Detection of protease and protease inhibitors during development of soybean crown nodules

By

LUFUNO ABIGAIL MASHAMBA

A thesis submitted in partial fulfilment of the requirement for the degree of

MAGISTER SCIENTIA

Department of Plant Science

Forestry and Agricultural Biotechnology Institute (FABI)

In the

Faculty of Natural and Agricultural Sciences

University of Pretoria

Supervisors : Prof KJ Kunert

: Dr U Schlüter

MAY 2010

DECLARATION

I, the undersigned hereby declare that the thesis submitted herewith, for the degree Magister Scientia, to the University of Pretoria, contains my own independent work.

This work has not been submitted for any degree at any other University.

Lufuno Abigail Mashamba

May 2010

TABLE OF CONTENTS

CHAPTER 2 MATERIALS AND METHODS

ABSTRACT

A symbiotic association between leguminous roots and soil fixing nitrogen bacteria is required for legume nodule formation. The primary function of nodules is the fixation of nitrogen from the atmosphere into an accessible form for plants. In this study, nodules of plants of the soybean cultivar Prima 2000 were characterized and their number and weight were determined during nodule development. Their nitrogen-fixing activity during nodule development was determined by color evaluation. A pink nodule color showed active leghemoglobin required for nitrogen fixation and a green nodule color nonfunctional leghemoglobin. Strong appearance of nonfunctional leghemoglobin in the later stages of nodule development during senescence was accompanied by an increase in protease activity within crown nodules demonstrated by gelatine-containing SDS PAGE. Cysteine protease activity was identified as a major protease activity during nodule senescence when the cysteine protease inhibitor E-64 was used to block total protease activity. Products, which may indicate the expression of cysteine protease inhibitors during nodule development, were detected with the reverse zymogram technique and Western blotting. However, these bands have not been characterized so far in more detail. Putative transgenic plants were produced using the Agrobacterium transformation technique to allow determining the activity of native and mutated papaya cysteine protease inhibitor coding sequences. These sequences will ultimately be used for soybean transformation to reduce cysteine protease activity in nodules. However, the presence of coding sequence in the genome of these putative transgenic plants could not be confirmed by gene amplification and protease activity testing. Overall, this study has contributed to establish parameters to measure nodule growth and performance during development.

THESIS COMPOSITION

Chapter 1 outlines the importance of nodules for biological nitrogen fixation and explains the difference between determinate and indeterminate nodules. Objectives of this study are development in soybean nodule; this chapter also outlines the current knowledge about leghemoglobin which is required for nitrogenase activity. Further, the possible role of the protease system in plant senescence and programmed cell death is reported. The aim and objectives of this study are finally provided at the end of this chapter. **Chapter 2** outlines the materials and methods used in this study. This includes the used plant growth and root nodulation technique, physiological evaluation of crown nodule growth and performance, various molecular biology techniques, SDS PAGE technique, zymogram technique and the tobacco transformation technique. **Chapter 3** outlines the results obtained from soybean plant growth and characterization of nodule growth and performance. It further reports on protease profiling during nodule development and the detection of expression of cysteine protease inhibitors during nodule development. Finally, the transformation of tobacco with a papaya cysteine protease inhibitor coding sequence using the Agrobacterium technique and the detection of inhibitor sequences in the plant genome is reported in this chapter. In **Chapter 4** results obtained are discussed and new results are highlighted which have contributed to a better understanding of measurement of nodule development in soybean nodules. **References** outlines list of relevant literature cited in this thesis.

ACKNOWLEDGEMENT

I would like to thank God for giving me strength, courage and patience to complete this MSc study. My sincere gratitude also to the Department of Plant Science of the University of Pretoria for granting me a wonderful environment to achieve this degree and the National Research Foundation for financial support that allowed me to conduct and accomplished my research. Special thanks to my supervisors: Dr Urte Schlüter, I thank her for the very good mentoring and useful suggestion that have led to compiling of this study, I'm very grateful for your advice and encouragement. To Prof. Karl Kunert, I thank you for installing such a confidence in me, and for being not only an excellent mentor but a caring and considerate person.

A special thanks to my parents-Mr M.G Mashamba and Mrs M.V Mashamba, my brothers (Dakalo and Mulalo) and sisters (Tshivhidzo, Muofhe, Iris, Lima, Thabelo and Phumudzo) for all years of support and encouragement, with your love, I couldn't ask for a better family, I 'm very grateful.

To my friends (Christina, Faith, Puleng, Olgar, Happy, Tumi and Tsholo), I would like to express sincere thanks for your helpfulness, friendliness, support and wonderful company you have provided me when days were dark during my studies. I am also grateful to Dr Muthumuni Sinthumule, for walking with me in this journey, your courageous words, lifted me, and gave me strength to go forward, you are such a wonderful partner, thank you.

A massive appreciation goes to my lab mates Juan, Rosita, Tumi, Tsholo, Celia, Priyen, Berhanu, Stefan, Eugene, Josephine and Endale, without you I would have been completely lost in lab.

ABBREVIATIONS AND SYMBOLS

%	Percentage
G	Grams
Mg	Milligrams
SA	South Africa
°C	Degree Celcius
rpm	Rotations per minute
Ph	Log hydrogen ion concentration
µL	Microlitre
UK	United Kingdom
Cm	Centimetre
FW	Fresh weight
LB	Luria broth
V	Voltage
mL	Millilitre
L	Liter
dH_20	Sterile distilled water
h	Hour
U	Unit
Bp	base pair
CO_2	Carbon dioxide
O_2	Oxygen
SOC	Super optimal broth with catabolites repression

mM	Millimolar
IPTG	Isopropyl-β-D-thiogalactoside
X-gal	5-bromo-4chloro-3indolyl-β-D galactoside
Ti-plasmid	Tumor inducing plasmid
OD	Optical density
M	Molar
BAP	6-benzyl-amino purine
IBA	Indole-butyric acid
SDS	Sodium dodecyl sulphate
PVDF	Polyvinylidene difluoride
sec	Seconds
BSA	Bovine serum album
nm	Nanometer
µg	Microgram
µM	Micromolar
TCA	Trichloro-acetic acid
mA	Milliamperes
$MgCl_2$	Magnesium chloride
OC-I	Rice cysteine protease inhibitor
SD	Standard deviation
Rt	Room temperature
$MgSO_4$	Magnesium sulpahte
H_2O	Water
KH_2PO_4	Potassium chloride
$CaCl_2$	Calcium chloride

KCl	Potassium chloride
$MnSO_4$	Manganase sulfate
$ZnSO_4$	Zinc sulphate
CuSO4	Copper sulphate
Na_2MoO_4	Sodium molybdate
Tris-HCl	2-Amino-2 (hydroxymethyl)-1, 3propernediol hydrochloride
TBS	Tris-buffered saline
MS	Murishage and Skoog medium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
TAE	Tris-acetate EDTA
EDTA	Ethylenediamine tetra acetic acid
Tris	2-amino-2-(hydromethyl) propane-1.3 diol
E. coli	*Escheria coli*
CaMV	Cauliflower mosaic virus
CPI	Cysteine protease inhibitor
SDS-PAGE	Sodium a dodecyl sulphate Poly-acrylamide gel
APS	Ammonium persulfate
E-64	(epoxysuccinyl)-L-leucylamino-4-guanidinobutane
Nt	Non transformed
TEMMED	N, N, N-tetramethyl ethylenediamine

(PCR)	Polymerase chain reaction
dNTPs	Deoxynucleosides triphoshates
Taq	Thermus aquaticus
BSA	Bovine Serum Albumin
kDa	Kilo Dalton
min	Minutes
Lb	Leghemoglobin
cDNA	Complimentary DNA
SDGs	Senescence down regulated genes
SAGs	Senescence associated genes
PCD	Programmed cell death
CPs	Cysteine protease
BrCys1	Phytocystatin from *Brassica rapa*
SrPI1	*Sesbania rostrata*, serine protease inhibitor
Asnodf32	Nodule specific cysteine protease
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
RNAi	RNA interference
WC5	Wheat cystatin gene
T1-T4	Putative transgenic papaya mutant plants

INDEX OF FIGURES

PAGES

activity gels were incubated after SDS-PAGE with 2 mg/ml papain overnight to degrade gelatine and tobacco proteins.

1.1 Importance of nodule for nitrogen metabolism

Nitrogen is one of the nutrients, plants require in the highest amount. In agriculture, nitrogen availability has a major influence on both yield and product quality (Devienne-Barret *et al.*, 2000). Generally, in all living organisms nitrogen is needed as a continual source for synthesis of new proteins. All inorganic nitrogen must first be reduced to ammonia before it can be assimilated into amino acids (Lea and Miflin, 1980).

In non-leguminous plants, adequate application of nitrogen to soil is necessary for high foliar protein and high crop yields (Sugiharto and Sugiyama, 1992), whereas inadequate supply of nitrogen to the roots and a prolonged nitrogen limitation result in a decrease of foliar protein (Crafts-Brandner *et al.*, 1998). Crop productivity and its quality are directly related to the availability of adequate nitrogen (Martindale and Leegood, 1997).

Leguminous plants, such as soybean, pea, cowpea, and peanut, can access nitrogen from dinitrogen in the air by means of a symbiotic association with soil fixing nitrogen bacteria known as *Rhizobium*. They are also central to environment-friendly agriculture; due to their ability of natural nitrogen fixation they provide a valuable alternative to chemical nitrogen fertilisation of the soil (Schultze and Kondorosi, 1998).

In this symbiosis, hosted *Rhizobium* bacteria fixes atmospheric nitrogen into accessible form to the plants by producing the nitrogenase complex known to reduce nitrogen to ammonia (Beringer *et al.*, 1979). The host plant on the other hand, provides the energy from photosynthesis and ensures the survival of bacteria within the potentially hostile environment

of the plant. During this symbiosis, the photosynthetic product sucrose is delivered to the symbiosome via the phloem where it functions as a source of reduced carbon source to the bacteria. This sucrose is required not only as a source of energy for nitrogen fixation and associated metabolism, but also as a source of carbon skeletons for ammonium assimilation exported from the symbiosome to the rest of plant (Colebatch *et al.*, 2002). The *Rhizobium* also manipulates the host respiration in early stage of symbiosis (Long *et al.*, 2001).

In accepting the *Rhizobial* partner, the plant lowers the endogenous defense that prevent invasion by foreign organisms, so that the bacteria can survive and grow closely to the plant cell cytoplasm (Puppo *et al.*, 2005). Plants also provide a unique micro-aerobic low oxygen environment for bacteria within the symbiosome, and control the expression of bacterial nitrogen fixation genes (Long *et al.*, 2001). In the plant, nitrogen is incorporated into essential macromolecules, such as amino acids and proteins, responsible for plant growth and development, in order to obtain a high crop yield (Lodwig *et al.*, 2003).

However, bacteria-based nitrogen fixation is not always sufficient for optimal plant growth because application of nitrogen to field grown legumes during pod filling often leads to the greater seeds yields with greater seed protein contents (Wesley *et al.*, 1998). Further studies indicated that leguminous plants housing soil fixing nitrogen bacteria also undergo nitrogen deficiency at pod filling, suggesting that legume-*Rhizobium* symbiosis breaks down too soon in the plant development cycle, requiring quicker remobilization from the leaves (Wesley *et al.*, 1998). Deficit in nitrogen rich compounds can lead to low protein content in seeds and generally to low seed production (Delgado *et al.*, 1994). In soybean seeds for instance high protein content requires large amounts of nitrogen, demanding high rates of nitrogen fixation

(King and Purcell, 2001). As a result, leguminous plants may derive less than 50 % of their nitrogen needs from symbiosis (Espinosa-Victoria and Graham, 2000).

1.2 Nodule development

The interaction between the symbiotic partners begins with the exchange of chemical signals between two species. During the establishment of symbiosis, plants secrete flavonoids and phenolic compounds (Irving *et al.*, 2000). In response, bacteria release lipochitooligosaccharide molecule called Nod factors through the expression of nod genes. These Nod genes are not expressed in free living cells of bacteria (Long *et al.*, 2001). In contrast, non-nodulating genes do not respond to bacterial signals (Mitra *et al.*, 2004).

Nod factors induce various responses in the plants including root hair deformation, and cortical cell-division, and finally specific organs called nodules are formed (Ehrhardt *et al.*, 1992). The Nod factors released by bacteria modify the plant hormone balance in such a way as to stimulate mitosis and to allow development of nodule that will house the bacteria within the plant (Puppo *et al.*, 2005). In the nodule, bacteria differentiate into a distinct cell type called bacteriod, which is capable of fixing nitrogen. Bacteroids are never free from the cytosol of plant host, but are surrounded by a host derived membrane called peribacteriod membrane (Long, 1989). This membrane effectively isolates the bacteria from the host cell cytoplasm. The peribacteroid membrane thus fulfils essential structural and metabolic roles, separating the bacteria from the plant cell cytoplasm and controlling the exchange of metabolites and signals (Puppo *et al.*, 2005). *Rhizobium* continues to divide until the infected cells are packed with thousand of bacteria, which are surrounded, either individually or in small groups, by symbiosome membrane (Colebatch *et al.*, 2002).

Super nodulation and hyper nodulation phenotypes have shown that the degree of nodulation is controlled through metabolite signals (Lodwig *et al.*, 2003) and hormones that control plant development (Ferguson and Mathesius, 2003). During nodule development the host plant expresses a certain number of proteins specific to nodule development and nitrogen fixation called nodulins. These genes have been subdivided into two major classes, early and late, based on the time point of expression during nodule development. Early nodulin genes are already expressed before the actual nitrogen fixation starts and activated in roots within hours or after few days of inoculation with *Rhizobium*. They are also suspected to be involved in nodule development. Late nodulin genes are activated in mature nodules around the time of nitrogen fixation (Yang *et al.*, 1993). Some of these encode enzymes involved in primary carbon and nitrogen metabolism. Some of these enzymes may help to maintain the structural integrity of the nodules, including the compartmentalization of bacteroids in symbiosomes (Colebatch *et al.*, 2002). The best known among these proteins is leghemoglobin (Lb), which is found in all legumes and regulates oxygen tension in nodules (Manen *et al.*, 1991).

Generally two developmental patterns of nodules can be distinguished into the determinate and indeterminate growth pattern (Oke and Long, 1999) as indicated in Fig. 1(a) and (b) below. Determinate nodules are characterized by a non persistent meristem that results in round-shaped organs with a homogeneous central fixation zone composed of infected bacteria cells, combined with some uninfected cells. This type of nodules is found in leguminous plant such as *Lotus japonicus* (miyakogusa), *Glycine max* (soybean), *Phaseolus vulgaris L* (common bean) and other tropical plants (Van de Velde *et al.*, 2006). The nodules are further classified into crown and lateral depending on the region of development within plant root system. Crown nodules develop from stem or tap root whereas lateral nodules develop from

lateral roots of the leguminous plant. Studies indicated that lateral root nodules may contribute to nitrogen fixation later in the growing season but often require specific *Rhizobia* strains for optimal fixation rates. Application of improved inoculum into the area around the lateral roots, suggested to have a significant positive effect on total nitrogen fixation especially if highly compatible inoculant strains are used (Pereira *et al.*, 1993).

Other leguminous plants, such as *Medicago truncatula,* form indeterminate nodules; these are long nodule surrounded by cortical cell layer, with five easily distinguishable developmental zones as shown in Fig. 1 (b). Zone (I) is made up of small meristematic cells, which are eternally dividing and do not contain any micro-symbionts. Zone (II) contains the infection zone where bacteria are captured for symbiosome formation. In Zone (III) the bacteria are housed in the symbiosomes and become capable in atmospheric nitrogen-fixation. Zone (IV) is an absence zone during early nodule differentiation, but appears as the nodule development becomes progressively larger as the plant ages. Proximal to the aging zone is a region (Zone V) where the bacteria are essentially free-living and do not show the ultra-structural features of symbiosomes (Timmers *et al.*, 2000). A plant controls nodule longevity in a similar manner to which it controls the extent of symbiotic root nodule formation through the signalling, metabolite and hormone pathways (Ferguson and Mathesius, 2003). Potential of nitrogen fixation per plant was documented to depend on the nodule number and weight of nodules per plant (Al Karaki *et al.*, 2000). The number of nodules per plant is reported to be positively correlated with plant height and with greater seed yield per plant (Al-Karaki *et al.*, 2000).

Ashraf and Iram (2005) reported that larger nodules also have a relatively greater potential of nitrogen fixation than smaller nodules. There is evidence that large nodules provide a greater

sink for phloem-derived unloading of photo assimilates and water than small nodules (Pereira *et al.*, 1993). Drought stress for instance can cause a marked decrease in number, size and mass of nodules thus, reducing nitrogen fixation capacity of the plants (Pereira *et al.*, 1993).

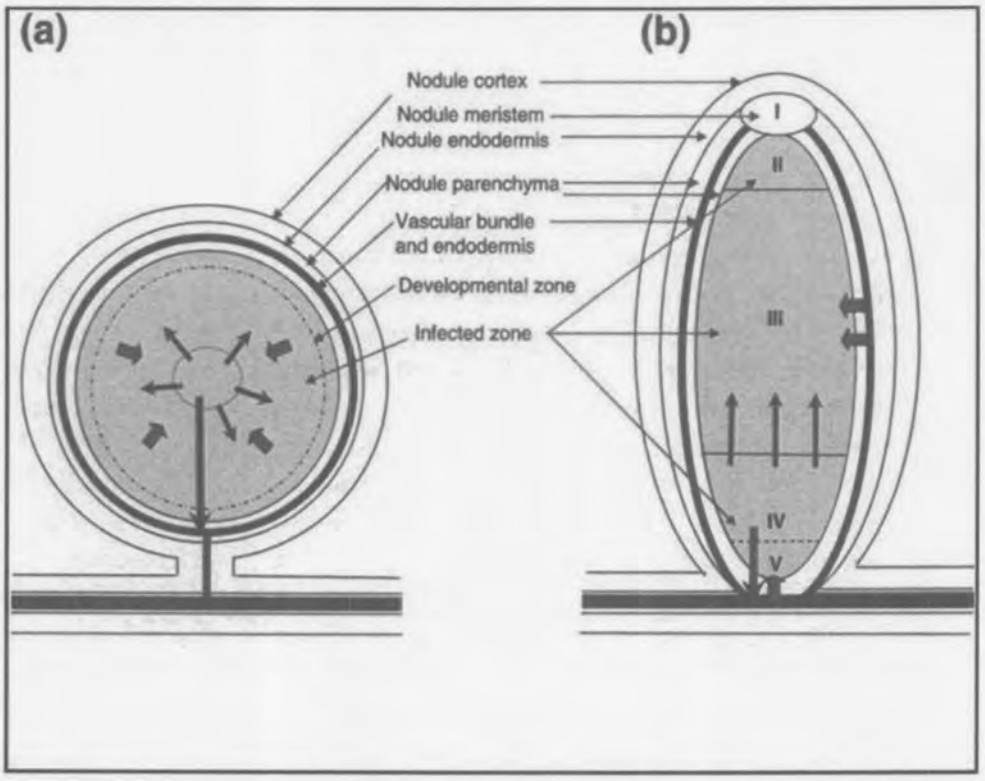


Figure 1.1: A diagrammatic representation of the structure nodule meristem of the determinate (a) indeterminate nodules with different zones (b). Arrows show an increase of senescent tissue within nodules (adapted from Puppo *et al.*, 2005).

The most abundant protein in nodules is leghemoglobin. The primary role of symbiotic leghemoglobin appears to be the facilitation of oxygen diffusion within infected tissues (Appleby *et al.*, 1992). This feature is evidenced by a great accumulation of functional hemoglobin in the target tissues with high affinity for oxygen and a relatively fast oxygen-dissociation rate, which permits low cellular concentrations of free oxygen and a reasonable flux of oxygen within tissues actively fixing nitrogen (Arredondo-Peter *et. al.*, 1997).

1.3 Nodule senescence

Root nodules have a limited functional lifespan, which determines their contribution to the host plant in terms of fixed nitrogen. Longevity of active functional period depends on the legume plant species, the Rhizobial strain and environmental conditions (Swaraj *et al.*, 1995). The nitrogen-fixing capacity of the nodules peaks early in the life of the nodule (Buchanan-Wollaston *et al.*, 2003). The lifespan of average nodule on a fast-growing herbaceous legume in the absence of stress is 10-12 weeks maximum from the point of initiation (Puppo *et al.*, 2005). It was suggested that shoot and root signals are major factors controlling nodule lifespan. Estimation of the factors causing the aging processes, it is logical to believe that the number of nodules present at any one time on the roots is dictated at least in part by the activity and sensing of processes in the shoot, because firstly plant nitrogen status is sensed in the shoots in such a way as to control the shoot: root ratio and secondly because the net assimilate produced and exported from the shoot determines the extent of nodulation (Scheible *et al.*, 1997).

Studies showed that inside a developing nodule all the cells are not at the same stage of development. The different stages are easily observed in indeterminate nodules (Fig. 1b) due to their well separated developmental zone but also exist in determinate nodules (Vance, 1986). During plant development, all plants experience a natural phase of senescence after flowering. At the final stage of development, senescence progresses. Senescence in plants is usually viewed as a developmentally programmed degeneration process that constitutes the final step of tissue development and is controlled by multiple developmental and environmental signals leading to tissue death and the whole plant (Lim *et al.*, 2003). During

senescence large scale of protein remobilization occurs, involving degradation of proteins leading to proteins loss. This allows the distribution of nitrogen reserves from leaves or other organs areas, and translocation into the reproductive parts such as seeds and fruits, this is most common in the wild plants where both nitrogen and water are the main growth limiting factors (Martinez *et al.*, 2007).

Senescence occurs in a specific and orderly manner, in terms of when, where and how it develops. Two types of senescence have been observed during plant growth, developmental or natural and induced or premature. Developmental senescence has been defined as the final phase of plant development and refers to both aging of specific tissues, organs and the whole plant, whereas induced senescence occurs due to environmental stresses and chemical application to the leguminous plants (Nooden *et al.*, 1997). It was reported that though senescence does progress with age, it is not simply an inactive aging process. It is controlled by internally and externally signals and it can also be delayed or accelerated by altering these signals. Lines of sorghum with properties of delayed senescence are being increasingly used to develop higher yielding cultivars (Huffaker *et al.*, 1990). In contrast, induced senescence due to environmental factors contributed to the postharvest loss of crop yield (Zimmerman and Zentraf, 2005).

Nodules are characterized by a relatively early senescence resulting in a decrease of nitrogen fixation (Alesandrini *et al.*, 2003). Premature nodule senescence is activated by various changes in environmental climate, including, water deficit, continuous darkness, and nitrate fertilization. Induced nodule senescence develops much more rapidly than natural senescence and shares some characteristics with developmental senescence (Matamoros *et al.*, 1999). During nodule senescence, the symbiosome organ structure changes, cytoplasm nodule cells

become progressively less dense and frequent vesicles appear. Furthermore the symbiosomes change in size and shape as the bacteroid deteriorates (Puppo *et al.*, 2005).

1.3.1 Determinate nodule senescence

In the case of determinate nodules, senescence begins at the centre of the tissue and then extends progressively reaching the periphery after a few short weeks (Puppo *et al.*, 2005). Previous studies suggested that symbiotic nitrogen fixation declines during pod-filling as a result of an inadequate supply of photosynthetic products to nodules. Fluctuations in nitrogen fixation are also observed during part of the growing season leading to loss of proteins, however both events that lead to loss of nitrogen fixation whether as a results of induced or aging senescence, appear to be irreversible unless new nodules are formed (Klucas *et al.*, 1974). Senescence involves the active turnover and recapture of cellular material for use in other organs (Pennell and Lamb, 1997). Onset of nodule senescence is accompanied by a decrease in soluble proteins and decrease in leghemoglobin content as illustrated in Fig. 1.2. Leghemoglobin degradation during nodule senescence is marked by a change in color from pink to green. Greening of the nodules has been attributed to conversion of Lb resulting in an organic molecule called biliverdin which has a green/brown color, which damages its function (Swaraj and Bishnoi, 1996). However, the rapid decrease in nitrogen fixation capacity that occurs following exposure to stress is observed before any observed decrease in leghemoglobin transcripts in pea nodules (Puppo *et al.*, 2005). Similarly, the rapid decrease in nitrogen fixation capacity that occurs following exposure to stress is observed well before a decline in leghemoglobin (Escuredo *et al.*, 1997). In most types of stress, the initial decrease of nitrogen fixation is associated with a decline in the oxygen concentration reaching the infected cells and bacteroids. The differentiated bacteria in the senescent tissue

may have a higher survival probability than free-living rhizobia in the rhizosphere, insuring the continuous infection of newly differentiated cells (Hernandez-Jimenez *et al.*, 2002).

It has been further shown that the nitrogen fixation process is very sensitive to stresses in general such as darkness, hydrogen peroxide and herbicide application (Fernandez *et al.*, 1996). When thirty days old common bean nodules were exposed to darkness, nodule senescence was initiated and already after 1 day of darkness nitrogenase activity, measured using a flow-through gas analysis system, decreased by 95 %. A general degradation of nodule metabolism occurred after 4 days with a large decline in leghemoglobin (Gorcena *et al.*, 1997). Further, reports indicated that when 5 weeks lupin nodules were sprayed with 5mM herbicide glyphosate of about after 3, 5 and 7 days, it was observed that after 7 days the nodule cytosol was reduced by about 20 % causing alteration in nodule and bacterial proteins. SDS-PAGE revealed that after 5 days of treatment leghemoglobin was reduced by about 47 % suggesting that the oxygen diffusion from nodule was also affected (De Maria *et al.*, 2005).

1.3.2 Indeterminate nodule senescence

During indeterminate nodule senescence, a matured zone (zone IV) is established proximal to zone III, this zone increasingly moves in aproximal-distal direction until it reaches the apical part and the nodule degenerates (Van de Velde *et al.*, 2006).

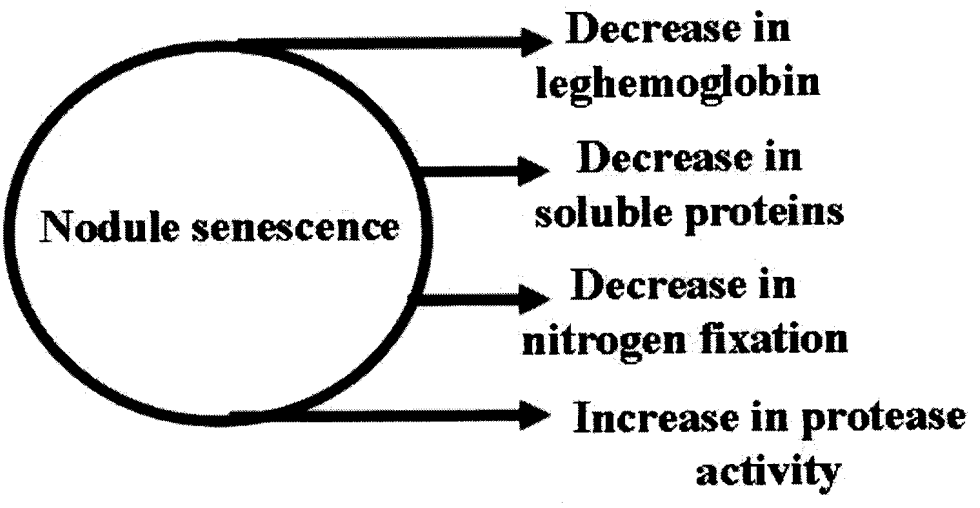


Figure 1.2: A schematic diagram illustrating the effect of nodule senescence during nodule development.

Though little investigation has been done in nodule senescence, more publication has been released from leaf senescence. Leaf senescence in *Arabidopsis* is affected not only by developmental factors, such as aging and hormones, but also with various environmental factors, such as stress and nutrients supply. There is considerable evidence from cDNA libraries derived from senescent leaf tissue that the expression of the vast majority of genes is down-regulated during senescence. Senescence down regulated genes (SDGs) includes photosynthesis genes. However a number of senescence associated genes (SAGs) are upregulated and have been identified in senescing leaves of *Arabidopsis* (Lohman *et al.*, 1994). It has been discovered that these genes are related to age dependent and senescence induced by other environmental factors. Like in any other plant, senescence found in *Arabidopsis* leaf is associated with the developmental aging process, and this particular senescence occurs after a developmental time point. One of the known SAGs in *Arabidopsis*

is upregulated in an age specific order and is also regulated by environmental factors. Plant hormones have been drawn in the regulation of senescence (Hensel *et al.*, 1993).

Studies showed that cytokinins are the most effective senescence-retarding growth regulator. Its exogenous application of cytokinins delays leaf senescence in *Araibidopsis* and other plants. However there is little information documented showing that genes related to cytokinin physiology are involved in leaf senescence. In contrast, ethylene, methylene jasmonate, brassionosteroids and salicylic acid promote the signs of senescence (Valpuesta *et al.*, 1995).

1.4 Senescence and Programmed Cell Death (PCD)

Senescence in higher plants, including leaf senescence is regarded as a type of programmed cell death (PCD). Plants exhibit PCD in a number of contexts during the hypersensitive response to pathogen attack and senescence (Estelle, 2001). Programmed cell death is a physiological cell death involved in the selective elimination of unwanted cell (Pennel and Lamb, 1997). Damaged cells also undergo PCD; this process removes potentially harmful cells and prevents them from multiplying and spreading. However not all damaged cells undergo PCD, cells damaged with stress at high level undergo necrosis. This is a nonphysiological phenomenal process involving cell swelling, lysis and linkage of cell contents. Moreover PCD occurs during development and is regulated by complex mechanism, necrosis is not normally associated with development, does not operate through gene-dependent signal pathways. Other types of cell death involve the localized cell death. During interactions with the biotic environment, cell death is known as hypersensitive response. Hypersensitive cell death occurs at the site of attempted attack by an avirulent

pathogen, and leads to the formation of dry injury that is clearly surrounded from healthy tissue (Dangl *et al.*, 1996).

Other authors suggested that senescence and cell death are under the control of the same coordinated signaling pathway since senescence involves PCD. Cell death during senescence occurs more slowly than in other types of programmed cell death associated with rapid cell death (Pennell and Lamb, 1997). The lower rate of cell death occurs during senescence is likely associated with efficient recycling of nutrients released during senescence.

1.5 General proteases

The life span of most cellular proteins is significantly shorter than the life span of the organism. Proteolysis is the degradation of proteins occurs via multiple pathways and is of fundamental importance for the normal development, homeostasis and death of a plant cell. The biochemical degradation of proteins through hydrolysis of peptide bonds is caused by the action of proteolytic enzymes (Habib and Fazili, 2007). These enzymes, also called proteases, catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins. These enzymes are widely distributed in nearly all plants, animals and microorganisms. Plant proteases are responsible for protein metabolism, a fundamental network of reactions required during the life cycle. Proteolytic enzymes are usually associated with the progression of cellular death during the senescence of the organ, and during the program of differentiation of the cellular components. Proteases mainly involve two groups of enzymes such as exoproteases, which act near the terminus of polypeptide chains, at the ends of protein substrates and designated as amino-or carboxypeptidases, and endoproteases acting

on the interior of protein substrates. Classification of endoproteases rests on the type of residue at the active site (Salas *et al.*, 2008).

Proteases are grouped into aspartic, cysteine, metallo and serine protease according to the essential amino acid residue at their active sites, the optimum pH range of activity and amino acid sequence similarities. The most common plant endopeptidases are cysteine proteases, otherwise known as thiol proteases, which are characterized by an active site formed by conserved Cys and His residues (Vincent *et al.*, 2000). Most plant cysteine proteases belong to the papain (C1) and legumain (C13) families.

Papain was the first cysteine protease to be discovered in the latex and fruit of *Carica papaya* and served for a long time as a model for mechanistic and structural studies on cysteine protease (Schaller, 2004). A number of genes encoding papain-like cysteine proteases have also been isolated from senescing organs including leaves, legume nodules and geminating seeds (Lohman *et al.*, 1994). The physiological functions assigned to plant cysteine proteases include, the building up and breaking down of storage proteins during seed germination, organ senescence and are implicated in a number of different cell deaths. Most important is their involvement in the proteolytic pathway affecting several metabolic processes, such as hormone signaling, cell cycle, embryogenesis, morphogenesis, flower development and oxidative stress. Plant cysteine proteases can be present in almost every organ. Cysteine protease in plants involved in wide range of developmental process such as response to biotic and abiotic stress.

During senescence, cellular organelles dismantled and macromolecules are degraded releasing nutrients for remobilization to other rapidly growing tissues in the plant (Beers *et al.*, 2000). Protein degradation has an important role in the process of senescence and in the

regulation of various plant hormone responses (Abraham *et al.*, 2006). Proteolysis is a natural way to erase mistakes occurring during biosynthesis and assembly of proteins or protein complexes in various cellular systems. Thus proteins that are not folded in a correct way, unable to gather their functional site in a multiprotein complex or being mistargetted to a wrong cellular compartment are all subjected to a rapid degradation (Andersson and Aro, 1997).

It is known that most proteins are degraded by cellular proteolytic enzyme of one sort or another. Some proteins are degraded when their constituents, carbon and nitrogen required supporting the life of the organism. Furthermore others are degraded in response to specific environment or cellular signals. In each case of degradation proteolysis is upregulated (Estelle, 2001). For the senescence program to proceed, there are genes that carried out the degeneration process. Proteolytic enzymes are involved in the digestive processes, pro-enzymes activation and releasing of physiologically active peptides (Oliveira *et al.*, 2003).

Limited proteolysis results in the maturation of enzymes, necessarily for protein assembly and subcellular targeting, and control of the enzyme activities, with regulation of proteins and peptides (Schaller, 2004). Activity of proteases has been correlated with the onset of cellular suicide induced by stress or pathogen attack (Alesandrini *et al.*, 2003).

1.5.1 Proteases and nodule senescence

Senescence is an integral part of development in nitrogen fixing nodules. Nodule senescence has been associated with increase in proteolytic enzymes (Asp *et al.*, 2004). Proteases have been identified in the cytoplasm of infected nodule cells and their activity appears to increase

markedly during nodule senescence. Previous studies indicated that plant proteolytic enzymes are age related during nodule senescence, resulting in rupture of peribacteriod membranes, lysis of bacteriods, and disorganization of host cell cytosol (Vance *et al.*, 1979).

The first target of these proteases is nodule cytosolic proteins, especially leghemoglobin, whose disappearance limits oxygen supply to bacteriods. Such proteolytic enzymes are also able to digest bacteriods after rupture of the peribacteriod membranes (Pladys and Vance, 1993). The fact that proteolytic enzyme activity and amount are strictly localized to the infected cells confirms their key role in the lytic process leading to the limitation of bacterial partners. It was reported from previous studies that after protoplast isolation of French bean nodules cells, lytic process led to elimination of bacterial partner (Pladys *et al.*, 1991). Early nodule senescence and reduced ammonia assimilating enzyme activities correlated with enhanced activity of acidic proteolytic enzymes. Such acidic protease has been implicated as factors affecting peribacteriod membrane stability and eliminating the microbial partner during incompatible plant microbe associations. Lack of ammonia production has been implicated in premature senescence and proteolysis (Pfeiffer *et al.*, 1983).

In root nodules, there is evidence that cysteine protease could be involved in nodule function, in the adaptation of host cells to physiological stresses, and in the control of nodule senescence (Manen *et al.*, 1991). Cysteine protease with acidic pH optima has been described in French bean nodules, its activity increased with the onset of senescence (Kardailsky and Brewin, 1996). Several of these cysteine protease genes show a highly specific temporal and spatial expression associated with senescence processes (Asp *et al.*, 2004). Cysteine protease expression has been reported from different stages of plant development. Many of the endogenous plant cysteine proteases identified have acidic pH

optima in *vitro,* suggesting that they are localized in vacuole in *vivo* (Callis, 1995). When a total RNA of Chinese milk vetch from nodules was subjected to Northern blot analysis, slight cysteine protease expression was firstly detected in young nodules (14 days) and the transcripts accumulated five times higher in matured nodules (30 days), localisation of the protease gene was not found before 11 days of nodule development. Its strong signals was observed in the senescence zone of the nodules and its transcript level decrease gradually from senescence zone to the inter zone. When the in situ hybridisation was conducted, these findings illustrated that gene expression is age dependent (Naitoi *et al.*, 2000). It was also reported that cysteine protease activity increased clearly with the onset of senescence (Pladys *et al.*, 1993), when effective and ineffective alfalfa proteins nodules were subjected to western blot, a bean primary cysteine protease antibody reacted with effective alfalfa nodule by detecting a slightly band of about 40 kDa in youngest nodule of 1-2 weeks. After 3-7 weeks, the band becomes more intense, indicating that cysteine protease is expressed increasingly in late developmental age of nodules. Similar studies with an increase of cysteine protease activity in senescing nodules have been documented during development of pea nodules (Kardailsky and Brewin, 1996). Their studies showed that northern blot analysis revealed that a cysteine protease expressed in senescent infected tissue at the mature region (base of indeterminate nodule) of pea nodules compared to other parts of plants (Kardailsky and Brewin, 1996). High expression of cysteine protease in senescence nodules was further documented when three cDNA libraries were constructed at different developmental stage of soybean nodules at 2, 5 and 10 weeks, from this cDNA libraries microarray analysis 81 from 382 genes showed a significant change in expression during nodule development, it was observed that after a week transcript level were not changed, however increased in matured and senescence soybean nodules at 5 and 10 weeks, one of cysteine protease was found expressed at this stage during the soybean root nodule

development (Lee *et al.*, 2004). It was reported that in nodules of Chinese milk vetch, *in situ* hybridisation showed that cysteine protease activity was observed only in aging or senescent infected tissue at the base of indeterminate nodule. It was further illustrated that same developmental process appeared in mature determinate nodules and suggested that the role of cysteine protease is likely to be involved in degradation and recycling of proteins and recovery of nitrogenous compounds from senescing tissue to the rest of the plant (Alesandrini *et al* ., 2002). It was reported that a function of nodule specific cysteine protease (Asnodf32) expression in senescence nodules can be inhibited with an application RNAi to hairy root of *Astaragalus sinicus* during plant transformation, as a results transgenic nodules with pRNAi 32 showed a distinct reduced expression of *Asnodf32* during nodule development. Furthermore 60 old days nodules harvested from control plant, showed larger senescence zone while transgenic nodules showed a relative smaller senescence zone. The nitrogenase activity of 60 days old nodules was higher compared to the control; this indicates that nodule senescence was delayed through silencing of the nodule specific cysteine protease in *Astragalus sinicus* (Li Y *et al.*, 2008).

Cysteine proteases are particularly abundant among proteases and well-known senescence-associated proteins found in late nodules (Lee *et al.*, 2004). Molecular analysis of *Glycine max* has led to the identification of a cysteine protease gene and showed cysteine protease expression in nodules. It was suggested that cysteine protease was involved in the adaptations to change in cell turgor as well as protein turnover in the symbioisome (Alesandrini *et al.*, 2003). Putative cysteine proteases were significantly upregulated during nodule senescence in *Medicago truncatula* and *Medicago sativa.* However no transcript of cysteine protease was identified in shoots and leaves. Based on southern blotting cysteine proteases were predicted to be part of a multigene family with five to seven members. As a

result specific cysteine protease performs essential function in nodule development, particularly in the senescing zone. This function may be evolutionary old and highly conserved since similar cysteine protease has been identified in *Alnus glutinosa* that is specifically expressed in nodules (Asp *et al.*, 2004).

In young functional nodules, the existence of cysteine proteases in cytosol has been reported with low proteolytic activities. Suggesting that maintenance of strict compartmentalization of proteases could constitute a strategy to limit the proteolytic process in fixing nodules (Pladys *et al.*, 1991). Plant cysteine proteases found in vacuoles and are responsible for the mobilization of endosperm storage proteins during seed germination (Oliveira *et al.*, 2003). This provides nitrogenous nutrients to support the growth of young seedlings. This class of proteases also found in the extracellular medium such as those of papaya and fig. Examples of these proteases have been identified in plants, insects, and microorganisms and are all similar to those found in mammalians.

As the aging process progresses, various hydrolytic enzymes are activated. Nodule senescence in soybean has been directly associated with elevated proteolytic activity resulting in the decrease of soluble protein content, including the disappearance of leghemoglobin (Espinosa-Victoria *et al.*, 2001). Degradation of proteins during senescence of the leaf could produce amino acids for translocation to the developing seed. During senescence, protein degradation has an important role in regulation of various plant hormones, nutrients are redistributed from the vegetative parts to the developing part of plants. Many of the enzymes, increased during senescence are clearly secondary or nonessential to senescence. These include the enzymes required to metabolize the amino acids and lipids released and to convert them to transportable forms (Nooden *et al.*, 1997).

Many genes are differentially expressed during senescence (Gepstein *et al.*, 2003), few genes have been isolated from senescent nodule tissue such as proteases. This includes those of the acid, thiol, serine, and cysteine types that have been isolated from senescing nodule tissue of alfalfa, soybean, French bean, and pea (Candace *et al.*, 2008). Leguminous root nodule protease could be involved in the nodule function, in the adaptation of host cells to physiological stresses, and in the control of nodule senescence (Kardailsky and Brewin, 1996). Whole spectrum of plant proteases including cysteine proteases (CPs) and aspartic proteases are involved in nodule protein breakdown and remobilisation of resources. Protease genes have now been cloned from a large range of senescent plant tissues, of these, thiol-proteases appear to be the most common proteolytic enzymes induced in senescent plant cells (Beers *et al.*, 2000). In particular, thiol- type protease activities appear to serve important roles in senescing soybean (Malik *et al.*, 1981), French bean (Playds *et al.*, 1991) and alfalfa nodules.

Despite the fact that protease enzymes are crucial to the cells and organisms that host them, they may be potentially damaging when over-expressed or present in higher concentrations. For this reason, the activities of these enzymes need to be strictly regulated and controlled. The synthesis of these enzymes as inactive pre-proteins, and their substrate specificity keeps a control on their activities, but it does not fulfill the desired level of regulation, and the fact remains, that cells and organisms require additional means of control. One important control mechanism involves interaction of the active enzymes with proteins that inhibit their activities (Laskowski and Qasim, 1999). Studies have already shown that the nodule lifespan could be prolonged through the silencing of nodule specific cysteine protease *(Asnodf32)* in

Astragalus sinicus. This further suggests that controlling of cysteine proteases will have great significance and potential application in crop plants and agricultural practice (Li *et al.*, 2008).

1.6 Protease inhibitors

Protease activity can be regulated by proteins known as protease inhibitors. These protease inhibitors are small proteins, generally present at high concentration in storage tissue, such as tubers and seeds, but they have also been found in the aerial parts of plants (De Leo *et al.*, 2002). All plant proteases inhibitors share a common trait such as the ability to form complexes with proteases, within which the protease enzymes lose their activity. Based on the characteristics of primary and spatial structure, including the number and the position of disulfide bonds within the active sites, the inhibitors are classified with families of related proteins (Mosolov and Valueva, 2005). Protease inhibitors from animals, plants and microbes have a common mode of action, this proteins bind with a protease in a substrate like manner but do not dissociate, thus inactivating the protease, as indicated in Fig. 1.3.

These proteins are part of the wide arrangement of performed defense mechanism in storage organs, where they block the growth and development of herbivorous predators by inhibiting digestive enzymes and provide protection against fungi and bacteria, using hydrolytic enzymes to gain entry (Johnson *et al.*, 1989). Furthermore, mechanical wounding or attack by pathogens or herbivorous causes a rapid accumulation of protease inhibitor transcript. Protease inhibitors are components of both performed and inducible defense mechanisms in plants. These particular proteins are known for their role in plant development. In storage tissue, they may prevent reserve proteins from premature hydrolysis by endogenous plant proteases. Since they are abundant and resistant to extreme of heat and pH protease

inhibitors also function as storage proteins that are immune to digestion to until germination (Richardson, 1991). The expression of protease inhibitors gene is developmentally regulated in various plant parts, suggesting that they participate in the control of proteolysis (Lievens *et al.*, 2004).

Plant protease inhibitors have been grouped into families and subfamilies and into different clans on the basis of sequence relationship and the relationship of protein folds of the inhibitory domains or units. An inhibitor domain is defined as the segment of the amino acid sequence containing a single reactive site after removal of any parts that are not directly involved in the inhibitor activity. On the basis of sequence homologies of their inhibitor domains, plant protease inhibitors have been classified into 48 families (Rawlings *et al.*, 2004).

Proteins containing a single inhibitor unit are termed simple inhibitors, and those that contain multiple inhibitor units are termed complex inhibitors. A total of 11 families contain between 2 and 15 inhibitory domains. Mainly of these are homo-typic, containing inhibitor units from a single family, some are however heterotypic, and contain inhibitor units from different families (Trexler *et al.*, 2001). On the basis of tertiary structure, 31 of the 48 families have been assigned to 26 clans, indicating that a large proportion of families show no relationships in their three dimensional structures (Rawlings and Barrett, 1994).

Among this family at least nine such families comprise protein inhibitors of plant origin known to date. Plant protease inhibitors are active against all the mechanistic classes of cysteine, serine, metallo and aspartic proteases that have been described in plants (Valueva and Mosolov, 1999). For example the plant Kunitz family (family 13) contains inhibitors of

serine proteases (family S1), but also includes inhibitors of cysteine proteases (C1) and the aspartic protease cathepsin D. Correspondingly, the serpin family of plant protease inhibitors, active against serine proteases also contains inhibitors of cysteine proteases, this results in plant inhibitors acting in specific class of proteases such as cysteine, serine, metallo and aspartic protease inhibitors (De Leo *et al.*, 2002).

There are eight families of serine protease inhibitors and one family of cysteine protease inhibitors in plants. Individual families of serine protease inhibitors differ significantly in the extent to which their members are widespread in the plant kingdom. For example, members of the families of soybean Kunitz inhibitor and potato inhibitor I are found in plants of 11 and 9 botanical families, respectively; other families of inhibitors are encountered less frequently, each family being characteristic of just one plant family.

Specific protease inhibitors for cysteine proteases are cysteine protease inhibitors called cystatins. A role for protease inhibitors has been proposed as modulators of programmed cell death in plants; the suggestion is that cell death may be regulated by the opposing activities of protease versus protease inhibitor enzymes (Coupe *et al.*, 2003). The over-expression of a cystatin in a soybean cell suspension blocked programmed cell death (PCD) (Solomon *et al.*, 1999). The over-expression of a cystatin that inhibits papain activity in *Arabidopsis* cell cultures blocked cell death in response to avirulent bacteria and nitric oxide (Van der Hoorn and Jones, 2004). The over-expression of this inhibitor in tobacco plants blocked the hypersensitive response induced by avirulent bacteria (Gopalan *et al.*, 1996). Cystatins are known as proteins inhibiting cysteine proteases by direct interaction with the active site. Cystatin refers to proteins that specifically inhibit the activity of papain and related cysteine

proteases such as cathepsin B, H and L. Their presence in microorganisms, animals and plant species might be ubiquitous (Massonneau *et al.*, 2005).

Cystatins have been found to be evolutionarily related, forming the cystatin super family, and members of super family are divided into groups. The classification in families is based on primary sequence similarities, molecular masses, number of disulfide bond and subcellular localization (Oliveira *et al.*, 2003). Cystatins are divided into the stefin, cystatin, kininogen and phytocystatin family. Phytocystatins includes almost all plant cysteine protease inhibitors. Among plant cystatins, the oryzacystatin from rice seeds was the first inhibitor of plant origin considered one member of the cystatin super family that is related by structure and a function to chicken egg white cystatin. Cysteine protease inhibitors of plant origin can be classified in two groups; one constituted by phytocystatin which posses a single domain, comprising the majority of the phytocystatins. The second group only posses multiple domains, such as the multicystatins isolated from potato tubers (Walsh and Strickland, 1993).

Molecular mass of phytocystatins purified showed a range of sizes between 5 to approximately 87 kDa and stability to temperature and extreme pH (Abe and Arai, 1991). It has been suggested that phytocystatin proteins regulate endogenous and heterologous cysteine protease (CPs), taking part in a variety of physiological process. This includes defense mechanisms by inhibition of digestive enzymes in the guts of insect pests and nematodes (Kiggundu *et al.*, 2006). It has been suggested that cysteine protease inhibitors play a regulatory role by inhibiting protein turnover (Hong *et al.*, 2006). Previous studies showed that wild-type Chinese cabbage and *Arabidopsis* do not express cystatins in mature leaves and constitutive expression of BrCys1 (phytocystatin from *Brassica rapa*) in *Arabidopsis* negatively affected leaf growth. Hong *et al.* (2006) suggests that there is also

suppression of action of endogenous cysteine protease inhibitor during plant development indicating a function in regulation of growth and development (Hong *et al.*, 2006).

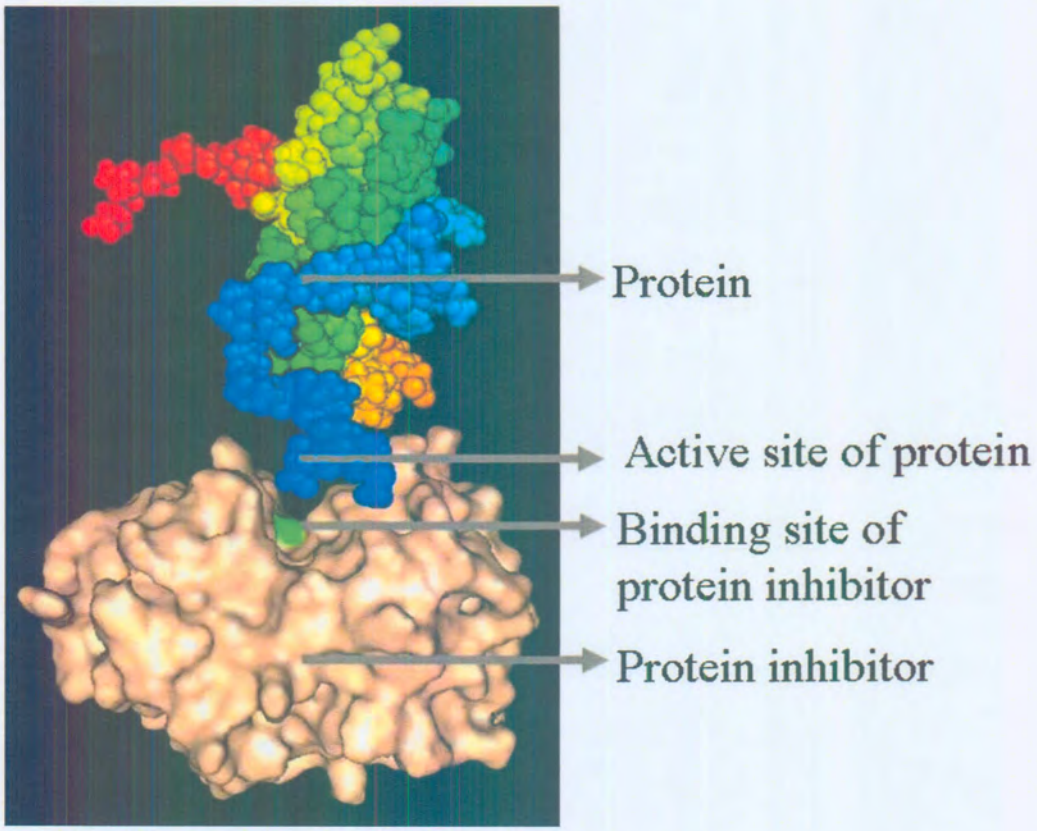


Figure 1.3: Protease inhibitor binding to a protease, adapted from (Hooper *et al.*, 2001).

Lievens *et al.* (2003) reported that at earliest stages of nodule development of *Sesbania rostrata*, a serine protease inhibitor (SrPI1) is expressed in the nodule primordium. Transcripts of the serine protease inhibitor were also found in regions surrounding the developing central nodule tissue. Also inside the central tissue, the gene is expressed in scattered cells of the infection zone and in uninfected cells of the young fixation zone. However, in mature nodules, lacking an active meristem, expression fades out. Lievens *et al.* (2003) suggested that the inhibitor activity of SrPI1 is involved in the control of plant

proteases related to nodulation, a function similar to that suggested for protease inhibitors in storage tissues or in sieve tube development. It was further concluded that SrPI1 could be another component of the mechanism that regulates invasion during nodule development. This mechanism protects the rest of the plant by a multi-layered barrier against escape of bacteria. When specific probes and primers were used to determine expression levels of (SrPI1), the transcript level was up-regulated during nodule development reaching a maximum at 2 days. However, the expression level dropped in mature nodules when they were 12 days old (Lievens *et al.*, 2003). In situ localisation also indicated that the protease inhibitor could be detected in the youngest part of the fixation zone, whereas expression of the protease inhibitor was absent in the older nitrogen-fixing nodule tissue.

1.7 Research aim and objectives

Nodules are specific organs fixing atmospheric nitrogen into an accessible form to plants during leguminous plant development. At plant maturity, leguminous plants experienced nodule senescence resulting in the loss of nodule activity. However, little is still known about the role of proteases and their natural inhibitors in nodule development. Therefore the aim of this study was to detect and measure protease and protease inhibitor activity during soybean nodule development and to test and design a cysteine protease inhibitor construct with tobacco used as a model plant which might be useful for transformation of soybean to block cysteine protease activity during nodule development. To achieve this aim, this study had the following objectives:

1) To establish the best growth condition for nodulating soybean plants

2) To monitor the nodule development process in soybean by measuring a variety of nodule performance parameters.

3) To measure protease activity and protease inhibitor expression during nodule development with a particular focus on cysteine proteases and cysteine protease inhibitors.

4) To design a cysteine protease inhibitor construct for transformation of soybean.

5) To test for activity of the designed cysteine protease inhibitor construct in tobacco used as a model plant.

CHAPTER TWO

Materials and methods

2.1 Plant growth conditions

2.1.1 Soybean growth for nodule formation

Soybean (*Glycine max* Merr) seeds of the variety Prima 2000 were placed into 5.5 inch pots (one seed/pot) containing vermiculite pre-soaked with de-ionised water. The seed was placed into a small hole containing 0.3 g powder of the *Bradyrhizobium japonicum* bacterium strain W734X purchased from Soygro SA. For soybean plant production and initiation of nodules, pots with inoculated seeds were placed into a green-house and exposed to a temperature of 25-27 °C, the experiment was repeated four times. Crown nodules developed on the tap root about 15 days after germination Lateral nodules developed from lateral roots at a later stage. Initially the pots were watered with 1000 ml dH_20, and provided with nitrogen-free Hoagland nutrient solution contained macro nutrients with 1 M $MgSO_4$. 7 H_2O, 1 M KH_2PO_4, FeDTA, 1 M $CaCl_2$ and 50 mM KCl, and micro nutrient solution (1.43 g H_2BO_3, 0.446 g $MnSO_4.4H_2O$, 0.575 g $ZnSO_4$, 0.124 g $CuSO4.2H_2O$ and 0.0125 g $Na_2MoO_4.2H_2O$ dissolved in 500 ml distilled water). This nutrient solution caused chlorotic leaves to mature plants from 6 to 8 weeks, then the concentration of KCl from macro nutrients was increased from 50 mM KCl. to 1 M KCl. Water supply to plants was reduced to 150 ml and provided once to three times per week depending on the developmental stage of soybean plants. Addition of artificial light for 2 h in the evening was also provided for the elongation of day length. Every two weeks new seeds were germinated to obtain nodules of different age for analysis. After a period of 14 weeks all crown nodules from soybean plants were hand harvested and morphologically characterised. Significant difference between comparable treatments means were determined by the use of student's t-test.

2.1.2 Growth of tobacco plants

Tobacco plants (*Nicotiana tobaccum L.*) of the variety Samsun were grown *in vitro* in a growth chamber at a 16 h/8 h light/dark cycle at 25 °C on half-strength MS medium (30 g/l, 2.2 g/l Murashige Skoog and 0.2 % gelrite, Sigma, UK). After the plants established a well developed root system in the medium and were about 10 cm high, the roots were washed with tap water to remove the growth medium. Plants were then transferred to small pots containing moist germinating soil (Culterra, SA) and grown in the green-house until maturity. Plants were provided with tap water three times per week and a Murashige and Skoog mineral salt mixture (MS salts, Murashige and Skoog, 1962) solution once per week. Tobacco plants were initially covered with a glass beaker for 14 days until the root system established and were then gradually exposed directly to the green-house condition. After two months plants were transferred to bigger pots (20 cm diameter) to allow enough space for plant growth.

2.2 Soybean nodule characterization

2.2.1 Nodule activity and fresh weight

Soybean crown nodules were harvested after a period of 4, 6, 8, 10, 12 and 14 weeks of plant growth. Crown nodules were cut into half to visualize the color of leghemoglobin. Red/pink color indicated an active nodule while a green/brown color indicated a senescent crown nodule. The two types of crown nodules were counted and used for further analysis. Crown nodule mass fresh weight (FW) was determined by weighing nodules on a scale (Fel-20005, Adam Equipment, UK).

2.3 DNA work

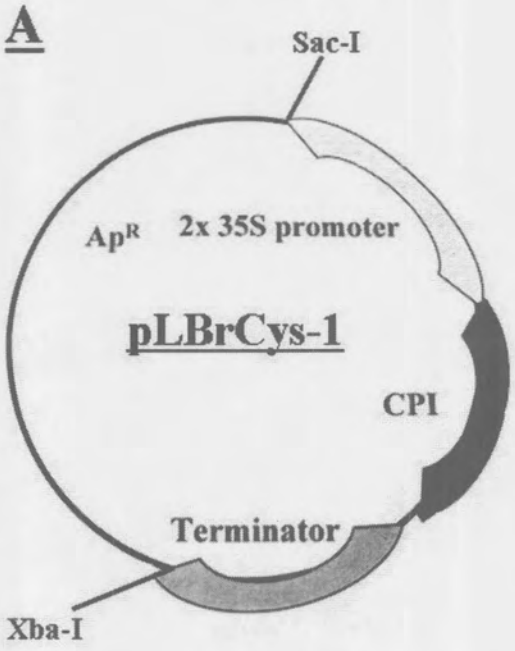
2.3.1 Vector design

The design of the vector was based on the selective of papaya cysteine inhibitor from plasmid pLBRCys1 and vector that can multiply both in plant and bacteria. The chosen plasmid vector for particular function was pBin19.

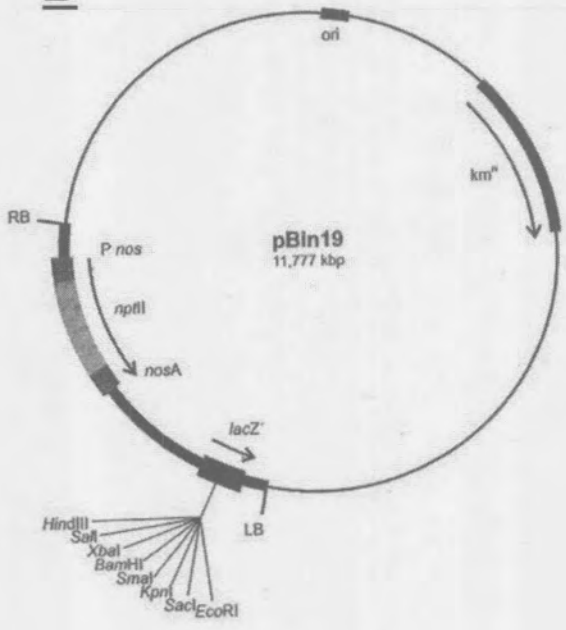
2.3.1.1 *Plasmid isolation*

Luria broth (LB) medium was prepared by mixing 5 g/l of yeast extract powder, 10 g/l of Bacto-tryptone powder, 10 g/l of NaCl and 12 g/l of agar powder. The pH of broth media was adjusted to 7.5 using 1 M NaOH before including agar and the medium was autoclaved for 20 min. *E. coli* cells (strain JM109) containing the plasmid pLBRCys1 carrying the 35S CaMV promoter, the papaya cystatin coding sequence and a CaMV terminator sequence were grown as colonies overnight on a petri-dish with 20 ml of LB medium containing 100 µg/ml ampicillin to select plasmid containing cells. *E. coli* cells containing a binary vector (pBin19) in which the papaya cysteine protease inhibitor sequence has been cloned under the control of the CaMV 35S promoter and CaMV terminator sequence (Fig. 2.1) were grown on solid LB medium with 100 µg/ml kanamycin added for selection of plasmid containing cells.

A


Sac-I
Ap^R
2x 35S promoter
pLBrCys-1
CPI
Terminator
Xba-I

B


ori
km^R
RB
P nos
pBIn19
11,777 kbp
nptII
nosA
lacZ'
LB
HindIII
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI

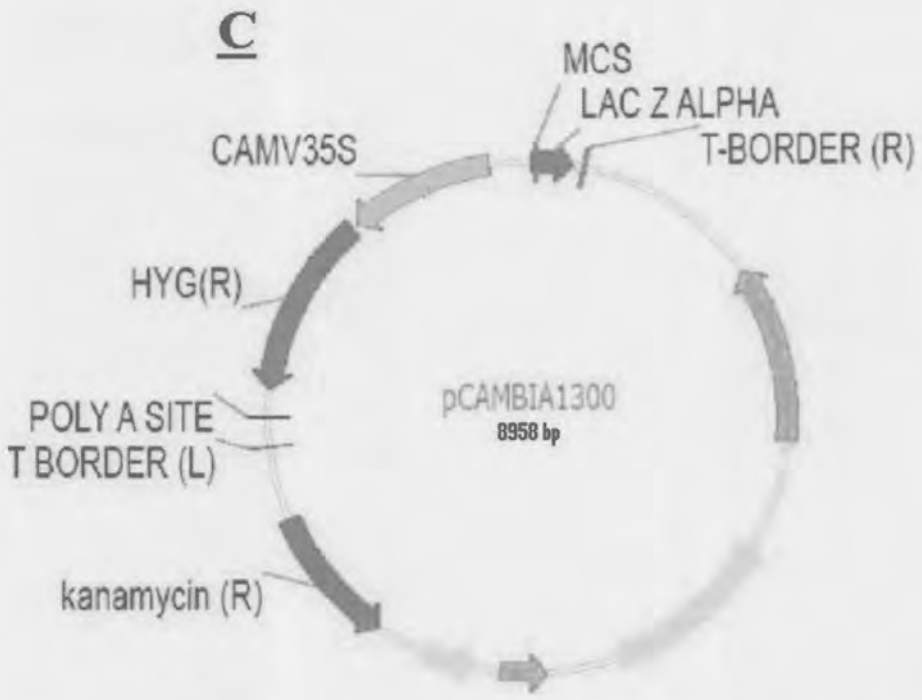


Figure 2.1: Vectors (A) pLBrCys1, (B) pBin19 (C) pCAMBIA 35S-Cys-mut 89 allowing transfer of either the native or a mutated cysteine protease inhibitor sequence (pCAMBIA 35S-Cys-mut 89) under the control of the 2 X CaMV 35S promoter and CaMV terminator sequence as a *XbaI/SacI* fragment into pBin19 and into the multiple cloning sites (MCS) of vector pCAMBIA 1300. Vector pBin19 allows transformed plant selection on kanamycin whereas vector pCAMBIA 1300 allows selection on hygromycin.

For plasmid isolation, bacterial colonies were grown on a petri-dish overnight at 37 °C. A single colony was then transferred into a 10 ml autoclaved bottle containing 4 ml liquid broth media (5 g/l yeast extract powder, 10 g/l Bacto-tryptone powder, 10 g/l of NaCl, pH 7.5). Cells were grown overnight at 37 °C on a shaker of 200 rpm with appropriate antibiotics.

Plasmid isolation was carried out by using the alkaline lysis method as outlined by Maniatis *et al.* (1982). Isolated plasmid DNA (5 µl) was mixed with an orange dye and loaded into 1 % agorose gel. The gel was prepared by mixing agarose (final concentration 1 %) with TAE

buffer (0.04 M Tris-base, 0.1 M of glacial acetic acid, 7 mM EDTA) and heating the solution for 3 min in a microwave oven. After cooling, 2 µl ethidium bromide was added to the solution. The agarose solution was poured into a casting system and a comb was inserted to produce wells into which the DNA was loaded. Electrophoresis was carried out for 15 min at 100 V.

2.3.1.2 *Cloning of cysteine protease inhibitor*

Since the mutated papaya cysteine proteinase inhibitor sequence was already cloned into the pCAMBIA 1300 vector, in this study only the cloning of the native papaya sequence into the vector pBin19 was carried out. Plasmid DNA of pLBRCys1 and pBin19 (Fig. 2.1) was digested for 1 h with 10 U of the restriction enzymes *XbaI* and *SacI* in an appropriate restriction enzyme buffer as recommended by the manufacturer (Fermentas, SA) to linearize the vector pBin19 and to obtain a 1800 bp fragment from pLBRCys1. Digested plasmid DNA was run on a 1 % agarose gel to monitor if digestion was complete and if the DNA fragment with the correct size of 1800 bp could be detected.

For isolation of the DNA fragment from the agarose gel, the DNA fragment with the correct size was cut out of the agarose gel using a sterile blade. The fragment was purified using a High Pure PCR product Purification kit (Roche, Switzerland). The linearized plasmid pBin19 was purified after digestion with the phenol/chloroform method according to Maniatis *et al.* (1982). Re-circulization of pBin19 was minimized by removing the 5'-phosphate from both DNA ends by alkaline phosphatase treatment (Maniatis *et al.*, 1982). Ligation of cut pBin19 with the purified 1800 bp fragment was carried in a reaction mixture (20 µl) containing the DNA fragment, linearized plasmid pBin19, 1 µl of ligase (Fermentas, SA) and 2 µl of ligase

buffer. The reaction was incubated at 16 °C for 16 h to facilitate ligation of insert into pBin19.

To insert the ligated plasmid into *E. coli*, a bacterial transformation procedure was carried out to multiply the plasmid within a bacterial cell. For transformation, commercially available competent *E. coli* cells (Roche, Switzerland) were removed from a -80 °C freezer and placed on ice for 10 min to defrost. Competent cells (50 µl) were mixed with 11 µl of the ligation reaction mixture and incubated on ice for 30 min. The mixture was exposed for 30 sec to a 42 °C heat shock treatment in the presence of 0.1 M $CaCl_2$. This treatment results in wounding of the bacterial membrane allowing the uptake of ligated DNA into bacterial cells. After heat shock, the cells were placed on ice and after 2 min 1 ml of SOC medium (20 g bacto-tryptone, 5 g bacto yeast, 0.5 g NaCl and 20 ml of 1 M glucose solution) was added to the sample and the mixture was shaken at 200 rpm at 37 °C for 60 min in a bench-top shaker. Cells (20 µl) were streaked out onto a LB plate containing 50 µg/ml kanamycin; 2.5 mM of IPTG (isopropyl-β-D-thiogalactoside) and 1 mM X-gal (5-bromo-4chloro-3indolyl-β-D galactoside) and plates were incubated overnight at 37 °C. Plasmid pBin19 contains the *LacZ* gene which codes for galactosidase which converts X-Gal into a colored blue product. In order for the gene to be actively transcribed from the DNA and for the enzyme to be produced, the activator called IPTG has to be added. Within the *LacZ* gene there is also a multiple cloning site and any insert cloned into the multiple cloning sites will disrupt expression and activity of the *LacZ* gene which prevents the formation of the blue color product (Chaffin and Rubens, 1998). pBin19 cell contained a cloned 1800 bp insert (pBinCys19) were identified by blue/white selection.

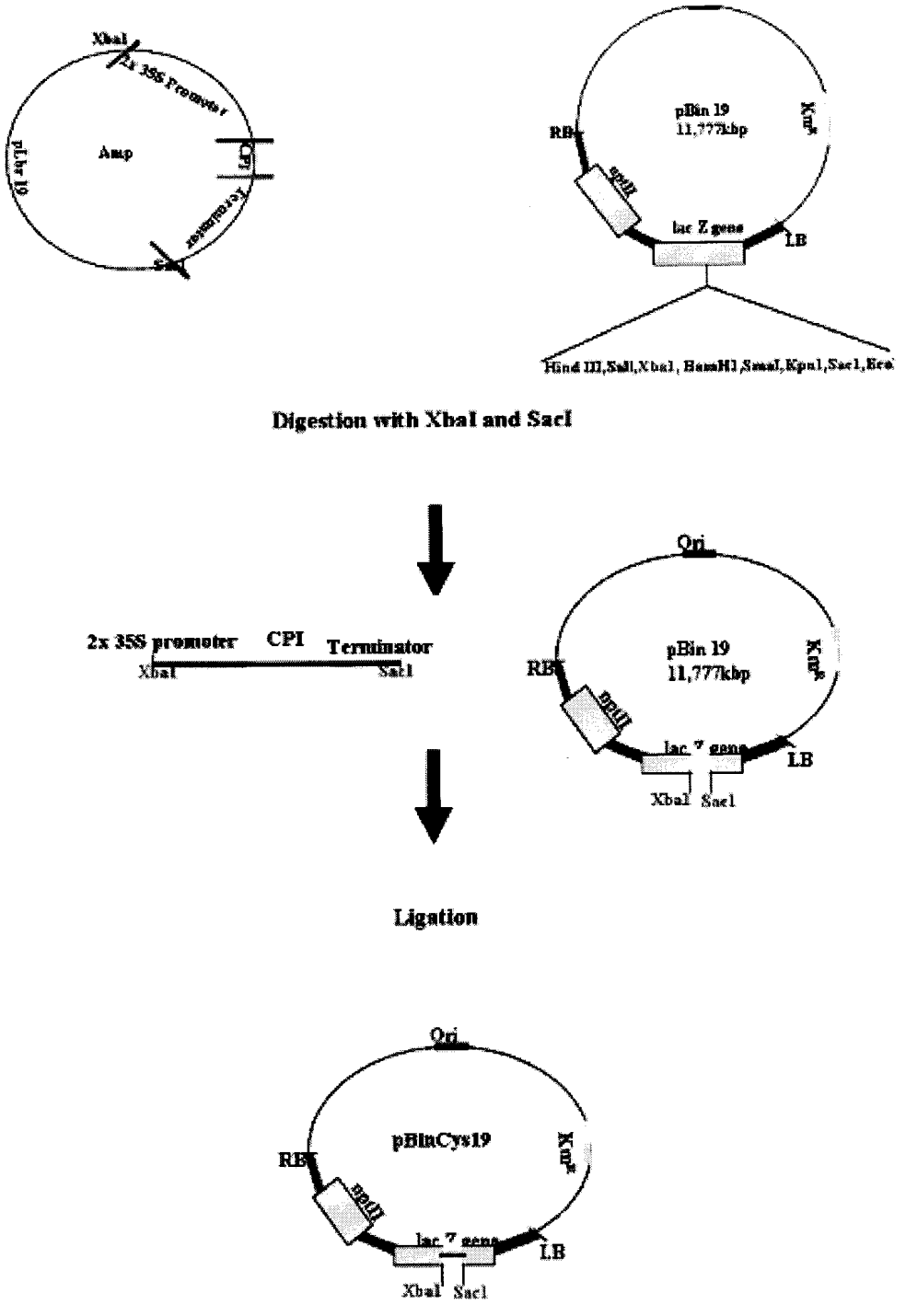


Figure 2.2: A flow diagram illustrating the creation of pBinCys19. A DNA fragment containing the native papaya cysteine protease inhibitor sequence (CPI) under the control of the 2 X CaMV 35S promoter and CaMV terminator sequence was excised from the vector pLBR19 vector using restriction enzymes *XbaI* and *SacI* and cloned into the pBin19 binary vector.

2.3.1.3 *Agrobacterium transformation*

Tri-parental mating was used for transferring plasmid pBin19 carrying the inserted 1800 bp fragment into *Agrobacterium tumefaciens* (LBA4404). This type of mating is a form of bacterial conjugation where a conjugative plasmid present in one bacterial strain assists the transfer of a mobilizable plasmid present in a second bacterial strain into a third bacterial strain. Tri-parental mating involves three bacterial strains (i) *E. coli* helper plasmid (pRK2013) for mobilizing the intermediate plasmid into *Agrobacterium*, (ii) *E. coli* carrying the recombinant intermediate vector (pBinCys19) and (iii) *Agrobacterium tumefaciens* strain (LBA4404) carrying the Ti-plasmid.

Agrobacterium cells were grown for 2 days at 25 °C on LB medium containing 50 µg/ml rifampicin. Cells of the *E. coli* helper strain pRK2013 were grown on LB media containing 50 µg/ml kanamycin overnight at 37 °C. Cells of *E. coli* carrying the recombinant vector pBinCys19 were grown on LB media with 50 µg/ml kanamycin. For mating, the two types of *E. coli* cells grown overnight were further sub-cultured in liquid media under shaking at 37 °C without addition of antibiotics until an optical density of 0.2 was reached.

For mating (Fig. 2.3), 1 ml of *Agrobacterium* cells, 0.5 ml of cells containing helper plasmid pRK2013 and 0.5 ml of *E. coli* cells containing pBinCys19 were mixed and introduced as a 20 µl drop onto a LB medium (S_1). Also on the LB medium plate were controls (20 µl) consisting of a mixture (K_1) of *Agrobacterium* cells and helper plasmid (PRK2013) containing cells (1 ml *Agrobacterium* cells and 0.5 ml helper cells) and a mixture (K_2) *Agrobacterium* cells with *E. coli* pBinCys19 cells (1ml cells *Agrobacterium* and 0.5 ml *E.*

coli pBinCys19 cells). The plate was then incubated at 28 °C for 1 day. Bacterial growth of S_1, K_1, and K_2 were scrapped from the solid medium using a toothpick and cells were separately diluted into 1.5 ml water in an Eppendorf tube. All three different samples (K_1, S_1 and K_2) were then streaked out (20 µl) onto separate solid LB plates containing 100 µg/ml rifampicin and 100 µg/ml kanamycin and plates were incubated for 48 h at 28 °C. After 2 days, colonies were visible on plate with S_1 sample and no growth was observed on K_2 and K_1 sample plates.

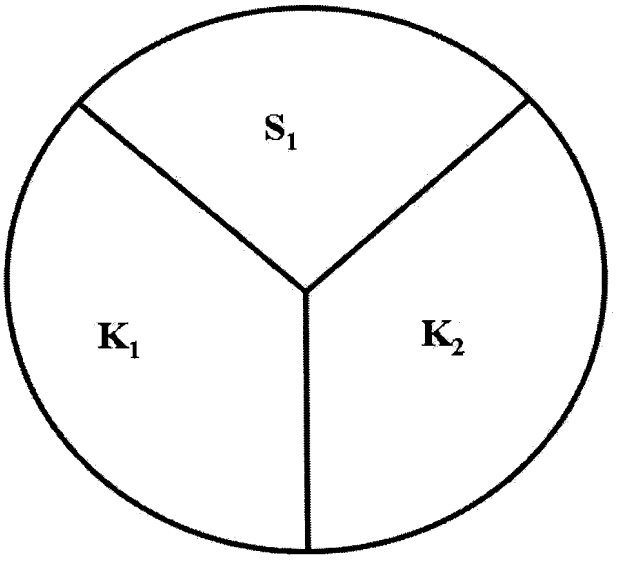


Figure 2.3 Arrangement of bacterial mixtures on solid LB medium plate.

A single colony from the S_1 plate was sub-cultured into liquid medium containing 100 µg/ml kanamycin and 100 µg/ml rifampicin. The plasmid pBinCys19 was extracted from *Agrobacterium* cells using the alkaline lysis method as described under paragraph 2.5.1.1. *E. coli* competent cells (strain JM109) were transformed with isolated plasmid DNA from *Agrobacterium* cells to confirm that *Agrobacterium* cells contain the plasmid pBinCys19. Verification of the plasmid was done by plasmid isolation from transformed *Agrobacterium* cells and restriction enzyme analysis of pBinCys19.

2.4 Plant transformation

2.4.1 Leaf sterilization

A fully expanded leaf from 2-3 months old, soil grown tobacco plant was sterilised in 70 % ethanol for 10 sec. To achieve a successful leaf sterilisation, the midrib from the central part

of the leaf was removed. The leaf was then cut into small pieces and further exposed for 20 min to a 1.2 % sodium hypochlorite solution (Bleach). The leaf was rinsed five times with sterilised distilled water. Sterilised filter paper was used to remove any excess water from the leaf. Leaf lateral veins alongside of the leaf ribs were sectioned with a sterilised cork borer into 0.5 cm leaf disks.

2.4.2 Infection with *Agrobacterium tumefaciens*

Agrobacterium tumefaciens cells (LBA4404) either containing plasmid pBinCys19 or pCambia-cys-mut 89 were grown on a liquid LB medium until and optical density of OD_{600} = 0.5 on a shaker at 28 °C in the presence of an appropriate antibiotic for selection. Cells were then centrifuged at 13000 rpm for 10 min to remove the antibiotics, and the pellet was re-suspended in liquid MS salt medium containing 0.08 M of sucrose at pH 5.8. Tobacco leaf discs were submerged into the *Agrobacterium* culture for 5 min with gentle shaking to improve infection. Leaf discs were then transferred to sterilised filter paper to remove any excess of bacterial suspension.

2.4.3 Shoot and root regeneration

Leaf discs were transferred to petri-dishes containing MS salts, 30 g/l sucrose, 10 mg/ml of benzyl-aminopurine (BAP) and 0.2 % gelrite. The pH was adjusted to 5.8 with 1 M of NaOH before adding gelrite (Sigma, UK). Petri dishes with discs were wrapped into alumina foil for 2 days to maintain darkness. Plates with discs were then transferred upside-down to new petri-dishes containing MS salts, 30 g/l sucrose and 10 mg/ml of BAP and 0.2 % gelrite (shoot regeneration medium) and antibiotics for suppression of *Agrobacterium* growth (500

mg/l cefotaxime) as well as for selection of transformed cells (100 mg/l kanamycin for pBinCys19 or hygromycin for pCAMBIA 35S-Cys-mut89. Shoot regeneration occurred after 6 weeks with one change of medium after 3 weeks. Regenerated shoots were excised and placed into a half-strength MS-salt medium containing 0.2 % gelrite, an appropriate antibiotic for selection and 0.25 mg/l indole-butyric acid (IBA) for inducing root formation. After 5 days, roots developed from shoots.

2.4.4 Screening of tobacco transformants

2.4.4.1 *Extraction of tobacco leaf genomic DNA*

Leaves from putative transformed tobacco plants expressing the cysteine protease inhibitor were harvested from green-house grown plants. The leaf midrib was removed from selected leaves using sterile blade. Five grams of tobacco leaves were homogenised in a pestle and mortar with 500 µl extraction buffer (0.3 M NaCl, 0.03 M tri-sodium citrate and 0.1 M EDTA, pH 8). The mixture was mixed gently with 10 µl of 20 % Sodium dodecyl sulphate (SDS) to dissolve the protein and lipids. An addition of 200 µl from 5 M NaCl was essential to keep DNA in the solution. The mixture was then centrifuged at full speed (13,000 rpm) for 5 min and the 500 µl of the resulting supernatant was precipitated with 1 ml of ethanol. The DNA precipitated as a fluffy precipitation. This mixture was then centrifuged shortly for 30 sec. The pellet obtained was dissolved in 50 µl of TE (10 mM Tris, 1 mM EDTA, and pH: 8) for 60 min at room temperature. Extracted genomic DNA (5 µl) from tobacco leaves was then separated on 1 % agarose gel as described under 2.3.1.1. Electrophoresis was carried out for 20 min at 100 V.

2.4.4.2 *Polymerase chain reaction (PCR) analysis*

PCR is a technique for an amplification of specific DNA sequence by simultaneous primer extension of complementary DNA strand. Primer is defined as a segment of DNA that is complementary to a given DNA sequence and that is required to initiate replication. These primers are designed so that each primer extension reaction directs to the synthesis of DNA strand. The requirements of the reaction are (dNTPs) deoxynucleosides triphoshates (providing both energy and nucleosides for the synthesis of DNA), *Taq* (*Thermus aquaticus*) polymerase (key enzyme component in the amplification reaction by adding free nucleotides to only the 3' end of the newly-forming strand resulting in elongation of the new strand in a 5'-3' direction), DNA template and buffer containing magnesium chloride (supplying Mg^{++} divalent cations as a cofactor for *Taq* polymerase enzyme) and primers (they direct the *Taq* DNA polymerase enzyme to move from 5' to 3' due to DNA direction).

In this study, putative tobacco transformants were screened by PCR to verify the presence of mutated papaya cysteine protease inhibitor sequence using the PapCys-1 right primer of sequence GGAATTGTGATCGGTGGTTT and PapCys-1 left primer ATCTGGTTGAAGCTCTGGG with sequence shown in Fig. 2.4. This reaction was performed for each of the 4 putative transformed tobacco plants. The PCR reaction was carried out in PCR tubes as follows, 0.5 µl of genomic DNA, 2.5 µl of PCR buffer, 2 µl of 50 mM $MgCl_2$, 2 µl of 1.25 µM dNTPs, 2.5 µl of 10 µM PapCys-1 R, 2.5 µl of 10 µM PapCys-1- L and sterile distilled water 27.6 µl) to obtain a 50 µl total reaction volume. Amplification of DNA was obtained using a Palm Cycler (Corbett Life Science cycler, Australia), the PCR cycling conditions setup were as follows: DNA denaturation at 94 °C for 5 min followed by

30 cycle of 94 °C for 30 sec, 58 °C for 30 sec. For primer annealing, 72 °C for 45 sec for DNA extension and a final DNA extension step after 30 cycles of 72 °C for 10 min to obtain fully-double stranded DNA. PCR products were then separated on a 1 % agarose gel, and the PCR specific products were identified on the basis of size and absence of similar products from a non-transformed tobacco plant.

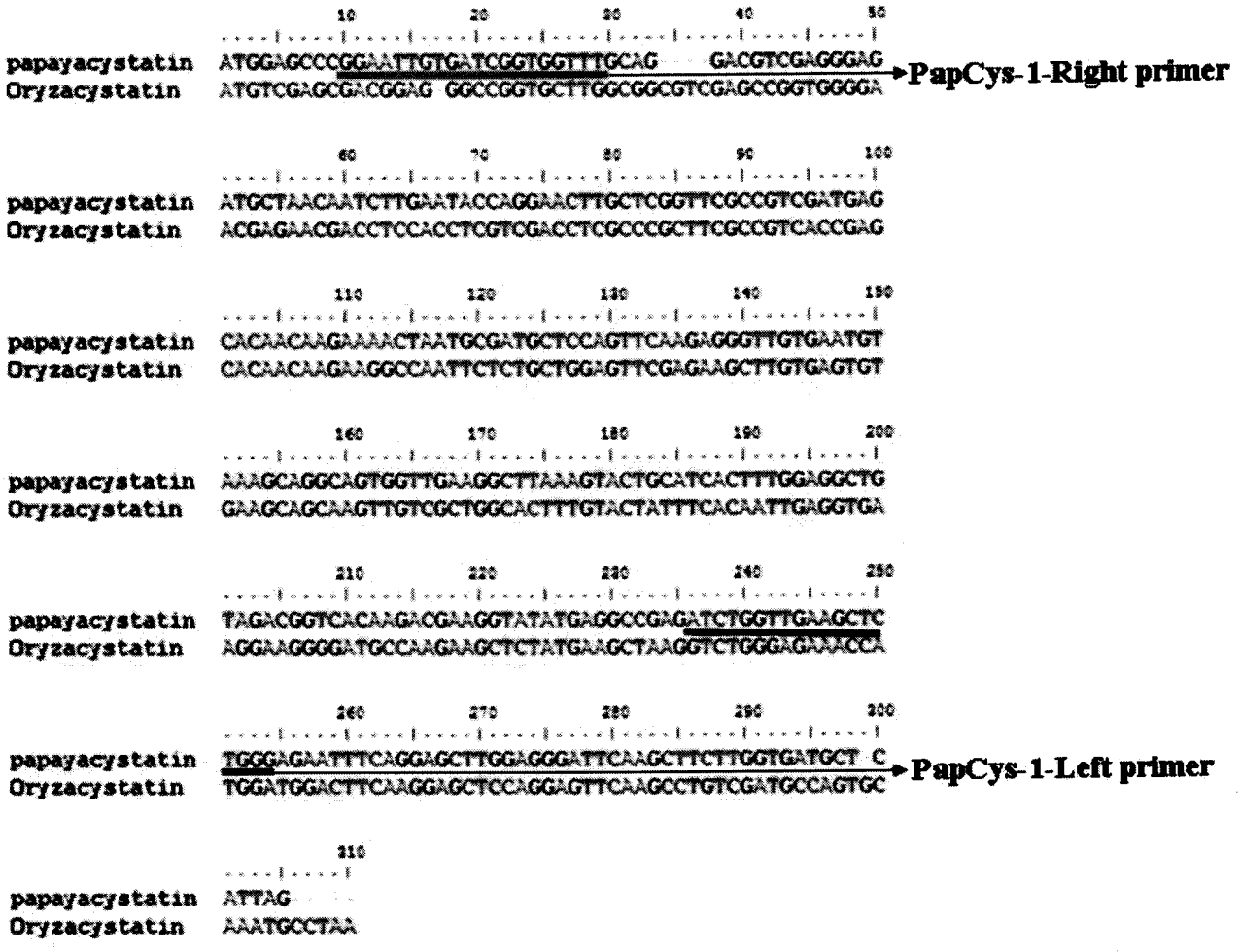


Figure 2.4: Sequences of papaya and rice cysteine protease inhibitor obtained from NCBI and aligned in Bio-Edit program with indication of right and left primer sequence (underlined) used during PCR.

2.5 Protein work

2.5.1 Protein extraction from nodules

Crown nodules were ground into powder in liquid nitrogen with a mortar and pestle for protein isolation. Proteins were dissolved by the addition of 1 ml of 50 mM Tris-HCl buffer pH 8.0, to the powder. The extract was centrifuged at 13,000 rpm for 10 min at 4 °C in an Eppendorf centrifuge. Resulting clear protein-containing supernatants were stored at -80 °C for further analysis.

2.5.2 Protein content determination

2.5.2.1 *Determination of nodule protein content*

Total protein content from crown nodule extracts was determined using a commercially available protein determination kit (Bio-Rad, UK) following the Bradford assay (Bradford, 1976). This assay involves the addition of an acidic dye (purchased from Bio-Rad, UK) to the protein solution and subsequent measurement of the reaction mixture at 595 nm with a spectrophotometer (Macince UK). Bovine serum album (BSA) from Sigma (South Africa) was used for obtaining a standard curve to provide a relative measurement for protein concentrations.

To obtain a standard curve, five concentrations of BSA (5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.3125 mg/ml) were prepared through serial dilutions with distilled H_20.

From each standard solution 10 µl was added to 200 µl of acidic dye (Bio-Rad, UK) and 790 µl of distilled H_20. Full color development of the mixture occurred after 30 min at room temperature. The same procedure was applied for crown nodule extracts. A blank sample was prepared by replacing the protein extract with water. Optical density of samples was measured in a 1 ml cuvette at 595 nm.

2.5.2.2 *Determination of tobacco protein content*

Crude protein extracts were prepared from plants by grinding the fourth, fifth and sixth leave of each plant in liquid nitrogen with a mortar and pestle The resulting leaf powder was transferred to a protein extraction buffer containing 50 mM Tris-HCl, 0.1 % TritonX-100 at pH 7.0 (0.74 g of leaf material per 1 ml of buffer) and the powder was homogenised on ice. Mixtures were then centrifuged at 4 °C for 10 min at 13,000 rpm in an Eppendorf centrifuge. The clear supernatant containing soluble proteins were then directly used for detection of protease activity. Protein contents were determined using the Bradford protein assay kit (Bradford, 1976) with bovine serum albumin as described in paragraph 2.3.2.1.

2.5.3 Detection of protease activity

2.5.3.1 *Gelatine-containing SDS-PAGE*

For the detection of protease activity in nodules and tobacco sodium a dodecyl sulfate polyacrylamide gel (15 % SDS-PAGE) was prepared containing 1 % gelatine, 0.01 % sodium dodecyl sulfate (SDS), 3 % polyacrylamide, 1 % ammonium persulfate (APS), 100 % TEMMED (Bio-Rad, UK) with 1.5 M Tris-HCl pH 8.8, and 1 M Tris-HCl pH 6.8. For

loading of the gel, 50 µg protein amount from nodule extracts or tobacco leaves were mixed with 5X sample buffer (31.25 ml 1M Tris-HCl pH 6.8, 1 % SDS powder, 25 ml 50 % glycerol, 75 µl of 1 mM bromo-phenol blue dissolved in 2 % ethanol and 5 µl of 100 % 2-mercaptoethanol). Protein separation on a Bio-Rad (UK) electrophoresis system was carried out using an SDS running buffer (0.038 M glycine, 0.1 M of Tris-HCl pH 8.0 and 1 % of SDS) at 100 V on ice.

After electrophoresis, the gel was incubated in 2.5 % Triton X-100 for 30 min at room temperature to re-nature the proteases. After 30 min, the gel was rinsed three-times for 10 min with distilled H_20 to remove the Triton-X 100 solution. The gel was then incubated overnight in a proteolytic buffer (0.1 M citrate phosphate buffer pH 6.0 containing 10 mM L-cysteine) at 37 °C for the developing the protease reaction. The gel was subsequently transferred overnight to a staining solution (25 % isopropanol, 10 % acetic acid and 0.5 % coomassie blue) at room temperature and then de-stained for 2 h with a de-staining solution (10 % acetic acid, 40 % dH_20 and 50 % methanol). The de-stained gel was recorded using a digital camera.

For detection of cysteine proteases, 30 µg of nodule protein extract was incubated with 5 µl of 1 % trans-(epoxysuccinyl)-L-leucylamino-4-guanidinobutane (E-64) on ice for 30 min before gelatine-containing SDS-PAGE. To detect serine protease activity, 5 µl of a 2 % serine protease inhibitor (soybean trypsin inhibitor) was mixed on ice for 30 min with 60 µg protein of a nodule extract for electrophoresis. As control, a protein sample incubated with water was used. All samples were mixed with 5X sample buffer (see paragraph 2.3.2) and proteins were separated by 15 % SDS-PAGE (see paragraph 2.3.2). Alternatively, inhibitors (1 % final concentration) were also added directly to proteolytic buffer used for the

development of the protease reaction.

2.5.3.2 *Azocasein test*

Protease activity was further determined with a spectrophotometer using azocasein as protein substrate as described by Michaud *et al.* (1995). In this assay, azocasein produces a red to orange color compound when hydrolyzed by proteases. The colored compound can be measured at 440 nm in a spectrophotometer. At this wavelength, one unit of protease activity is defined to be the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvette under the conditions of the assay. For the assay, 50 µg soluble proteins from 200 µg of protein nodules were mixed with 450 µl of assay buffer (0.1 M citrate phosphate buffer, 5 mM cysteine, 5 mM β-mercaptoethanol at pH 6.0). Azocasein (Sigma, UK) was pre-activated for 10 min at 37 °C by adding an equal volume of a 2.5 % azocasein solution to the reaction mixture. The protein extract was then added to the pre-activated azocasein and this mixture was then incubated at 37 °C for 3 h. To stop the reaction, 300 µl of 10 % (w/v) trichloro-acetic acid (TCA) was added to the mixture and the mixture was incubated for a further 30 min at 4 °C for complete color development. Any azocasein residuals were removed by centrifugation at 12000 rpm for 5 min at 4 °C in an Eppendorf centrifuge. To 1.0 ml of the supernatant, 1.0 ml of 1 N NaOH was added to neutralise the TCA. Finally the absorbance of the solution was determined at 440 nm in a spectrophotometer.

2.5.3 Detection of cysteine proteinase inhibitors

Endogenous cysteine protease inhibitors in crown nodules and tobacco leaves were detected

by using a reverse zymogram. For that, a gelatine-containing gel was placed into proteolytic buffer containing 2 mg / 100 ml of the plant cysteine protease papain (Roche, Switzerland) for hydrolyzing the gelatine. Nodule protein extracts were mixed with 5X sample buffer and loaded onto a 15 % SDS-PAGE containing 1 % gelatine for protein separation. Electrophoresis was run on ice at 100 V for 3 h. The gel was incubated overnight in a proteolytic buffer containing papain and then stained with 0.5 % coomassie blue. Regions where cysteine protease inhibitors have reacted with papain remained blue after gel de-staining.

2.5.4 Western blot analysis

Nodule and tobacco leaves protein samples were mixed with 5X sample buffer as described in 2.4.3.1. Extracts were boiled at 95 °C for 15 min. and shortly centrifuged for 30 sec at full speed (13,000 rpm) and then subjected to a 15 % SDS-PAGE for 3 h at 100 V at room temperature. The separated proteins were then transferred onto a nitrocellulose Immun Blot™ PVDF membrane (Bio-Rad, UK). The transfer buffer contained 0.18 M of glycine, 0.02 M Tris base and 15 % methanol at pH 8.2. Transfer was done on ice for 180 min at 45 V and 200 mA.

After 3 h of protein transfer, the membrane was incubated overnight on ice in a 5 % skimmed milk powder solution in TBS (0.5 M of Tris-HCl, 1 M NaCl, 0.1 % Tween 20 pH 7.6) to saturate the membrane with proteins. The membrane was then incubated on ice for 5 h with TBS buffer containing 5 % skimmed milk powder and an antibody raised against the rice cysteine protease inhibitor OC-I in a rabbit (1:500 dilution). After incubation, the membrane was washed three-times for 10 min with 5 % skimmed milk powder, 0.1 % Tween 20 and

TBS (Tris-base saline). A goat anti-rabbit antibody conjugated to alkaline phosphatase (Santa Cruz Biotechnology, USA), which was used as a secondary antibody, was added (1:2000 dilution) to the membrane, and the membrane was incubated with the rabbit antibody for 60 min at room temperature in 5 % skimmed milk powder, 0.1 % Tween 20 and TBS. Finally the membrane was washed three times for 30 min with 10 min intervals in 0.1 % Tween 20 containing 30 ml of TBS. The presence of the cysteine protease inhibitor was detected using the alkaline phosphatase detection kit (Bio-Rad, UK).

CHAPTER THREE

Results

3.1 Soybean plant and nodule characterization

3.1.1 Soybean growth

3.1.1.1 *Growth medium optimization*

The first trial of soybean growth under green-house conditions in vermiculite and inoculation with *Rhizobium* resulted in chlorotic leaves after 6 to 8 weeks of growth and slow development of plants. This was found when a nitrogen-free Hoagland solution containing 50 mM of potassium chloride (KCl) was used as mineral nutrition of soybean plants. In the second trial when 1 M KCl was added to the Hoagland nutrient solution, plant leaves were healthier and not chlorotic. Since slow plant growth still occurred at this KCl concentration, a third trial was carried out where the water supply of 1000 ml/day/pot (500ml in the morning and afternoon) was reduced to 150 ml/day/pot in young soybean plant and to 200 ml/day/pot for mature plants. This resulted in a faster growth of soybean plants over a period of 14 weeks (Fig. 3.1).

3.1.1.2 *Soybean growth under greenhouse conditions*

Soybean plants of Prima 2000 (Fig. 3.1) were grown over a period of 21 weeks and subjected under green-house conditions at 25 °C, this experiment was repeated four times. However in this experiment only plants grown from a period of 4 to 14 were used. Due to fluctuations in

the natural light condition, there were some differences in the growth pattern of plants among different individual experiments regarding growth characteristics of plants. These included differences in height, water absorbance and leaf number in particular of the oldest plants (14 weeks). Therefore for further analysis, only plants which were similar regarding their growth characteristics were used for the different time intervals of growth.

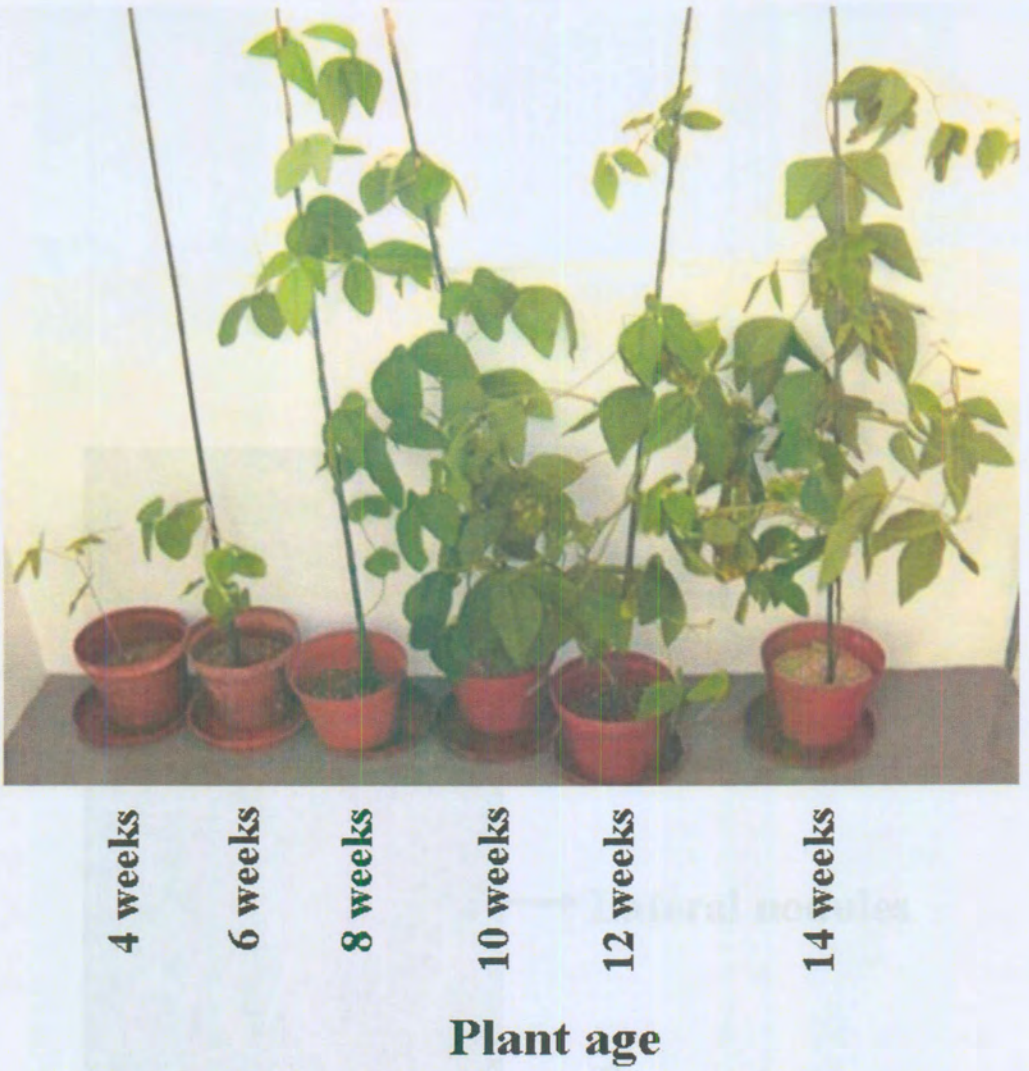


Figure 3.1: Growth of soybean plants from 4 to 14 weeks after germination under green-house conditions. Prima 2000 plants were grown and provided with an extended light period (2 h artificial lights in the evening). Seedlings were watered three times with de-ionised water and a twice a week with nitrogen-free Hoagland solution.

3.1.1.3 *Soybean nodule characterization (Azocasein test)*

Nodules growing on the tap root (Fig. 3.2), which are also called crown nodules, were harvested from soybean plants after 4, 6, 8, 10, and 12 weeks of plant growth. When the fresh weight (FW) of these crown nodules was determined, a continuous increase in crown nodule weight over the growth period from 4 to 12 weeks was found (Fig. 3.4A). Crown nodule weight was significantly higher ($p<0.05$) after 8 weeks when compared to 4 weeks old crown nodules and 12 weeks old nodules had further a significantly higher ($p<0.05$) weight than 8 weeks old crown nodules. Results also showed that the weight increase from 6 weeks until 12 weeks was primarily due to growth of existing nodules and not due to formation of new nodules on the tap root.

The crown nodule number increased during the first 6 weeks of plant growth while the number of crown nodules remained stable from 6 weeks to 12 weeks of nodule development (Fig. 3.4B). The nodule number was significantly higher ($p<0.05$) after 8 weeks of growth when compared to the number after 4 weeks of plant growth. However, there was no significant difference ($p<0.05$) in the nodule number after 10 and 12 weeks of growth when compared to 8 weeks of growth.

Crown nodules (4, 6, 8, 10 and 12 weeks old) were dissected into halves for visual observation of the colouration of leghemoglobin in the nodules (Fig. 3.3, 3.4C). Active leghemoglobin, which delivers oxygen from the plant cytosol to *Rhizobium* bacteria, has a pink color and indicates active nitrogen fixation. A greenish to brown color indicates degraded non-active leghemoglobin. From 4 weeks to 6 weeks all crown nodules showed a

pink color indicating fully active nodules. Degradation of leghemoglobin started after 8 weeks of soybean growth and the percentage of non-active nodules was significantly lower in 12 weeks old nodules than in 8 weeks old nodules ($p<0.05$). After 10 weeks, 30 % of crown nodules had degraded leghemoglobin and after 12 weeks at least 40 % of crown nodules were not active.

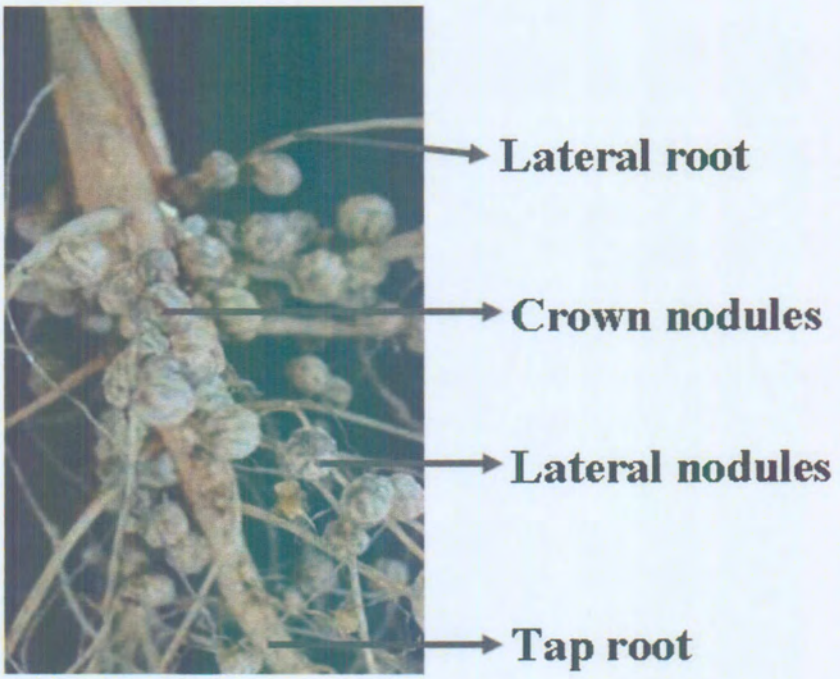


Figure 3.2: Crown and lateral nodules from soybean plants. Two types of nodules developed at different parts of soybean roots. Crown nodules developed at an early age from a tap root whereas lateral nodules developed at a later age from the lateral root hair of the soybean plant (photo taken by Elsie Cruywagen).

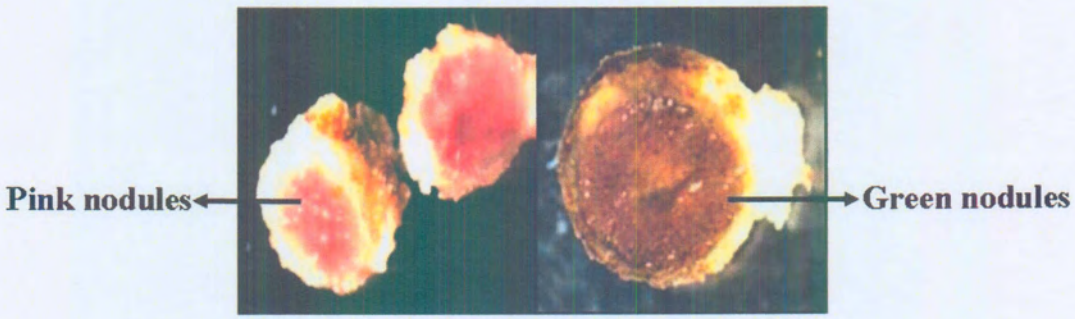


Figure 3.3: Color of crown nodules. Healthy nodules display a pink color due to the heme cofactor of leghemoglobin indicating active nodule fixing nitrogen whereas brown/greenish color shows a breakdown of leghemoglobin resulting in an organic molecule called biliverdin which has a green/brown color (photo taken by Dr MC Mathabe).

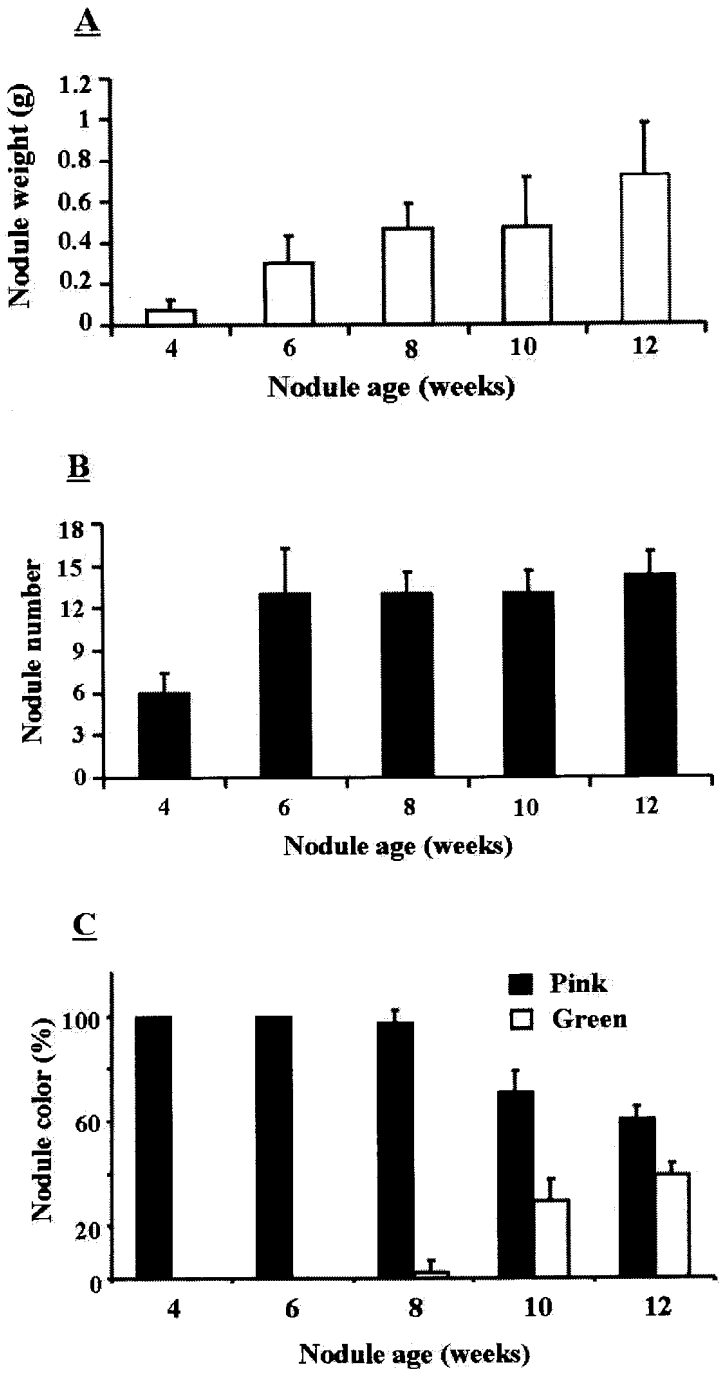


Figure 3.4: Weight (A), number (B) and color of crown nodules (C). Pink color indicates active leghemoglobin, greenish/brown color indicates inactive leghemoglobin. Pink color demonstrates active nodule fixing nitrogen with functional leghemoglobin whereas green/brown color shows degraded non-functional leghemoglobin crown nodules. Values shown represent the mean of 4 individual experiments ± standard deviation (SD).

3.1.2 Nodule protease activity

3.1.2.1 *Detection of total protease activity in nodules using the azocasein test*

Since Azocasein is very sensitive to protein content, older crown nodules were used for this experiment. When the total protease activity from soybean crown nodules was analyzed by using azocasein as a target protein, a continuous increase of protease activity from 7 to 21weeks old nodules was found (Fig. 3.5) and protease activity was significantly higher in 21 weeks old crown nodules when compared to 7 weeks old crown nodules ($p<0.05$).

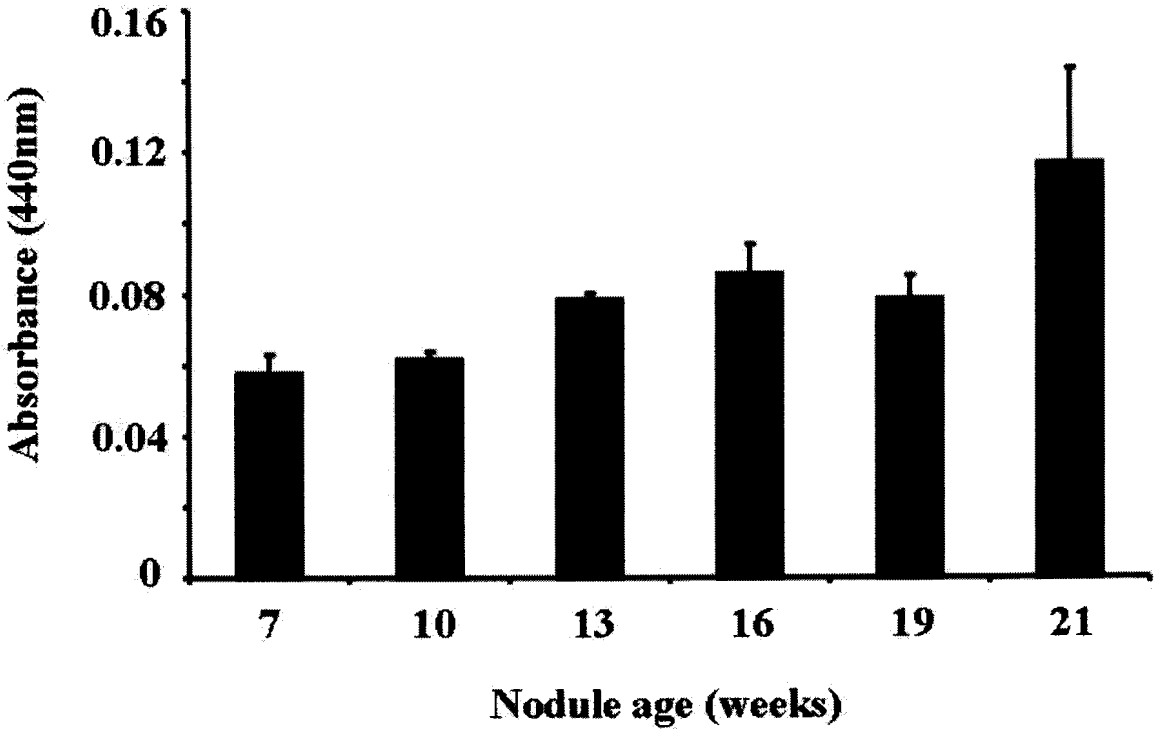


Figure 3.5: Protease activity of crown nodules of different age measured by the azocasein test at 440 nm where protease containing extracts of crown nodules were incubated with azocasein for 3 h at 37 °C and formation of the azo-dye was measured. Bars represent the mean of four individual samples ±SD with three replications.

3.1.2.2 *Detection of class-specific proteases in nodules*

To visualize specific protease activities present in soybean nodules of different age gelatine SDS-PAGE was used where gelatine is degraded by proteases resulting in clear bands on a stained poly-acrylamide gel. When soluble protein extracts from soybean of different age were separated by gelatine SDS-PAGE, three different protease activities were detected (Fig.3.6A). Young crown nodules (4 to 10 weeks old) had an almost identical pattern of protease activity (protease activity P2 and P3). In contrast, mature crown nodules (10 to 14 weeks old) revealed an additional protease activity during nodule development (Fig. 3.6A; P1) and also had higher protease activity visible as more extensive visible clearance of the stained gel.

When nodule protein extracts were pre-incubated with the cysteine protease inhibitor E-64, this treatment did not affect protease activity (P2) in 4 and 10 weeks old nodules (Fig. 3.6B). This indicates that cysteine protease activity was not a major component of P2 activity but very likely of P3 due to the disappearance of protease activity observed. However, when an extract of 14 weeks old nodules was pre-incubated with E-64, protease activity (P1) significantly decreased indicating the existence of cysteine protease activity in 14 weeks old nodules and cysteine protease as a component of P1. Furthermore a slight inhibition of protease activity P2 also indicated possible presence of cysteine protease activity in this band.

When the same experiment was done for detection of serine protease activity in nodule extracts, where extracts were pre-incubated with a soybean trypsin-chymotrypsin inhibitor,

only in younger (6 weeks old) nodules, a decrease in the protease activity (less intense bands) was found after treatment with an inhibitor (Fig. 3.7B). Such a decrease was not found in 10 and 14 weeks old nodules indicating that serine protease activity is not a major activity in mature nodules.

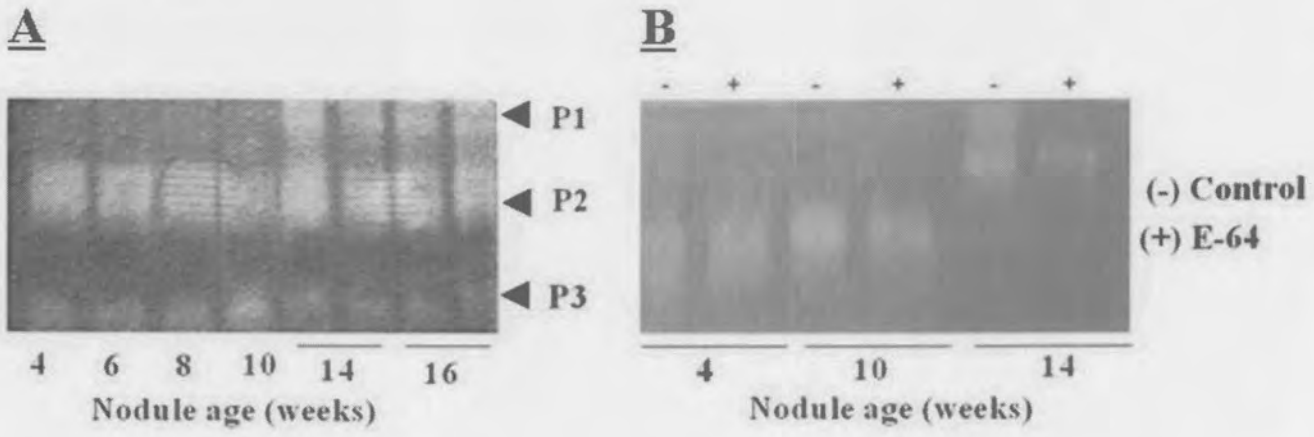


Figure 3.6: Gelatine-containing SDS-PAGE of crown extracts from different age (A). Different proteases activities (P1, P2, and P3) are shown by clearance of stained gel. Inhibition of cysteine protease activity in crown nodule by specific cysteine protease inhibitor (E-64) during nodule development (B).This indicates that cysteine protease is the major activity in mature crown nodules.

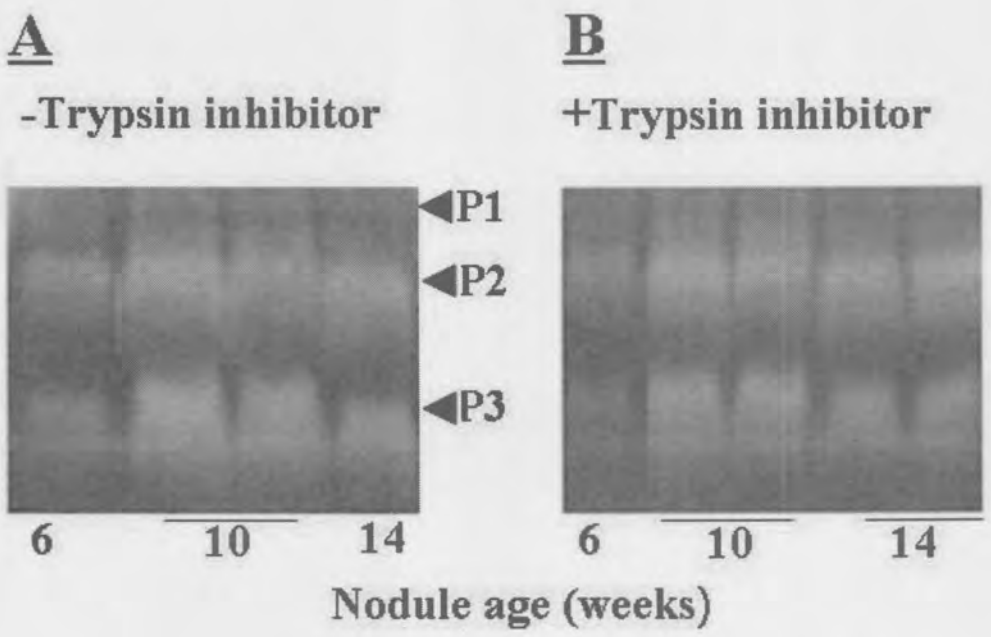


Figure 3.7: Gelatine-containing SDS-PAGE for detection serine protease activity in extract from crown nodules of different age and incubation of extracts with a trypsin inhibitor to block serine protease activity. Serine protease activity in nodules without inhibitor pre-incubation at pH 8.0 (A) and after pre-incubation with an inhibitor at pH 8.0 (B).

3.1.2.3 *Detection of protease inhibitor activity in nodules*

Identification of cysteine protease inhibitor activity in nodules during development was carried out using a reverse zymogram. This was done by incubating the gelatine-containing SDS gel after protein separation into a papain-containing acidic proteolytic buffer. Adding papain to the proteolytic buffer degrades gelatine and all other nodule-derived proteins with the exception where cysteine protease inhibitor activity has blocked papain action and inhibitor containing protein bands are not de-stained (still blue) after de-staining. Reverse

zymogram analysis revealed two putative cysteine protease inhibitor activities (Fig. 3.8). A first activity of a possible cysteine protease inhibitor (PI-2) could be detected in extracts of 4 and 8 weeks old crown nodules. A second possible protease inhibitor activity (PI-1) could be further detected in extracts of 4 to 10 weeks old crown nodules. However, no cysteine protease inhibitor activity was found in 12 and 14 weeks old mature crown nodules.

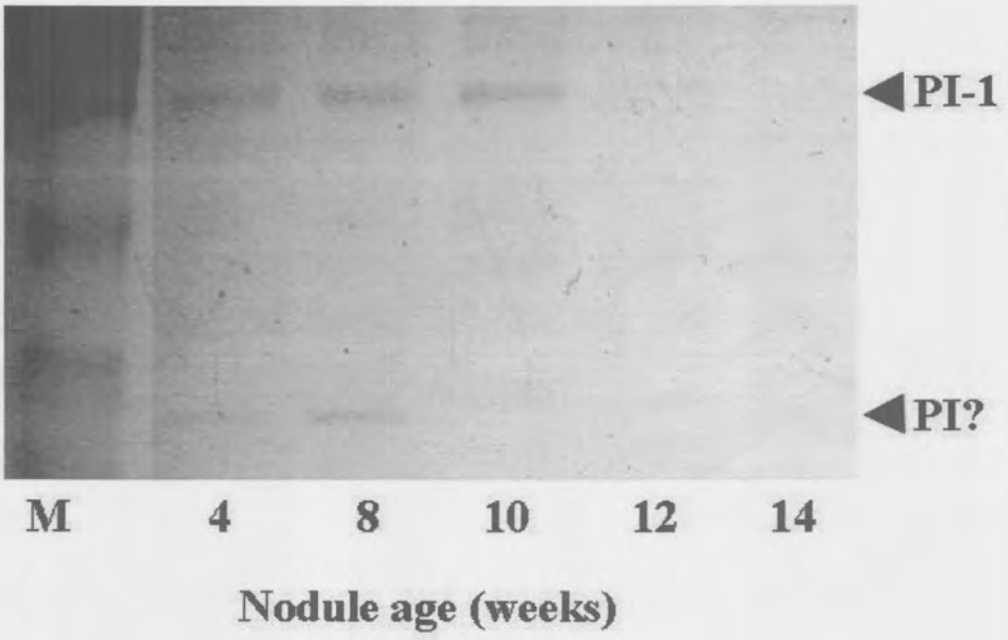


Figure 3.8: Reverse zymogram indicating location of putative cysteine protease inhibitor activity (blue band) on a gelatine-containing SDS-PAGE gel after treating the gel with the cysteine protease papain.

3.1.2.4 *Detection of cysteine protease inhibitors in nodules by Western blotting*

When protein extracts from nodules of different age were separated by gelatine SDS-PAGE and analyzed by immuno-blotting using a cysteine protease inhibitor anti-rabbit antibodyserum raised against a cysteine protease inhibitor of rice (OC-I), a single hybridization product was detected by Western blot analysis in both young and mature soybean nodules (Fig. 3.9). However, in mature nodules (14 weeks old) the detected band was weaker after detection with an alkaline phosphatase labeled secondary antibody when compared to the band detected in young nodules (4 and 10 weeks old). This possibly indicates that a cysteine protease inhibitor is continuously expressed in soybean crown nodules during nodule development.

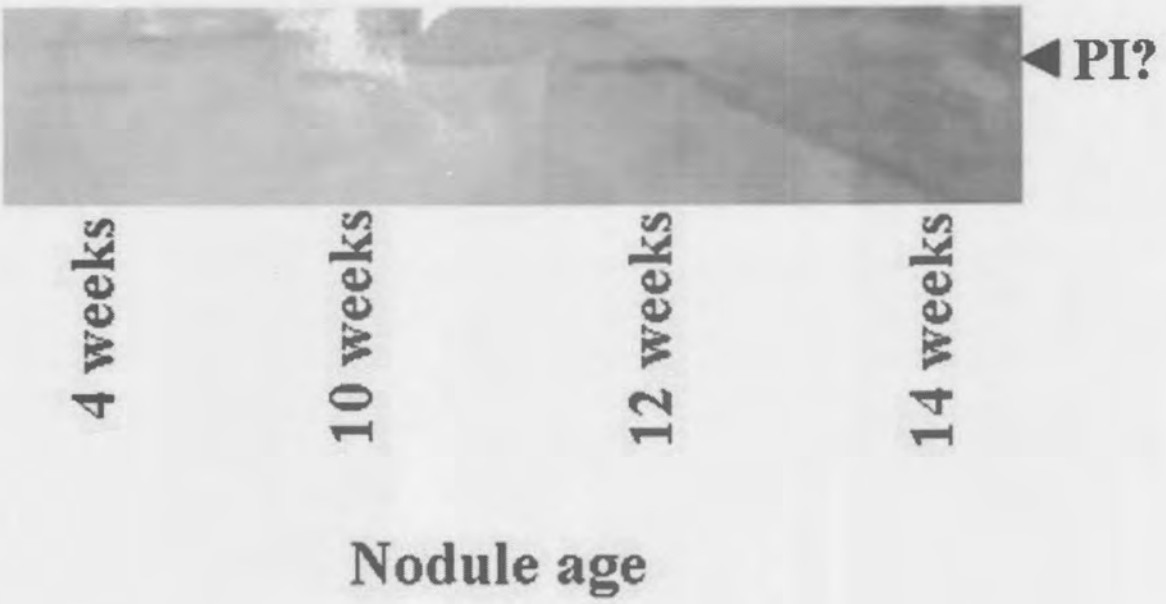


Figure 3.9: Western blot analysis to detect possible cysteine protease inhibitor expression during nodule development using an anti-rabbit antibody raised against the rice cysteine protease inhibitor OC-I. Nodule protein extracts (50 µg total protein) were electrophoretically separated by gelatine SDS-PAGE and the gel was treated with papain for protein degradation and bands containing possible cystatin protease inhibitor activity were hybridized with the rabbit antibody for detection.

3. 2 Tobacco transformation

3.2.1 Production of transformed tobacco plants

3.2.1.1 *Tobacco transformation*

Tobacco leaf discs were submerged into a suspension of *Agrobacterium tumefaciens* (LBA4404) cells carrying either the plasmid pBin19 carrying the native papaya cysteine protease inhibitor coding sequence or the plasmid pCAMBIA 1300 in which a mutated papaya cysteine protease inhibitor coding sequence had been inserted (Fig. 2.2; vector pCAMBIA mut-Cys-89). The pCAMBIA vector further carried a DNA coding sequence to provide hygromycin resistance and pBin19 kanamycin resistance in transformed plants. Since tobacco leaf discs were always contaminated after *Agrobacterium* infection which could not be controlled by antibiotic application, only leaf discs infected with *Agrobacterium* carrying pCAMBIA 1300 were used. For tobacco shoot formation, discs were placed onto agarose plates containing Murashige and Skoog medium (MS medium; Murashige and Skoog, 1962), cefotaxime for the suppression of *Agrobacterium* growth and hygromycin for selection of transformed shoots carrying the inhibitor coding sequence. Only 4 putative transformed shoots could be regenerated after 6 weeks of cultivation on selection medium.

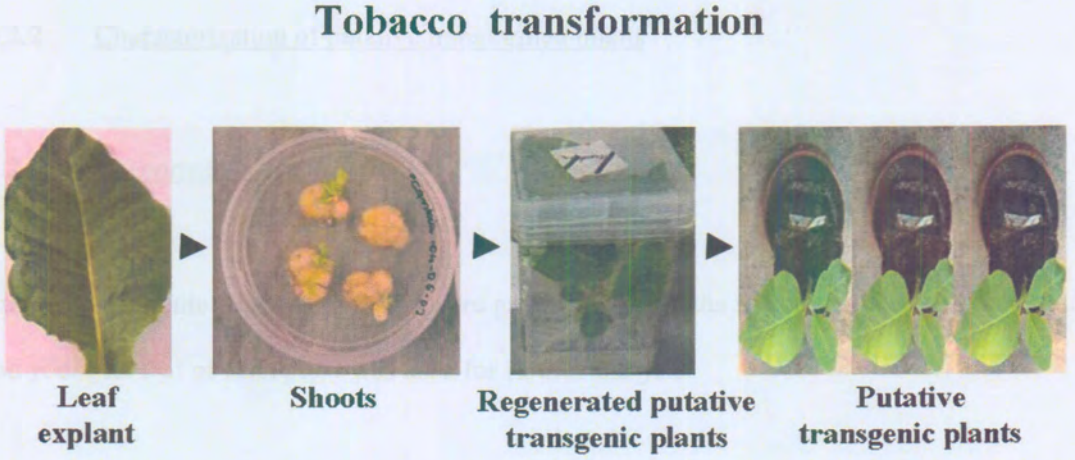


Figure 3.10: Tobacco transformation using *Agrobacterium tumefaciens*. Leaf discs were excised from a leaf of a tobacco plant with a cork borer and submerged into an *Agrobacterium tumefaciens* culture (LBA4404). *Agrobacterium* cells carried the vector pCAMBIA 35S-Cys-mut 89. Shoots regenerated after 4 weeks on MS medium containing hygromycin for selection and cefotaxime for suppression of *Agrobacterium* growth. Putative transgenic shoots were placed on ½ strength MS medium containing hygromycin for root formation. Rooted in vitro-grown plants were finally transferred to green-house for analysis and seed production.

3.2.2 Characterization of putative transformed plants

3.2.2.1 *Phenotypic analysis*

Putative transgenic plants (Fig. 3.11) were grown for 3 months in a green-house at 25 °C and the youngest leaf of each plant was used for further analysis.

Figure 3.11: Growth of putative tobacco plants (T1-T4) in the green-house at 25 °C. Plants were watered with distilled water 3 times a week and fertilized twice a week with a MS nutrient solution.

3.2.2.2 *PCR analysis*

The presence of the isolated genomic DNA (Fig. 3.12), from 4 putative transformed plants was confirmed on 1 % agarose gel that was stained with ethidium bromide. Primer pair (PapCy1-left and PapCys1-right) was then used for the amplification of the inhibitor coding sequence (encodes cysteine proteinase inhibitor). Amplification of coding sequence was not successful in plants T1-T4 (Fig. 3.13). However, the inhibitor coding sequence could be amplified from the isolated plasmid pCAMBIA -Cys- mut 89 with a predicted fragment size of 250 bp (Fig. 3.13).

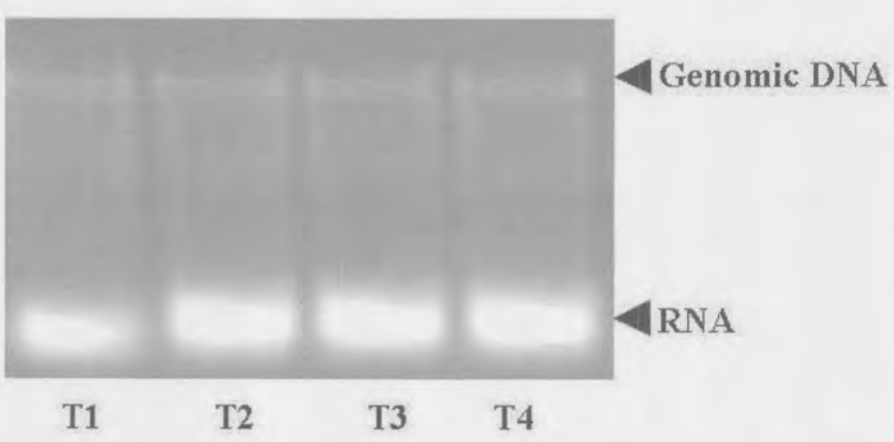


Figure 3.12: Genomic DNA from different putative transformed tobacco lines (T1-T4). Position of genomic DNA and RNA on a 1 % agarose gel is shown.

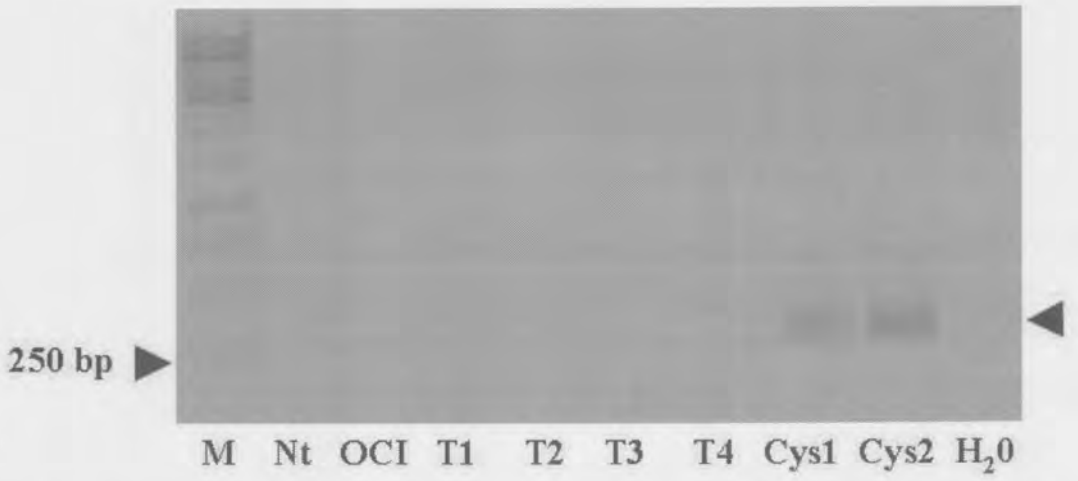


Figure 3.13: PCR analysis to identify transgenic tobacco lines carrying the mutated papaya cysteine protease inhibitor sequence. T1-T4, Nt (non-transformed tobacco) and tobacco expressing rice cystatin represents PCR amplification of a 250 bp DNA fragment corresponding to the coding sequence of the papaya cysteine protease inhibitor from DNA plasmid of Cys1 and Cys2 represent positive controls (plasmid pCAMBIA -Cys- mut-89and pBinCys19) with papaya cysteine protease inhibitor respectively, a negative control (PCR product from genomic DNA of a non-transformed tobacco plant).

3.2.2.2 *Western blot analysis*

To detect the expression of the mutated cysteine protease inhibitor coding sequence in putative transgenic tobacco plants protein extracts from tobacco leaves were separated by SDS-PAGE. After transferring proteins to a membrane any presence of the expressed inhibitor in the protein extracts was detected by immuno-blotting using an rabbit antibody raised against the rice cysteine protease inhibitor OC-I. Using this rabbit antibody, no expression of the inhibitor was found in the 4 transformed plants (Fig. 3.14). In contrast, a band of about 14 kDa corresponding to the rice inhibitor was found when a protein extract from a tobacco expressing OC-I was detected.

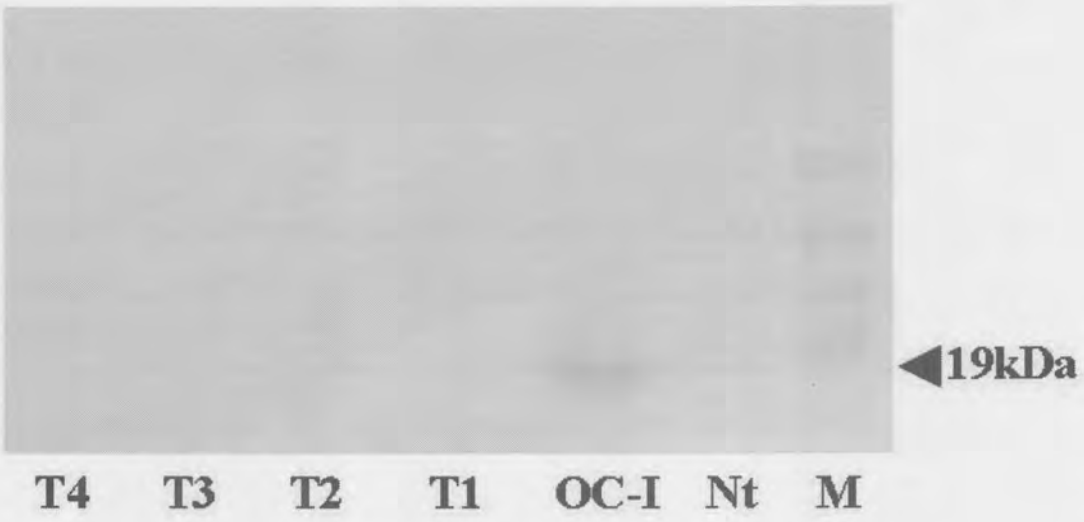


Figure 3.14: Western blot analysis to detect mutated cysteine protease inhibitor expression in a non-transformed (Nt) tobacco plant and putative transformed plants (T1-T4). OC-I represents detection of expression of the rice cysteine protease inhibitor OC-I in a transformed OC-I expressing tobacco plant. Protein extracts from tobacco leaves (60 µg protein) were separated by SDS-PAGE and analyzed by an rabbit antibody raised in rabbits against OC-I.

3.2.2.3 *Protease activity*

When protein extracts from putative transformed (T1-T4) and tobacco expressing OC-I were separated by gelatine SDS-PAGE on an poly-acrylamide gel, protease activities were detected (non-stained clear bands) in all tobacco plant extracts after staining with coomassie blue (Fig. 3.15). A clear band of protease activity (Fig. 3.15; P) was found in all putative transformed plants (T1-T4). However, this activity was not found in tobacco expressing OC-I. This activity band possible represents cysteine protease activity which was blocked in the OC-I expressing tobacco plant.

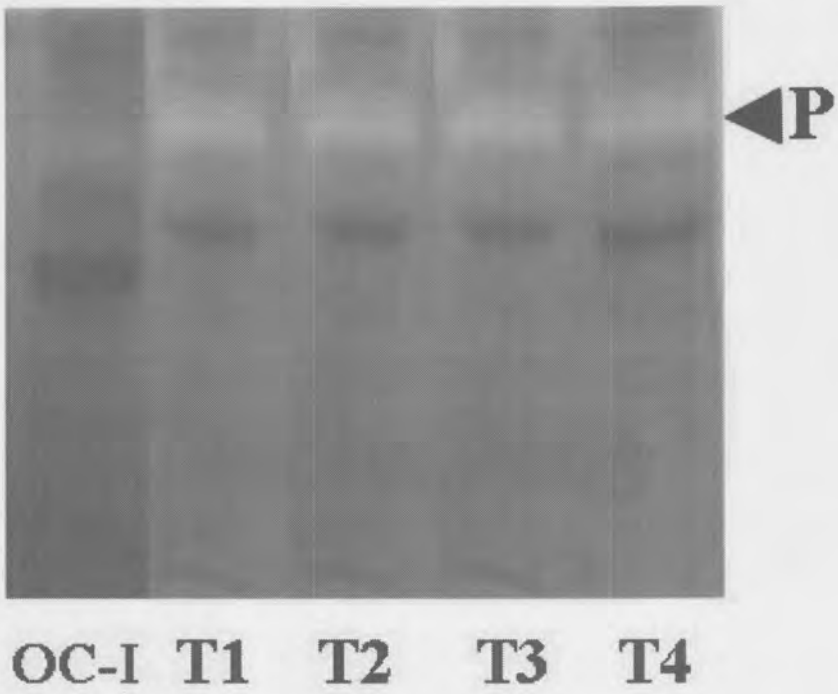


Figure 3.15: Protease activities in putative transformed tobacco plants using gelatine SDS-PAGE. T1-T4 represents different protease activities from putative transformed plants. OC-I represents protease activities of a transgenic plant expressing the rice cysteine protease inhibitor OC-I. For detection of protease activities, leaf protein extracts (25 µg) were loaded onto the poly-acrylamide gel.

3.2.2.4 *Cysteine protease inhibitor detection*

Detection of cysteine protease inhibitors in tobacco leaves was carried out using a reverse zymogram. This was done by incubating the gelatine containing poly-acrylamide gel after SDS-PAGE into a papain containing acidic proteolytic buffer. Adding papain to the proteolytic buffer degrades gelatine and all other papain sensitive tobacco proteins with the exception of cysteine protease inhibitors which block protease action. These inhibitor containing areas are therefore still stained blue indicating a papain in-sensitive protein. For protein extracts derived from tobacco OC-I expressing plant and the 4 putative transgenic

plants, reverse zymogram showed two stained bands (PI-1 and PI-2), possibly containing proteins that prevented papain activity (Fig. 3.16). However, these bands were not detected when a protein extract from a non-transgenic plant was analyzed plants.

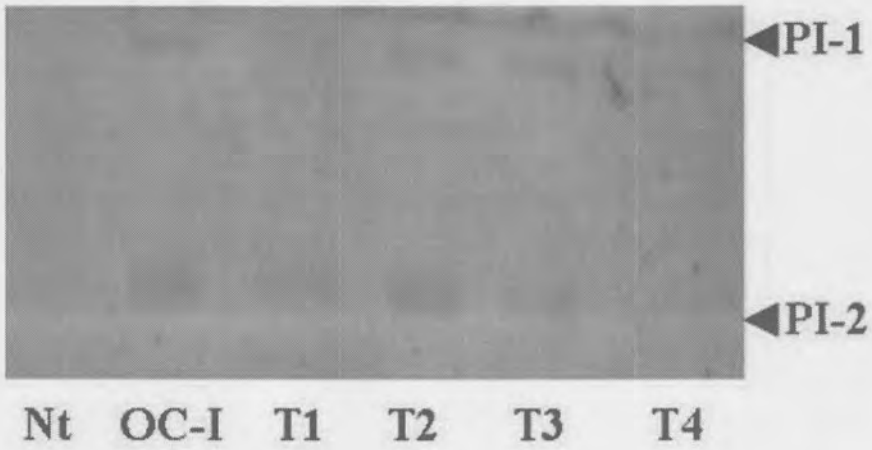


Figure 3.16: Detection of cysteine protease inhibitor activity using a reverse zymogram. T1-T4 represents inhibitor activity from putative transgenic plants. OC-I represents inhibitor activity of a transgenic plant expressing the rice cysteine protease inhibitor OC-I and Nt represents inhibitor activity of a wild-type non-transformed tobacco plant. For detection of inhibitor activity gels were incubated after SDS-PAGE with 2 mg/ml papain overnight to degrade gelatine and tobacco proteins.

CHAPTER FOUR

Discussion

4.1 Proteolytic activity in soybean nodules

This study has first focused on the characterisation of crown nodule development in soybean plants under greenhouse conditions. Capability of leguminous plants to establish endosymbiosis relationship with soil fixing nitrogen *Rhizobium* bacteria results in specialized determinate or indeterminate organ structure known as nodules (Van de Velde *et al.*, 2006). Determinant nodules exist from tropical leguminous crops, such as soybean, and are characterised by a non-persistent meristem resulting in a round-shaped organ with homogenous central fixation zone, whereas indeterminate nodules are long and continuously grow due to an apical meristem. Al-Karaki *et al.* (2000) showed that both nodule weight and nodule number of *Tetragonolobus palaestinus* are critical for the ability of the plant to fix atmospheric nitrogen in association with *Rhizobium* bacteria. It has been suggested that an improvement in the nitrogen availability would turn to improve the quality and crop yield of plants (Akunda, 2001). In this study investigating soybean nodule development, it was found that the number of crown nodules increased only in the first 4 to 6 weeks and then remained stable until the end of the experiment at 12 weeks. Increase in soybean nodule weight was observed continuously with increasing age. This indicates that soybean plants do not continuously form new crown nodules, but that they continue to increase in weight in the later stage of plant growth.

A further aspect of the study was to obtain a better understanding of protease and protease activity in soybean nodules. Pladys and Vance, (1993) found that senescing in pea nodule is characterized by high protease activity. Protease activity in aging of alfalfa nodules also increased and effectively degraded the leghemoglobin protein (Pladys and Vance, 1993).

However active soybean crown nodules, which fix nitrogen and indicated by the pink leghemoglobin color of nodule cross sections, were only identified in young nodules when they were 4 to 6 weeks old. More mature crown nodules had a green color associated with leghemoglobin degradation. Degradation, started from the centre of the nodule. A decrease in activity of the nodules due to the degradation in active leghemoglobin is balanced by an early increase in size of nodules. Ashraf and Iram (2005) found that larger nodules have a relatively greater proportion of fixing nitrogen than smaller nodules. A higher number of nodules per plant also have a greater potential for nitrogen fixation ultimately resulting in a greater yields.

This study has also shown that mature crown nodules had higher protease activity when compared to young crown nodules. Pfeiffer *et al.* (1983) reported that mature soybean nodules, harvested after full seed development and after the physiological growing phase, had a high protease activity when compared to younger nodules which were harvested from soybean in the vegetative growth phase. Further, when proteolytic activity from French bean nodules was measured using the azocasein assay, increased proteases activity was found with increasing nodule age (Playdys *et al.,* 1991) and protease activity at an acidic pH optimum was mainly responsible for high activity (Playdys *et al.,* 1991). Further, when protease activity from effective and ineffective alfalfa nodules from increasing age was conducted with the azocasein assay, protease activity was low throughout nodule development in effective nodules, but slowly increased after three weeks. In contrast, ineffective nodules had an early senescence after 11 days (Pladys and Vance, 1993). An increase of protease activity at a later stage of nodule development was also found at full seed development and physiological maturity which correlated with decrease in soluble protein and leghemoglobin in senescing soybean nodule (Pladys and Vance, 1993). In this study, it was observed that

almost identical pattern of protease activity was obtained when soybean crown nodules were used, until nodules were 10 weeks old and increased dramatically when nodules were 14 and 16 weeks old.

In this study, the visible onset of senescence occurred when degradation of leghemoglobin started during nodule development and the nodule color changed from pink to green. Leghemoglobin functions in oxygen transportation from the plant cytosol to the *Rhizobium* bacteria providing a micro-site oxygen environment to the nodule (Becana and Klucas, 1992). When soybean crown nodules of increasing age were dissected into halves, almost 30 % of crown nodules showed signs of degraded leghemoglobin after 10 weeks. Moreover, this study has indicated that leghemoglobin color is a useful marker for nodule development confirming an earlier report by Pfeiffer *et al.* (1983). When soybean root nodules were grouped according to vegetative growth, flowering, early seed development, full seed development, physiological maturity and harvest maturity, nodule greening was an indication of leghemoglobin loss (Pfeiffer *et al.,* 1983). Furthermore, when total soluble protein was determined and total leghemoglobin content in the nodule cytosol fraction was determined by the pyridine hemochrome assay, both soluble proteins and leghemoglobin decreased with increasing age (Pfeiffer *et al.*, 1983). Arredondo-Peter *et al.* (1997) also reported that expression of leghemoglobin could only be detected in young nodules of cowpea at 20 days when compared to mature nodules of 29 days.

It has further been reported that exposure of *Lotus japonicus* to hydrogen peroxide (H_2O_2) results in the degradation of leghemoglobin. However this decrease in leghemoglobin was accompanied with an increase in protease activity (Günther *et al.*, 2007). Leghemoglobin degradation and increase in protease activity was also found in this study with soybean crown

nodules. At 14 weeks of age, the green color of nodules indicated considerable leghemoglobin degradation. This was accompanied by the appearance of dramatically increased protease activity detected in a protease activity gel. These nodules had most likely also lost much of their nitrogen-fixing capacity (Günther *et al.*, 2007). It has also been reported that during development and senescence of ineffective and effective alfalfa nodules, increase in proteolysis can be blocked by the cysteine protease inhibitor E-64 indicating a particular involvement of cysteine proteases in this process (Pladys *et al.*, 1991). When the cysteine protease inhibitor E-64 was added to protease extracts from soybean crown nodules, as done in this study, cysteine protease activity was significantly blocked in mature crown nodules at the age of 14 weeks. This shows that cysteine proteases also play an important role in nodule senescence. A specific cysteine protease (*Asnodf32*) involved in metabolism of aging nodules has been identified by Naito *et al.* (2000). Strong transcription of *Asnodf32* was observed in the senescence zone of the nodules and the transcript level decreased gradually from senescence zone to the inter zone.

When a reverse zymogram technique was used as an effective tool for characterisation of natural protease inhibitor two bands appeared with extracts from young nodules on a protease activity gel indicating inhibition of cysteine protease activity. These bands were not found when older crown nodules were analyzed. Using an oryzacystatin antibody and Western blotting only a weak band was detected after 14 weeks but some stronger bands were detected in younger nodules. However, there is no further evidence so far that these bands correspond to cysteine protease inhibitors. In the literature, there is only one report of detection of Kunitz-type serine protease inhibitor during nodulation but no expression of a cysteine protease inhibitor has, to my knowledge, been reported.

A final objective of this study was to test if a cysteine protease inhibitor coding sequence, which might be ultimately used in soybean transformation, is active in a plant system. Expression of this inhibitor in soybean nodules might allow the evaluation of the function of cysteine proteases during nodule development. Since a soybean transformation has not yet been developed in our group, the activity of such potential expressed cysteine protease inhibitor coding sequence was first tested in tobacco. As an inhibitor coding sequence, a mutated sequence was used which had already shown increased inhibitory activity in an *in vitro* papain enzyme assay (Kiggundu, unpublished data). Research has already shown that the expression of the rice cysteine protease inhibitor (OC-I) in tobacco plants increases the growth rate of plants and further had a greater biomass and showed earlier flowering as well as higher numbers of flowers and seeds (Gutiérrez-Campos *et al.*, 2001). Results obtained by Van der Vyver *et al.* (2003) also showed that photosynthesis was better protected against chilling in plants expressing OC-I indicating that cysteine protease inhibitor expression in plants offers benefit in terms of better growth and protection against stress conditions.

In this study, due to bacterial contamination after *Agrobacterium* transformation, putative transgenic plants could, however, only be obtained for the mutated inhibitor sequence. Putative transgenic plants showed expression of cysteine protease inhibitors in reverse zymograms and also a change in the protease profile of plants. However, when specific DNA primers were used for amplification of the inhibitor in genomic DNA of putative transgenic plants, no amplification product with the expected size was identified. Furthermore, when Western blotting was used to detect inhibitor expression in putative transgenic plants, no inhibitor expression was found in all putative transformed tobacco plants while a single band with the expected size of about 14 kDa corresponding to OC-I was found in OC-I expressing tobacco leaves. Since no transgenic tobacco plants were produced this experiment is currently

repeated.

Overall, this study allowed establishing the growth of nodulated soybean plants over a period of 14 weeks. In general, to obtain healthy fully grown soybean plants, growth during winter should be avoided under the conditions outlined in this thesis because it results in early flowering. In this study several parameters to measure soybean nodule performance during development were established. Further, the protease profile in nodules during development was identified with a high cysteine protease activity during nodule senescence. A first attempt has also been made to identify cysteine protease inhibitor expression during nodule development and putative bands indicating possible inhibitor expression have been identified by gel electrophoresis and Western Blotting. Unfortunately, an attempt to express a cysteine protease inhibitor in a transgenic tobacco plant was so far unsuccessful.

Future actions might include the repeat of the transformation experiment and to pay specific attention to control contamination and also the quality of explants (time of the year and plant age). Further, to identify the possible expression of cysteine protease inhibitor and also the technique of capturing and enriching the inhibitor during the nodule development via antibody-antigen precipitation in the presence of Protein A-Sepharose which binds to an rabbit antibody followed by SDS-PAGE and Western Blotting. A further suggested future action might include a more specific protease profiling using specific fluorescence-labelled substrates for the different types of proteases expressed during nodule development.

References

Abe, M. and Arai, S. (1991) Some properties of a cysteine proteinase inhibitors from corn endosperm. Agricultural and Biological Chemistry. 55, 2417-2418.

Abraham, Z., Martinez, M., Carbonero, P. and Diaz, I. (2006) Structural and functional diversity within the cystatin gene family of *Hordeum vulgare*. Journal of Experimental Botany. 57, 4245-4255.

Akunda, E. M. W. (2001) Improving food production by understanding the effect of intercropping and plant population on soybean nitrogen fixing attributes. Journal of Food Technology in Africa. 6, 110-115.

Alesandrini, F., Mathis, R., Van de Sype, G., Herouart, D. and Puppo, A. (2003) Possible roles for cysteine protease and hydrogen peroxide in soybean nodule development and senescence. New Phytologist. 158, 131-138.

Alesandrini, F., Pierre, F., Puppo, A. and Hérouart, D. (2003) Isolation of a molecular marker of soybean nodule senescence. Plant Physiology and Biochemistry. 41, 727-732.

Al-Karaki, G. N. (2000) Morphological and yield traits of wild legume (*Tetragonolobus palaestinus* Boiss) populations. Journal of Agronomy and Crop Science. 184, 267-270.

Andersson, B. and Aro, E. (1997) Proteolytic activities and proteases of plant chloroplasts. Physiologia Plantarum. 100, 780-793.

Appleby, C. A. (1992) The origin and functions of haemoglobin in plants. Science Progress. 76, 365-398.

Arredondo-Peter, R., Moran, F. J., Sarath, G. and Klucas, R. V. (1997) Analysis of *Vigna unguiculata* leghemoglobin genes. Revista Brasileira de Fisiologia Vegetal. 9, 143-149.

Ashraf, M. and Iram, A. (2005) Drought stress induced changes in some organic substances in nodules and other plant parts of two potential legumes differing in salt tolerance. Flora. 200, 535-546.

Asp, T., Bowra, S., Borg, S. and Holm, B. P. (2004) Molecular cloning, functional expression in *Escherichia coli* and enzymic characterization of a cysteine protease from white clover (*Trifolium repens).* Biochimica et Biophysica Acta. 1699, 111-122.

Beers, E. P., Woffenden, B. J. and Zhao, C. (2000) Plant proteolytic enzymes: possible roles during programmed cell death. Plant Molecular Biology. 44, 399-415.

Beringer, E. J., Brewin, N., Johnston, A. W., Schulman, H. M. and Hopwood, A. D. (1979) The *Rhizobium*-legume symbiosis. Proceedings of the Royal Society Biological Sciences. 204, 219-233.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72, 248-254.

Buchanan-Wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpur, S., Page, T. and Pink, D. (2003) The molecular analysis of leaf senescence - a genomics approach. Plant Biotechnology. 1 , 3-22.

Callis, J. (1995) Regulation of protein degradation. The Plant Cell. 7, 845-857.

Candace, J. W., Chan-Weiher, C. and Johnson, A. D. (2008) Isolation of a novel family of genes related to 2-oxoglutarate-dependent dioxygenases from soybean and analysis of their expression during root nodule senescence. Journal of Plant Physiology. 163, 1736-1744.

Chaffin, D. O. and Rubens, C. E. (1998) Blue /White screening of recombinant plasmids in Gram-positive bacteria by interruption of alkaline phosphatase gene *(phoZ)* expression. Gene. 291, 91-99.

Colebatch, G., Kloska, S., Trevaskis, B., Freund, S., Altmann, T. and Udvardi, M. K. (2002) Novel aspects of symbiotic nitrogen fixation uncovered by transcript profiling with cDNA arrays. Molecular Plant-Microbe Interaction. 15, 411-420.

Corre-Menguy, F., Cejudo, F. J., Mazubert, C., Vidal, J., Lelandais-Brière1, C., Torres, G., Rode, A. and Hartmann, C. (2002) Characterization of the expression of a wheat cystatin gene during caryopsis development. Plant Molecular Biology. 50, 687-698.

Coupe, A. S., Sinclair, B. K., Watson, L. M., Heyes, A. J. and Eason, J. R. (2003) Identification of dehydration-responsive cysteine proteases during post-harvest senescence of broccoli. Journal of Experimental Botany. 54, 1045-1056.

Crafts-Brandner, J. S., Hölzer, R. and Feller, U. (1998) Influence of nitrogen deficiency on senescence and the amounts of RNA and proteins in wheat leaves. Physiologia Plantarum. 102, 192-200.

Dangl, J. L., Dietrich, R. A. and Richberg, M. H. (1996) Death don't have no mercy: cell death programs in plant-microbe interactions. Plant Cell. 8, 1793-1807.

De María, N., De Felipe, M. R. and Fernández-Pascual, M. (2005) Alterations induced by glyphosate on lupin photosynthetic apparatus and nodule ultrastructure and some oxygen diffusion related proteins. Plant Physiology and Biochemistry. 43, 985-996.

De-Leo, F., Volpicella, M., Licciulli, F., Liuni, S., Gallerani, R. and Ceci, L. R. (2002) Plant-PIs: a database for plant protease inhibitors and their genes. Nucleic Acid Research. 30, 347-348.

Delgado, E., Mitchell, R. A. C., Parry, M. A. J., Driscoll, S. P., Mitchell, V. J. and Lawlor, D. W. (1994) Interacting effects of CO_2 concentration, temperature and nitrogen supply on the photosynthesis and composition of winter-wheat leaves. Plant, Cell and Environment. 17, 1205-1213.

Devienne-Barret, F., Justes, E., Machet, J. M. and Mary, B. (2000) Integrated control of nitrate uptake by crop growth rate and soil nitrate availability under field conditions. Annals of Botany. 86, 995-1005.

Ehrhardt, D. W., Atkinson, E. M. and Long, R. S. (1992) Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science. 256, 998-1000.

Escuredo, P. R., Minchin, F. R., Gogorcena, Y., Iturbe-Ormaetxe, I., Klucas, R. V. and Becana, M. (1996) Involvement of activated oxygen in nitrate-induced senescence of pea root nodules. Plant Physiology. 110, 1187-1195.

Espinosa-Victoria, D., Vance, C. P. and Graham, P. H. (2000) Host variation in traits associated with crown nodule senescence in soybean. Crop Science. 40, 103-109.

Espinosa-Victoria1, D. and Graham, H. P. (2001) Accumulation of coumestrol, daidzein, and genistein is not related to soybean crown nodules senescence. Terra. 19, 345-351.

Estelle, M. (2001) Proteases and cellular regulation in plants. Current Opinion in Plant Biology. 4, 254-260.

Ferguson, B. J. and Mathesius, U. (2003) Signaling interactions during nodule development. Journal of Plant Growth Regulation. 22, 47-72.

Fernandez-Pascual, M., De Lorenzo, C., De Felipe, M. R., Rajalakshmi, S., Gordan, A. J., Thomas, B. J. and Minchini, F. R. (1996) The possible reasons for relative salt stress tolerance in nodules of white lupin cv. Multolupa. Journal of Experinmental Botany. 47, 1709-16.

Gepstein, S., Sabehi, G., Carp, M-J., Hajouj, T., Nesher, M. F. O., Yariv, I., Dor, C. and Bassani, M. (2003) Large scale identification of leaf associated genes. The Plant Journal. 36, 629-642.

Gogorcena, Y., Gordon, A. J., Escuredo, P. R., Michini, F. R., Witty, J. F., Moran, J. F. and Becana, M. (1997) N_2 fixation, carbon metabolism, and oxidative damage in nodules of dark-stressed common bean plants. Plant Physiology. 11 3, 1193-1201.

Gopalan, S., Wei,W. and He, S,Y. (1996) *hrp* gene induction of *HN1*, a plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signals. Plant Journal. 10, 591-600.

Gunther, C., Schlereth, A., Udvardi, M. and Ott, T. (2007) Metabolism of reactive oxygen species is attenuated in leghemoglobin-deficient nodules of *Lotus japonicus*. Molecular Plant Microbe Interactions. 20, 1596-1603.

Gutiérrez-Campos, R., Torres-Acosta, J. A., Pérez-Martínez, J. D. and Gómez-Lim, M. A. (2001) Pleiotropic effects in transgenic tobacco plants expressing oryzacystatin I gene. Hortscience. 36, 118-119.

Habib, H. and Fazili, K. M. (2007) Plant protease inhibitors: a defense strategy in plants. Biotechnology and Molecular Biology Reviews. 2, 068-085.

Hensel, L. L., Grbic, V., Baumgarten, D. A. and Bleecker, A. B. (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis*. Plant Cell. 5, 553-564.

Hernandez-Jimenez, M. J., Lucas, M. M. and De Fellipe, M. R. (2002) Antioxidant defence and damage in senescing lupin nodules. Plant Physiology and Biochemistry. 40, 645-657.

Hong, J. K., Hwang, J. E., Lim, C. J., Yang, K. A., Jin, Z-L., Kim., C. Y., Koo, J. C., Chung, W. S., Lee, K. O., Lee, S. Y., Cho, M. J. and Lim, C. O. (2007) Over-expression of Chines cabbage phytocystatin 1 retards seed germination in *Arabidopsis*. Plant Science. 172, 556-563.

Huffaker, R. C. (1990) Proteolytic activity during senescence of plants. New Phytologist. 116, 199-231.

Irving, H. R., Boukli, N. M., Kelly, M. N. and Broughton, W. J. (2000) Nod factors in symbiotic development of root hairs. In Cell and Molecular Biology (eds), Springer, Tokyo, 241-265.

Johnson, R., Narvaez, J., An, G. and Ryan, C. (1989) Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. Proceedings of the National Academy of Sciences.86, 9871-9875.

Kardailsky, I. V. and Brewin, N. J. (1996) Expression of cysteine protease genes in pea nodule development and senescence. Molecular Plant Microbe Interactions. 9, 689-695.

Kiggundu, A., Pillay, M., Viljoen, A., Gold, C. Tushemereirwe, W. and Kunert, K. (2003) Enhancing banana weevil (*Cosmopolites sordidus*) resistance by plant genetic modification: A perspective Modulating the proteinase inhibitory profile of plant cystatin by a single mutation at positively selected amino acid sites. African Journal of Biotechnology. 2, 563-569.

King, C. A. and Purcell, L. C. (2001) Soybean nodule size and relationship to nitrogen fixation response to water deficit. Crop Science. 41, 1099-1107.

Klucas, R. (1974) Studies on soybean nodule senescence. Plant Physiology. 54, 612-616.

Laskowski, J. M. and Qasim, M. A. (2000). What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes: Biochemica Biophysica. Acta. 1477, 324 -337.

Lea, P. J. and Miflin, B. J. (1980) Transport and metabolism of asparagine and other nitrogen compounds within the plant. In PK Stumpf and EE Conn (eds), The Biochemistry of Plants. 5, Academic Press, New York , 569-607.

Lee, H., Hur, C. G., Oh, C. J., Kim, B. H., Park, S. Y. and An, C. S. (2004) Analysis of the root nodule-enhanced transcriptome in soybean. Molecules and Cells. 18, 53-62.

Li, Y., Zhou, L., Li, Y., Chen, D., Tan, X., Lei, L. and Zhou, J. (2008) A nodule-specific plant cysteine proteinase, AsNODF32, is involved in nodule senescence and nitrogen fixation activity of the green manure legume *Astragalus sinicus*. New Phytologist. 180, 185-192.

Lievens, S., Goormachtig, S. and Holsters, M. (2004) Nodule-enhanced protease inhibitor gene: emerging patterns of gene expression in nodule development on S*esbania rostrata.* Journal of Experimental Botany. 55, 89-97.

Lim, P. O., Woo, H. R. and Nam, H. G. (2003) Molecular genetics of leaf senescence in *Arabidopsis* . Trends in Science. 8, 272-278.

Lodwig, E. M., Hosie, A. H., Bourdes, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, L. A. and Poole, P. S. (2003) Amino acid cycling drives nitrogen fixation in the legume -*Rhizobium* symbiosis. Nature. 422, 722-726.

Lohman, K. N., Gan, S., John, M. C. and Amasino, R. M. (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. Physiologia Plantarum. 92, 322-328.

Long, R. S. (1989) *Rhizobium*-legume nodulation: life together in the underground. Cell. 56, 3203-3214.

Long, R. S. (2001) Genes and signals in the *Rhizobium*-legume symbiosis. Plant Physiology. 125, 69-72.

Malik, N. S. A., Pfeiffer, N. E., Williams, D. R. and Wagner, F. W. (1981) Peptidohydrolases of soybean root nodules. Identification, separation and partial characterization of enzymes from bacteroid-free extracts. Plant Physiology. 68, 386-392.

Manen, J. F., Simon, P., Van Slooten, J. C., Osteras, M., Frutiger, S. and Huhges, G. J. (1991) A nodulin specifically expressed in senescent nodules of winged bean is a protease inhibitor. Plant Cell. 3, 259-270.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning, a Laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory)

Martinez, D. E., Bartoli, C. G., Grbic, V. and Guiamet, J. J. (2007) Vacuolar cysteine proteases of wheat (*Triticum aestivum L.*) are common to leaf senescence induced by different factors. Journal of Experimental Botany. 58 1099-1107.

Martindale, W. and Leegood, R. C. (1997) Acclimation of photosynthesis to low temperature in *Spinacia oleracea* L. II. Effects of nitrogen supply. Journal of Experimental Botany. 48, 1873-1880.

Massonneau, A., Condamine, P., Wisniewski, J. P., Zivy, M. and Rogowsky, P-M. (2005) Maize cystatins respond to developmental cues, cold stress and drought. Biochimica et Biophysica Acta. 1729, 186-199.

Matamoros, M. A., Baird, L. M., Escuredo, P. R., Dalton, D. A., Minchin F. R., Iturbe-Ormaetxe, I., Rubio, M. C., Moran, J. F., Gordon, A. J. and Becana, M. J. (1999) Stress induced legume root nodule senescence, physiological, biochemical, and structural alteration. Plant Physiology. 121, 97-111.

Michaud, D., Bernier-Vadnais, N., Overney, S. and Yelle, S. (1995) Constitutive expression of digestive cysteine proteinase forms during development of the Colorado potato beetle, Leptinotarsa decemlineata say (Coleoptera: Chrysomelidae). Insect Biochemistry Molecular and Bioliogy. 25, 1041-1048.

Mitra, R. M., Shaw, L. S. and Long, R. S. (2004) Six nonnodulating plant mutants defective for Nod factor-induced transcriptional changes associated with the legume-*Rhizobia* symbiosis. Proceedings of the National Academy of Sciences. 101, 10217-10222.

Mosolov, V. V. and Valueva, T. A. (2005) Proteinase inhibitors and their function in plants: a review. Applied Biochemistry and Microbiology. 41, 227-246.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiology. 15, 473-497.

Naito, Y., Futjie, M., Usami, S., Murooka, Y. and Yamada, T. (2000) The involvement of a cysteine proteinase in the nodule development in Chinese milk vetch infected with *Mesorhizobium huakuii* subsp. *rengei*. Plant Physiology. 124, 1087-1095.

Nooden, L. D., Guiamet, J. J. and John, I. (1997) Senescence mechanisms. Physiologia Plantarum. 101, 746-753.

Oke, V. and Long, R. S. (1999) Bacteroid formation in the *Rhizobium*-legume symbiosis. Current Opinion Microbiology. 2, 641-646.

Oliveira, A. S., Xavier-Filho, J. X. and Sales, M. (2003) Cysteine proteainases and Cystatins. Brazilian of Archives Biology and Technology. 46, 91-104.

Pennell, R. and Lamb, C. (1997) Programmed cell death in plants. American Society of Plant Physiologists. 9, 1157-1168.

Pereira, P. A. D., Miranda, B. D., Attewell, J. R., Kmiecik, K. A.and Bliss, F. A. (1993) Selection for increased nodule number in common bean *(Phaseolus vulgaris L.)*. Plant and Soil. 148, 203-209.

Pfeiffer, N. E., Torres, C. M., and Wagner, F. W. (1983) Proteolytic activity in soybean root nodules. Plant Physiology. 71, 797-802.

Pladys, D. and Vance, C. P. (1993) Proteolysis during development and senescence of effective and plant gene-controlled ineffective alfalfa Nodules. Plant Physiology. 103, 379-384.

Pladys, D., Dimitrijevic, L. and Rigaud, J. (1991) Localization of a protease in protoplast preparations in infected cells of French bean nodules. Plant Physiology. 97, 1174-1180.

Puppo, A., Groten, K., Bastain, F., Carzaniga, R., Soussi, M., Lucas, M. M., De Felipe, M. R. Harrison J., Vanacker, H. and Foyer, C. H. (2005) Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process. New Phytologist. 165, 683-701.

Rawling, N. D. and Barret, A. J. (1994) Families of cysteine peptidases. Methods Enzymology. 244, 461-486.

Rawlings, N. D., Tolle, D. P. and Barrett, A. J. (2004). Evolutionary families of peptidase inhibitors. Biochemical Journal. 378, 705-716.

Richardson, M. (1991) Seed storage proteins: the enzyme inhibitors. Methods in Plant Biochemistry. 5, 259-305.

Richardson, M. (1991) Seed storage proteins: the enzyme inhibitors. Methods in Plant Biochemistry. 5, 259-305.

Salas, C. E., Gomes, M. T. R., Hernandez, M. and Lopes, M. T. P. (2008) Plant cysteine proteinases: evaluation of the pharmacological activity. Phytochemistry. 69, 2263-2269.

Schaller A. (2004) A cut above the rest: the regulatory function of plant proteases. Planta. 220, 183-197.

Scheible, W-R., Lauerer, M., Schulze, E. D., Caboche, M. and Stitt, M. (1997) Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco. The Plant Journal. 11, 671-691.

Schultze, M. and Kondorosi, A. (1998) Regulation of symbiotic root nodule development. Annual Review of Genetics. 32, 33-57.

Solomon, M., Belenghi, B., Delledonne, M., Menachem, E. and 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.

Stougaard, J. (2000) Regulators and regulation of legume root nodule development. Plant Physiology. 124, 531-540.

Sugiharto, B. and Sugiyama, T. (1992) Effects of nitrate and ammonium on gene expression of phosphoenolpyruvate carboxylase and nitrogen metabolism in maize leaf tissue during recovery from nitrogen stress. Plant Physiology. 98, 1403-1408.

Swaraj K., Dhandi, S. and Sheokand, S. (1995) Relationship between defense mechanism against activated oxygen species and nodule functioning with progress in plant and nodule development in *Cajanus cajan L.* Millsp. Plant Science. 112, 65-74.

Swaraj, K. and Bishnoi, N. R. (1996) Physiological and biochemical basis of nodule senescence in legumes: a review. Plant Physiology and Biochemistry. 23, 105-116.

Timmers, A. C. J., Soupene, E., AuriacM., C., De Billy, F., Vasse, J., Boistard, P. and Truchet, G. (2000) Saprophytic intracellular *Rhizobia* in alfalfa nodules. Molecular Plant-Microbe Interactions. 13, 1204-1213.

Trexler, M., Banyai, L. and Patthy, L. (2001). A human protein containing multiple types of protease-inhibitory modules. PNAS. 98, 3705-3709.

Valpuesta, V., Lange, N. E., Guerrero, C. and Reid, M. S. (1995) Up-regulation of a cysteine protease accompanies the ethylene-insensitive senescence of daylily (*Hemerocallis*) flowers. Plant Molecular Biology. 28, 575-582.

Valueva, A. T. and Mosolov, V. V. (1999) Protein inhibitors of the proteinases in seeds: 1 classification, distribution, structure and properties. Russian Journal of Plant Physiology. 46, 362-378.

Van de Velde, W., Guerra, J. C. P., Guerra, J. C. P, De Keyser, A., De Rycke, R., Rombauts, S., Maunoury, N., Mergaert, P., Kondorosi, E., Holsters, M. and Goormachtig, S. (2006) Aging in legume symbiosis: a molecular view on nodule senescence in *Medicago truncatula.* Plant Physiology. 141, 711-720.

Van der Hoom, R, A, L. and Jones, J, D, G. (2004) The plant proteolytic machinery and its role in defence. Current Opinion in Plant Biology. 7, 400-407.

Van der Vyver , C., Schneidereit, J., Driscoll, S., Turner, J., Kunert, K. and Foyer, C. H. (2003) Oryzacystatin I expression in transformed tobacco produces a conditional growth phenotype and enhances chilling tolerance. Plant Biotechnology Journal. 1, 101-112.

Vance, C. P., Heichel, G. H., Barnes, D. K., Bryan, J. W. and Johnson, L. E. (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa *(Medicago sativa* L.) following harvest. Plant Physiology. 64, 1-8.

Vance, C. P., Reibach, H. P. and Ellis, R. W. (1986) Proteolytic enzymes of legume nodules and their possible role during nodule senescence. In: Plant Proteolytic Enzymes. Vol 2, M.J. Dalling, Boca Raton: CRC Press. 103-124.

Vincent, J. L., Knox, M. R., Ellis T. H. N., Kaló, P., Kiss, B. G. and Brewin, N. J. (2000) Nodule expressed Cyp15a cysteine protease genes map to syntenic genome regions in *Pisum and Medicago* spp. Molecular Plant Microbe Interactions. 13, 715-723.

Waldron, C., Wegrich, L. M. P., Merlo, A. O. and Walsh, T. A. (1993) Characterization of a genomic sequence coding for potato multicystatin, an eight domain cysteine proteinase inhibitor. Plant Molecular Biology. 23, 801-812.

Walsh, T. A. and Strickland, J. A. (1993) Proteolysis of the 85-Kilodalton crystalline cysteine proteinase inhibitor from potato release functional cystatin domains. Plant Physiology. 103, 1227-1234.

Wesley, T. L., Lamond, R. E, Martin, V. L. and Duncan, S. R. (1998) Effects of late-season nitrogen fertilization on irrigated soybean yield and composition. Journal of Production Agriculture. 11, 331-336.

Wolfson, J. L. and Murdock, L. L. (1990) Growth of *Manduca sexta* on wounded tomato plants: role of induced proteinase inhibitors. Entomologia Experimentalis et Applicata. 154, 257-264.

Yang, W. C., Katinakis, P., Hendriks, P., Smolders, A., De Vries, F., Spee, J., Van Kammen, A., Bisseling, T. and Franssen, H. (1993) Characterization of GmENOD40, a gene showing novel pattern of cell specific expression during soybean nodule development. The Plant Journal. 3, 573-583.

Zimmerman, P. and Zentraf, U. (2005) The correlation between oxidative stress and leaf senescence during plant development. Cellular and Molecular Biology Letters. 10, 515-534.