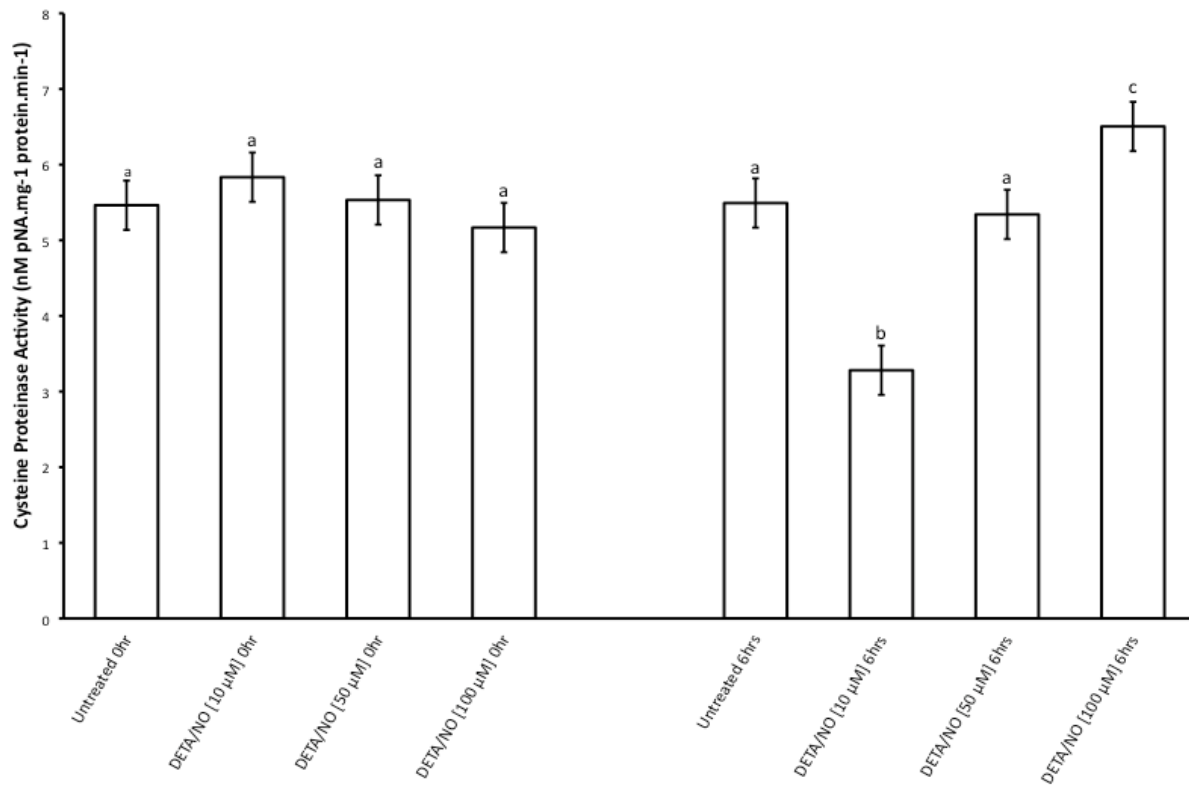
Figure 2.1: Cysteine protease activity (CPA) in response to various concentrations (10 μM, 50 μM and 100 μM) of (DETA/NO) in root nodules at 6 hours of treatment. Extract (50 μg of protein) was incubated with cysteine protease substrate of BAPNA, and the cysteine protease activity was determined by measuring the hydrolysis of BAPNA at 405 nm.

Figure 2.2: Cysteine protease activity (CPA) in response to 10µM of DETA/NO and DETA in root nodules at 6 hours of treatment. Extract (50 μg of protein) was incubated with cysteine protease substrate BAPNA, then the cysteine protease activity was determined by measuring the hydrolysis of BAPNA at 405 nm.
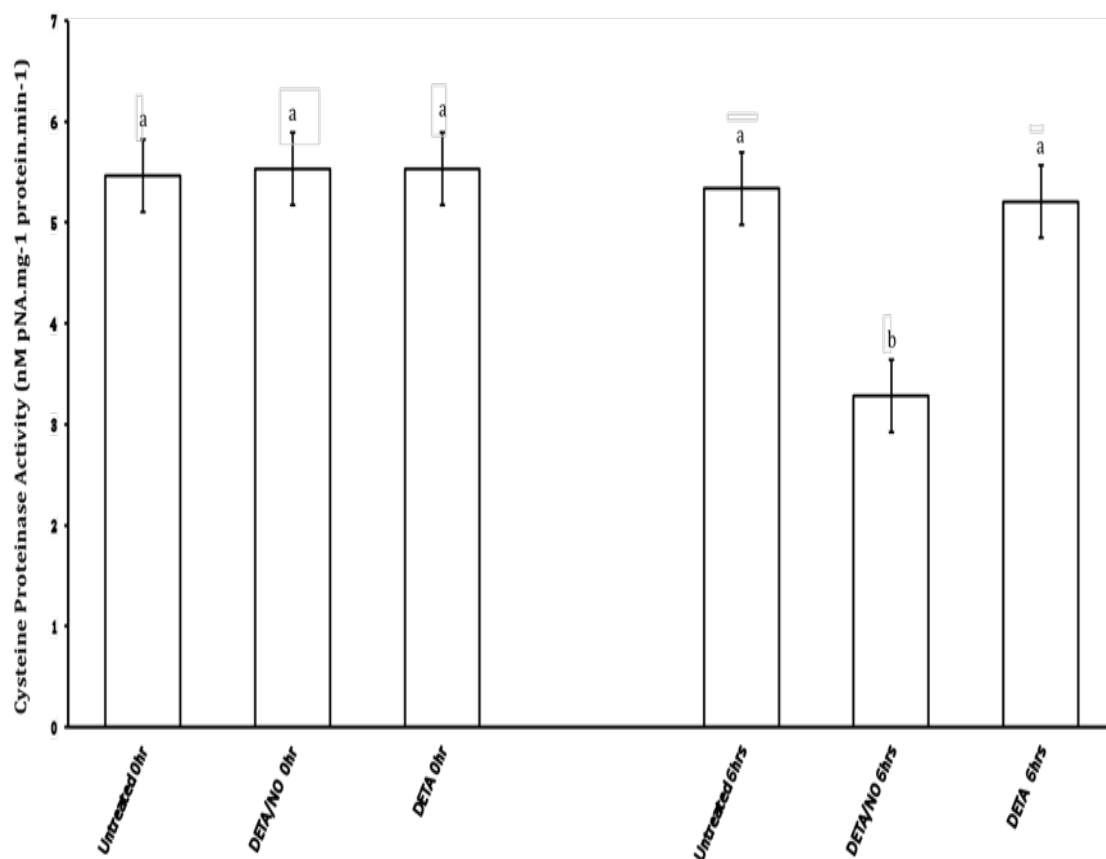
2.4 Discussion

This is the first report on the role of nitric oxide in regulating root nodule CPA. The report further signifies the occurrence and functioning of cysteine proteases across the animal and plant kingdoms (Loukas *et al*., 1998; Wiederanders, 2003). The papain-like activity in plants is well established, although data on the regulation of CPA by NO in root nodules has never been reported before.

The occurrence of NO in root nodules is well documented (Cueto *et al*., 1996). In mammalian tissues, nitric oxide is synthesized from three isofroms of nitric oxide synthase (NOS) consisting of two isofroms that are constitutively expressed in cells, namely neuronal (nNOS) and endothelial (eNOS), and one isoform named inducible (iNOS) that is not constitutively expressed (Green *et al*., 1981; Wagner *et al*., 1983; Sakuma *et al*., 1988; Bredt and Snyder, 1989). Both cytoprotective and cytotoxic effects were observed in root nodules because nodules incubated for 6 hours in 100 μM of DETA/NO induced an increase in CPA while 10 μM of DETA/NO indicates reduced CPA where's 50 μM of DETA/NO showed no significant different with untreated CPA. This concentration (50 μM of DETA/NO) also reduced cell viability whereas 10μ M DETA/NO did not alter cell viability (Keyster, 2010, PhD thesis, Stellenbosch University, South Africa) but lead to a decrease in CPA in root nodules. Botella *et al*., 1996 demonstrated that soybean cysteine proteinase inhibitor genes are expressed different organs in response to wounding and methyl jasmonate, presuming a role of the cysteine proteinase inhibitor genes in plant defense.

The regulated CPA from 50 μM of DETA/NO treated roots nodules might be due to S-nitrosylation or inducing of cysteine protease inhibitors. Cysteine protease inhibitors regulate PCD activated by ROS in plants (Solomon *et al*., 1999). NO directly activates guanylyl cyclase to produce cGMP, which has a role in leaf expansion and elongation of roots (Gouvêa *et al*., 1997; Durner *et al*., 1998; Beligni and Lamattina, 2001).

The different effects of the various concentrations of DETA/NO (10 μM, 50 μM and 100 μM) indicate an NO concentration dependence of the activity of cysteine proteases. The NO concentration dependence of the activity of cysteine protease is in agreement with previously observed cytotoxic and cytoprotective role of NO in plants (Beligni and Lamattina, 2001). iNOS in animals produce elevated NO levels that function in cGMP-independent responses involved in immunity and inflammation (Coleman *et al*., 2002). Cell viability of root nodules incubated with DETA/NO at 100 μM for 6 hours was reduced, along with increased cysteine protease activity at this concentration of the NO

donor (Keyster, 2010, PhD thesis, Stellenbosch University, South Africa). This is in agreement with previous observations where it was shown that increased cysteine protease activity is accompanied by increased PCD and also that high NO levels induce PCD (Natal *et al*., 2008). During root nodule senescence leghemoglobin and bacteroids are degraded and nitrogen fixation is inhibited (Pladys and Vance, 1993).

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Chapter 3

The role of cGMP in modulating soybean root nodule cysteine protease activity

3.1 Introduction

Recent progress in plant science revealed that guanylate cyclase (GC) has vital roles in diverse biological processes (Meier *et al*, 2009). GCs are divided into two major families, namely NO activated soluble guanylate cyclase (sGC) and a transmembrane receptor class that contains a catalytic domain within the intracellular portion of the protein (McDonald and Murad, 1995). The biological production of guanosine 3', 5'-cyclic monophosphate (cGMP) is via enzymatic hydrolysis of guanosine triphosphate (GTP) catalyzed by GC. NO activates soluble guanylate cyclase by binding to the heme domain of the GC, resulting in an increase in the activity of the enzyme, thus elevating the levels of cGMP in cells by up to a 100-fold more than when NO is not bound to the GC (Mur *et al*., 2006). At least one GC has been identified in higher plants but this GC appears to be NO-insensitive (Ludidi and Gehring, 2003). Polverari *et al*. (2003) has demonstrated that the cysteine protease RD21 is inhibited by a cysteine protease inhibitor protein (cystatin). Also Durner *et al*. (1998) showed that NO induces defense responses through a cGMP-dependent pathway in tobacco plants. It has been reported that NO alleviates programmed cell death by inhibition of caspase activity, possibly via a cGMP-dependent pathway (Kim *et al*., 1999). In this chapter, we evaluated the influence of cGMP in CPA and cell viability in roots nodules.

3.2 Materials and Methods

NO activates soluble guanylate cyclase (cGC) to produce cGMP **(**Pacher *et al*., 2007). It was thus investigated if cGMP has any role in regulating the activity of cysteine protease activity in soybean root nodules. Soybean plants were grown and treated as previously described in Chapter 2, except that all treatments were replaced by either 50 μM cGMP (as the cell-permeable form of cGMP, i.e. 8-(4-chlorophenylthio)-[CPT]-cGMP) or 100 μM of the sGC irreversible inhibitor 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) or a combination of both the cGMP and the ODQ. Assays for cysteine protease inhibitor activity were done on root nodules at 0 or 6 hours after treatment as described in Chapter 2. In summary, for 'Untreated' samples, intact plants in vermiculate were treated with 100ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 and used for assaying for Cysteine

protease activity at 0 and 6 hours after treatment. For 'cGMP' samples, intact plants in vermiculate were incubated in 100 ml of 100mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 50 μM of 8-(4-chlorophenylthio)-[CPT]-cGMP and used for evaluating the cysteine protease activity at 0 and 6 hours after treatment. For inhibition of sGC activity, the sample were subject to treatment with ODQ by treating intact plants in vermiculate with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3, containing 100 μM ODQ. A treatment ('cGMP + ODQ') to determine if the ODQ effect on Cysteine protease activity was specifically a result of attenuation sGC activity, was set up by incubating intact plants in vermiculate with 100ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 supplemented with 50 μM cGMP plus 100 μM of (ODQ). These 'cGMP + ODQ'-treated plants were used for cysteine protease activity at 0 and 6 hours of treatment. Cysteine protease activity was determined only from the nodules of the nodulated roots as previously described (Zhang *et al*, 2008).

3.2.1 Measurement of cell viability

Loss of cell viability, indicated as loss of plasma membrane integrity, was measured spectrophotometrically as Evans Blue uptake. Root nodule tissue (10 mg fresh weight) from all treatments (at 0 hour and 6 hours of treatment) was transferred to Evans Blue solution (0.25% [w/v] Evans Blue in water) for 20 min. Root nodules were then washed for 15 min with distilled water to remove unbound Evans Blue. Evans Blue taken up by nodule cells was released by homogenizing the tissue with 500 μL of 1% (w/v) SDS followed by incubation at 55°C. The absorbance of the supernatant was determined at 600 nm using FLUOstar OPTIMA (BMG LABTECH) spectrophotometer operated via OPTIMA Software.

3.3 Results

3.3.1 The response of root nodule cysteine protease activity to exogenously applied cGMP. Exogenously applied cGMP did not significantly ($p < 0.05$) alter nodule cysteine protease activity (figure 3.1). Similarly, inhibition of endogenous cGMP biosynthesis from sGC did not affect cysteine proteinase activity in the root nodules (figure 3.1).

3.3.2 Exogenous cGMP does not induce cell death in soybean root nodules.
Pharmacological studies indicate that NO can react with the haem group in the haem-binding domain of sGC to induce a transient increase in cGMP, which is in turn

implicated in the regulation of gene expression in plants (Maathuis 2006). We investigated if cGMP would elicit cell death in root nodules after treatment with cGMP for 0 hour and 6 hours (figure 3.2). Exogenously applied cGMP did not induce an absorbance increase at 600 nm (compared to untreated nodules) indicative of the inability of nodule cells to take up Evans Blue, suggesting that nodule cells were viable and not dying (figure 3.2). A similar trend was observed for both the ODQ and the cGMP plus ODQ treatments.

3.3 Discussion

Pharmacological studies using an inhibitor of NO-sensitive guanylyl cyclase, namely 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), implicate cGMP as a downstream second messenger molecule in NO-mediated signaling in plants (Neill *et al*., 2008; Ederli *et al*., 2009; Suita *et al*., 2009). Guanosine 3', 5'-cyclic monophosphate (cGMP) is an important signaling molecule with essential roles in cellular functions as a second messenger and functions in plant growth and development, including auxin induced adventitious rooting and stomatal opening as well as responses to both abiotic and biotic stresses such as responses to salt and osmotic stress (Neill *et al*., 2008; Ederli *et al*., 2009; Suita *et al*., 2009). Constitutive NOS causes a transient increase NO and is implicated in cytoprotection through a cGMP-dependant pathway, whereas inducible NOS produces high concentrations of NO that induce PCD (Green *et al*., 1981; Wagner *et al*., 1983; Sakuma *et al*., 1988; Bredt and Snyder, 1989). Modolo *et al*. 2002 suggested that NO and cGMP might have a function in the establishment of symbiosis between leguminous plants and nitrogen-fixing bacteria. The results presented in this chapter indicate that cGMP has no critical role in cysteine protease activity during normal physiological function of root nodules in soybean plants. It documented that sGC activity in tomato is a regulator of 1-aminocyclopropane-1-carboxylic acid synthase gene expression (Matarasso *et al*., 2005). The exogenously applied cGMP did not affect cysteine protease activity in root nodules, neither did the inhibition of soluble guanylyl cyclase (sGC) by ODQ.
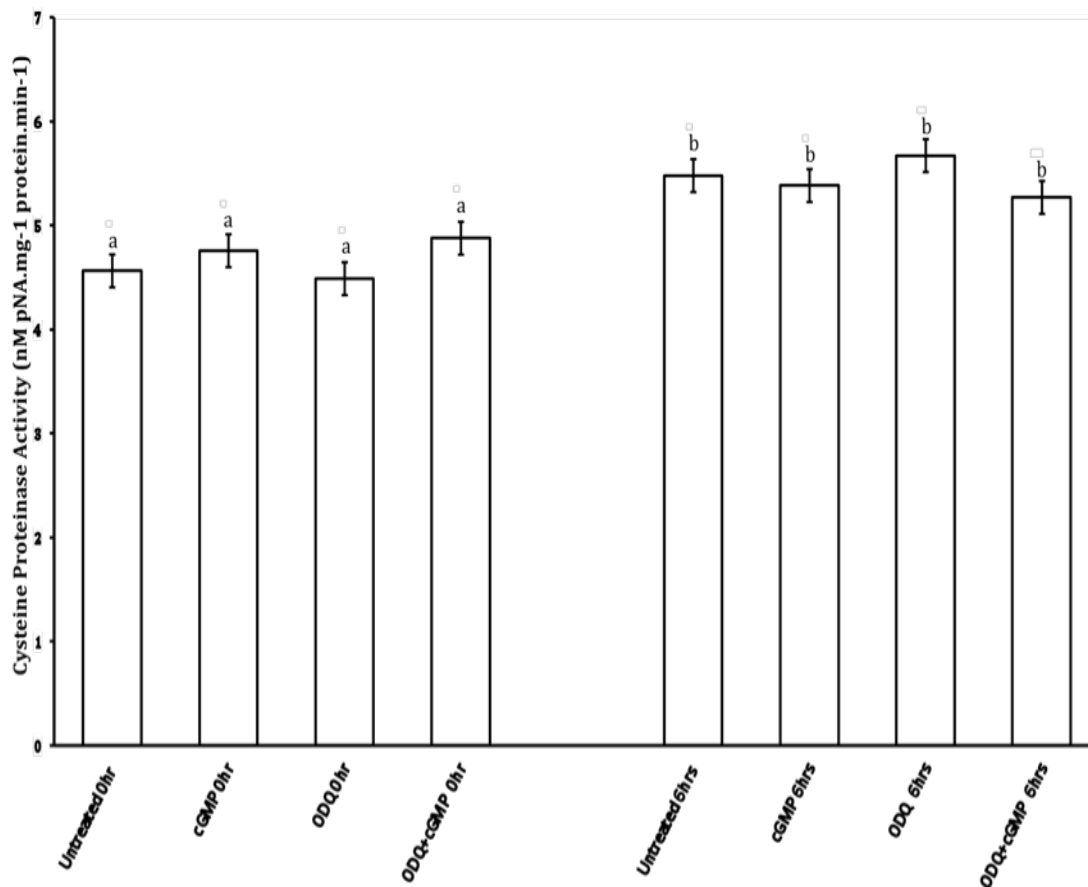
Figure 3.1: Cysteine protease activity in response to (cGMP, ODQ and ODQ + cGMP) in root nodules. Extract (50 μg of crude protein) was incubated with cysteine protease substrate (0.5 mM BAPNA), and the cysteine protease activity was determined by measuring the hydrolysis of BAPNA at 405 nm.
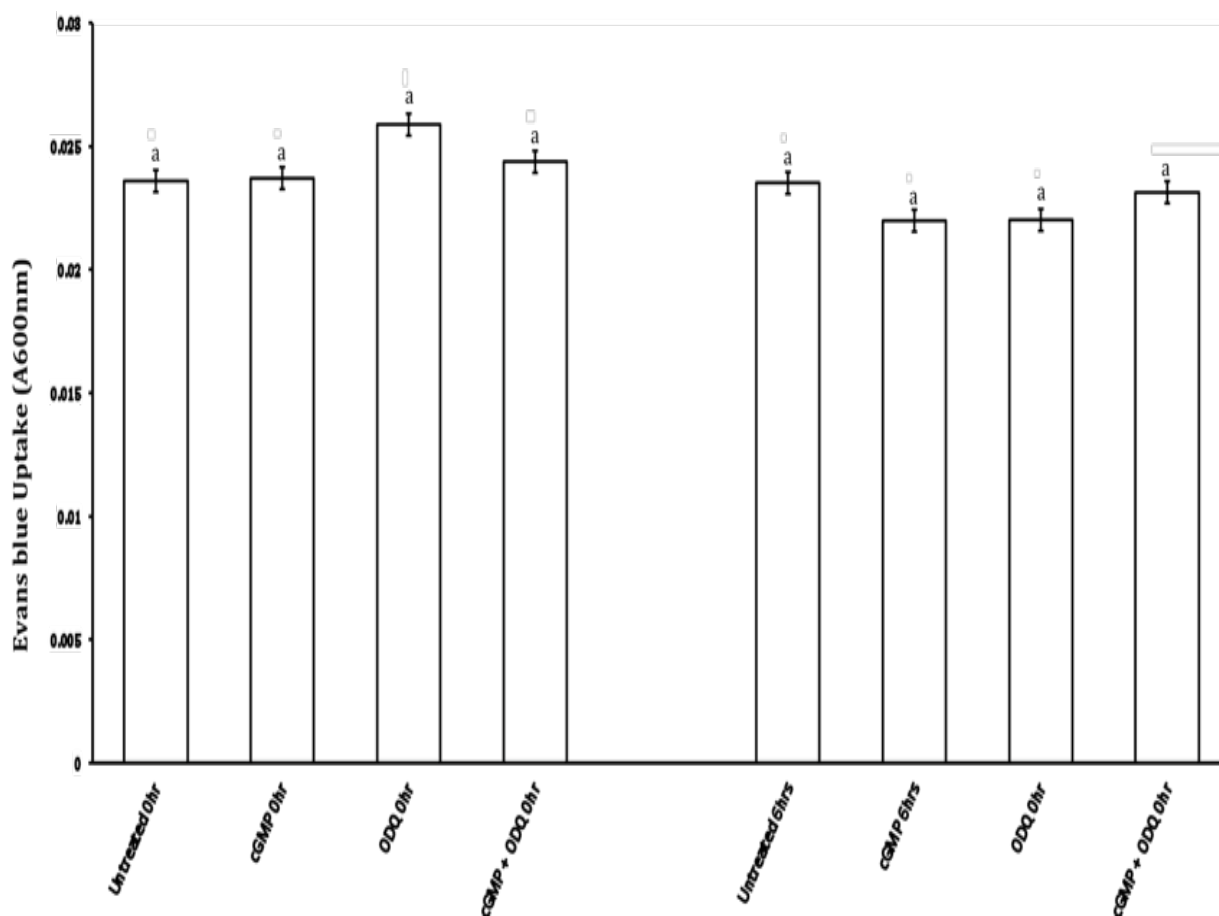
Figure 3.2. Effect of cGMP on cell death evaluated by Evans Blue staining in soybean root nodules treated with 50 μM of [CPT]-cGMP, 100 μM of ODQ and a combination of 50 μM of [CPT]-cGMP plus 100 μM of ODQ.

3.4 References

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Chapter 4

4.Regulation of nodule cysteine protease activity by nitric

oxide under abiotic stress

4.1 Introduction

Legume root nodules are organs that emerge from the symbiotic association between legumes and nitrogen-fixing soil bacteria of the Rhizobium family. In general, nodules senescence is understood as an age-dependant physiological process that occurs when certain molecular events elicit programmed cell death (Jones *et al.*, 2001). The phenotypic feature of nodule senescence is a change in nodule color from red (functional leghemoglobin) to green (degradation of leghemoglobin). At cellular level cysteine proteases are believed to take part in senescence and are the most common proteolytic enzymes identified in this process in the senescence zone in pea, Chinese milk vetch and soybean (Kardailsky and Brewin, 1996; Naito *et al.*, 2000; Alesandrini *et al.*, 2003). Increased production of reactive oxygen species (ROS) has crucial role in plant senescence although steady-state accumulation to moderate levels of ROS is important in regulating plant development (Matamoros *et al.*, 2003). Abiotic stress conditions increase the accumulation of ROS to concentrations that activate PCD and signal transduction molecules responsible for the activation of defense mechanisms (Alesandrini *et al.*, 2003).

4.2 Material and Methods

4.2.1 Effect of exogenous NO˙ on nodule cysteine protease activity during salinity stress

For 'Untreated' samples, intact soybean plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 and used for cysteine protease activity assays at 0 and 6 hours of treatment. Intact plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 100 mM NaCl (these were regarded as 'NaCl') and used for cysteine protease activity assays at 0 and 6 hours of treatment. For 'NaCl + NO' samples, intact plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 100 mM NaCl and 10 μM DETA/NO. These plants were used for cysteine protease activity at 0 and 6 hours after treatment. Cysteine protease activity was determined only from the nodules of the nodulated roots as previously described (Zhang *et al.*, 2008).

4.2.2 Effect of exogenous NO˙ on nodule cysteine protease activity during osmotic stress

For 'Untreated' samples, intact soybean plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 and used for cysteine protease

activity assays at 0 and 6 hours of treatment. Intact plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 200 mM sorbitol (these were regarded as 'Sorbitol') and used for cysteine protease activity assays at 0 and 6 hours of treatment. For 'Sorbitol + NO' samples, intact plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 200 mM sorbitol and 10 μM DETA/NO. These plants were used for cysteine protease activity at 0 and 6 hours after treatment. Cysteine protease activity was determined only from the nodules of the nodulated roots as previously described (Zhang *et al*., 2008).

4.2.3 Effect of cGMP on nodule cysteine protease activity during salinity stress

For 'Untreated' samples, intact soybean plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 and used for cysteine protease activity assays at 0 and 6 hours of treatment. Intact plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 100 mM NaCl (these were regarded as 'NaCl') and used for cysteine protease activity assays at 0 and 6 hours of treatment. For 'NaCl + cGMP' samples, intact plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 100 mM NaCl and 50 μM 8-(4-chlorophenylthio)-[CPT]-cGMP. These plants were used for cysteine protease activity at 0 and 6 hours after treatment. Cysteine protease activity was determined only from the nodules of the nodulated roots as previously described (Zhang *et al*., 2008).

4.2.4 Effect of cGMP on nodule cysteine protease activity during osmotic stress

For 'Untreated' samples, intact soybean plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 and used for cysteine protease activity assays at 0 and 6 hours of treatment. Intact plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 200 mM sorbitol (these were regarded as 'Sorbitol') and used for cysteine protease activity assays at 0 and 6 hours of treatment. For 'Sorbitol + cGMP' samples, intact plants growing in vermiculite were treated with 100 ml of 100 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.3 containing 200 mM sorbitol and 50 μM 8-(4-chlorophenylthio)-[CPT]-cGMP. These plants were used for cysteine protease activity at 0 and 6 hours after treatment. Cysteine protease activity was determined only from the nodules of the nodulated roots as

previously described (Zhang *et al*., 2008).

4.3 Results

4.3.1 Nitric oxide minimizes the effect of sorbitol and sodium chloride soybean root nodules

At 0 hour the activity of cysteine protease was similar in all the samples, while after 6 hours there were significant differences in cysteine protease activities among the various treaments (figure 4.1). After 6 hours of treatment, sorbitol at 200 mM caused an increase in cysteine protease activity by approximately 28% compared to the untreated sample (figure 4.1). Treatments with 10 μM DETA/NO in combination with 200m M Sorbitol revealed a decrease in nodule cysteine protease activity. The effect of exogenously applied 10 μM DETA/NO on sorbitol-treated plants caused a significant decrease in cysteine protease activity (11% lower compared to treatment with 200 mM sorbitol, *p* < 005). On other hand, the cysteine protease activity of soybean nodules from plants treated with 100 mM was 36% higher than that of untreated plants and 11% higher than the sorbitol-treated plants. Exogenously applied 10 μM DETA/NO caused a decrease in the cysteine protease activity of nodules of plants treated with 100 mM NaCl (figure 4.1).

4.3.2 cGMP inhibits salinity stress-induced cysteine protease activity.

The NaCl treatment increased cysteine protease activity in root nodules. At 6 hours of treatment with a combination of 50 μM of -(4-chlorophenylthio)-[CPT]-cGMP and 100 mM NaCl, a decrease in cysteine protease activity was observed (figure 4.2). Treatment with 100 μM of the soluble guanylyl cyclase inhibitor 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) in combination with cGMP and NaCl caused a reduction in cysteine protease activity.

4.3.3 cGMP inhibits induction of cysteine protease activity by osmotic stress.

At 0 hour of treatment, the 200 mM Sorbitol and 200 mM Sorbitol + 50 μM cGMP, 200 mM Sorbitol +100 μM ODQ and 200 mM Sorbitol + 50 μM cGMP + 100μM ODQ treatments at 0 hour showed similar cysteine protease activity. At 6hours, the root nodule treatments with 50 μM of [CPT]-cGMP combined with 200 mM sorbitol caused a decrease in cysteine protease activity compared to treatments with 200 mM sorbitol alone (figure 4.3). Sorbitol causes osmotic stress in plant cells and is thus useful for mimicking drought stress. The results show that ODQ in combination with sorbitol induce cysteine protease activity while a combination of ODQ, cGMP and sorbitol alleviates the

sorbitol-induced osmotic stress. These results suggest that cGMP is involved in the inhibition of osmotic stress-induced cysteine protease activity.

4.3.4 cGMP alleviates sorbitol-induced cell death in soybean root nodules.

Abiotic stresses such as cold, drought and salinity increase the level of reactive oxygen species that induce programmed cell death (PCD) in plants. Having discovered in this study (Chapter 2) that high NO concentrations induce cell death and given that some NO-mediated processes are affected through cGMP, we investigated if exogenously applied cGMP would alleviate the cell death caused by abiotic stress (NaCl and sorbitol) after 0 and 6 hours of treatment. The results in figure 4.4 and 4.5 suggest that cGMP alleviates the cell death caused by NaCl and sorbitol, as reflected by absorbance decrease at 600 nm (indicative of a decrease in Evans Blue uptake because of a reduction in the number of dying cells). Application of sorbitol in combination with ODQ and also NaCl in combination with ODQ induces nodule cell death (as indicated by Evans Blue uptake by dead cells).
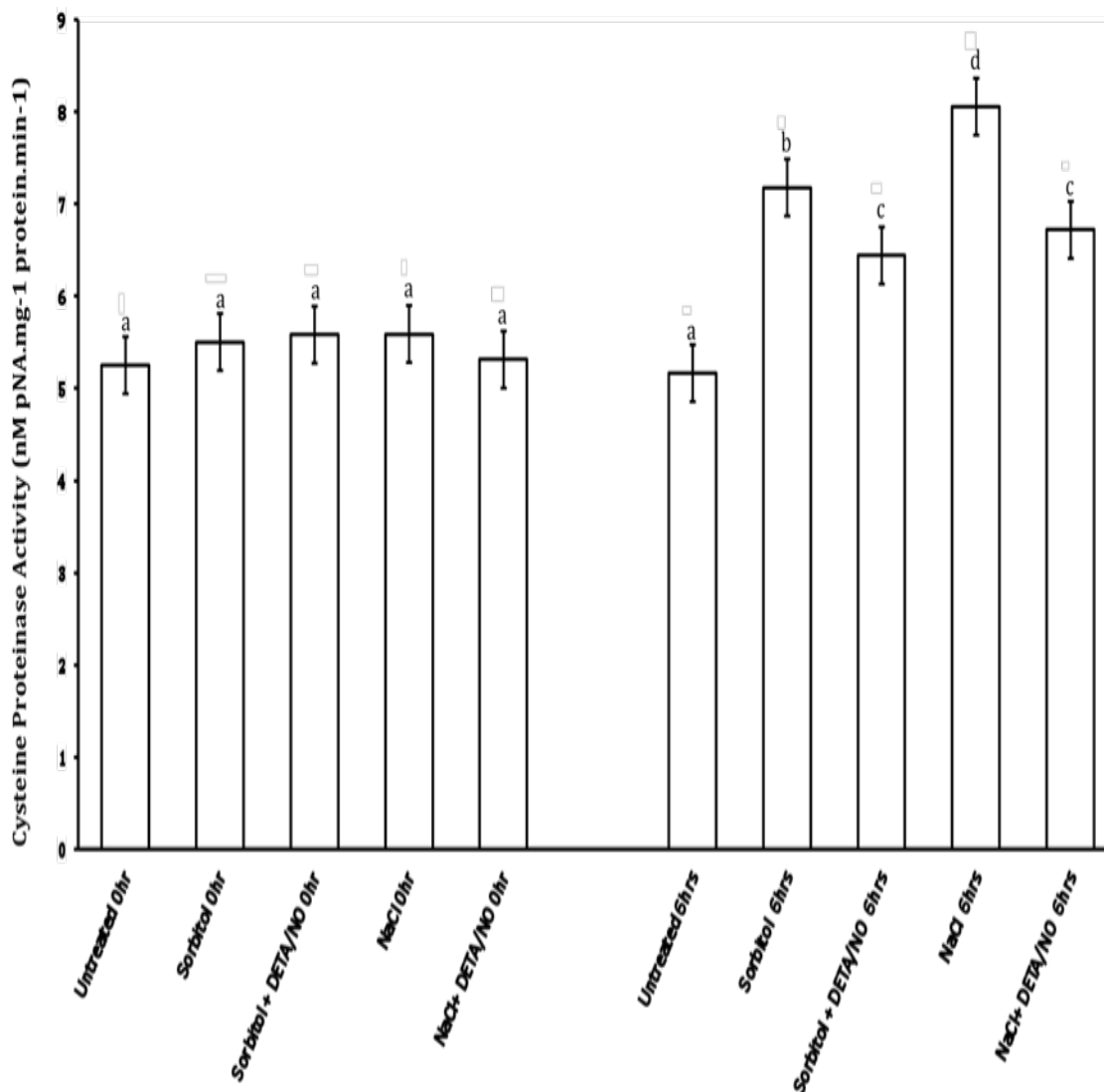
Figure 4.1. Soybean root nodule cysteine protease activity in response to sorbitol, sorbitol + DETA/NO, NaCl and NaCl + DETA/NO at 0 and 6 hours of treatment. Extract (50 μg of protein) was incubated with cysteine protease substrate (0.5 mM BAPNA) and the cysteine protease activity was determined by measuring the hydrolysis of BAPNA at 405 nm.
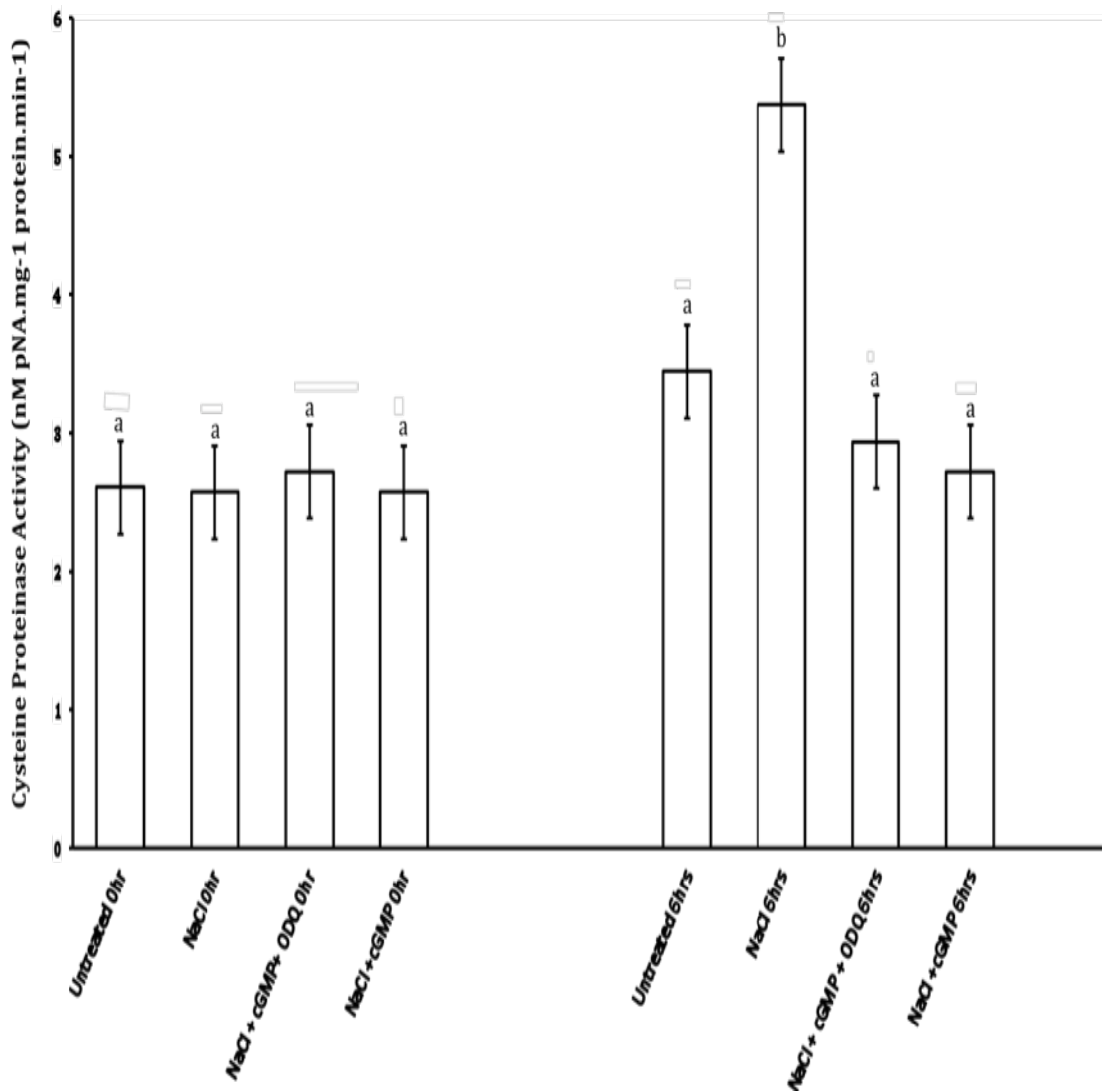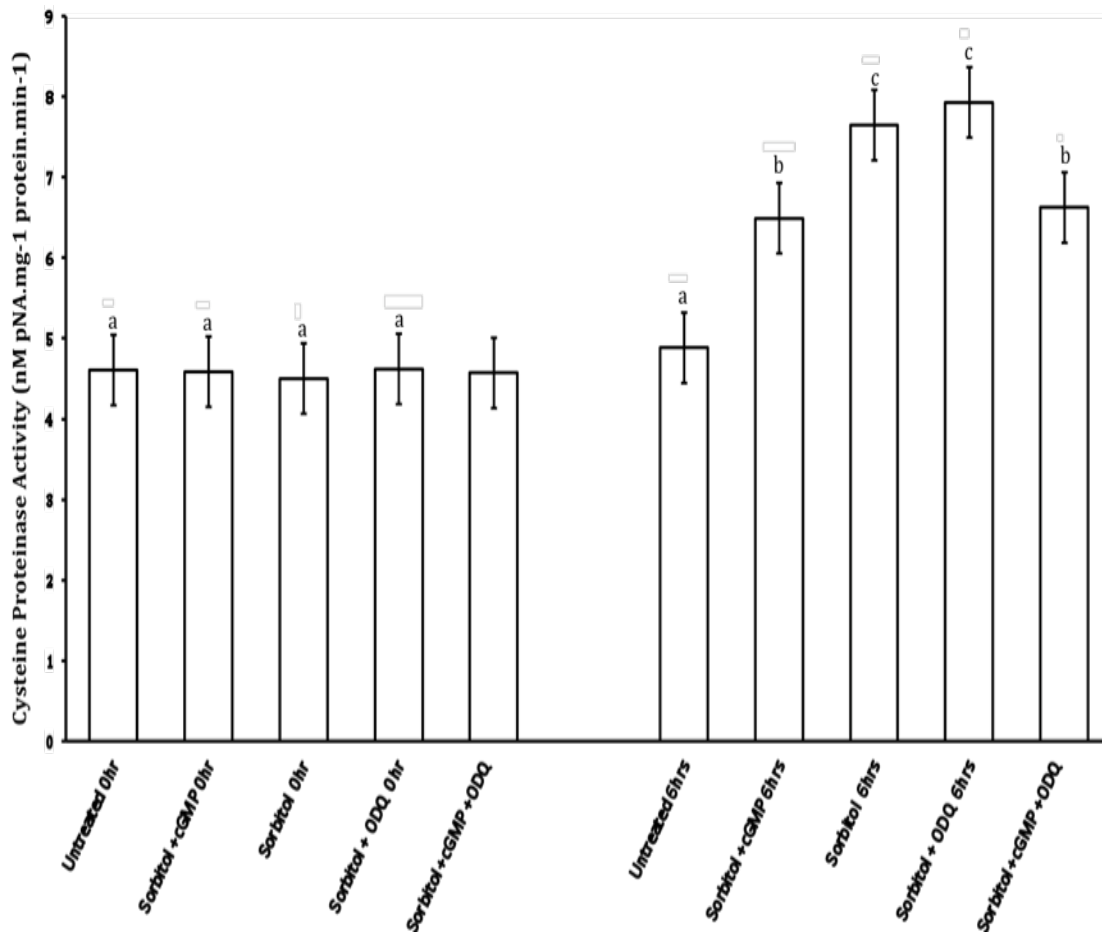
Figure 4.2: Cysteine protease activity in response to NaCl, NaCl + cGMP and NaCl + cGMP + ODQ in root nodules after 6 hours. Extract (50 μg of protein) was incubated with cysteine protease substrate (0.5 mM BAPNA) and the cysteine protease activity was determined by measuring the hydrolysis of BAPNA at 405 nm.
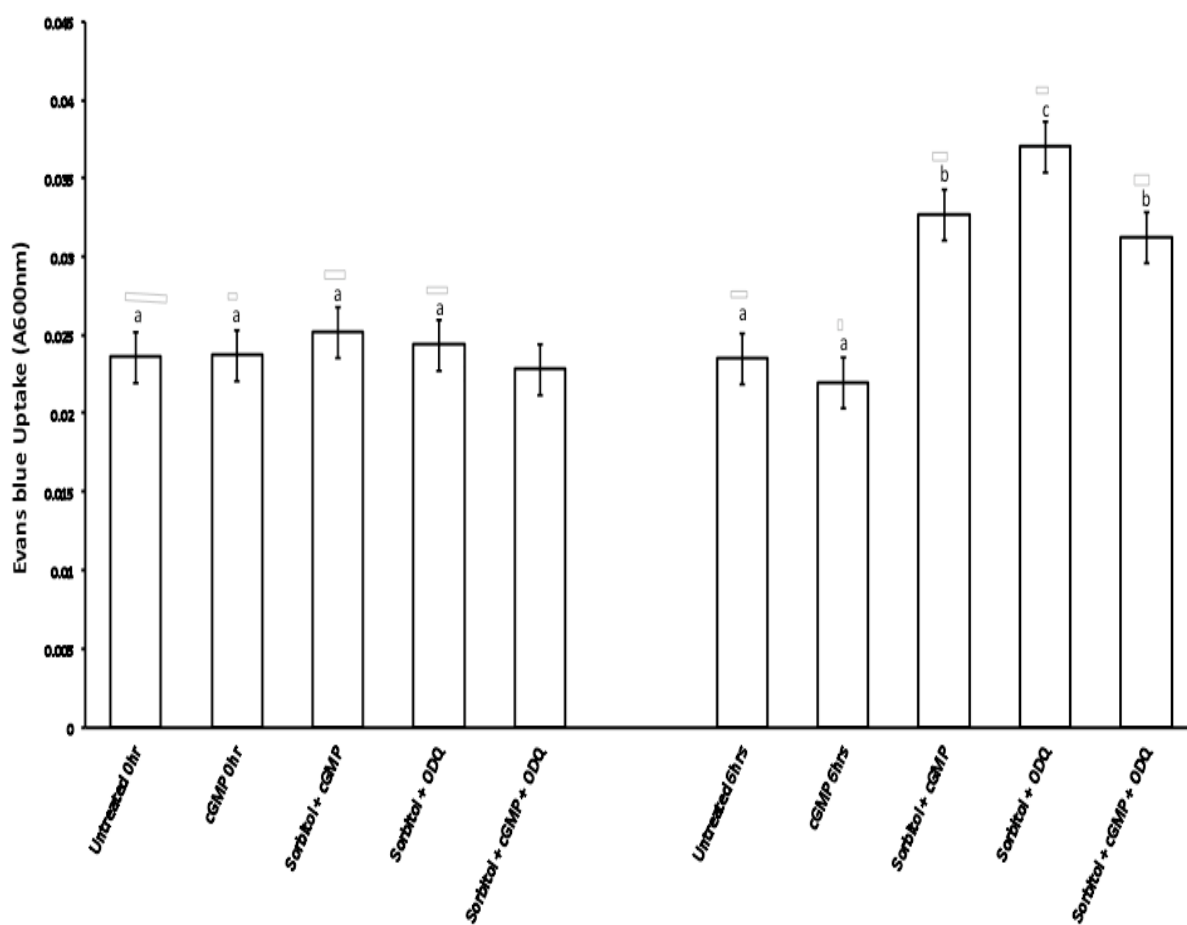
Figure 4.3. Cysteine protease activity in response to sorbitol, sorbitol + cGMP, sorbitol +ODQ and sorbitol + cGMP + ODQ in root nodules after 6 hours. Extract (50 μg of protein) was incubated with cysteine protease substrate (0.5 mM BAPNA) and the cysteine protease activity was determined by measuring the hydrolysis of BAPNA at 405 nm.

Figure 4.4. Effect of sorbitol and cGMP on cell death evaluated by Evans Blue staining in soybean root nodules treated with 50 μM of [CPT]-cGMP plus 200 mM Sorbitol, 100μM ODQ and 50 μM of [CPT]-cGMP plus 100μM of ODQ plus Sorbitol.
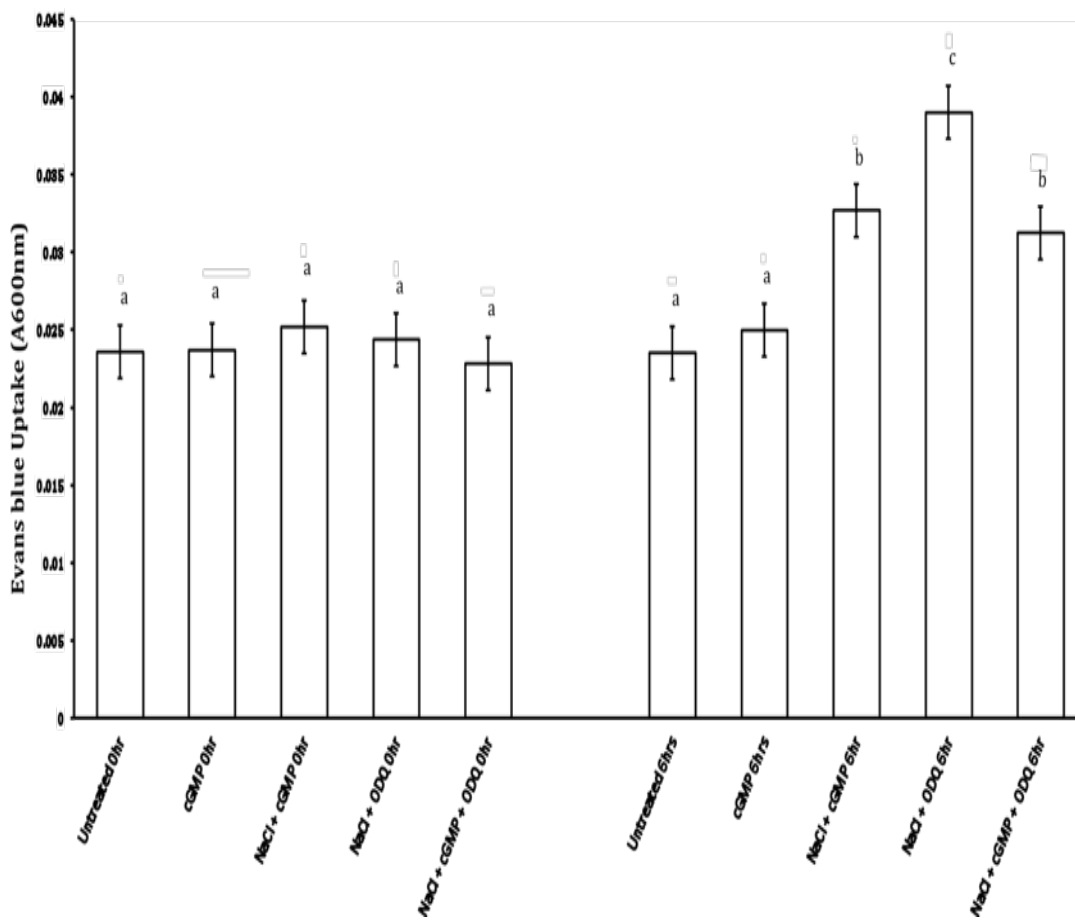
Figure. 4.5. Effect of NaCl and cGMP on cell death evaluated by Evans Blue staining in soybean root nodules treated with 50 μM of [CPT]-cGMP plus 150 mM NaCl, 100 μM of ODQ and 50 μM of [CPT]-cGMP plus 100 μM of ODQ plus NaCl.

4.4 Discussion

Abiotic stress has adversely effects on a wide array of plant processes including disturbances to morphology, anatomy, physiology and biochemistry during development and these disturbances limit the productivity of crop plants (Mittler 2006). Drought and salt stress lead to the accumulation of ROS, which have toxic effects on cell metabolism, causing a decline in the photosynthetic capacity therefore halting plant growth (Mittler 2002). ROS has dual function in plants as they can act both as toxic and regulatory compounds where they regulate various physiological and biochemical processes including plant growth and development, cell cycle, programmed cell death, hormone signaling, biotic and abiotic cellular responses (Mittler *et al*., 2004; Foyer and Noctor 2005; Fujita *et al*., 2006). Drought stress stimulates an earlier or accelerated senescence and some drought specific cysteine protease that are not induced under natural aging process. Salinity imposes both an ionic and osmotic imbalance on plant cells. The results reported here show that sorbitol and NaCl induced cysteine protease activity in soybean root nodules. Vacuolar processing enzymes (VPEs), which are caspase-like cysteine proteases that cleave peptide bond at the C-terminal side of asparagine and aspartate, are found in the vacuole in which they mediate in the execution of plant PCD (Hatsugai *et al*., 2004). Abiotic stress induces PCD through ROS-mediated signals (Palavan-Unsal *et al*., 2005). Sorbitol and NaCl treatment of nodulated legumes cause high Evans Blue uptake in nodule tissue, which is an indication of cell death in root nodules (Marshall Keyster, PhD thesis, Stellenbosch University). DETA/NO and cGMP reduce the induction of cysteine protease activity in response to both Sorbitol and NaCl. This may suggest that NO alleviates the effects of abiotic stress (sorbitol and NaCl) through a cGMP-mediated pathway. However, further experimentation using a combination of nitric oxide synthase inhibitors and guanylyl cyclase inhibitors is required to unequivocally support such hypotheses. Sang *et al*.,(2008) demonstrated that nitric oxide mediates the antioxidant defense system in maize to reduce hydrogen peroxide accumulation involved in water stress-induced cellular damage. Cystatin, an endogenous cysteine protease inhibitor gene, blocked PCD that was triggered by oxidative stress and this is thought to occur via inhibition of oxidative stress-induced cysteine protease activity in soybean (Solomon *et al*., 1999). It is thus possible that nitric oxide acts via complex pathways to protect plants against abiotic stress; such pathways including cGMP-mediated signaling, modulation of antioxidant defenses and alteration of cysteine protease activity either directly through s-nitrosylation or via regulation of cystatin gene expression.

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Solomon,M., Belenghi,B., Delledonne,M., Menachem,E., Levine, A., 1999. The Involvement of Cysteine Proteases and Protease Inhibitor Genes in the Regulation of Programmed Cell Death in Plants. *The Plant Cell* 11: 431–443,

Chapter 5

5. Identification of two maize genotypes with varying tolerance to abiotic stress

5.1 Introduction

Botanists, molecular biologists, and plant breeders recognize cereals (maize, wheat and sorghum) as moderately sensitive to a wide range of abiotic stresses (Tester and Bacic, 2005; Ngara *et al*., 2008; Zhang *et al*., 2008). Drought and salinity poses great environmental constraints to plant survival and agricultural crop yield. Abiotic stress induces ROS-mediated PCD with characteristic features such as ion leakage, shrinkage of the nuclei, chromatin condensation, nuclear DNA fragmentation and lesion formation. In cells from root tips of rice and in tobacco protoplasts, salt stress-induced PCD is preceded by disturbance of Ca^{2+}, K^+, and H^+ ion equilibrium and increased production ROS such as hydrogen peroxide (Huh *et al*., 2002; Shabala *et al*., 2007). Shabala *et al*., (2007) showed that expression of an animal anti-apoptotic gene named CED-9 in tobacco during salt stress maintains K^+ homeostasis and protects the plant from salt- and oxidative stress-induced cell death. Zhang *et al*., (2008) demonstrated that two cysteine proteinase inhibitors from *Arabidopsis thaliana*, AtCYSa and AtCYSb, increase the salt, drought, oxidation and cold tolerance; suggesting a possibility of using these cysteine protease inhibitors to genetically improve environmental stress tolerance in plants. Recent studies have revealed parts of the molecular mechanisms of abiotic stress tolerance in plants, which have been extensively studied through transcriptomic and proteomic profiling between stressed and unstressed plants (Tester and Bacic, 2005; Vinocur and Altman, 2005; Ngara *et al*., 2008). However, considerable progress in understanding plant stress tolerant mechanisms has been made in *Arabidopsis thaliana* (Vinocur and Altman, 2005; Ngara *et al*., 2008). In this study, we investigated the effect of abiotic stress treatments on selected growth, physiological and biochemical aspects of maize.

5.2 Materials and methods

Maize (*Zea mays*) seeds (cultivars Grace, CAP9001, Mac Medium Pearl, Silverking, CAP444NG and Okavango) were kindly donated by Capstone Seeds (Pty) Ltd, Howick, South Africa. The seeds were germinated in vermiculate (pre-soaked in distilled water) in

15 cm diameter for 7 days and then seedlings were transferred to sterile potting soil for yellow Maize (CAP444NG and Okavango). All seedlings were maintained under the following growth chamber conditions: on a 25/19°C day/night temperature cycle and 16/8 hours light/dark regime with a photon flux density of 300 μmol photons.m^{-2}.s^{-1} during the day (light) period.

5.2.1 Treatment of maize for abiotic stress response analysis

The maize treatment started at stage Z11 for the following samples: Untreated, Salinity and Drought. For 'Untreated' samples, intact plants in potting soil were watered with 100 ml of distilled water. For salinity samples, intact plants in potting soil were supplied with 100 ml of distilled water containing 150 mM NaCl and 15 mM CaCl$_2$. Lastly, for the drought samples, intact plants in potting soil were not supplied with any water source. The treatments were done each day for 3 weeks. Growth parameters were measured along with cysteine protease activity. CPA was measured by the method of Zhang *et al*, 2008.

5.3 Results

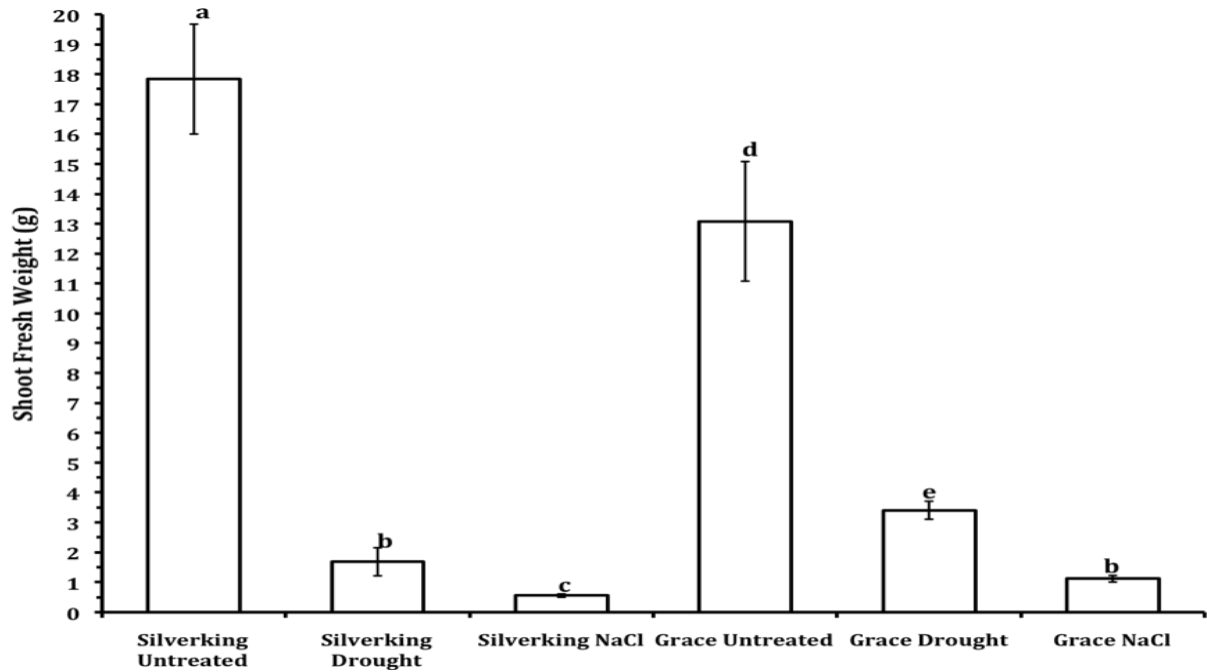### 5.3.1 Effect of Abiotic stress (drought and salinity) on maize growth

To examine the effect of abiotic stress on the maize growth, total root and shoot biomass, shoot and root length, stem thickness and cysteine protease activity of the seedlings was measured. In response to drought and NaCl stress, the fresh and dry weights of shoots in cultivar Silverking were reduced more drastically than those of cultivar Grace (figure 5.1). A similar trend in response to drought and NaCl stress occurred to the fresh and dry weights of roots because cultivar Silverking roots were reduced more drastically than those of cultivar Grace (figure 5.2). There was a significant loss in shoot lengths for both Silverking and Grace in response to both drought and salinity stress but the reduction in the shoot length of Silverking was more pronounced than that of Grace for both types of stress (figure. 5.3). Stem thickness was reduced by both drought and salinity but the reduction was more severe in Silverking than in Grace (figure. 5.4).

5.3.2 Cysteine protease activity

Cysteine protease activity was measured after salt and drought stress. In response to these stresses, cysteine protease enzyme activity in Silverking leaves was increased when compared to controls. The cysteine protease activity increased in leaves of

Silverking by 57% in response to drought and 88% in response to NaCl stress, while for Grace leaves about the cysteine protease activity increased by 25% in response to drought and 65% in response to NaCl stress (figure 5.5 a). Cysteine protease activity was also increased in roots when compared to the controls. In roots of Silverking, cysteine protease activity increased by about 70% in response to drought and increased by 82% in response to NaCl in comparison to the cysteine protease activity of untreated plants, whereas the cysteine protease activity of Grace roots increased by only 57% in response to drought and by up to 78% in response to NaCl when compared to untreated plants of the same cultivar (figure 5.5 b).
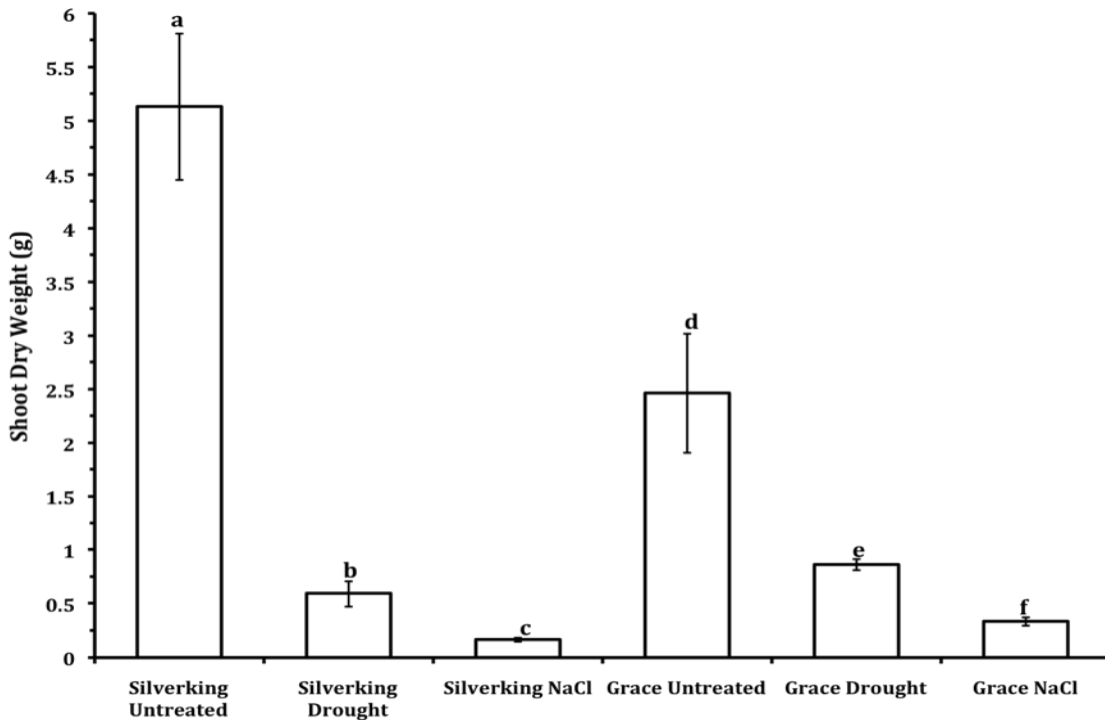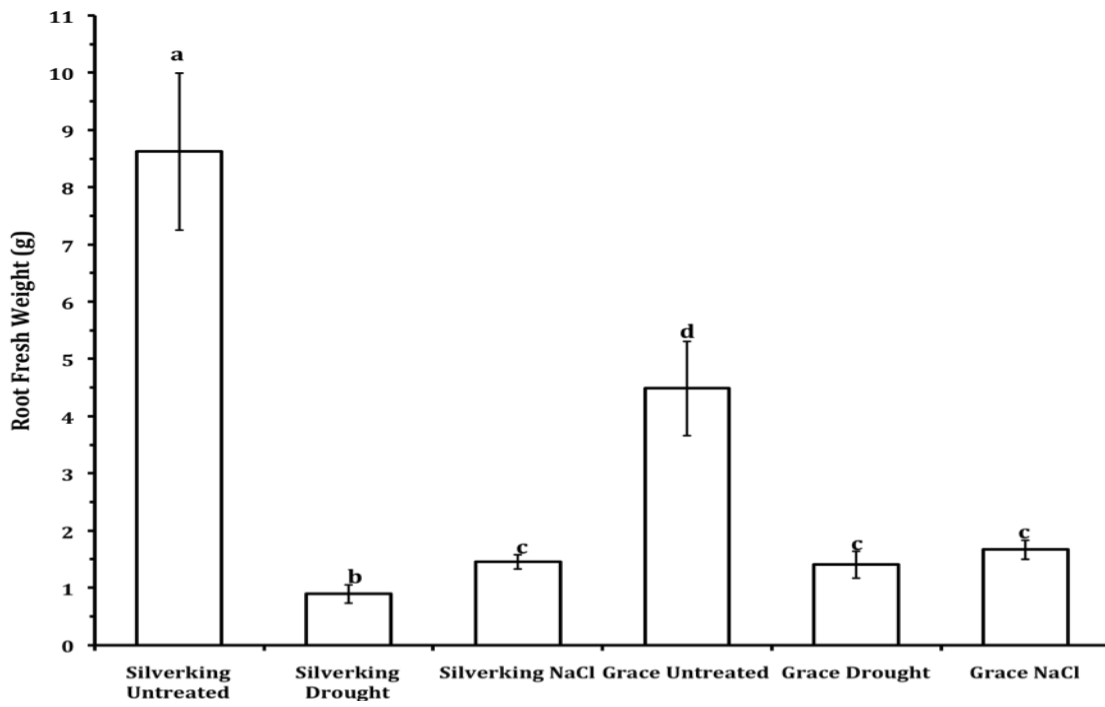
Figure 5.1. Effect of drought (osmotic stress applied by withholding irrigation) and salinity stress (150 mM NaCl) on Silverking and Grace biomass. (A) Shoot fresh weight, (B) Shoot dry weight.
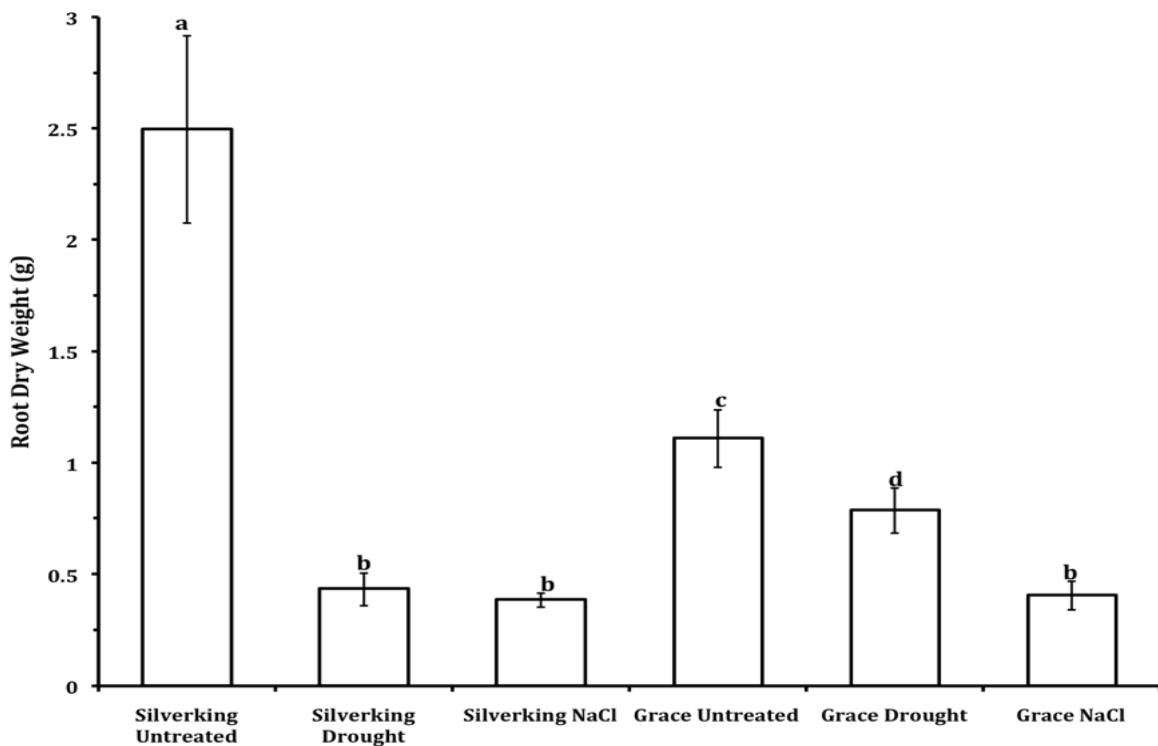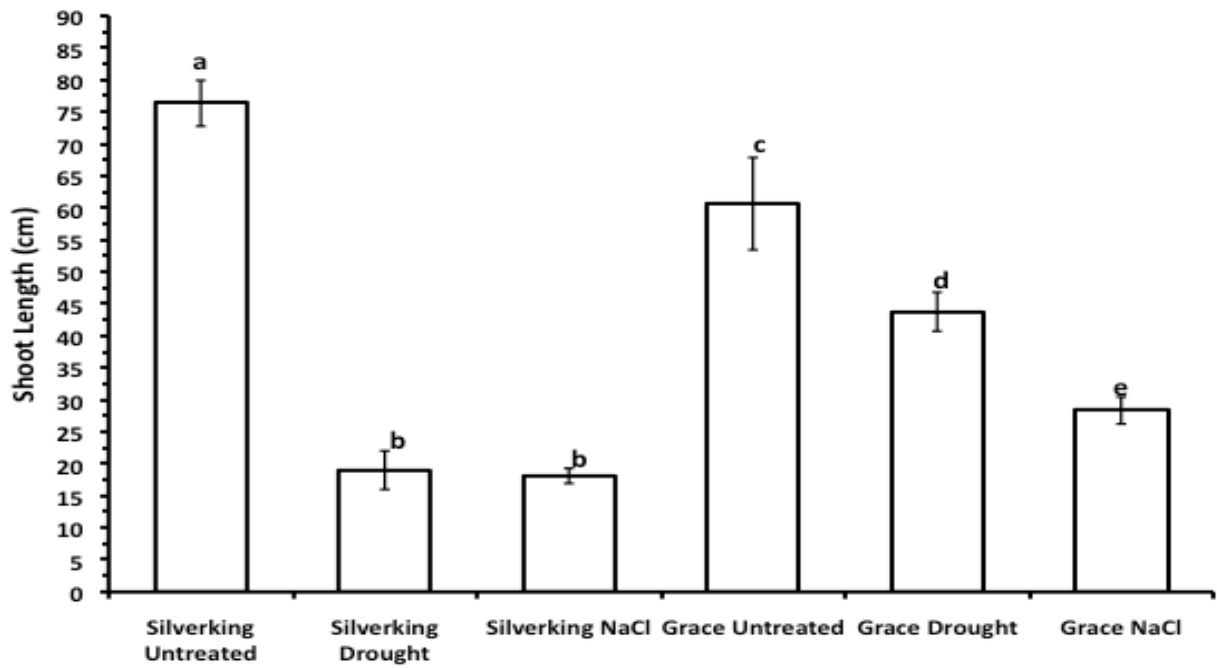
A)

B)

Figure 5.2. Effect of drought (osmotic stress imposed by withholding irrigation) and salinity stress (150 mM NaCl) on Silverking and Grace biomass. (A) Root fresh weight, (B) Root dry weight.
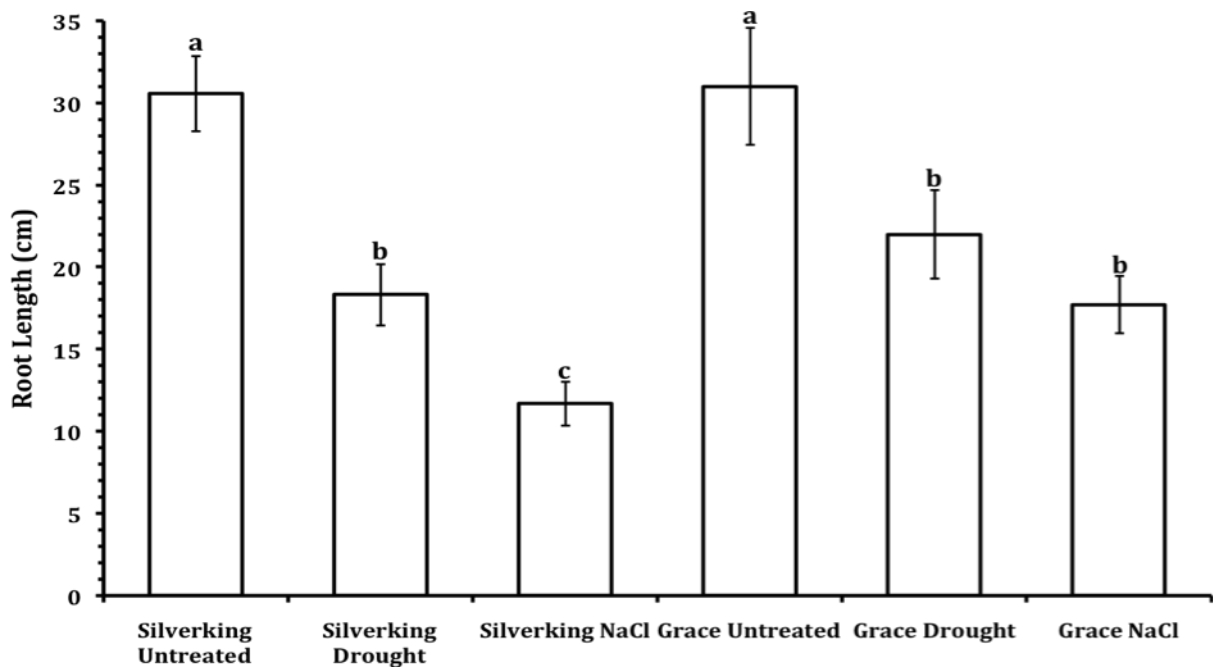
A)

B)

Figure 5.3. Effect of drought (osmotic stress imposed by withholding irrigation) and salinity stress (150 mM NaCl) on Silverking and Grace biomass. (A) Shoot length, (B) Root length.
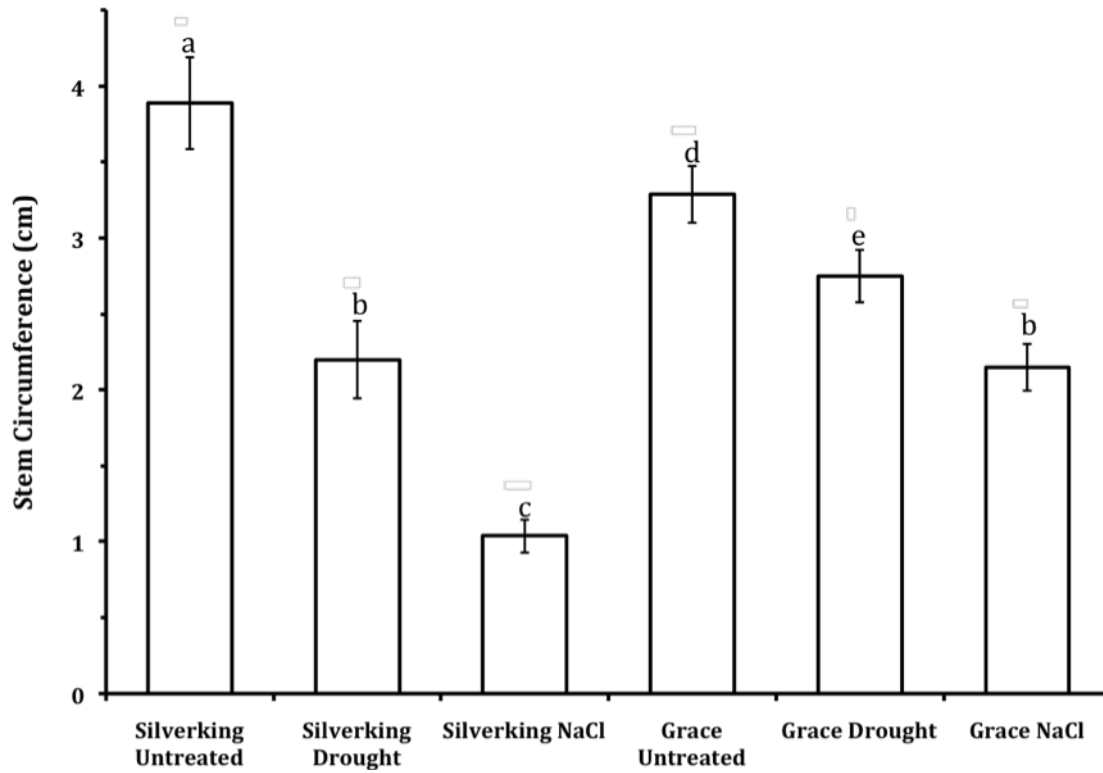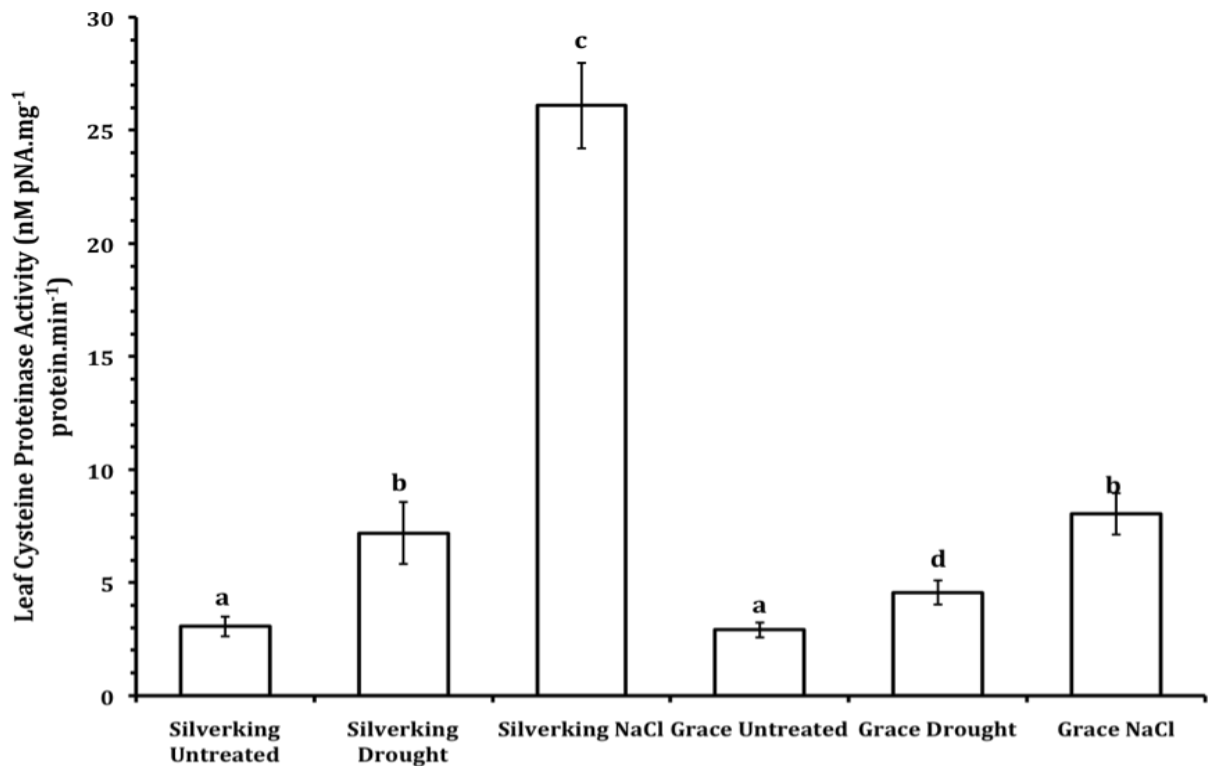
Figure 5.4. Effect of drought (osmotic stress imposed by withholding irrigation) and salinity stress (150 mM NaCl) on Silverking and Grace stem thickness.
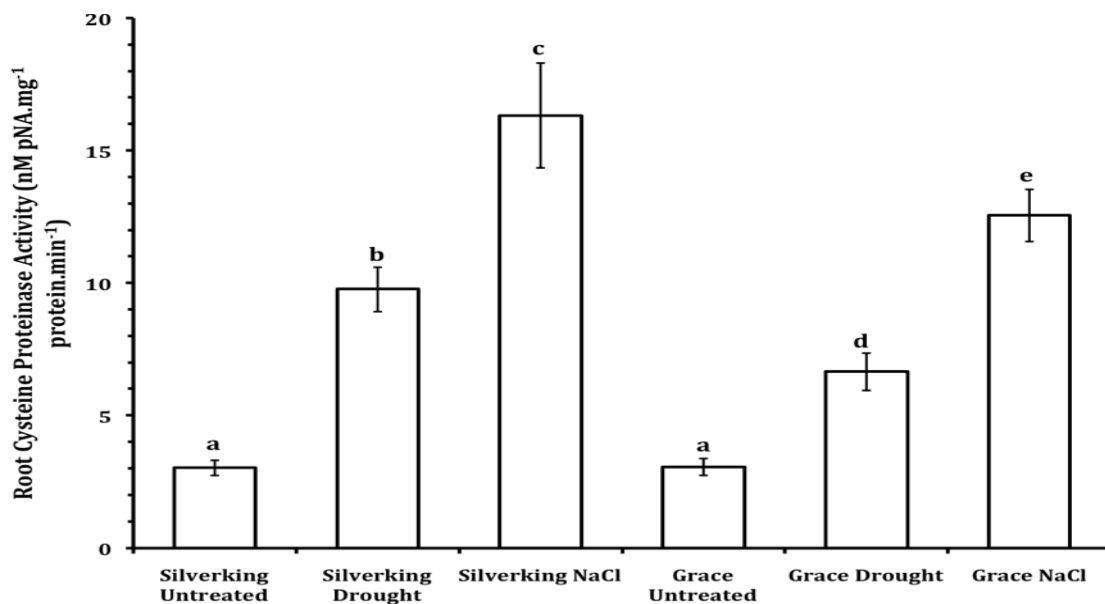
A)

B)

Figure 5.5. Effect of drought (osmotic stress imposed by withholding irrigation) and salinity stress (150 mM NaCl) on cysteine protease enzyme activity in Silverking and Grace cultivars.

5.4 Discussion

Maize is a commercially important crop and main staple food in Southern Africa where it is grown on over 12 million hectares (FAOSTAT, 2003). However, salt stress and drought impose constraint on maize production. There are attempts to genetically introduce traits that my confer drought and salt stress tolerance in maize (Tester and Bacic, 2005; Ngara *et al*., 2008; Zhang *et al*., 2008). This study has investigated the level of sensitivity/tolerance to drought and salinity in several maize genotypes (Grace, CAP9001, Mac Medium Pearl, Silverking, CAP444NG and Okavango). In this study, Grace and Silverking were found to have contrasting levels of performance under abiotic stress conditions. The performance of these two contrasting genotypes was evaluated on the basis of changes in their growth (measured by determining various growth parameters) to drought and salinity. The two abiotic stresses result in a decrease in overall plant growth. Grace exhibits betters tolerance to both salt and drought stress while Silverking appears to be highly sensitive to both of the abiotic stresses. Drought and NaCl adversely affected the lengths of shoots and roots, stem thickness and the weights of shots and roots and this negative impact was more severe in Silverking than in Grace. Earlier work demonstrated that leaf area is reduced when drought or salt stresses occur before and after flowering and this reduces photosynthesis rate (Demetriou *et al*., 2007). PCD plays several key roles that regulate plant growth and development including differentiation of tracheary elements, endosperm development and leaf senescence (Fukuda, 2000; Young and Gallie, 2000; van Doorn and Woltering, 2004). Cysteine proteases are instrumental in the execution of PCD in animals and are involved in distinctive cell activities including DNA degradation processes (Grudkowska and Zagdańska, 2004). In plant cells, high caspase-like activity occurs during PCD (Segovia *et al*., 2003; Zuppini *et al*., 2007). In this study, drought and salinity stress elevated cysteine protease activity of shoots and roots and this increased activity was more pronounced in Silverking than in Grace.

Taken together, the results of this study show that Grace is more tolerant to the abiotic stresses investigated here than Silverking and also shows that cysteine protease activity is inversely proportional to the level of abiotic stress tolerance. This observation suggests that the level of cysteine protease activity during exposure of maize to abiotic stress could serve as an indicator/a screening tool for sensitivity/tolerance to the abiotic stress.

5.5 References

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Chapter 6

6.1 General Conclusion

In summary, we conclude that NO regulates cysteine protease enzyme activity in root nodules of soybean in an NO concentration-dependent manner. This is in agreement with observations that implicate NO as a cytotoxic molecule at high concentrations and a cytoprotective molecule at relatively low concentrations in plants (Beligni and Lamattina, 2001). The lower NO concentration halts cell death in root nodules by diminishing the cysteine protease activity. The molecular mechanism for the reduction in cell death remains to be identified and elucidated. Besides S-nitrosylation, NO can act via induction of cGMP biosynthesis through activation of soluble guanylate cyclase. In consideration of this effect of NO on cGMP biosynthesis, we investigated the effect of exogenously applied cGMP on root nodule cysteine protease activity and found that it does not influence the cysteine protease activity under unstressed conditions. It appears that, under normal physiological conditions of root nodules, the cysteine protease activity in response NO operates in a cGMP-independent pathway. Interestingly, both NO and cGMP reduce the induction of cysteine protease activity caused by either sorbitol or NaCl. Low concentrations of exogenously applied NO reduce the induction of cysteine protease activity caused by abiotic stress in root nodules and this appears to be mediated through a cGMP-dependent pathway.

Osmotic stress imposed by sorbitol and salinity stress imposed by NaCl treatments adversely affected the stem thickness, shoot length and root length in maize and this negative impact was more severe in the maize cultivar Silverking than in the cultivar Grace. Furthermore, osmotic stress and salinity treatments elevated cysteine protease activity of shoots and roots and this activity was more prominent in Silverking than in Grace. When comparing the two genotypes, it is clear that Grace is more tolerant to abiotic stress than Silverking.