PROTEOMIC APPROACHES TO PROFILING OF CYSTEINE PROTEASES EXPRESSED IN LEAVES AND ROOT NODULES DURING NATURAL SENESCENCE OF THE SOYBEAN PLANT

by

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RESEARCH DISSERTATION
Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOCHEMISTRY

in the

FACULTY OF SCIENCE& AGRICULTURE
(School of Molecular &Life Sciences)

at the

UNIVERSITY OF LIMPOPO

SUPERVISOR: Prof I. Ncube

2011

DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree of Master of Science in Biochemistry has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

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ACKNOWLEDGEMENTS

I owe my deepest gratitude to my supervisor, Prof Ignatious Ncube, whose encouragement, tenacity, perseverance, guidance and support from the initial to the final level enabled me to develop an understanding of the research project. Your patience, interest, constructive comments and useful suggestions enabled me to complete this thesis.

I am greatly indebted to my lab colleagues Reagile, Thembekile, Emmanuel, Gloria and Tipson, who supported me every step of the way. Thank you guys for being so kind and willing to share ideas.

It gives me great pleasure to thank Prof Karl Kurnet of Forestry and Agricultural Biotechnology Institute for useful advice towards the project and those who made this thesis possible such as my family and all the people who stood by me giving me the moral support I needed.

I would like to express gratitude to:
The Royal Society and National Research Foundation (NRF) project for providing me with a scholarship, the Flemish Interuniversity Council (VLIR-UOS) Own Initiative project and VLIR-IUC project for funding the research.

Many thanks also to the whole Department of Biochemistry, Microbiology and Biotechnology for the assistance in the form of advice or suggestions.

Lastly, I would like to acknowledge the Almighty God for the guidance and wisdom he has provided me for with God nothing is impossible

DEDICATION

This thesis is dedicated to my entire family, my mother, Sinodia and my father, Lovemore who have supported me all the way, my sisters, Tsitsi and Christina, who have always had faith in me and my brothers Gashirai and Kudzai who stood by me even when all hope was lost.

ABSTRACT

Soybean is one of the most cultivated legume plants in developing countries. Nodule senescence is a major limitation in producing high yields of soybean as it coincides with the pod filling stage. Delaying nodule senescence could be a way of increasing the yield of soybean therefore determination of the role of cysteine protease in soybean is of vital importance. In this study, soybean plants were grown under controlled temperature and light conditions. Leaves and root crown nodules were collected at 4, 6, 10, 12 and 16 weeks of age. In a comparative 1-dimensional SDS-PAGE analysis of soybean nodule proteomes as the plant matured, it showed differences in proteins expressed as shown by different banding patterns with less variation between the younger soybean nodule extracts (4, 6 and 10 weeks old) as compared to the older ones (12 and 16 weeks old). As determined by azocasein assay and protease zymography, the protease activity of the nodule extracts generally decreased with an increase in the age of the nodules whereas that of the leaves increased as the plants grew older. Cysteine proteases in the soybean nodule extracts readily cleaved the Z-Arg-Arg-AMC substrate with the highest activity shown in the younger nodules as compared to the older ones. In the leaf extracts, cysteine protease activity increased with age of the leaves. DCG-04, a biotinylated irreversible inhibitor, proved to be an effective label in profiling of activity of cysteine proteases in 1-dimensional and 2-dimensional systems. The labelling was inhibited specifically by cysteine protease inhibitor, E-64. In root nodules, the DCG-04 probing demonstrated that the expression of cysteine proteases is higher in early stages of development of the soybean nodules as compared to the later stages whereas in the leaves, there is higher expression of cysteine proteases in the old leaves (16 weeks). Using 2-dimensional polyacrylamide gel electrophoresis, five cysteine protease isoforms were visualised with the size ranging from approximately 25 to 30 kDa and a pI range of 4-6. In older nodules (12 and 16 weeks old) the higher pI isoforms are down-regulated with the 26 kDa and pI 4.5 protease being the predominant isoform. Affinity precipitation of the cysteine proteases yielded a strong band with the size of about 26 kDa. All assays used show that while in leaves, the expected trend of high expression of cysteine proteases in senescing leaves is observed, in soybean nodules the expression of cysteine proteases decreases with senescence. There is, therefore, no correlation between senescence and cysteine proteases in nodules. The highly expressed cysteine protease in young nodules could play a developmental or regulatory role during the early stages of development.

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BCA	Bicinchoninic acid
BSA	Albumin bovine serum
DMSO	Dimethyl sulfoxide
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBST	tris buffered saline with tween 20
LDS	Lithium dodecyl sulphate
DTT	Dithiotreitol
TEMED	N,N,N',N'-Tetramethyl-ethylendiamine
SDS	Sodium dodecyl sulphate
CHAPS	3-[(Cholamidopropyl)dimethylammonio]-1-propanesulfonate
APS	Ammonium persulphate
DCG-04	L-Lysinamide,N-[[(2S,3S)-3-(ethoxycarbonyl)-2-oxiranyl]carbonyl]-L-leucyl-L-tyrosyl-6-aminohexanoyl-N6-[5-[(3aS,4S,6aR)-hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl]-1-oxopentyl]-
E-64	*Trans*-(Epoxysuccinyl)-L-leucylamino-4-guanidinobutane
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
TCA	Trichloroacetic acid
Z-Arg-Arg-AMC	Z-L-Arginyl-L-Arginine-7-Amido-4-Methyl-Coumarin
HRP	Horse radish peroxidise

CHAPTER 1

1 INTRODUCTION

1.1 Background information

Developing countries are facing malnutrition challenges due to lack of protein rich food. There is an increase in demand for protein rich food in these countries due to the ever increasing population and scarcity of fertile lands (Sridhar and Seena, 2006). Leguminous plants are the third largest group of higher plants with over 20,000 species which provide about one third of all dietary protein nitrogen and one third of processed vegetable oil for human consumption (Graham and Vance, 2003). Legumes are also known to have desirable characteristics such as abundance of carbohydrates, ability to lower the serum cholesterol, high fibre, low fat (except for oilseeds) and high concentration of polyunsaturated fatty acids (Sridhar and Seena, 2006).

Soybean has proven to be the most cultivated legume plant in most developing countries (Mateos-Aparicio *et al*., 2008). It falls in one of the largest subfamilies of legume plants known as *Faboideae.* The interest in this legume has increased because of its functional components (Mateos-Aparicio *et al*., 2008). Soybean provides significant amounts of fatty acids and proteins for human and animal nutrition. Soybean also has non-food uses, for example in producing industrial feed stocks and combustible fuels. Several studies have correlated consumption of soy products with high antioxidant activity, cholesterol reduction and prevention of chronic diseases and cancer (Lee *et al*., 2011). Soybean also has the ability to undergo a symbiotic interaction with the soil microorganism, *Bradyrhizobium japonicum*, to form a nitrogen-fixing symbiosis which takes place in tumour-like growths, referred to as nodules, on the roots of the plant (Komatsu *et al*., 2009). About 70% of the potential yield is lost as a result of unfavourable physiochemical environments, even in developed agricultural systems (Komatsu *et al*., 2009). To meet

these challenges some biochemical processes like nodule and leaf senescence of the plant need to be controlled during growth of the plant.

Senescence is one of the main factors that contribute to poor yield of the soybean plant (Puppo *et al*., 2004). Factors that lead to senescence have received relatively little attention (Puppo *et al*., 2004). Accumulating evidence suggests that proteases contribute to the establishment of symbiosis and senescence (Puppo *et al*., 2004). Senescence has a tremendous impact on agriculture. For example, nodule senescence limits crop yield and biomass production, and contributes substantially to post harvest loss in vegetable and ornamental crops during transportation, storage and on shelves (Gan, 2007). Delaying the onset or reducing the rate of nodule senescence may be one means of increasing crop productivity. Several studies on soybean leaf senescence have shown an inverse relationship between grain yield and delayed leaf senescence, suggesting that maximum yields can only be achieved in plants whose leaves senesce during pod-filling (Araujo *et al*., 2007). However, before senescence can be controlled, it must first be characterized.

The activity of proteolytic enzymes is an essential element of senescence (Wagstaff *et al*., 2002). The most commonly identified proteolytic enzymes are the cysteine proteases (Esterbian-Garcia *et al*., 2010). Cysteine proteases are comprised of a number of different classes, but the majority of senescence-associated cysteine proteases behave as typical papain-family members (Granell *et al*., 1998). Cysteine proteases are involved in almost all aspects of plant growth and development including germination, circadian rhythms, senescence and programmed cell death. They are also involved in mediating plant cell responses to environmental stress (such as water stress, salinity, low temperature, wounding, ethylene, and oxidative conditions) and plant-microbe interactions (including nodulation) (Shoekand and Brewin, 2003).

A lot of genes encoding cysteine proteases have been isolated and characterized during the senescence process. mRNA levels of two cysteine proteases related genes, SAG2 and SAG12, were found to be significantly higher in degreening tissues than in green tissues during senescence of *Arabidopsis* leaves, and were thought to be involved in the

progression of senescence in somatic tissues (Lohoman *et al*., 1994). PsCyp15a, a gene that encodes a vacuolar cysteine protease was also found to be expressed in wilt-induced shoots of *Pisum sativum* (Pea) and in root nodules (Vincent and Brewin, 2000). Research on transcripts encoding cysteine proteases in tomato SENU2 and SENU3 (Drake *et al*., 1999), in sweet potato SPG31 (Chen *et al*., 2002), in brinjaul SmCP (Xu and Chye, 1999) and in lettuce LsCP2 (Klosterman *et al*., 2011) has shown an up regulation during leaf senescence. Cysteine proteases play an important role in proteolysis and nitrogen remobilization during the senescence process. Analysis of the protein degradation and determination of function of cysteine proteases that increase during senescence is one strategy that can be used to increase understanding of this complex developmental stage.

1.2 Motivation of study

Soybean is a very important source of protein and oil in the whole world. Researchers have long sought to improve and optimize pod filling in the soybean plant which is mainly affected by stress causing poor yields. Nodule senescence is one the main causes of poor yields because it happens during the pod filling phase. There has been accumulating evidence which reveals the involvement of cysteine proteases in nodule senescence. It is therefore, very important to conduct more research on soybean and activity of cysteine proteases during development of the plant so as to pave a way to increase the yield of the crop.

1.3 Aims and objectives

The main aim of this study is to profile and isolate cysteine proteases in root nodules during natural senescence of the soybean plant in comparison with the leaves.

In order to achieve the main aim of the study, the following objectives had to be met:

- Growth of soybean plants under controlled conditions and collection of leaves and root crown nodules

- Preparation of sample extracts and determination of total protein content by the BCA assay
- Measurement of the proteolytic activity of the cysteine proteases
- Separation of proteins by polyacrylamide gel electrophoresis (1-dimensional and 2-dimensional)
- Zymography of cysteine proteases
- DCG-04 Probing for cysteine proteases
- Isolation of cysteine proteases

1.4 Significance of the study

Early nodule senescence has been a major limitation in agriculture in the production of high yields of soybean. Several studies have related to the involvement of cysteine proteases in this process. The current study is therefore intended to identify the roles played by cysteine proteases specifically in the soybean plant as it undergoes nodule senescence. Creating a better understanding of the roles played by cysteine proteases will allow delaying of the senescence process by either deleting the cysteine protease genes or co-expressing cystatins which will in turn inhibit the activity of cysteine proteases. Knowledge of the cysteine protease profiles in plants, mainly food crops, will also enable the selection of varieties of plants that have suitable cysteine protease profiles thereby reducing the chances of a poor yield. Because cysteine proteases have a central importance in plant physiology, they could serve as important targets for the study of nodule development and functioning at the molecular level. And also because of their widespread occurrence in nodulating plants they could also serve as candidate genes for targeted plant breeding programmes.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Legumes

Legumes are the third largest family of higher plants with over 20,000 species. They are second to cereal crops in agricultural importance based on area harvested and total production (Doyle and Luckow, 2003). Most legumes have seed pods that are seamed on two sides. The seeds are arranged in a single row and attached to the pod along one seam. The pods are extremely variable, ranging from small single-seeded types to woody pods a metre long. The pods of most legumes are dehiscent i.e they split along both seams when ripe. Others have indehiscent pods, called loments that are constricted between the seeds. These split into single seed segments when ripe. Others have a single seed in a winged pod and are dispersed by wind (Graham and Vance, 2003). The taxonomic classification of legumes is *Leguminosae***,** which can be further divided into three sub-families, *Faboideae*, *Mimosoideae* and *Caesalpinioideae*.

2.1.1 Uses of legume plants

Grain legumes provide about one third of all dietary protein nitrogen and one third of processed vegetable oil for human consumption (Graham and Vance, 2003). Seeds of grain legumes contain at least 20% to 40% of protein. In many places of the world, legumes complement cereals or root crops, the primary source of carbohydrates, in terms of amino acid composition. Because of their nitrogen-fixing abilities, legumes are used to help stabilize the soil and prevent erosion and also as green manure, a natural fertilizer (Doyle and Luckow, 2003). Legumes are also an important food crop for other animals such as chicken, sheep and cattle. They are generally low in fat and high in protein, and contain important micronutrients such as folate, potassium, iron and magnesium (Doyle and Luckow, 2003). Many legumes are used extensively as shade trees or for ornamental plants because of their attractive foliage or flowers. Some are fast growing and used as a

source of timber, charcoal and wood pulp. Others, including numerous Australian wattles, provide attractive wood for timber or ornamental woodwork. Many food additives, such as gums for thickeners like gum arabic, guar gum and tragacanth gum, are also derived from legumes. Soybean derivatives are used extensively in processed foods such as soybean lecithin. Other uses for legumes include production of insecticides like rotenone, dyes like the indigo dye and also medicines like phytoestrogens (Doyle and Luckow, 2003).

Legumes are highly nutritious. They are a staple food all over the world and are one of the best sources of soluble fibre. The soluble fibre helps lower levels of damaging low density lipoprotein (LDL) cholesterol in the blood, thus lowering heart-disease risk (Messina *et al*., 1999). Researchers have observed that eating legumes four times a week can reduce the risk of coronary heart disease by 22% (Jenkins *et al*., 2003). The legumes' contribution to heart health lies not just in their fibre, but in the significant amounts of antioxidants and essential minerals like iron, copper, folic acid, vitamin B6, and magnesium (Trinidad *et al*., 2009). Grain legumes generally also have a hypoglycaemic effect, reducing the increase in blood glucose after a meal and, hence, blood insulin. Legumes are, therefore, included in the diet of insulin-dependent diabetics (Nevin and Young, 2005). In addition to that, legumes are low in fat and high in good quality protein they also provide health-promoting secondary compounds known as phyto-chemicals that can protect against human cancers and protect the plant against the onslaught of pathogens and pests (Gepts *et al*., 2005).

2.1.2 Soybean *(Glycine max)* as a legume plant

Soybean is a legume plant from the *Faboideae* subfamily. This is the largest subfamily with over 12,000 species which include peas, sugar beans, alfalfa and peanuts. The soybean plant is characterized by a flower that has one large creased petal (called the banner or standard), two side petals (wings), and two bottom petals joined to form a boat shaped structure (keel). The growth of a soybean plant varies, it grows from a height of

20 cm to 2 metres (Doyle and Luckow, 2003). The pods, stems, and leaves are covered with fine brown or gray hairs and the leaves have 3 to 4 leaflets per leaf. The leaflets are 6–15 cm long and 2–7 cm wide. The leaves fall before the seeds are mature. The fruit is a hairy pod that grows in clusters of 3–5, each pod is 3–8 cm long and usually contains 2–4 seeds (Figure 1).

Figure 1. A mature soybean plant (Doyle and Luckow, 2003).

2.1.3 Importance of soybean

Soybean is mainly used as a source of bean sprouts, milk, soy sauces, flour and cooking oil (Duke, 1983). Soybean seeds are used to make texturised vegetable protein and soybean oil which is low in saturated fat and high in polyunsaturated fatty acids. The oil is ideal to use when making margarine, salad dressing, beverage powders, cheese spreads, ice creams and coffee creamers. Soy oil cakes, made from the fibrous residue left over after the beans have been crushed for oil extraction provide rich protein feed for cattle, sheep, pigs and poultry (Lee *et al.*, 2011). Soybean is also used as a rotational plant to fertilize the soil because of its ability to fix nitrogen due to the symbiotic interaction with the soil microorganism, *Bradyrhizobium japonicum*, to form a nitrogen-fixing symbiosis. In industries when the by-products of soy are processed, they can be used in the manufacturing of plastics, shoes, floor tiles, cosmetics, paints, electrical insulation and soap (Hymowitz, 1990).

The increasing popularity of soy foods is mainly attributed to the large amount of health benefits associated with the use of soybeans. Soybeans potentially have multifaceted health-promoting effects, including cholesterol reduction, improved vascular health, preserved bone mineral density, reduction of menopausal symptoms (Anderson *et al*., 1999), anti-carcinogenic effects, and protective effects against diabetes, irritants of the digestive tract, and kidney diseases (Friedman *et al*., 2001). Soy foods have high protein content, vitamins, minerals and fibres. The interest in this legume has increased because of its great amount of dietary fibre and its high isoflavone content which makes it singular among other legumes (Mateos-Aparicio *et al*., 2008).

2.2 Biochemical processes in soybean plants

2.2.1 Nitrogen Fixation

Biological nitrogen fixation is the process that changes inert N_2 to biologically useful NH_3. Legume nitrogen fixation starts with the formation of a nodule. A common soil bacterium, *rhizobium*, invades the root and multiplies within the cortex cells. A lot of research has been done to investigate the interaction between *rhizobia* and leguminous plants (Cullimore *et al*., 2001). This interaction leads to the formation of nodules on the root. There is extensive signal exchange between the partners as early signals secreted by *rhizobia*, called nod factors indicate the ability to nodulate the particular plant species. After a series of coordinated events, *rhizobium* cells are taken up by the plant cells where they differentiate into structures called bacteroids which have two membranes one from the plant and the other from the *rhizobium* (Ackay and Roughgarden, 2007). More work has been done which suggests that nitrogen fixation is dependent upon continuous shuttling of amino acids in and out of the bacteroids (Ackay and Roughgarden, 2007). The legume-*rhizobium* symbiosis is a finely regulated process that involves significant carbon and energy metabolism by the plant.

The plant supplies all the necessary nutrients and energy for the bacteria. If legumes are planted with an inoculum, within a week after infection, small nodules are visible with the naked eye. In the field, small nodules can be seen 2-3 weeks after planting, depending on legume species and germination conditions. When nodules are young and not yet fixing nitrogen, they are usually white or gray inside. As nodules grow in size, they gradually turn pink or reddish in colour, indicating nitrogen fixation has started. The pink or red colour is caused by leghemoglobin (similar to hemoglobin in blood). Leghemoglobin is a heme protein that buffers the concentration of free oxygen in the cytoplasm of infected plant cells to ensure the proper functioning of the oxygen sensitive nitrogenase enzyme that is responsible for fixation of the atmospheric nitrogen (Gunther *et al*., 2007). Legume nodules that are no longer fixing nitrogen usually turn green and may actually be discarded by the plant. Pink or red nodules should predominate on a

legume in the middle of the growing season. The plant must contribute a significant amount of energy in the form of photosynthesis derived sugars and other nutritional factors for the bacteria (Schuzle, 2004). Any stress that reduces plant activity will reduce nitrogen fixation. Factors like temperature and water may not be under the farmer control. But nutrition stress (especially phosphorus, potassium, zinc, iron, molybdenum and cobalt) can be corrected with fertilizers. When a nutritional stress is corrected, the legume responds directly to the nutrient and indirectly to the increased nitrogen nutrition resulting from enhanced nitrogen fixation. Poor nitrogen fixation in the field can be easily corrected by inoculation, fertilization, irrigation or other management practices (Lindemann and Glover 2008).

Soybean can also undergo symbiotic interaction with the soil microorganism, *Bradyrhizobium japonicum*, to form a nitrogen-fixing symbiosis. The structural organisation of soybean nodules is quite complex, composed of different zones and various cell types each with different functional attributes (Oehrle *et al.*, 2008). Determinant, or spherical nodules, such as those found on soybean do not have persistent meristems. Within each infected cell of determinate-type soybean nodules, the *B. japonicum* bacteroids are distributed within membrane envelopes called symbiosomes. The bacteroids never come into direct contact with the host cell cytoplasm as the symbiosome membrane acts as a selective permeability barrier for the transport of plant metabolites to the bacteroids and fixed nitrogen to the plant (Oehrle *et al*., 2008). A soybean plant may divert 20 to 30% of its photosynthesis derived sugars to the nodule instead of other plant functions when the nodule is actively fixing nitrogen (Lindemann and Glover 2008).

Nitrogen fixation by *rhizobia* is of great importance in agriculture in several ways. After harvesting, the legume roots left in the soil decay, releasing organic nitrogen compounds for uptake by the next generation of plants. Farmers take advantage of this natural fertilization by rotating a leguminous crop with a non leguminous one. Nitrogen fixation by natural means cuts down on the use of artificial fertilizers. This not only saves money but helps to prevent the many problems brought about by excessive use of commercial

nitrogen and ammonia fertilizers such as eutrophication of rivers and lakes, generation of acid rain, and overgrowth of agricultural land by non-food crops (Burdass, 2002).

2.2.2 Senescence in plants

Plant senescence is the aging in plants which involves structural, biochemical and molecular changes. Plants seem to have both unintended and programmed aging which is controlled by genetic, hormonal, chemical and environmental changes (Webb *et al*., 2008). It remains unclear how the process is initiated and can be regulated. Senescence in legumes has also been defined as an integral part of development of nitrogen fixing nodules. The process involves the expression of genes whose products are required to carry out senescence-related processes. Most of the genes expressed encode enzymes, which are involved in the degradation of proteins, nucleic acids, lipids, and polysaccharides, although genes that encode proteins involved in nutrient recycling, defence, transcriptional regulation, and signal transduction have also been isolated (Webb *et al*., 2008). In addition, proteins, antioxidants and other nutritional compounds are degraded during senescence. The main types of senescence are leaf and nodule senescence which take place almost at the same time involving common stages in both processes (Figure 2).

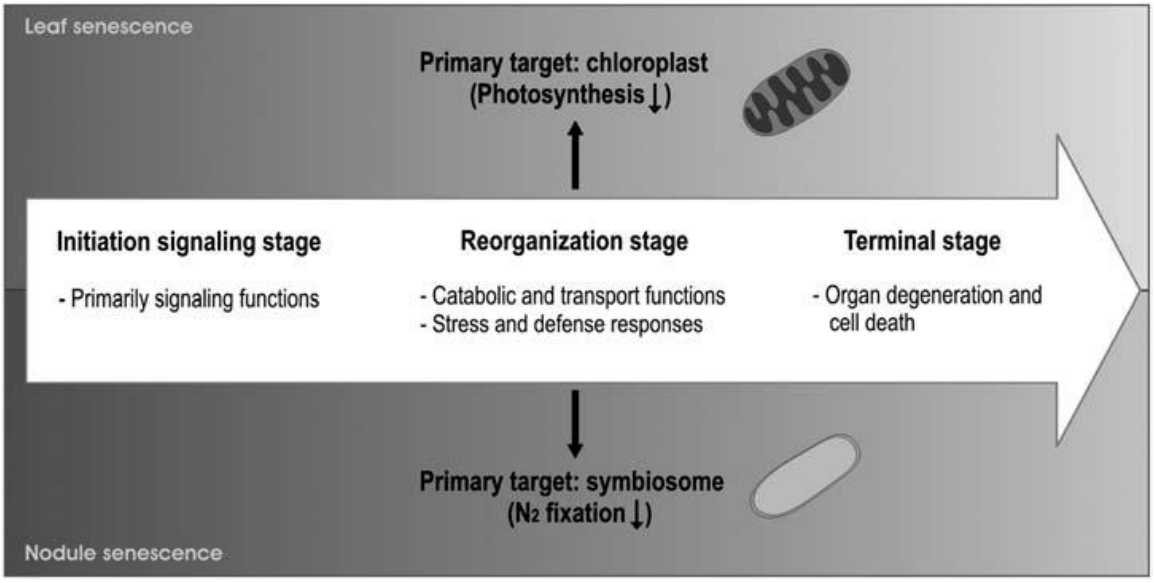

Figure 2. Schematic representation of common stages between nodule and leaf senescence (Van de Velde *et al*., 2006).

2.2.3 Nodule senescence

Although degenerative in nature, nodule senescence is an active process programmed in development of a plant. Nodules exhibit natural or induced aging which coincide with the drop in nitrogenase activity at the flowering period or at pod filling stage which greatly affects the grain field. Relatively little information is available concerning the physiological and biochemical events associated with root nodule senescence. Researchers have suggested that symbiotic nitrogen fixation declines during pod-filling as the result of an inadequate supply of photosynthetic products to nodules (Puppo *et al*., 2004). It has also been reported that nodule senescence could be a delayed reaction of the host plant against *rhizobia* establishment (Pladys and Vance, 1993). The process coincides with senescence of the whole plant and occurs naturally at the start of anthesis due to synthesis of signals generated in the shoot (Puppo *et al*., 2004). Soybean nodules result from a complex but orderly interaction between legume root cells and *Bradyrhizobium japonicum*. The interaction commences when *rhizobia* penetrate the epidermal cells of root hairs and terminate when remnants of the nodules are separated

from the roots. The amount of nitrogen fixed by nodules is a function of the central tissue volume and the active life of this tissue (Klucas, 1974).

Senescence in nodules is visible by a colour change in the nitrogen-fixing zone from pink, associated with the functional leghemoglobin protein, to green which is associated with the degradation of its heme-group. A distinctive characteristic of nodule senescence is the triggering of a wide range of proteolytic activities that cause large scale protein degradation (Pladys and Vance, 1993). The final outcome is death of both bacteroids and nodule cells. For many leguminous crops, nodule senescence has been found to coincide with pod filling and exogenous application of nitrogen during pod filling increases both yield and seed protein content (Puppo *et al*., 2004). Therefore extending the period of active nitrogen fixation by delaying the nodule senescence process might have a beneficial effect on crop yield and seed quality. The existence of soybean *(Glycine max)* varieties with delayed nodule senescence and a longer active nitrogen fixation period (Espinosa-Victoria *et al*., 2000) indicates genetic control of the onset of senescence, suggesting a molecular basis to modify timing and progression of developmental nodule senescence. Signals that trigger the formation of a senescence zone need to be identified to modulate the active lifespan of a nodule (Van de Velde *et al*., 2006).

2.2.4 Leaf senescence

Leaf senescence is the degradation of cell constituents in the leaf, which allows the plants to remobilize the materials released during this process from leaves to reproductive organs. The onset of leaf senescence can also be defined as the time at which apparent photosynthesis begins to decline (Lohoman *et al*., 1994). Leaf senescence can be genetically controlled to occur at a given time in the leaf lifespan, even when growth conditions are optimal, but it can also be environmentally induced, when inputs such as water and nutrients are restricted. Leaf maturity and senescence are associated with biochemical changes such as rapid degradation of ribosomes and rRNA, a moderately rapid breakdown of total protein followed by accelerated chlorophyll degradation (Araujo *et al*., 2007). The common feature for leaf senescence is loss of protein through catalysis

by proteases. The derived amino acids are transported to areas of growth or storage. During senescence the cellular structure, metabolic activities, and physiological role of the leaf are greatly altered. Prior to senescence the leaf serves as a source of photosynthetic products. As senescence progresses, there is a degeneration of the chloroplasts, and the photosynthetic apparatus is disassembled. Thus, senescence has a negative influence on the yield due to loss of the leaf photosynthetic assimilatory capacity (Weibo *et al*., 2002).

Delaying leaf senescence in order to extend the seed-filling period is a great strategy for increasing grain yield. Several studies on soybean leaf senescence have shown an inverse relationship between grain yield and delayed leaf senescence, suggesting that maximum yields can only be achieved in plants whose leaves senesce during pod-filling. Therefore, crop improvement should balance the extension of the mature phase of canopy as a carbon source and the rapid recovery of mineral nutrients during leaf senescence (Araujo *et al*., 2007).

2.3 Proteases

Proteases refer to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins which means that they are essential for the protein turnover and hence ability of cells to respond to changing environmental conditions. They are also called proteolytic enzymes or proteinases. Genomic studies have revealed a striking diversity of protease genes which reflects specific functions of regulating the fate of many different proteins (Simova-Stoilova *et al*., 2009). Proteases differ in their ability to hydrolyze various peptide bonds. Generally proteases are synthesized as pre-pro-peptides with auto-inhibitory prodomains and undergo strictly controlled maturation which results in active enzyme forms. Protease activity is subjected to complex control at various levels by transcriptional and translational regulation, by post-translational processing and activation, and through the action of specific protease inhibitor proteins (Simova-Stoilova *et al*., 2009). There is great interest in studying these proteases because an increase in proteolytic activity during nodule senescence has been observed and thiol

type proteases appear to play an important role in this process (Alesandrini *et al*., 2003). There has also been a report on the expression of a cysteine protease gene in senescing soybean nodules (Alesandrini *et al*., 2003). Very little is known about the specific biological functions of proteases. Each type of protease has a specific kind of peptide bond that it breaks. Proteases are classified according to their catalytic mechanisms and active site residues. There are serine, cysteine, aspartic and metalloproteases. Among the largest protease families are subtilisin like serine proteases and papain-like cysteine proteases (Van der Hoorn *et al*., 2004).

2.3.1 Serine proteases

Serine proteases are one of the four major classes of proteolytic enzymes and have been studied in great detail in several physiological systems including digestion, coagulation, immune and endocrine function (Morris and Sakanari, 1994). Serine proteases are digestive enzymes which include trypsin, chymotrypsin and elastase which differ in their substrate activity. For example, chymotrypsin prefers an aromatic side chain on the residue whose carbonyl carbon is part of the peptide bond to be cleaved. Trypsin prefers a positively charged lysine or arginine residue at this position. During catalysis, there is nucleophilic attack of the hydroxyl oxygen of a serine residue of the protease on the carbonyl carbon of the peptide bond that is to be cleaved. An acyl-enzyme intermediate is transiently formed. Hydrolysis of the ester linkage yields the second peptide product (Morris and Sakanari, 1994).

2.3.2 Aspartate proteases

Aspartate proteases are a large class of endopeptidases with acidic pH optimum around pH 3 widely distributed among microorganisms, plant and animal cells. They are specifically inhibited bypepstatin A and contain two Asp residues in their active site and the nucleophilic agent is a water molecule (Simova-Stoilova *et al*., 2009). During catalysis of an aspartate protease, one aspartate accepts a proton from an active site H_2O, which attacks the carbonyl carbon of the peptide linkage. Simultaneously, the other

aspartate donates a proton to the oxygen of the peptide carbonyl group. Examples of aspartate proteases include the digestive enzyme pepsin, some proteases found in lysosomes, the kidney enzyme renin, and the HIV-protease (Diwan, 2008). Aspartate proteases have also been associated with the development, senescence, diseases resistance and stress response of many plants (Simoes and Faro, 2004).

2.3.3 Zinc proteases

Zinc proteases also known as metalloproteases include the digestive enzymes carboxypeptidases, various matrix metalloproteases (MMPs) that are secreted by cells, and one lysosomal protease. Some MMPs (e.g., collagenase) are involved in degradation of the extra cellular matrix during tissue remodeling and some have roles in cell signaling relating to their ability to release cytokines or growth factors from the cell surface by cleavage of membrane-bound pre-proteins. Some are also involved in protein turnover and are induced in germination, senescence, host pathogen interactions and stress conditions (Simova-Stoilova *et al*., 2009). Based on the catalytic mechanism most of the aminopeptidases are metalloproteases, catalyzing the release of amino acid residues from the N-terminus of proteins. They have neutral pH optimum and are localized in cytoplasm, chloroplasts and mitochondria. A zinc binding motif at the active site of a metalloprotease includes two histidine residues whose imidazole side-chains are ligands to the Zn^{2+}. During catalysis, the Zn^{2+} promotes nucleophilic attack on the carbonyl carbon by the oxygen atom of a water molecule at the active site. An active site base (a glutamate residue in carboxypeptidase) facilitates this reaction by extracting a proton from the attacking water molecule (Diwan, 2008).

2.3.4 Cysteine proteases

Cysteine proteases have been implicated in many regulatory and degradative processes in animal and plant cells. These proteases include papain, which is a well-studied plant cysteine protease (Azarkan, 2003), cathepsins which are a large family of lysosomal cysteine proteases with varied substrate specificities. Cathepsins are promising drug

targets for many diseases such as osteoporosis, rheumatoid arthritis, arteriosclerosis, cancer, and inflammatory and autoimmune diseases (Grudkowska and Zagdanska, 2004). Caspases are another class mainly involved in the activation and implementation of apoptosis and calpains which cleave intracellular proteins involved in cell motility and adhesion (Diwan, 2008).

Cysteine proteases, also known as thiol proteases are wide spread in nature as they are present in all living organisms. More than twenty families of cysteine proteases have been described many of which are of industrial importance. The implication of cysteine proteases in numerous vital processes and pathologies has made them highly attractive targets for drug design. The proper functioning and regulation of activity of cysteine proteases is a delicate balance of many factors, one of the most crucial being the protease inhibitors (Turk *et al.*, 2002). In plants cysteine proteases are involved in a wide range of developmental processes such as responses to biotic and abiotic stress, processing and degradation of storage proteins in seeds, programmed cell death during plant growth and senescence in flowers and leaves (Asp *et al*., 2004).

2.3.2 Classification of cysteine proteases

Most plant cysteine proteases (EC.3.4.22) are proteins of molecular mass of about 21-30 kDa with a cysteine residue in their active site. These proteases are comprised of more than 40 families grouped into at least six superfamilies. A lot these plant cysteine proteases are classified under the papain, legumain, caspase and calpain families. Ubiquitin C-terminal hydrolases and ubiquitin-specific proteinases have also been detected in plants. They are components of the ubiquitin proteosome dependant pathway that catalyze de-ubiquitination of proteins (Vierstra, 2003). They are classified based on the effect of their active site inhibitors (Barrett, 1994). Molecular analysis of *Glycine max* has led to the identification of a cysteine protease gene that shows expression in nodules. In 5-week-old nodules, a stage where nitrogen fixation begins to decline, in situ hybridization experiments detected expression in the periphery of the central zone of the determinate nodule. At 10 weeks, the gene was strongly expressed in the centre of the

infected tissue where necrosis was progressing. Some sequences of the gene were identified in a range of libraries from different tissues as well as libraries generated from tissues subjected to abiotic and biotic stress indicating ubiquitous expression pattern in soybeans (Asp *et al*., 2004).

2.3.3 Types of cysteine proteases

Papain is a cysteine protease that is a proteolytically active constituent in the latex of tropical papaya fruit. It is stable and active under a wide range of conditions. The latex of *Carica papaya* is a rich source of four cysteine endopeptidases including papain, chymopapain, glycyl endopeptidase, and caricain. The proteins are synthesized as inactive precursors that become active within two minutes of the plant being wounded and the latex expelled. Papain is a minor constituent, but has been more widely studied because it is more easily purified (Azarkan, 2003). Papain has fairly broad specificity; it has endopeptidase, amidase, and esterase activities. The active site consists of seven subsites that can each accommodate one amino acid residue of a substrate. Specificity is controlled by the S_2 subsite, a hydrophobic pocket that accommodates the P_2 side chain of the substrate. Papain exhibits specific substrate preferences primarily for bulky hydrophobic or aromatic residues at this subsite. Outside of the S_2 subsite preferences, there is a lack of clearly defined residue selectivity within the active site.

The mature forms of all papaya proteinases are between 212 and 218 amino acids, and exhibit a strong degree of homology. Papain is synthesized as a zymogen with a 133 amino acid N-terminal region that is not part of the active enzyme (Azarkan, 2003). Papain is a single-chained polypeptide with three disulfide bridges and a sulfhydryl group necessary for the activity of the enzyme. Papain is expressed as an inactive precursor, prepropapain. The formation of active papain requires several cleavage steps including an initial cleavage of the 18 amino acid preregion (the signal sequence), followed by further cleavage of the glycosylated 114 amino acid pro-region. The pro-region serves as an intrinsic inhibitor and folding template. It has been found that members of the papain group of proteinases preferentially cleave peptide bonds with arginine or phenylamine (Fischer *et al.*, 2000).

Calpains are cytoplasmic, calcium-dependent cysteine proteinases requiring micro- or millimolar concentrations of Ca^{2+} for activity, with a highly conserved molecular structure in the catalytic site. There have been reports on the identification of calcium-dependent enzymes in plants i.e. in *Arabidopsis* roots, and in root tips and the grain aleurone layer of *Zea mays* (Grudkowska and Zagdanska, 2004). Legumains are a group of cysteine proteinases isolated from maturing *Ricinus communis* seeds, *Glycine max* cotyledons, germinating *Vicia sativa* seeds and in different organs of *A. thaliana* (Grudkowska and Zagdanska, 2004). These enzymes belong to the asparaginyl-specific subclass of the cysteine endopeptidase family which cleaves peptide bonds with Asn or Asp. They are active only at acidic pH. Plant legumains are usually called vacuolar processing enzymes (VPE) but they are also present in the cell wall and their function is not restricted to precursor protein processing but also includes protein breakdown in the vacuole or cell wall (Muntz *et al.,* 2002). Caspases are a class of cysteine proteases that exhibit a high specificity with an absolute requirement for an Asp residue adjacent to the cleavage site and a recognition sequence of at least four amino acids N-terminal to the cleavage site. Caspase-like proteinases in plants are inhibited by specific caspase inhibitors and are resistant to typical cysteine proteinase inhibitors (Grudkowska and Zagdanska, 2004).

2.3.4 Catalytic mechanism of cysteine proteases

The catalytic mechanism of cysteine proteases have involves a nucleophilic cysteine thiol in a catalytic triad. The first step is deprotonation of a thiol in the enzyme's active site by an adjacent amino acid with a basic side chain, usually a histidine residue. The next step is nucleophilic attack by the deprotonated cysteine's anionic sulfur on the substrate carbonyl carbon. In this step, a fragment of the substrate is released with an amino terminal, the histidine residue in the protease is restored to its deprotonated form, and a thioester intermediate linking the new carboxy-terminal of the substrate to the cysteine thiol is formed. The thioester bond is subsequently hydrolyzed to generate a

carboxylic acid moiety on the remaining substrate fragment, while regenerating the free enzyme (Barret, 1994).

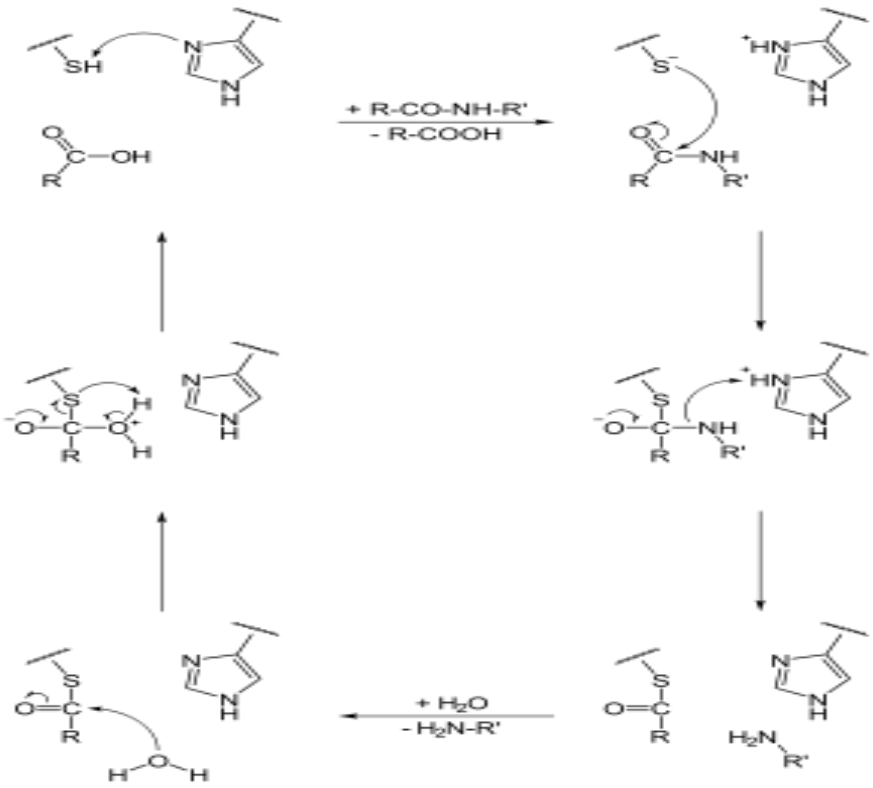

Figure 3. Reaction mechanism of the cysteine protease mediated cleavage of a peptide bond (Barret, 1994).

2.4 Protease inhibitors

Protease inhibitors are proteins regulating activity of proteases by binding tightly to their active site. Protease activity has been correlated with specific events involving plant development and regulation of programmed cell death. Plant protease inhibitors play an important role in mobilization of stored reserves to provide amino acids to the development parts of plants and defence against insect attacks (Mashamba *et al.,* 2009). The presence of these protein inhibitors in the seeds of many plants in considerably high

amounts has been the subject of much speculation as to whether these inhibitors have any role in the control of proteolysis during plant development (Filho, 1992).

There are two types of protease inhibitors which are endogenous and exogenous inhibitors. Endogenous protease inhibitors are proposed to regulate the proteolytic activity in a wide variety of physiological and pathological processes *in vivo* (Saitoh *et al*., 2007). Endogenous inhibitors of papain-like cysteine proteases have been proposed to regulate inappropriate proteolysis in intracellular and extracellular physiological processes. Protease inhibitors can also be classified according to the type of protease they inhibit of by their mechanism of action. According to proteases they inhibit, there are cysteine protease inhibitors of which papain inhibitors are the most studied, serine protease inhibitors of which trypsin inhibitors are the most well known, threonine protease inhibitors, aspartic protease inhibitors and metallo-protease inhibitors (Saitoha *et al*., 2005). Classes of inhibitor mechanism of action include suicide inhibitors, transition state inhibitors, protein protease inhibitor and chelating agents. In addition to preventing inappropriate proteolysis that could be harmful or even lethal, the inhibitors of this class of proteases are thought to control the activity of proteases released by various microorganisms or inflammatory cells. They are generally categorized according to class of protease they inhibit (Turk *et al*., 2002).

2.4.1 Inhibitors of cysteine proteases

Inhibitors of the cysteine protease family are widely distributed in animals and plants. These are mainly involved in the regulation of physiological and pathological processes caused by cysteine proteases. Research has shown that inhibitors of the cysteine protease family are a major component of larval digestive systems for pests of important legume food crops. Well-known inhibitors of cysteine proteases are the cystatin superfamily members including the stefins, the cystatins, the kininogens, and the phytocystatins (Turk *et al*., 2002). Cystatin is a name that was given to a protein present in chickens' egg whites that inhibit papain and other cysteine endoproteinases. Cystatins are competitive reversible tight binding inhibitors of cysteine protease family (Gerhartz *et al*., 1997).

Type 1 cystatins, also known as stefins do not have disulphide bonds and are intracellular whereas type 2 cystatins are secreted and contain two disulphide bonds. These two types are low molecular weight proteins, of about 100 residues for type 1 and 115 residues for type 2 proteins. Type 3 cystatins, or kininogens have more complex structures, containing three type 2 domains and an unrelated C-terminal sequence with an overall of about 355 residues. Inhibitors of the cystatin family have been of particular interest because they form complexes with cysteine proteases even after their catalytic sites have been inactivated by bulky active-site reagents which distinguish cystatins from any other inhibitors studied (Anastasi *et al*., 1983). Cystatin type cysteine proteases have also been identified in a range of plants including pineapple, rice, maize and a number of legumes including soybean and cowpea (Zhang *et al*., 2010).

Trans-(Epoxysuccinyl)-L-leucylamino-4-guanidinobutane (E-64) is a non-competitive irreversible inhibitor of cysteine proteases (Figure 4). It is produced by the fungus *Aspergillus japonicus* and its activity as an inhibitor of cysteine proteases has been described and characterized. The compound has been widely used as a specific inhibitor of cysteine proteases both *in vitro* and *in vivo*. E-64 inhibits the activity of cysteine proteases by forming hydrogen bonds hydrophobic interactions with the protease. The irreversibility of the inhibitor action is due to the reaction of the epoxy group at the position C-2 on the inhibitor molecule with the cysteine residue of the catalytic site of the protease. E-64 forms a thio-ether bond with the sulf-hydryl group in the active center of the enzyme. It is very useful for active site titration as one mole of E-64 inhibits one mole of protease. E-64 is mainly stable as well as soluble in DMSO (Govrin and Levine, 1999). Researchers have revealed that E-64 inhibits the cysteine proteases specifically, cathepsin B and cathepsin L from rat liver with IC_{50} values of 8.7 and 3.5 nM respectively (Murata *et al.*, 1991).

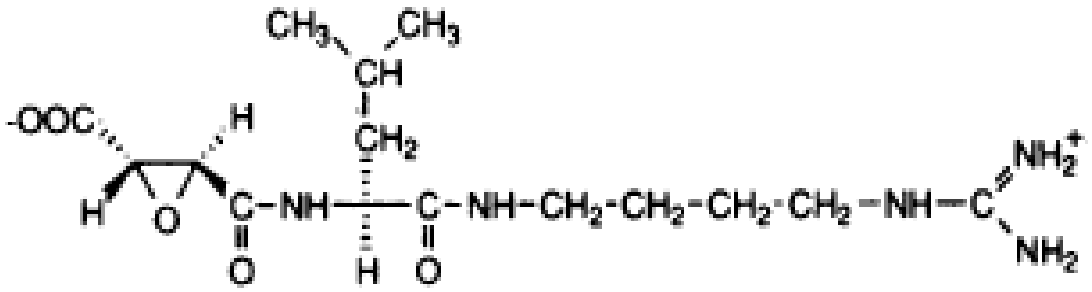

Figure 4. Structure of E-64 n-(n-(1-hydroxycarboxyethyl-carbonyl)leucylamino- butyl)-guanidine (Murata *et al.,* 1991).

DCG-04 is a modified cysteine protease inhibitor, resulting from biotinylation of E64. It is a mechanism-based probe that covalently targets cysteine proteases from the papain family hence it is mainly used for affinity labelling of cysteine proteases (van der Hoorn *et al.*, 2004). It has a molecular weight of 903 and its full name is L-Lysinamide, N-[[(2S,3S)-3-(ethoxycarbonyl)-2-oxiranyl]carbonyl]-L-leucyl-L-tyrosyl-6-aminohexanoyl-N6-[5-[(3aS,4S,6aR)-hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-yl]-1-oxopentyl] ($C_{43}H_{66}N_8O_{11}S$) (Figure 5).

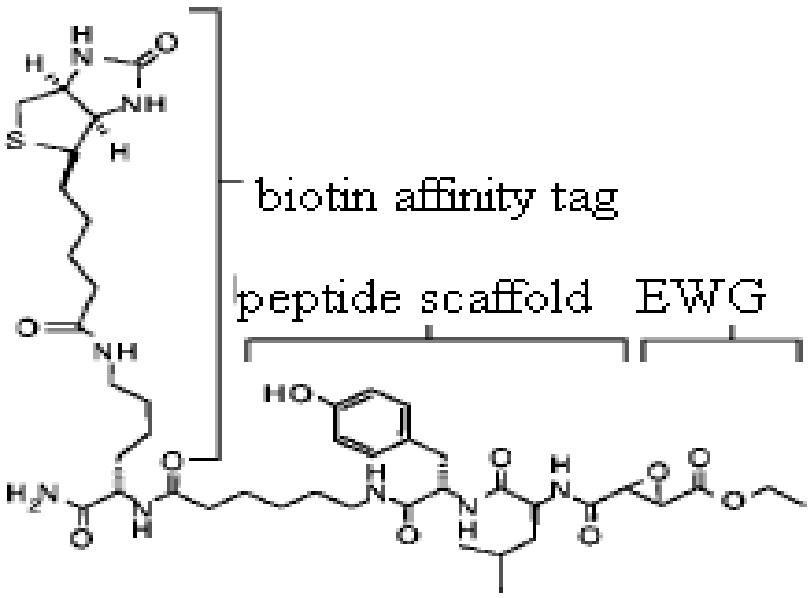

Figure 5. Structure of the epoxide inhibitor DCG-04 composed of a biotin affinity tag, a peptide scaffold and an epoxide electron withdrawing group (EWG) (van der Hoorn *et al.,* 2004)

2. 6 Proteomic studies

Proteomics is the study of the expressed protein complement of a genome at a specific time. The drivers of proteomic analyses are the technological achievements of the past years that have enabled the quantitative analysis of DNA sequences, mRNA and protein expression in cells and tools such as DNA microarrays, two-dimensional gel electrophoresis and mass spectrometry. The main reasons for carrying out proteomic analysis are to explore cellular functions to enhance productivity or influence desired properties of biological products, to learn and apply knowledge of cell function in response to environmental change, including exposure to normal or unusual substrates and to exploit knowledge of cell function and properties to improve product purification and characterization (Gupta and Lee, 2007). Proteome analysis is also required to determine which proteins have been conditionally expressed, how strongly, and whether any posttranslational modifications are affected. Two or more different states of a cell or

an organism (e.g., healthy and diseased tissue) can be compared and an attempt made to identify specific qualitative and quantitative protein changes.

2.6.1 Two-dimensional electrophoresis for proteomics

One of the greatest challenges of proteome analysis is the reproducible fractionation of these complex protein mixtures while retaining the qualitative and quantitative relationships. Currently, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is the only method that can handle this task (Gorg *et al*., 2000), and hence has gained special importance. Since 2-D PAGE is capable of resolving over 1,800 proteins in a single gel (Choe and Lee, 2000), it is important as the primary tool of proteomics research where multiple proteins must be separated for parallel analysis. It allows hundreds to thousands of gene products to be analyzed simultaneously. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a technique in which proteins are separated according to their isoelectric point (pI) in the first dimension and molecular weight (Mr) in the second dimension (Gorg *et al*., 2000). The separation of proteins takes an advantage of electrophoretic mobility of individual constituents of a complex mixture of proteins fractionating by charge in one and by mass in the other.

Proteins are first separated on the basis of their pI, the pH at which a protein carries no net charge and will not migrate in an electrical field. The technique is called isoelectric focusing (IEF). For 2-D PAGE, IEF is best performed in an immobilized pH gradient (IPG). The IPG method has numerous advantages over the older tube gel method (Gorg *et al*., 2000). IPG strips offer the advantage of gradient stability over extended focusing runs. A conditioning step is applied to proteins separated by IEF prior to the second-dimension run. This process reduces disulfide bonds and alkylates the resultant sulphur-hydryl groups of the cysteine residues. Concurrently, proteins are coated with SDS for separation on the basis of molecular weight. For the SDS-PAGE second-dimension, the ability to run many gels at the same time and under the same conditions is important for the purpose of gel-to-gel comparison. The proteins will be separated based on their mass

in which a vertical slab gel with stacking and separating gels of polyacrylamide (SDS-PAGE) is used (Gorg *et al*., 2000). The result of using these two independent techniques is a high degree of resolution within a protein population and is the fundamental tool of proteomics and allows thousands of proteins to be analyzed simultaneously (Salekdeh *et al*., 2002).

In order to visualize proteins in gels, they must be stained and the choice of staining method is determined by several factors including desired sensitivity, linear range, ease of use, expense, and the type of imaging available. Some software packages have been developed to detect and quantify protein spots on the gels and to compare replicate 2D gels or identify protein expression changes across plant anatomy changes (Wilkins *et al*., 1995). Some of them include Kepler, PD-Quest and Melanie.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

The seeds of *Glycine max Knapp* cultivar were a gift from the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. Immobilized pH gradient (IPG) strips and carrier ampholytes were purchased from Bio-Rad Laboratories, Hercules, California, USA. NuPAGE Antioxidant, LDS sample buffer, NuPAGE carrier ampholytes, iodoacetamide, di-thiothreitol (DTT), ZOOM IPG strips, IPGRunner cassettes and IPGRunner mini-cell apparatus were purchased from Invitrogen, Carlsbad, California, USA. Protein molecular weight markers were purchased from Inqaba Biotech, Pretoria, SA. Urea, β-mercaptoethanol, thiourea, trizma base, protease inhibitor cocktail, N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED), sodium dodecyl sulphate (SDS), 3-[(Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), bromophenol blue, ammonium persulphate (APS), *trans*-(Epoxysuccinyl)-L-leucylamino-4-guanidinobutane (E-64), cysteine, sodium acetate, bovine serum albumin (BSA), azocasein, trichloroacetic acid (TCA), sodium hydroxide, papain, Z-arginyl-L-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-AMC), sodium chloride, bicinchoninic acid reagent (BCA), acrylamide, Bis-acrylamide, dimethyl sulfoxide (DMSO), agarose, tween 20, triton X100, and coomassie brilliant blue R-250 were obtained from Sigma Aldrich, St. Louis, Missouri, USA. Glycerol, ethanol, acetone, acetic acid, methanol, hydrochloric acid (HCl) and isopropanol were obtained from Rochelle Chemicals. All other chemicals were basic laboratory reagents of highest available purity.

3.1.2 Equipment

Equipment used included Invitrogen ZOOM IPGRunner Mini-cell, Invitrogen ZOOM ElectroBlotting Mini-cell, Biorad Mini-protean systems II and III, Biorad Protean isoelectric focusing (IEF) cell, Biorad PS250-2, Beckman Coulter Allegra X-22R centrifuge, Beckman Coulter DTX 800 Multimode Detector micro-plate reader, Beckman Coulter DU 720 UV/Vis spectrophotometer and Syngene GeneSnap Imaging System in this study.

3.2 Methods

3.2.1 Growth of the soybean plants and collection of nodules

Glycine max Knapp cultivar seeds (Figure 6) were grown in a growth chamber at 24 ± 2°C under 18 h light regime in 15 cm pots. The pots were filled with moist vermiculite and an innoculant which contained *Bradyrhizobium japonicum* bacteria. Initially, the pots were watered every two days with 100 mL of distilled water until the first true leaves developed. After germination, the plants were watered once every week with 200 mL of nitrogen free nutrient solution (Table 1) and two times a week with 200 mL distilled water. Root crown nodules (located at the first 4 cm segment of the tap root, including the first 1-4 cm of lateral roots arising from the segment) and leaves (located 10 cm from lateral roots) were collected at 4, 6, 10, 12 and 16 weeks post germination. The plant material was frozen in liquid nitrogen immediately and stored at -80°C.

Figure 6. Mature seeds of *Glycine max Knapp* cultivar

Table 1: Nitrogen-free nutrient solution (Ahmed and Evans, 1960).

Chemical	Stock solution (g/L)	Volume of stock for 1L solution with dH_20)
1M KCl	74.557	10ml
1M $CaCl_2$	147.02	10ml
1M $MgSO_4$	246.48	2ml
1M KH_2PO_4	136.09	2ml
Micronutrients		2ml
H_3BO_3	2.86	
$MnCl_2.4H_2O$	1.81	
$ZnCl_2$	0.11	
$CuCl_2.2H_2O$	0.05	
$Na_2MoO_4.2H_2O$	0.0025	
FeEDTA		2ml
$FeSO_4.7H_2O$	25.0	
Na_2EDTA	34.0	

3.2.2 Extraction of proteins and tagging of cysteine proteases for activity profiling

Protein extraction was carried out according to a method by van der Hoorn *et al.* (2004). Proteins were extracted by grinding 0.5 g of root nodules or leaves in liquid nitrogen using a mortar and a pestle. Two millilitres of sodium acetate buffer, (50 mM, pH 6) containing 10 mM L-cysteine and 2 µM DCG-04 was added to the ground material. The mixture was incubated for 5 h at room temperature to ensure tagging of all cysteine proteases. Control samples contained 0.2 mM E-64 in addition to DCG-04. Each suspension after grinding (0.5 mL) was precipitated using ice cold acetone followed by incubation for 30 minutes on ice and by centrifugation at 10 625 x g for 5 minutes. The acetone pellet was washed three-times by adding 70% acetone. The washed pellet was dissolved in 250 µL TBS (50 mM Tris, pH 7.5, 150 mM NaCl) containing 2 µM protease inhibitor cocktail composed of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, E-64, leupeptin, bestatin, EDTA and pepstatin A. The extracts were then stored at -80°C.

3.2.3 Determination of total protein concentration

Bicinchoninic acid assay (BCA) was used to estimate the protein quantity in soybean nodule and leaf extracts according to Smith *et al*. (1985). Bovine serum albumin (BSA) was used as a standard, where solutions of different concentrations (10-1000 µg/mL) were prepared in TBS. Ten microlitres of the nodule or leaf extract was added to the 96 well flat-bottomed microtitre plate after 20 times dilution of the sample with the TBS buffer, and 200 µL of the BCA working reagent (prepared by mixing 50 parts of Bicinchoninic acid solution with 1 part of 4% copper sulphate solution) was then added. The flat-bottomed microtitre plate was incubated for 30 minutes at room temperature. Absorbance was read at 550 nm on Beckman Coulter DTX 800 multimode detector. The protein concentration of the nodule and leaf extracts was estimated from the BSA standard curve.

3.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was perfomed according to Laemmli (1970) using 12% and 15% polyacrylamide gels and Biorad mini-protean III apparatus to determine the protein subunit profile of extracts of the root nodules and the leaves. Twenty five micrograms of the nodule protein extracts and SDS sample buffer (2.5 ml glycerol, 1.25 ml of 0.5 M Tris-HCl, 2 ml of 10% (w/v) SDS, and 0.2 ml of 0.5% (w/v) bromophenol blue) were mixed and boiled for 5 minutes to denature the proteins before loading onto the polyacrylamide gels. The electrophoresis apparatus was then connected to a Biorad PS 250-2 Techware power supply which was initially run at 100 V for 15 minutes and then at 200 V constantly until the protein extracts reached the sealing gel. Protein bands were visualized by staining using 0.1% Coomasie Brilliant Blue R250 (w/v), in methanol/ acetic acid/ water (50:10:40) and destained by boiling in an excess of water using a microwave oven. Visualisation and densitometric analysis of the protein bands was perfomed using a Syngene GeneSnap imaging system.The band density was expressed as a fraction of the total of all band densities for each sample extract.

3.2.5 Proteolytic activity of the leaf and nodule extracts

3.2.5.1 Azocasein assay

Azocasein assay was perfomed to measure the total proteolytic activity of the protein extracts according to a method by Arnon (1970). Proteins were extracted by grinding 0.5 g of the root nodules and leaves in liquid nitrogen using a mortar and a pestle. One millilitre of 50 mM sodium acetate, pH 6 buffer containing 10 mM L-cysteine was added to the ground material. The suspension after grinding was then centrifuged for 10 minutes at 8 850 x g. One hundred microlitres of the protein extract was added to 100 µL of sodium acetate buffer, (50 mM, pH 6) and 900 µL of reaction buffer containing 1% (w/v) azocasein in 0.1 M sodium acetate buffer, pH 6 and 5 mM DTT. The mixture was

incubated for 10 minutes at 37°C followed by addition of 600 µL of 20% TCA. The reaction mixture was then allowed to stand for 10 minutes at room temperature and then centrifuged for 5 minutes at 12 389 x g. All the sample extracts were analysed in triplicate. The supernatant was collected and the absorbance of the released azo dye was then measured using a Beckman Coulter DU720 UV/Vis spectrophotometer at 436 nm.

3.2.5.2 Fluorometric assay for cysteine proteases

Cysteine protease activity of the soybean leaf and nodule extracts was measured by a fluorometric method according to Barrett (1994). Z-L-arginyl-L-arginine-7-amido-4-methyl-coumarin (Z-Arg-Arg-AMC) was used as the synthetic peptide fluorescent substrate for cysteine proteases. The proteins were extracted as described in section 3.2.5.1. The protein extract (10 µL) was added to 80 µL of 50 mM sodium acetate buffer containing 80 µM of the peptidyl substrate and 10 mM L-cysteine. The amount of amido-4-methyl-coumarin (NH_2AMC) released from hydrolyzed substrates at excitation and emission wavelengths of 355 and 460 nm respectively was estimated by the increase in fluorescence in a Beckman Coulter DTX 800 Multimode Detector micro-plate reader over 10 minutes. Controls contained an equal volume of the buffer instead of enzyme solution. To confirm that the activity measured was that of cysteine proteases, E-64 was used as a specific inhibitor for cysteine proteases. In this case, extracts were pre-incubated with 0.2 mM E-64 for 1 h before addition of 50 mM sodium acetate buffer containing 80 µM of the peptidyl substrate and 10 mM L-cysteine. Also to monitor the activity of endogenous protease inhibitors in the protein extracts, 0.2 mM of E-64 was added to 50 µL of the nodule extracts and this was incubated at room temperature for 1 h to complete inhibition of cysteine proteases in the nodule extracts. The nodule protein was precipitated with 200 µL acetone and the acetone pellet was washed 5 times with 70% acetone to remove unreacted E-64. The acetone pellet was then redissolved in 20 µL of 50 mM sodium acetate buffer. Twenty microlitres of 0.5 mM papain was added to the inhibited nodule extracts and incubated for an hour at room temperature. To monitor the activity of papain activity in the presence of endogenous protease inhibitors, 10 µL of the nodule extracts and papain mixture was added to 80 µL of 50 mM sodium acetate buffer

containing 80 μM of the peptidyl substrate and 10 mM L-cysteine. The amount of amido-4-methyl-coumarin (NH_2AMC) released from hydrolyzed substrates was measured as described above.

3.2.5.3 Zymography

Nodule and leaf proteins were extracted as described in section 3.2.5.1. Equal volumes (15 μL) of the protein extracts were mixed with 5 μL of non-SDS sample buffer (2.5 ml glycerol, 1.25 ml of 0.5 M Tris-HCl, and 0.2 ml of 0.5% (w/v) bromophenol blue). Polyacrylamide gels (10%) containing 0.1% gelatin were then prepared according to the method by Laemmli, (1970). After electrophoresis the gels were washed for 30 minutes in 2.5% Triton-X 100 to remove SDS. For protease zymography, the gels were then incubated overnight at 37°C in 50 mM acetate buffer pH 6 containing 10 mM cysteine. For reverse zymography, the polyacrylamide gels were incubated overnight in 50 mM acetate buffer pH 6 containing 10 mM cysteine and 0.02 mg/ml papain. Protein bands were visualized by staining using 0.1% Coomasie Brilliant Blue R250 (w/v), methanol/ acetic acid/ (50:10:40) and destained by boiling in an excess of water using a microwave oven. The protease bands were visualised using a Syngene GeneSnap imaging system.

3.2.6 Two Dimensional Polyacrylamide Gel Electrophoresis

3.2.6.1 First Dimensional Polyacrylamide Gel Electrophoresis- Isoectric focusing

Isoelectric focusing is separation of proteins on the basis of their isoelectric point (pI), the pH at which a protein carries no net charge and will not migrate in an electrical field. The nodule or leaf extracts were concentrated 20 times using acetone precipitation. Forty micrograms of the nodule or leaf protein extracts was added to 130 μL of the sample rehydration solution (8 M urea, 2 M thiourea, 2% CHAPS as a detergent, 50 mM DTT as a reducing agent, 0.02% ampholyte solution and 5% bromophenol blue as a dye) in order

to denature and solubilise the sample proteins for isoelectric focusing. The mixture was then loaded into sample loading wells of the ZOOM IPG Runner Cassette. IPG Strips of appropriate pI range were gently inserted into the cassette with the acidic end first, ensuring that there is no introduction of air bubbles. Sample loading wells were then sealed with Invitrogen sealing tape and the strips were incubated overnight at room temperature to rehydrate the strips. The strips were then focused in ZOOM IPG Runner Core and a mini-cell at low current of 200 V for 20 minutes increasing the voltage gradually to 2000 V over a period of 60 minutes. The final focusing voltage was maintained at 2000 V for 100 minutes

3.2.6.2 Second Dimensional Gel Electrophoresis

Second dimension separation is based on protein molecular weight using SDS-PAGE. The proteins resolved in IPG strips in the first dimension were applied to second-dimension gels. The IPG strips were first incubated on a rotary shaker for 15 minutes in lithium dodecyl sulphate (LDS) sample buffer with 0.2 M DTT as a reducing agent in preparation for SDS- PAGE. The IPG strips were again incubated on a rotary shaker for 15 minutes in LDS sample buffer containing 125 mM iodoacetamide as an alkylating agent. The IPG strips were then loaded on the 15% SDS polyarylamide gels (section 3.6) and overlaid with 400 µL of 0.5% molten agarose in distilled water. The electrophoresis apparatus was connected to a PS 250-2 Techware power supply which ran as described in section 3.2.4.

3.2.7 Probing for cysteine proteases

Probing was performed to specifically detect DCG-04 tagged cysteine proteases in the soybean nodule and leaf extracts. The protein extracts were resolved by 1-dimensional and 2-dimensional SDS-PAGE as described in section 3.2.4 and 3.2.6 respectively. The protein bands or spots depending on the electrophoresis method were electro-blotted onto a nitrocellulose membrane at 30 V for 1 h using a ZOOM Electro-Blotting Mini-cell. The

transfer buffer contained 25 mM Tris, 192 mM glycine and 10% methanol. 50 mL of Ponceau's solution (0.1% Ponceau S (w/v) in 5% acetic acid (v/v)) was used to reversibly stain proteins on the nitrocellulose membranes in order to ensure that the proteins were transferred. The molecular weight markers were highlighted using 1 mg/mL of horseradishperoxidase (HRP) "as the ink" in a felt pen. The membranes were washed 3 times using distilled water to remove the stain and blocked by incubating overnight in 4% BSA and 0.02% Tween 20 in TBS at room temperature on a rotary shaker. The nitrocellulose membranes were then washed three-times at 5 minutes per wash using 0.02% Tween 20 in TBS. After washing, the membranes were incubated for 1 h using 1 mg/ml streptavidin peroxidase in TBS at a dilution of 1: 1000. The membranes were then washed five-times at 5 minutes per wash using 0.02% Tween 20 in TBS followed by a single wash in TBS for 5 minutes. Lastly, the membranes were developed using the Thermoscientific enhanced chemiluminescent kit according to manufacturer's instructions and visualised on a Syngene GeneSnap imaging system. The isoelectric points of the spots were estimated based on the positioning of the IPG strip on the gel and the corresponding pH gradient of the strip.

3.2.8 Isolation of cysteine proteases

3.2.8.1 Affinity precipitation

Affinity precipitation was performed to isolate specifically the labelled cysteine proteases from the extracted as described in section 3.4. The protein extracts were first concentrated at least 10 times using 15 mL Amicon Ultra centrifugal filter devices (10 kDa cutout membranes). The streptavidin magnetic beads were separated from the suspension using a magnetic stand and washed three-times with TBS. The concentrated samples (100 µL) were then added to the pure streptavidin magnetic beads. The sample and bead mixture was incubated overnight on a rotary shaker at room temperature. The unbound proteins were washed off the streptavidin magnetic beads five-times using 0.02% Tween 20 in TBS. The beads were then boiled in 50 µL SDS sample buffer (2.5

ml glycerol, 1.25 ml of 0.5 M Tris-HCl, 2 ml of 10% (w/v) SDS, and 0.2 ml of 0.5% (w/v) bromophenol blue) for 10 minutes for the streptavidin beads to release the bound cysteine proteases. SDS-PAGE polyacrylamide gels were performed on the crude protein extracts and purified extracts using 12% (section 3.2.4).

CHAPTER 4

4 RESULTS

4.1 Developmental changes in the growth of the soybean plants

The soybean crown nodules and leaves were harvested at 4, 6, 10, 12 and 16 weeks. At 4 weeks, the soybean plants were at a vegetative stage whereby the plants had fully developed trifiolates. The leaves were green and nitrogen fixing zone of the nodules was pink in colour, which is an indication that the nodules were actively fixing nitrogen (Figure 7). At 10 weeks, the plants were now at a reproductive stage as the plants had open flowers at the nodes of the main stems (Figure 8). The leaves were still green and the nitrogen fixing zone of the nodules had turned to a reddish colour. At 16 weeks the soybean plants had developed pods which were still green but with seeds that were still to develop. Some of the leaves had turned yellow and some were drying which signifies leaf senescence. The nitrogen fixing zone of the nodules was green and the nodules were bigger in size as compared to the 4 week and 10 week crown nodules (Figure 9).

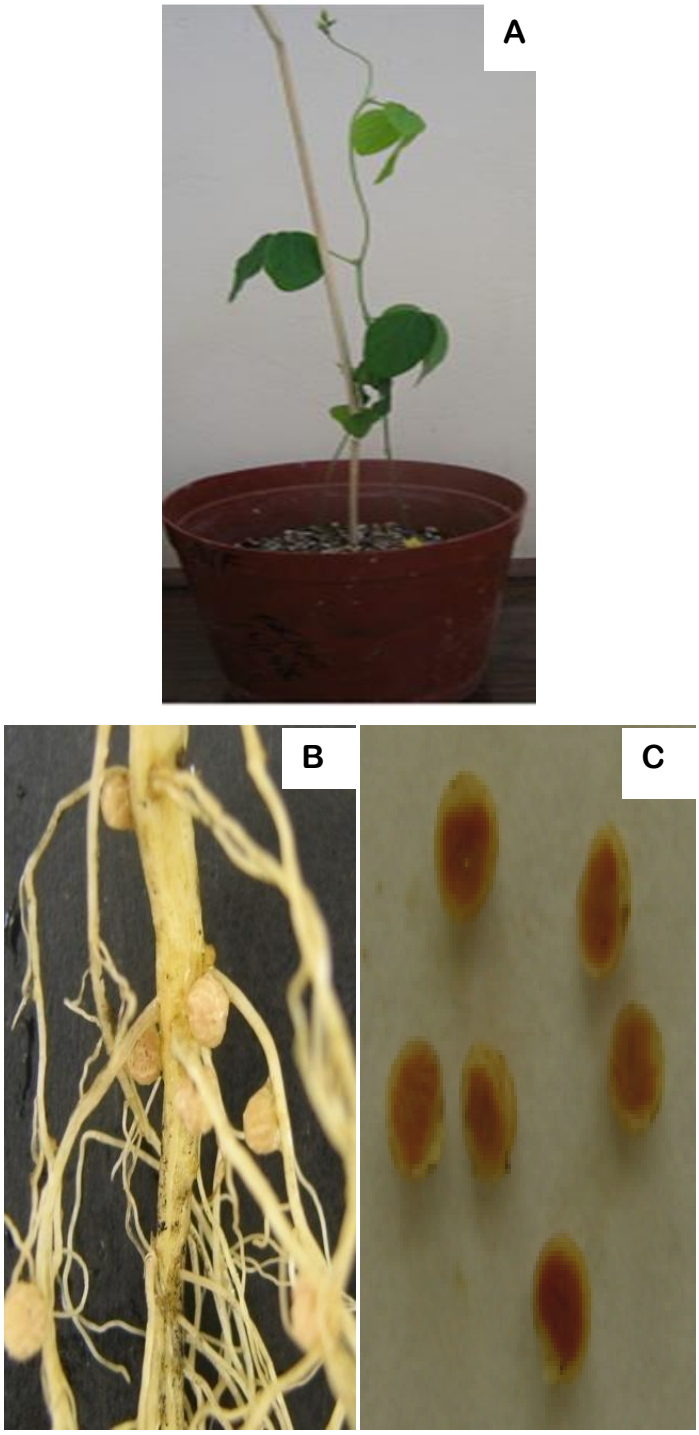

Figure 7. Four weeks old soybean plant (A), root nodules (B) and cut sections of the crown root nodules (C).

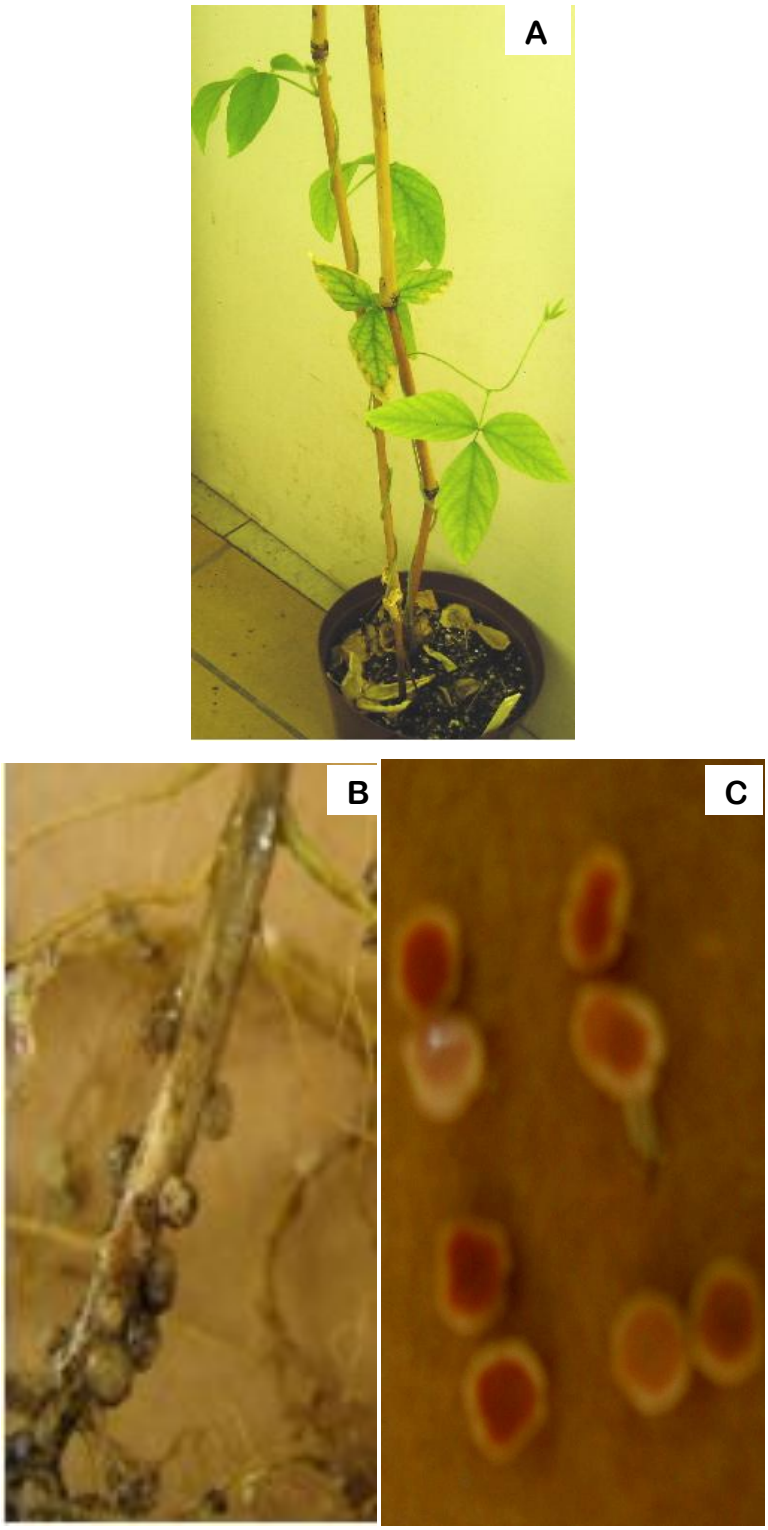

Figure 8. Ten weeks old soybean plant (A), root nodules (B) and cut sections of the crown root nodules (C).

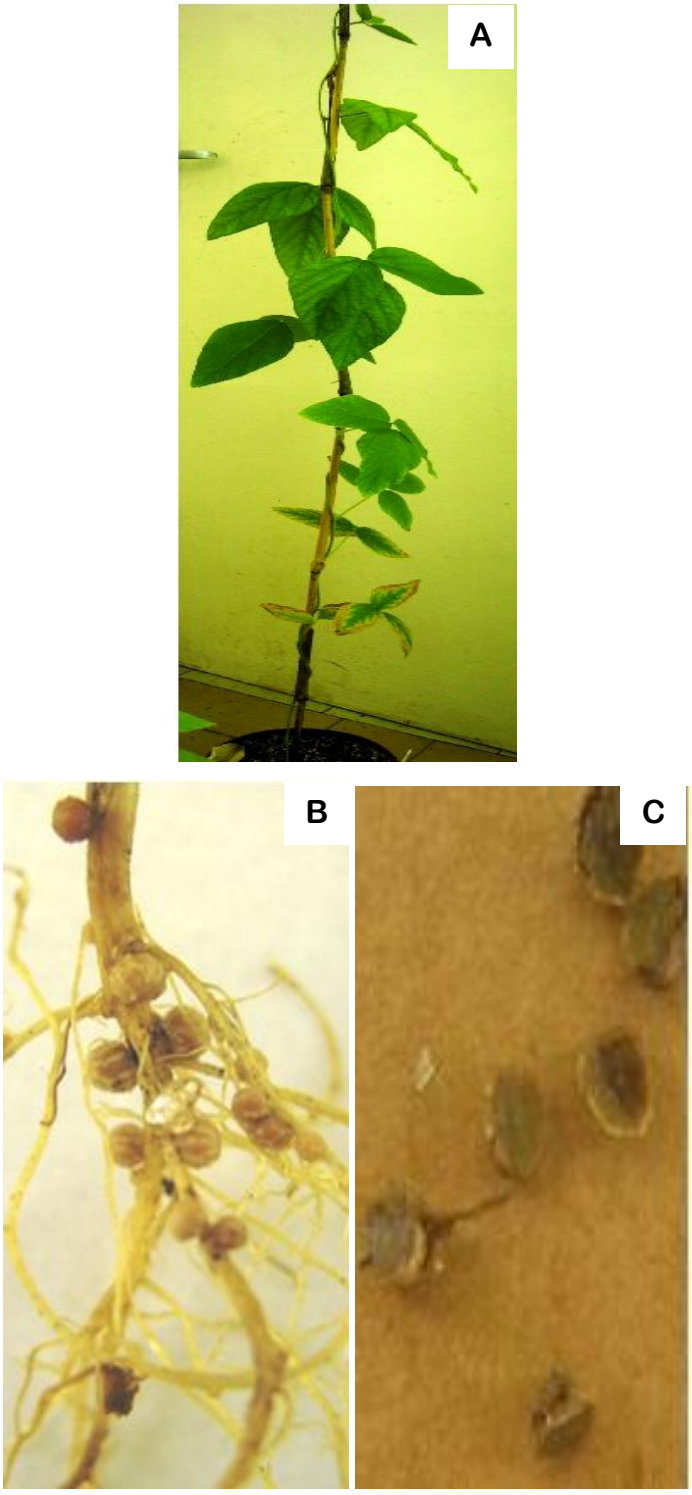

Figure 9. Sixteen weeks old soybean plant (A), root nodules (B) and cut sections of the crown root nodules (C).

4.2 Quantitation of total soluble protein

Bicinchoninic acid assay (BCA) was used to estimate the quantity of protein in the soybean nodule extracts using bovine serum albumin (BSA) as a standard (Smith *et al*., 1985). The soluble protein concentration in the soybean nodule protein extracts was estimated from the BSA standard curve. Total soluble protein in the nodule extracts decreased with an increase in the age of plants (Figure 10). The 4 week soybean nodules had the highest soluble protein content of 2.3 µg/µL and 16 week nodules had the lowest protein content of 1.4 µg/µL.

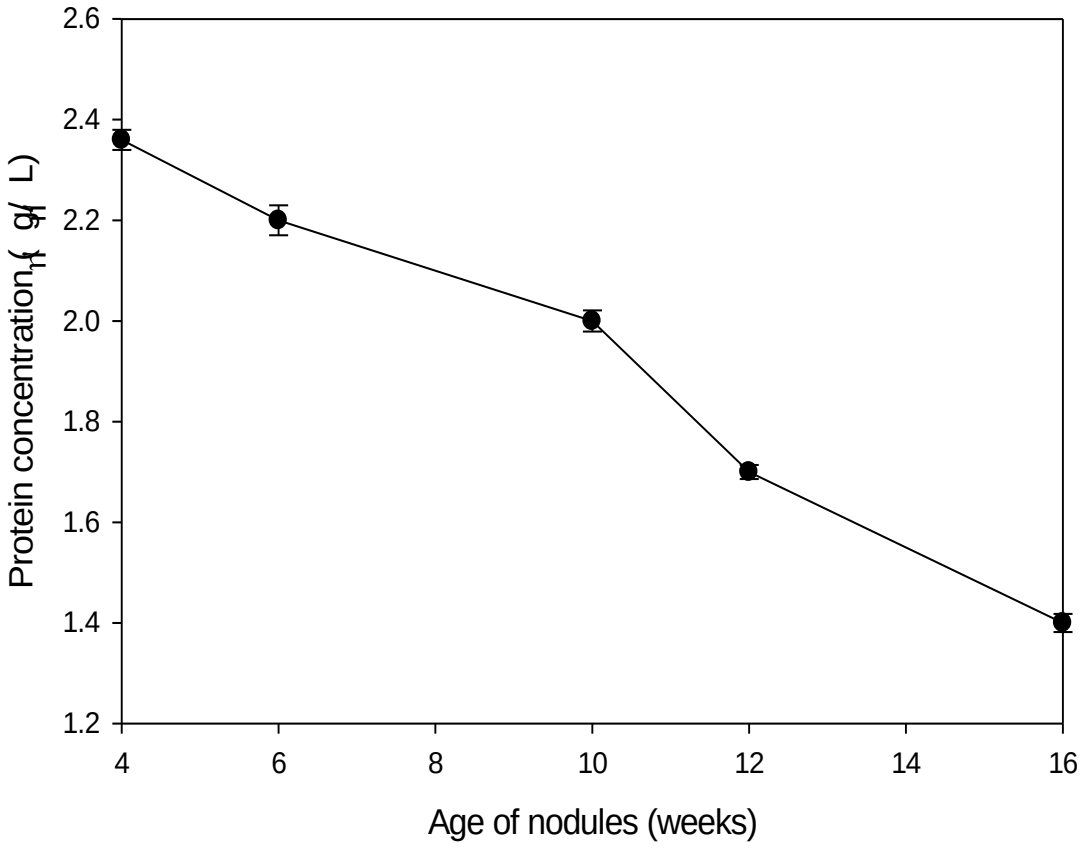

Figure 10. Total soluble protein concentration of the soybean nodule extracts during development, maturity and senescence of the soybean plants.

4.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the soybean nodule extracts during development, maturity and senescence of the soybean plants.

SDS-PAGE using 12% acrylamide gels was used to separate the proteins expressed in soybean nodule extracts in-order to compare the banding pattern of the soybean nodule protein extracts. The soybean root nodule extracts exhibited different banding patterns with less variation between the younger soybean nodule extracts (4, 6 and 10 weeks old) as compared to the older ones (12 and 16 weeks old) (Figure 11). Protein bands 1, 3, 4 and 5 were highly expressed in the 4, 6 and 10 weeks nodule protein extracts as evidenced by the high percentage density in the densitometric analysis. The percentage density for protein extracts was lower in 12 and 16 weeks old nodules signifying a lower expression of the proteins (Figure 11). The younger plants seemed to have a higher number of visibly intense protein bands, as compared to 12 and 16 weeks. Sixteen week nodule protein extract had the highest expression of bands 6, 7 and 8 as evidenced by the high percentage density.

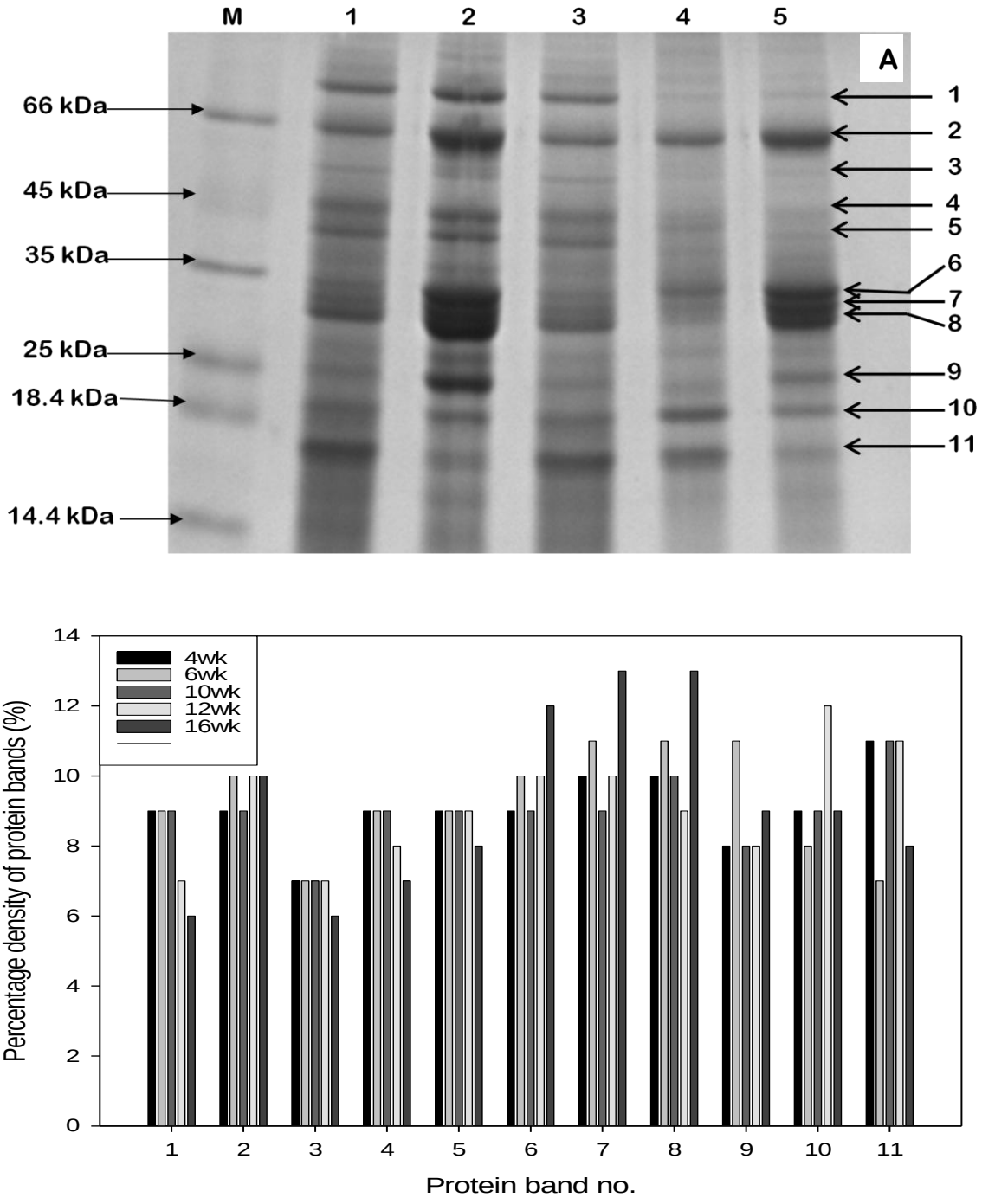

Figure 11. SDS-PAGE analysis (A) of soybean root nodule extracts and densitometry of the major protein bands labelled 1 to 11 (B). Lane M: Molecular weight marker; lane 1: Extract from 4 week nodules; lane 2: Extract from 6 week nodules; lane 3: Extract from 10 week nodules; lane 4: Extract from 12 week nodules and lane 5: Extract from 16 week nodules.

4.4 Proteolytic activity of the plant and nodule extracts

4.4.1 Azocasein assay

The total proteolytic activity of the soybean nodule and leaf extracts was determined by azocasein assay which measured the extent to which the extracts hydrolyzed azocasein. The proteolytic activity of the nodule extracts generally decreased with an increase in the age of the nodules with the 4 week nodules having the highest proteolytic activity and 16 week nodules with the lowest (Figure 12). The 10 week soybean nodule extract had a high total proteolytic activity of 91%. The total proteolytic activity of the soybean leaf extracts increased with an increase in the age of the leaves (Figure 13). The 4 week leaf extract showed a relatively high total protease activity of 82%.

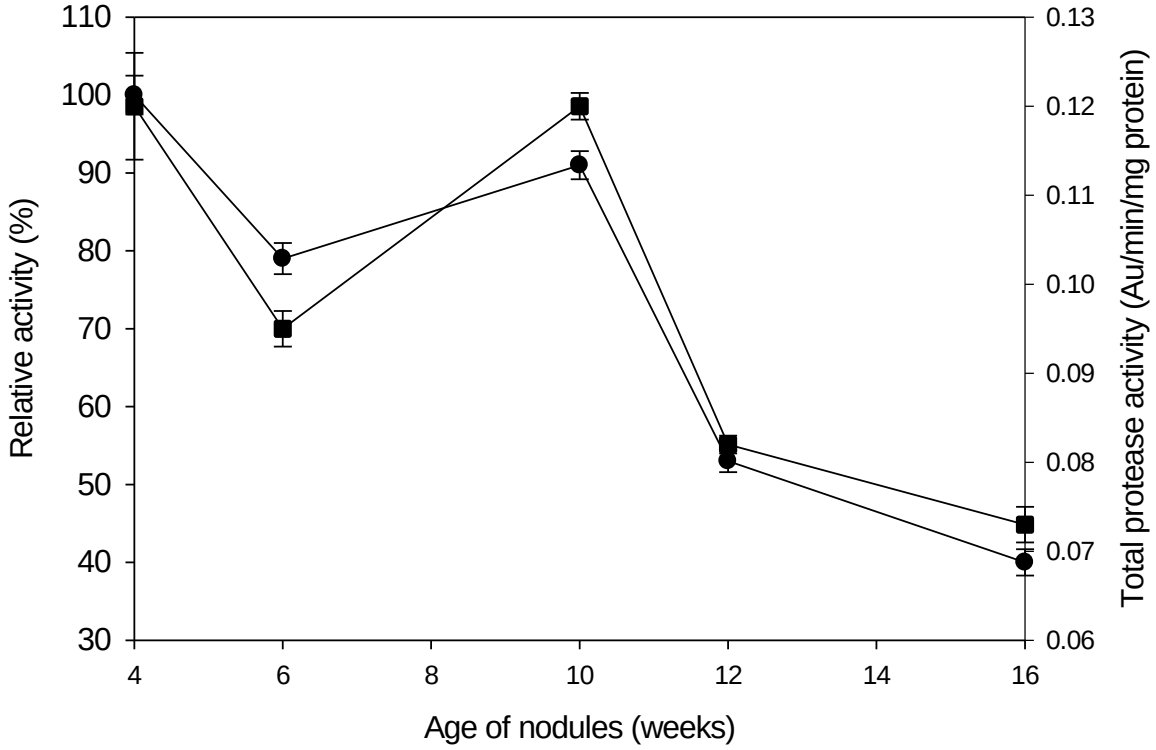

Figure 12. Total proteolytic activity of soybean nodule extracts as determined using azocasein assay. Results are expressed relative to the nodule extracts that gave the highest protease activity (●) and as total protease activity per milligram of protein (■).

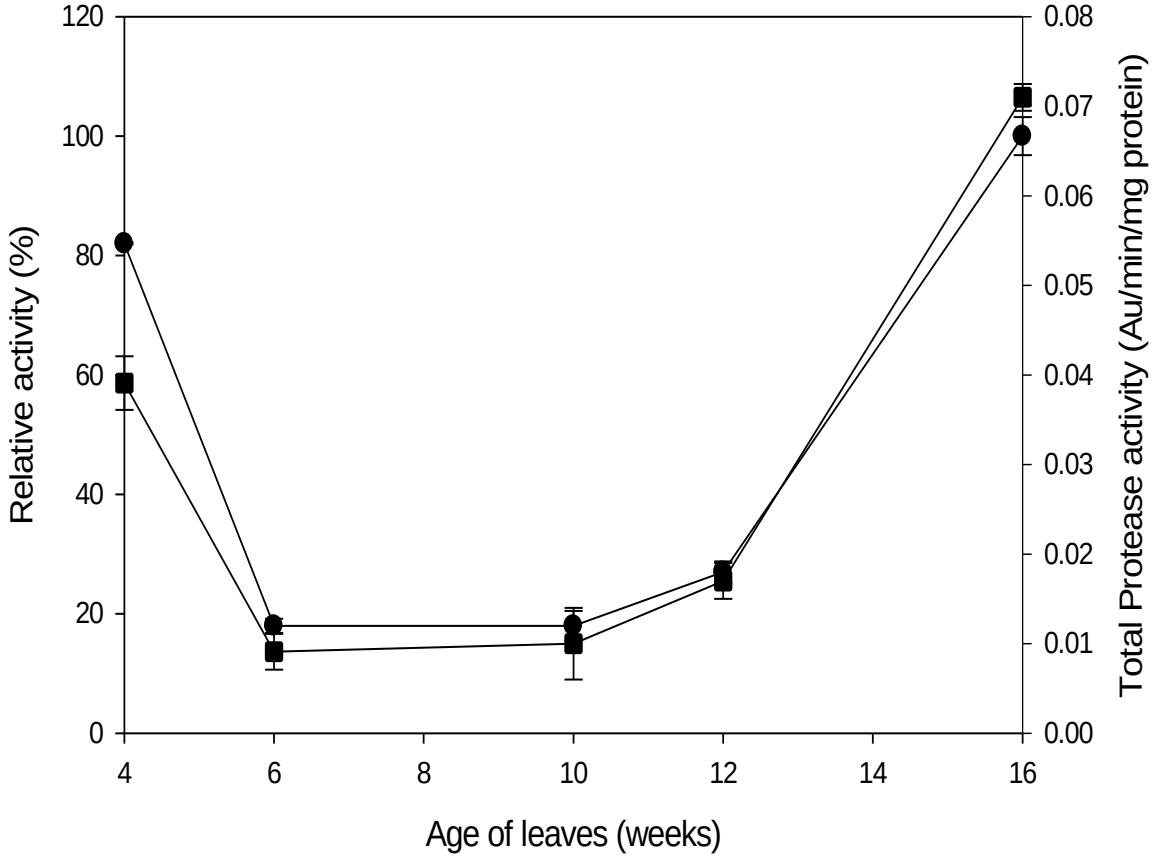

Figure 13. Total proteolytic activity in the soybean leaf extracts as determined using azocasein assay. Results are expressed relative to the leaf extracts that gave the highest protease activity (●) and as total protease activity per milligram of protein (■).

4.4.2 Flourometric assay for cysteine protease activity

Z-Arg-Arg-AMC is a known peptidyl substrate that is used specifically for detection of cysteine proteases. Addition of the peptidyl substrate Z-Arg-Arg-AMC to the soybean nodule and leaf extracts detected activity of cysteine proteases which were able to cleave the substrate and activity was measured as the generated fluorescence. The soybean nodule extracts showed a gradual decrease in cysteine protease activity as the soybean plant grew older. The decrease in the cysteine protease activity was correlated to the total soluble protein content in the soybean leaf extracts as the plant matured (Figure 14). In

soybean leaf extracts, cysteine protease activity increased as the plants were aging. The increase in the cysteine protease activity was not correlated to the total soluble protein content in the soybean leaf extracts as the plant matured (Figure 15).

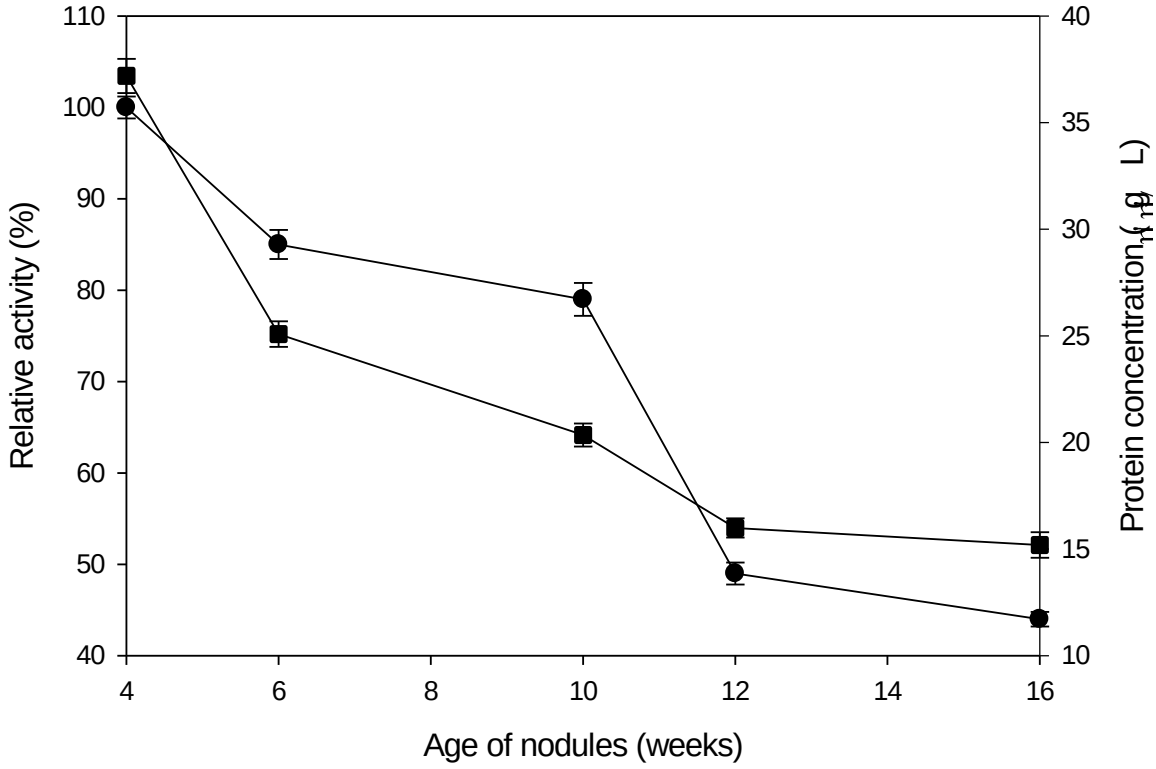

Figure 14. Total protein concentration of the soybean nodule extracts as determined using BCA assay (■) and cysteine protease activity of the soybean nodule extracts as assayed using Z-Arg-Arg-AMC as a substrate (●). Results are expressed relative to the nodules extracts that gave the highest cysteine protease activity.

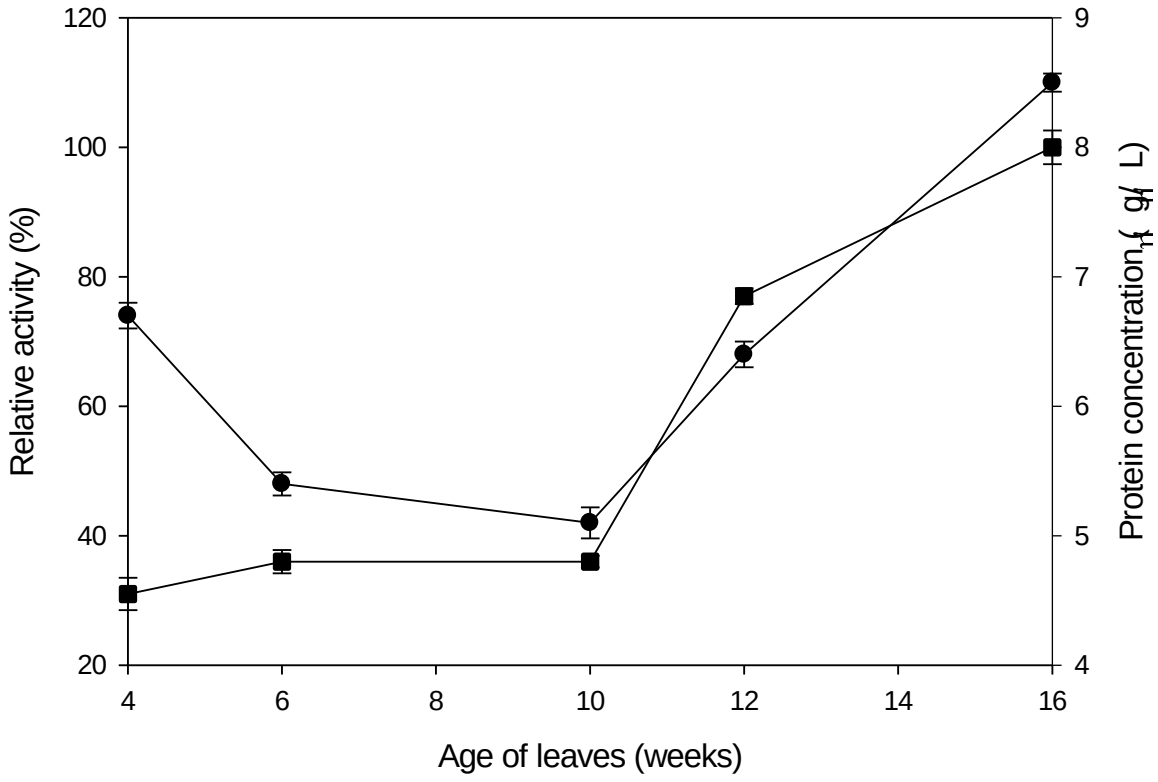

Figure 15. Total protein concentration of the soybean leaf extracts as determined using BCA assay (●) and cysteine protease activity of the soybean leaf extracts as assayed using Z-Arg-Arg-AMC as a substrate (■). Results are expressed relative to the leaf extracts that gave the highest cysteine protease activity.

4.4.3 Flourometric assay- cystatin activity

Papain (10 µg) was also added to the soybean nodule extracts which had been pre-incubated with E-64 in order to determine the activity of the protease inhibitors contained in the soybean nodule extracts. Nodule protein was precipitated with acetone and thoroughly washed with 70% acetone to remove excess E-64. The inhibitors contained in the nodule extracts inhibited the activity of papain as the proteolytic activity of papain was lowered in all the soybean nodule extracts. The inhibition increased with an increase in the age of the nodules as evidenced by the very low papain activity in the 12 and 16 week nodule extracts. The 6 week soybean nodule extract had the highest inhibition of papain activity which indicates its high content in protease inhibitors (Figure 16).

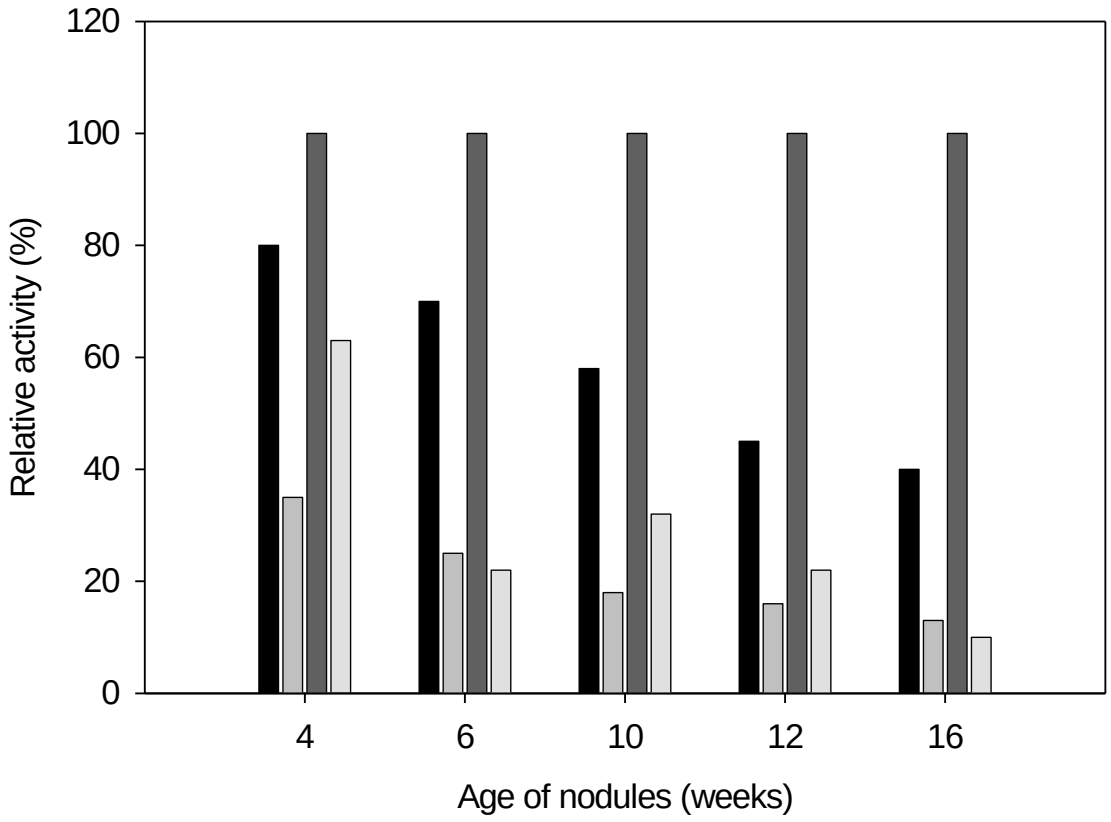

Figure 16. Cysteine protease activity of the soybean nodule protein (■), nodule protein with E-64 (■), papain (10 µg) (■) and nodule protein with E-64 and papain (■).Results are expressed relative to the extracts that gave the highest cysteine protease activity.

4.4.4 Protease Zymography

Protease zymograms were performed to obtain a profile of the proteolytic activity of proteases in the soybean extracts. Ten percent acrylamide gels which contained 0.1% gelatin in the resolving gel where used. The protease bands observed were named P1-P5. A broad band (P3) was visualised as the major band in all the soybean leaf (Figure 17) and nodule (Figure 18) extracts. As the plant matured, the protease activity of the P3 band increased from the 4 to 16 weeks old soybean leaf extracts whereas in nodule extracts the protease activity decreased in the older plants (Figure 18). The 10, 12 and 16 weeks soybean leaf and nodule extracts showed high expression of other active proteases (P4 and P5) with lower apparent molecular weights. The zymograms also included extracts pre-incubated with E-64, a specific inhibitor for cysteine proteases. E-64 reduced the proteolytic activity of the P3 band in all the extracts but not activity of other bands (P4 and P5) which were observed the 10, 12 and 16 weeks leaf and nodule extracts. In reverse zymography, Ten percent acrylamide gels which contained 0.1% gelatin in the resolving gel also where used. After incubation of the gel in papain solution, papain digested the entire gelatin that was in the resolving gel and resolved proteins from the nodule extracts. Cysteine protease inhibitors were observed as bands on a clear gel which were more visible in the 4 and 6 weeks old soybean nodule extracts compared to the 10, 12 and 16 weeks old soybean nodule extracts (Figure 19). Protein bands expressed in the 4 and 6 weeks old soybean nodule extracts had a lower intensity when compared to those in a native coomassie stained acrylamide gel (Figure 19).

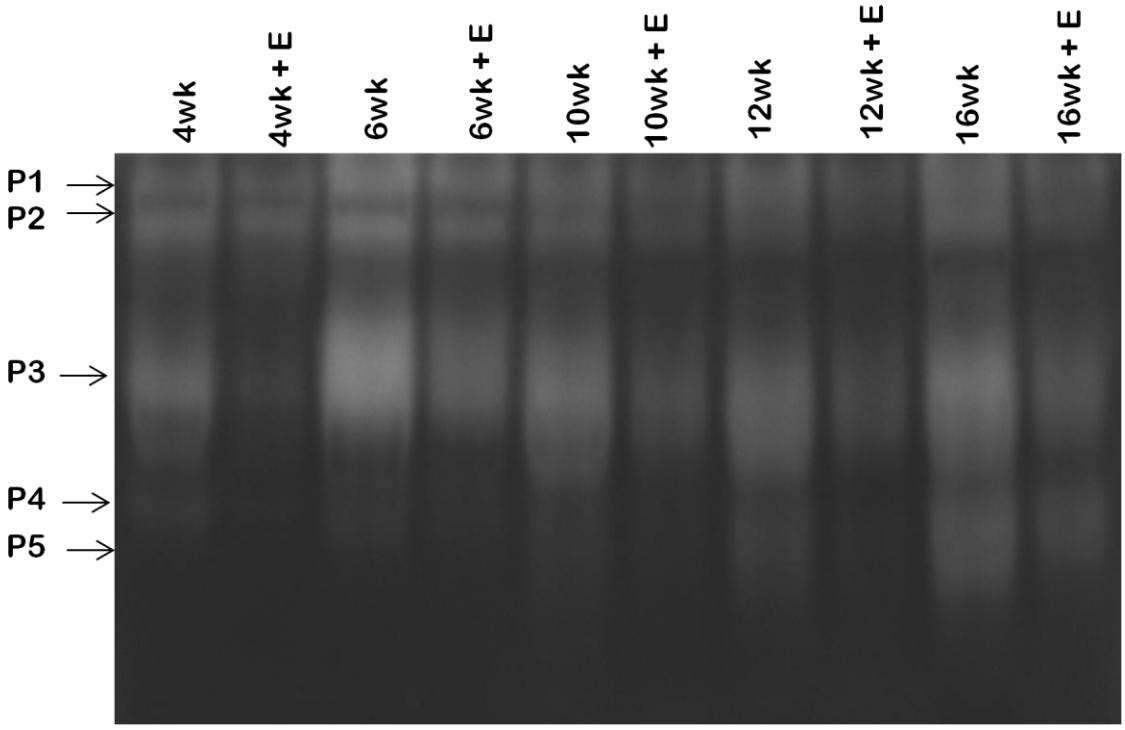

Figure 17. Protease zymogram of the soybean leaf extracts. 4wk: Extract from leaves of a 4 week old plant; 4wk+E: Extract from leaves of a 4 week old plant with excess E-64; 6wk: Extract from leaves of a 6 week old plant; 6wk+E: Extract from leaves of a 6 week old plant with excess E-64 and 10wk: Extract from leaves of a 10 week old plant; 10wk+E: Extract from leaves of a 10 week old plant with excess E-64; 12wk: Extract from leaves of a 12 week old plant; 12wk+E: Extract from leaves of12 week old plant with excess E-64; 16wk: Extract from leaves of a 16 week old plant and16wk+E: Extract from leaves of a16 week old plant with excess E-64.

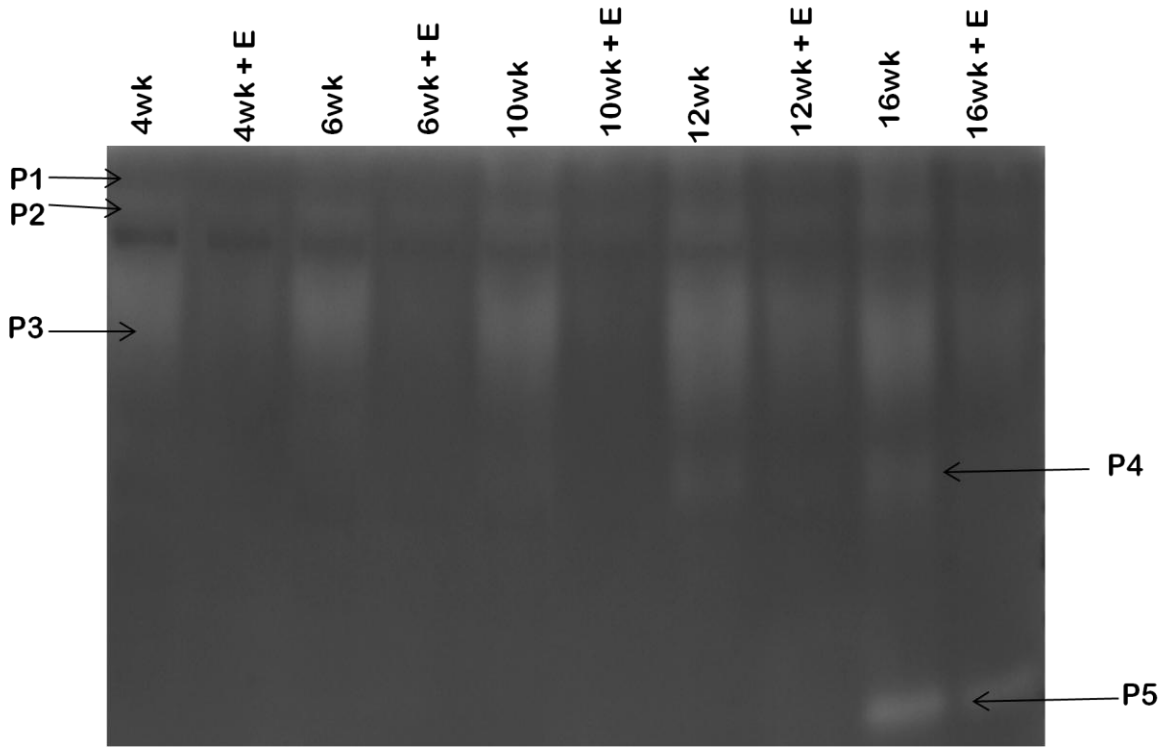

Figure 18. Protease zymogram of the soybean nodule extracts. 4wk: Extract from nodules of a 4 week old plant; 4wk+E: Extract from nodules of a 4 week old plant with excess E-64; 6wk: Extract from nodules of a 6 week old plant; 6wk+E: Extract from nodules of a 6 week old plant with excess E-64 and 10wk: Extract from nodules of a 10 week old plant; 10wk+E: Extract from nodules of a 10 week old plant with excess E-64; 12wk: Extract from nodules of a 12 week old plant; 12wk+E: Extract from nodules of12 week old plant with excess E-64; 16wk: Extract from nodules of a 16 week old plant and16wk+E: Extract from nodules of a16 week old plant with excess E-64.

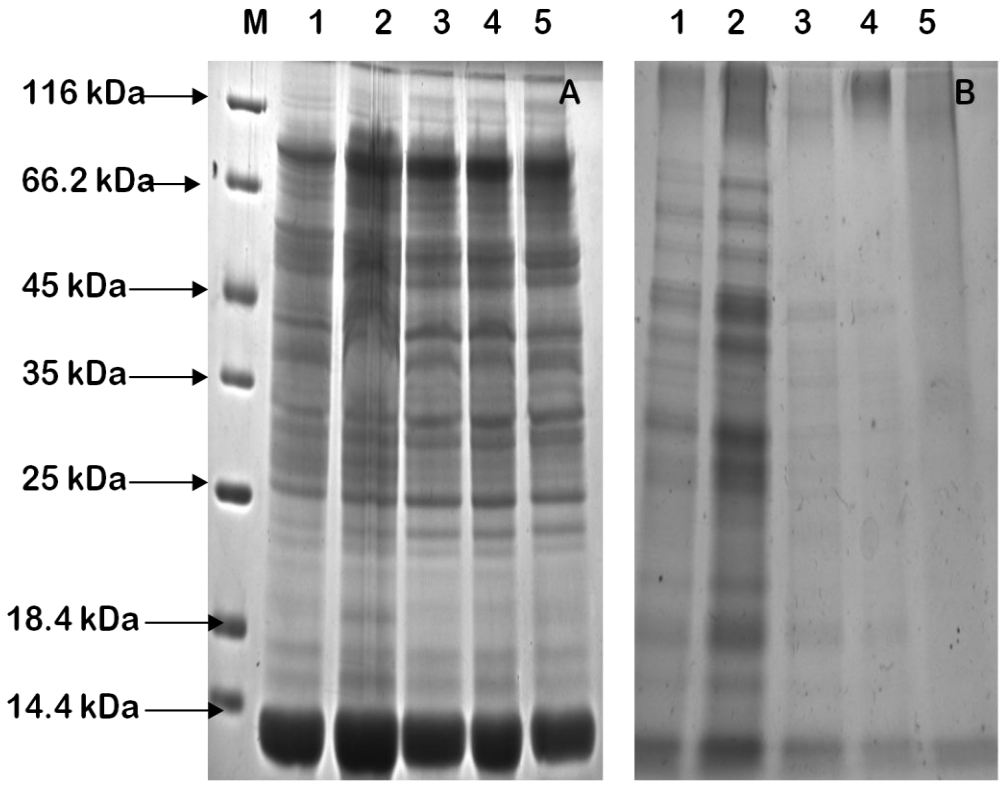

Figure 19. SDS-PAGE analysis (A), Reverse zymography (B) of soybean nodule extracts. Lane M: molecular weight marker: Extract from nodules of a 4 week old plant; lane 2: Extract from nodules of a 6 week old plant; lane 3: Extract from nodules of a 10 week old plant; lane 4: Extract from nodules of a 12 week old plant and lane 5: Extract from nodules of a16 week old plant.

4.5 Probing for cysteine proteases

Cysteine protease-specific labelling was used to detect and profile cysteine protease isoforms without purifying them from the crude protein extracts. Cysteine proteases in the soybean extracts were tagged with DCG-04 which is a biotinylated modified cysteine protease inhibitor. The nodule protein extracts were resolved by 1-dimensional and 2-dimensional PAGE. The proteins were then transferred onto nitrocellulose membranes. Biotinylated proteins were detected using streptavidin peroxidase and chemiluminescence. A comparative analysis of the content of active cysteine proteases in the soybean nodule (Figure 20) and leaf (Figure 21) extracts from 4 to 16 week old soybean plants was performed. A coomassie stained gel for the soybean nodule extracts that demonstrates the total extracts is shown in figure 11, The active site affinity tagging with DCG-04 confirmed the presence of cysteine proteases in all the soybean extracts which appeared to be a broad band ranging approximately from 25 to 30 kDa. The signal intensity of the band for nodule extracts decreased with an increase in age of the nodules indicating that the cysteine proteases were more expressed in the younger nodules and the expression decreased with age (Figure 20). Tagged papain was used as a positive control to ensure that the signal visualised was that of cysteine proteases. A band with high signal intensity was visualised for papain at about 26 kDa. For soybean leaf extracts, the signal intensity of the broad band increased with an increase in the age of the leaves which is an indication that active cysteine proteases are more expressed in the older leaves (Figure 21).

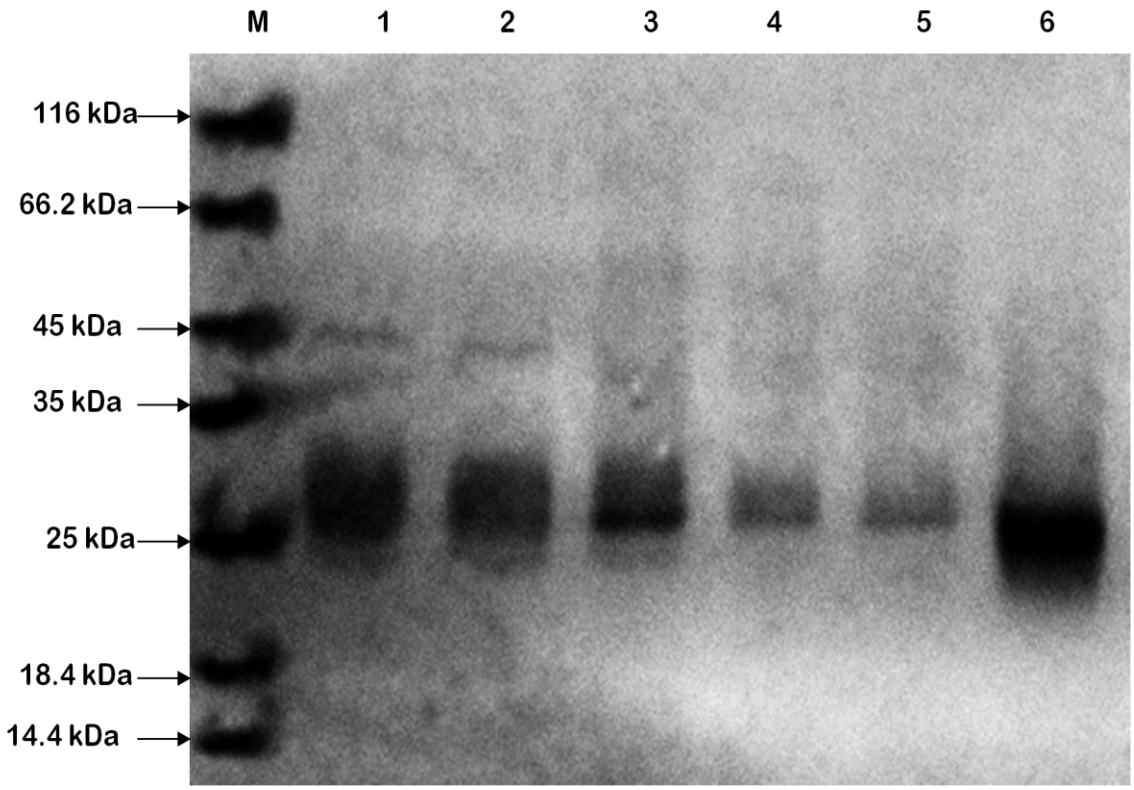

Figure 20. Probing of DCG-04 labelled soybean nodule cysteine proteases, Lane M: molecular weight marker; Lane 1: Extract from nodules of a 4 week old plant; lane 2: Extract from nodules of a 6 week old plant; lane 3: Extract from nodules of a 10 week old plant; lane 4: Extract from nodules of a 12 week old plant; lane 5: Extract from nodules of a16 week old plant and lane 6: papain.

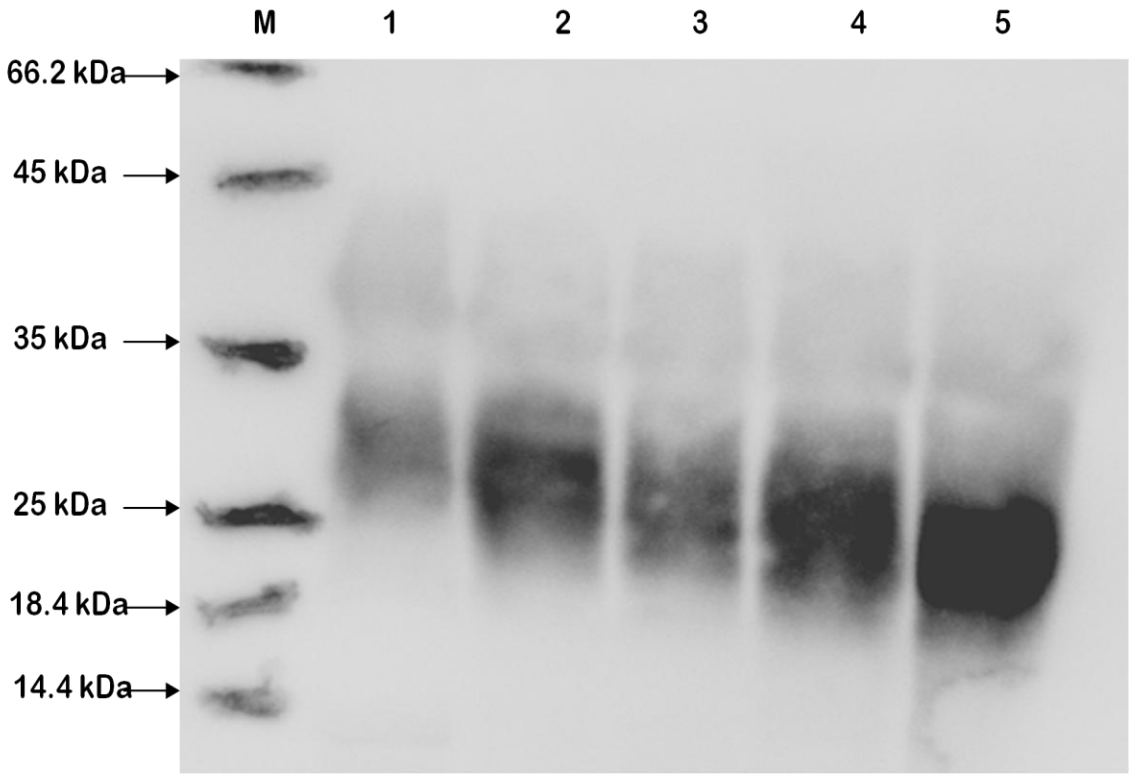

Figure 21. Probing of DCG-04 labelled soybean leaf cysteine proteases, Lane M: molecular weight marker; Lane 1: Extract from leaves of a 4 week old plant; lane 2: Extract from leaves of a 6 week old plant; lane 3: Extract from leaves of a 10 week old plant; lane 4: Extract from leaves of a 12 week old plant and lane 5: Extract from leaves of a16 week old plant

To confirm that the signals on the protein blots represent cysteine proteases, labelling of the soybean nodule extracts was performed in the presence of an excess of a known cysteine protease inhibitor, E-64 which would compete with DCG-04 for the active site of cysteine proteases and significantly reduce labelling of cysteine proteases. DCG-04 labelling was reduces by E-64 as the signal was drastically reduced in extracts that were pre-incubated with E-64 (Figure 22).

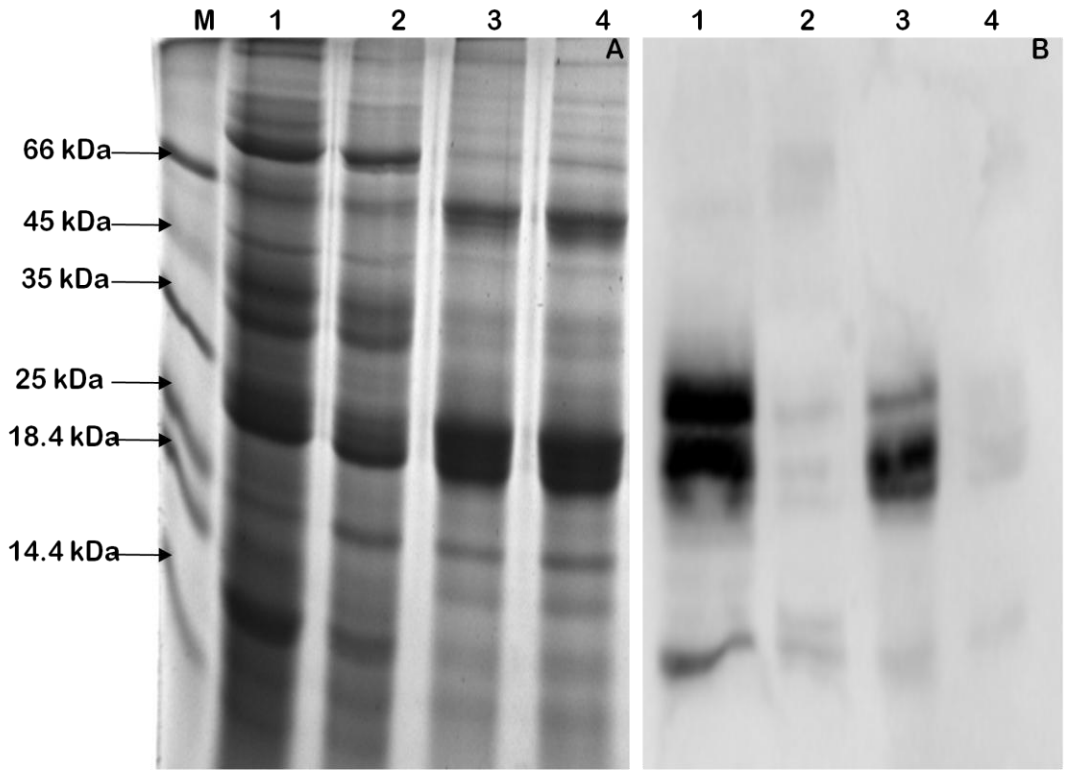

Figure 22. Coomasie stained (A) and DCG-04 (B) probing of 4 and 16 weeks old labelled soybean nodule extracts and soybean nodule extracts labelled in the presence of excess E-64. Lane M: Molecular weight marker; lane 1: 4 week old labelled soybean nodule extract; lane 2: 4 week old soybean nodule extract labelled in the presence of E-64; lane 3: 16 week old labelled soybean nodule extract and lane 4: 16 week old soybean nodule extract labelled in the presence of E-64.

4.6 Two dimensional polyacrylamide gel electrophoresis of the soybean nodule and leaf extracts

Nodule and leaf proteins were separated more effectively according to their isoelectric point and subunit molecular weight using 2-dimensional gel electrophoresis (2D-PAGE). A number of spots were observed for the 4 week soybean nodule proteome and the area where cysteine proteases are expected is highlighted (Figure 23). Cysteine proteases in soybean nodules may beat very low concentrations as shown by the absence of clear spots in the area highlighted (Figure 23).

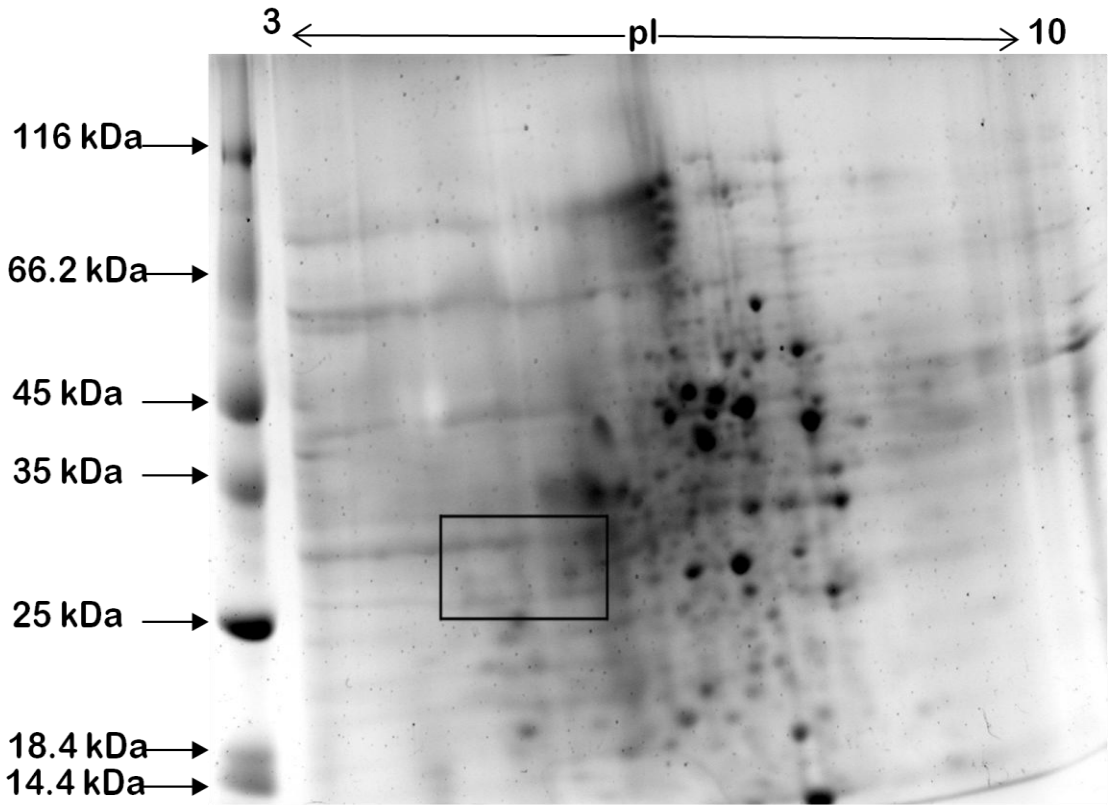

Figure 23. 2D- PAGE of a 4 week old soybean nodule proteome. The rectangle shows area where cysteine proteases are expected.

4.6.1 Probing for cysteine proteases in soybean nodules

Active cysteine proteases were tagged with DCG-04, separated using 2D-PAGE and detected using streptavidin peroxidase and chemiluminescence as described in section 3.2.7. Use of the horseradish peroxidise to highlight the molecular weight markers enabled visualisation of the markers after probing. In all the soybean nodule extracts, all the spots had a molecular weight ranging from approximately 25 to 30 kDa. A major spot with a low pI of about 4, labelled “a” was observed from 4 to 16 weeks soybean nodule extracts (Figures 24, 25 and 26). The 2-D blots also showed expression of more cysteine proteases in the young nodules (4 weeks) as evidenced by the cluster of spots with higher signal intensity. Less acidic cysteine protease isoforms labelled ”b”, “c”, “d” and “e” with a pI of about 5-6.8, were more expressed in the 4, 6 and 10 weeks old nodules and had molecular weight close 30 kDa (Figures 24 and 25). To confirm specificity of the labelling, in the control experiment E-64 was added in excess to the 4 weeks soybean nodule extract in order to compete with DCG-04 for the binding site. The blot only showed 4 spots “g”, “h” and “i” with very low signal intensity at about 14.4 kDa (Figure 24) which could be a result of unspecific binding.

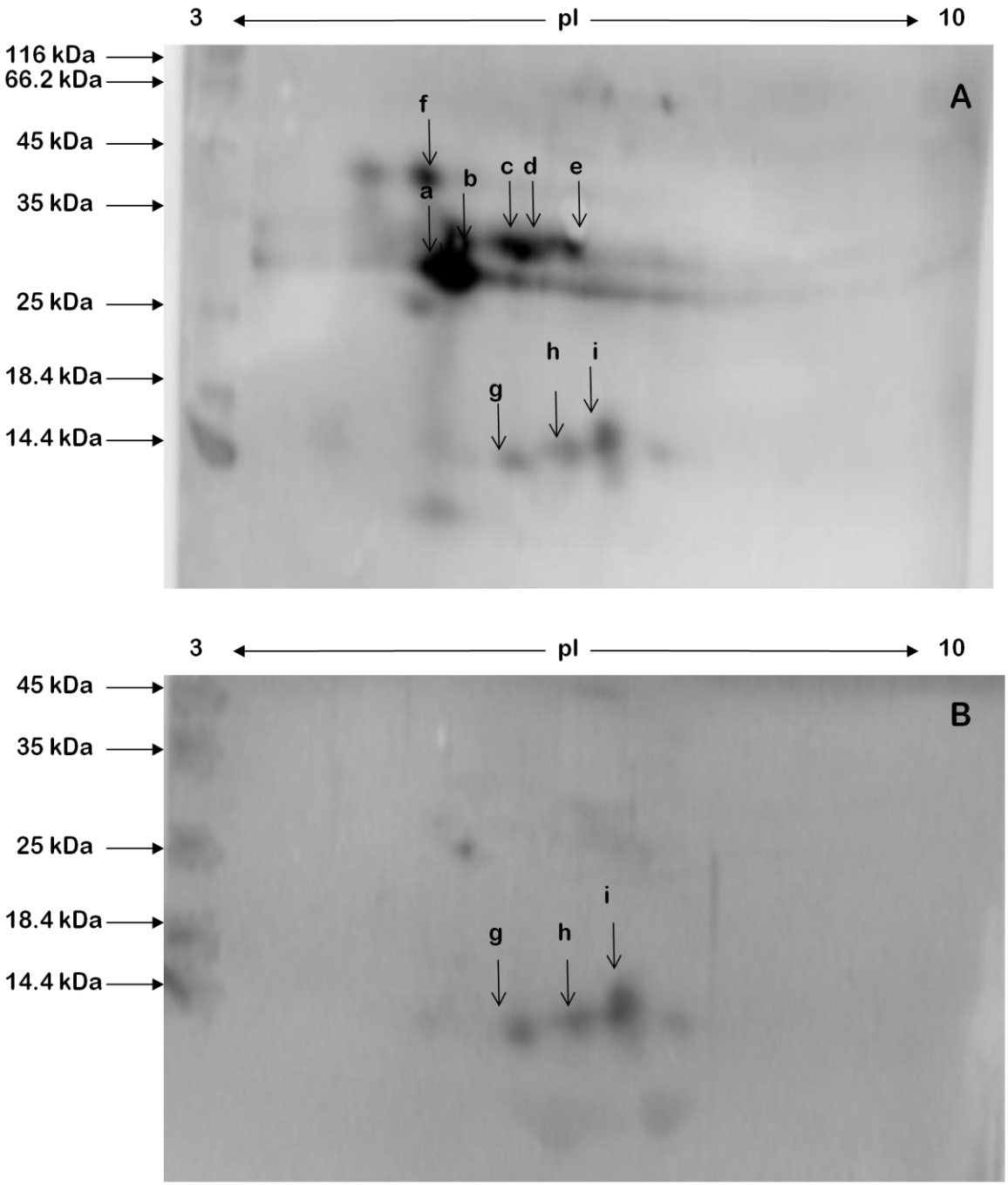

Figure 24. Probing of DCG-04 labelled extract from nodules of a 4 week old soybean plant(A), labelled extract from nodules of a 4 week old soybean plant in excess of E-64 (B), after separation by 2D-PAGE. Non-specific labelling (g, h and i) after separation by 2D- PAGE.

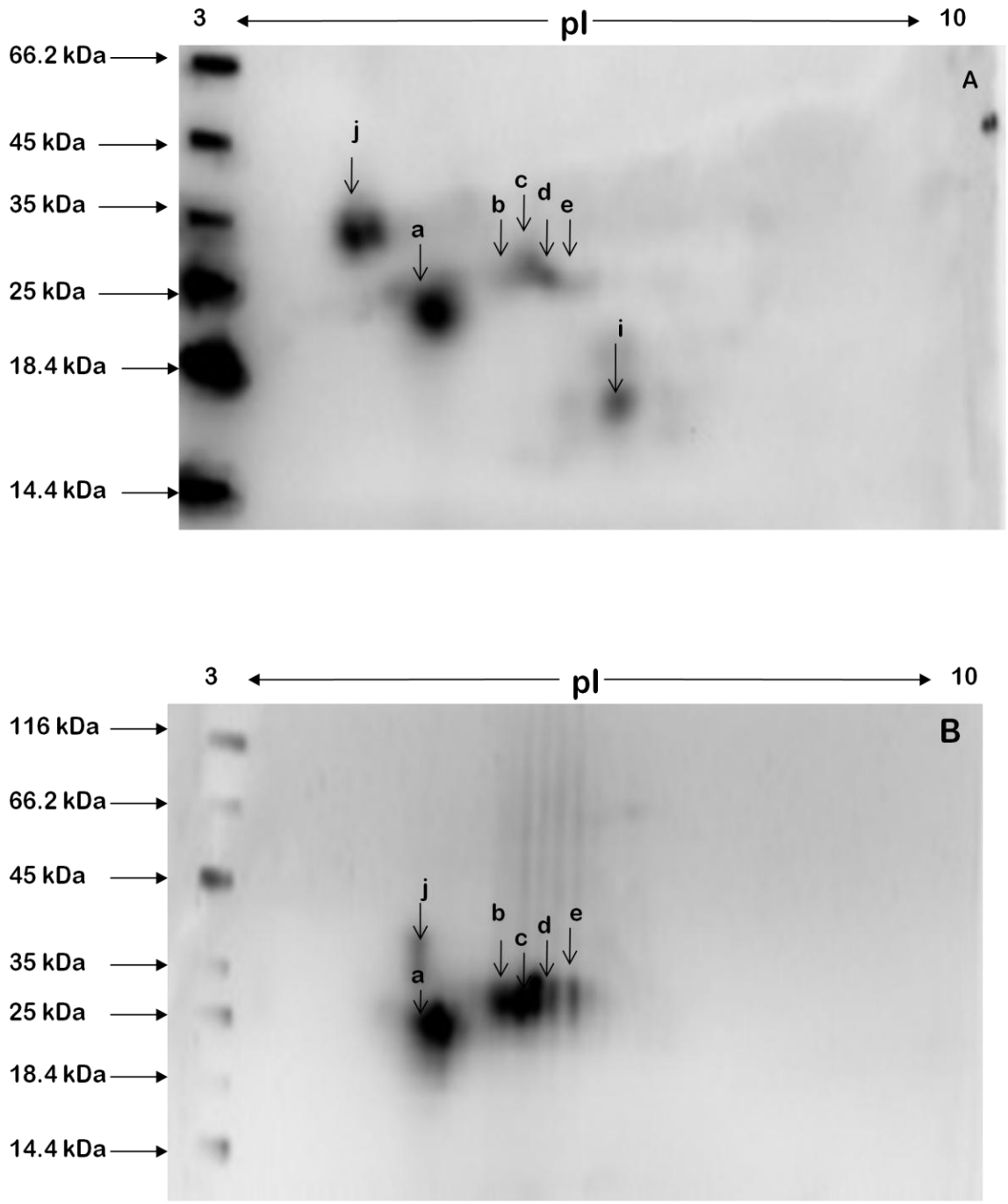

Figure 25. Probing of DCG-04 labelled extracts from nodules of a 6 week old soybean plant (A) and 10 week old soybean plant (B) after separation by 2D-PAGE.

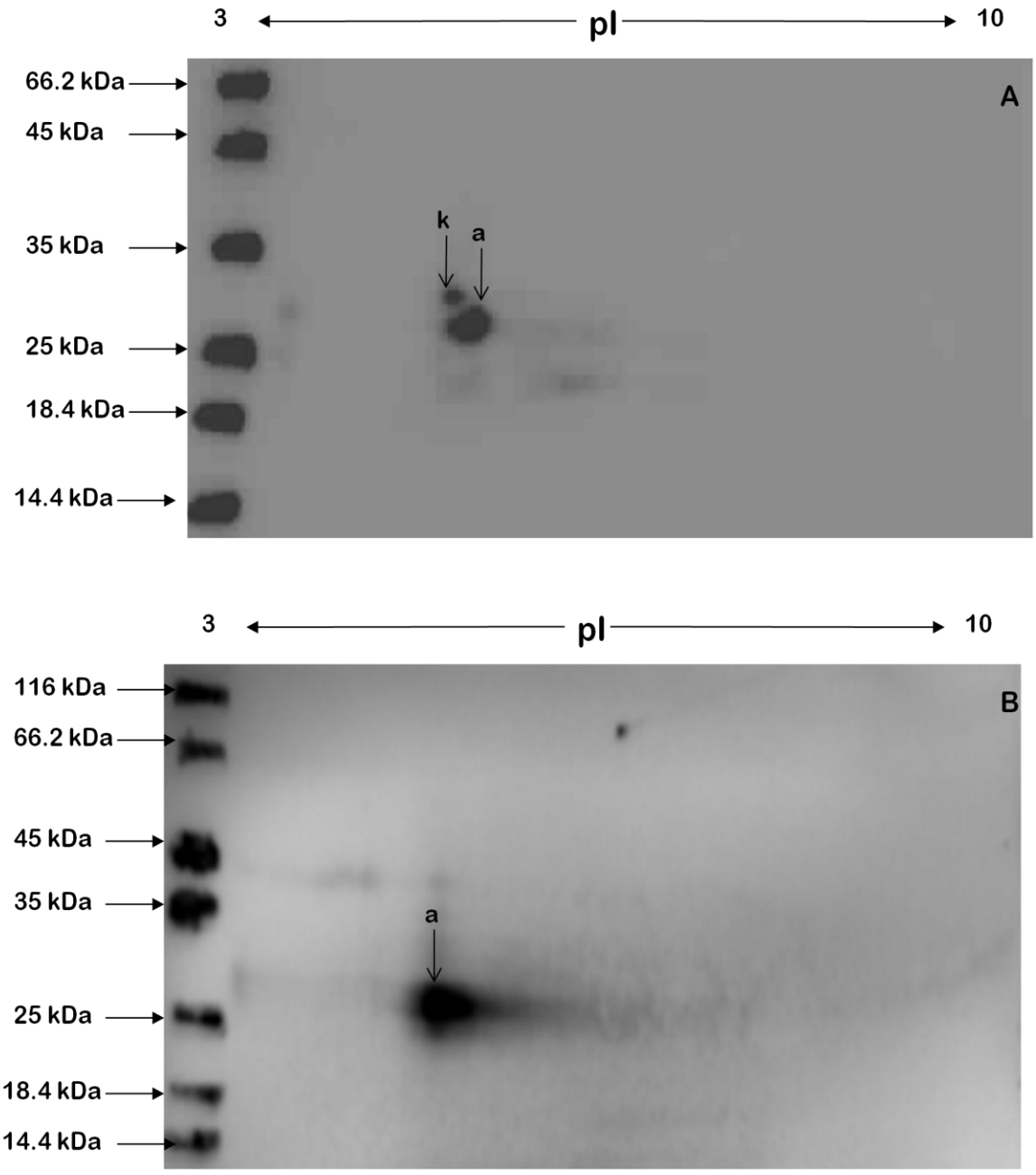

Figure 26. Probing of DCG-04 labelled extracts from nodules of a 12 week old soybean plant (A) and 16 week old soybean plant (B) after separation by 2D- PAGE.

4.6.2 Probing for cysteine proteases in soybean leaves

Soybean leaf extracts were also tagged with DCG-04, separated using 2D-PAGE and active cysteine proteases were detected using streptavidin peroxidase and chemiluminescence as described in section 3.2.7. All the spots had a molecular weight ranging from approximately 25 to 30 kDa. A major spot labelled "a" with a pI of 4 was observed in all soybean leaf extracts. The intensity of the spot increased with an increase in the age of the leaves (Figure 27, 28 and 29) which is an indication that the expression of cysteine proteases increases with an increase in the age of the leaves. The 2-D blots showed a higher expression of cysteine proteases in the old leaves (16 weeks) as evidenced by a cluster of spots with high signal intensity (Figure 29). Less acidic cysteine protease isoforms with isoelectric points ranging from 5 to 7 labelled "c", "d", "e" and "f" were highly expressed in the 10 weeks old leaves (Figure 28) when compared to the same isoforms in the 12 and 16 week leaf extracts (Figure 28 and 29 respectively).

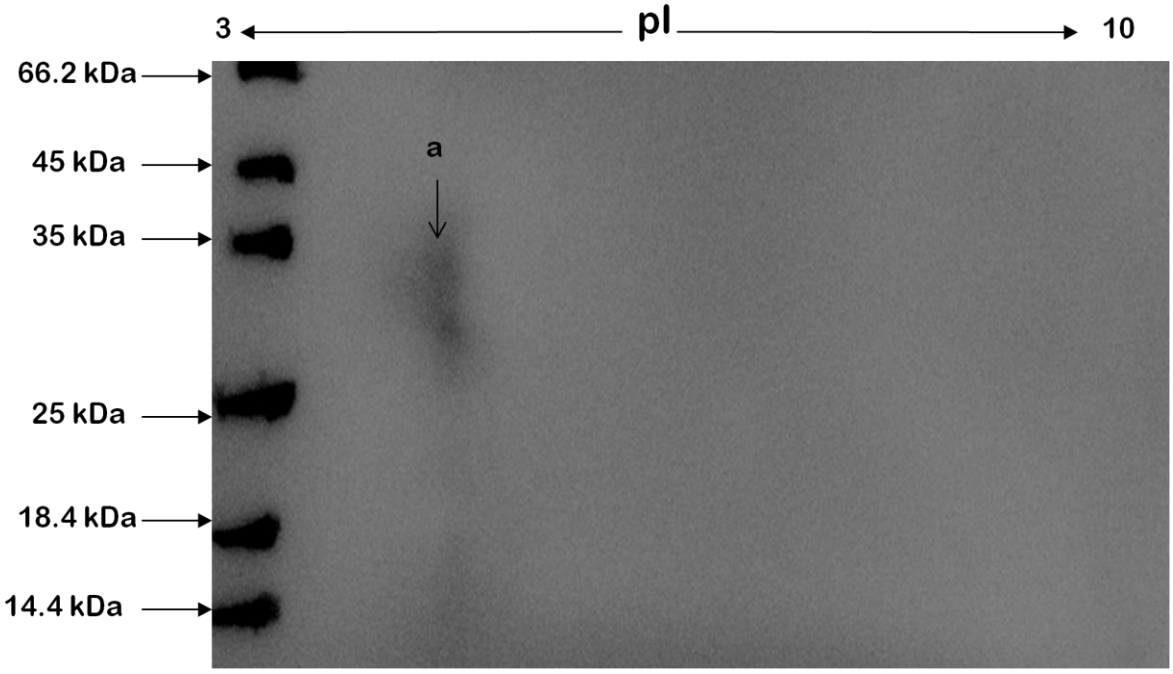

Figure 27. Probing of DCG-04 labelled extract from the leaves of a 4 week old soybean plant after separation by 2D- PAGE.

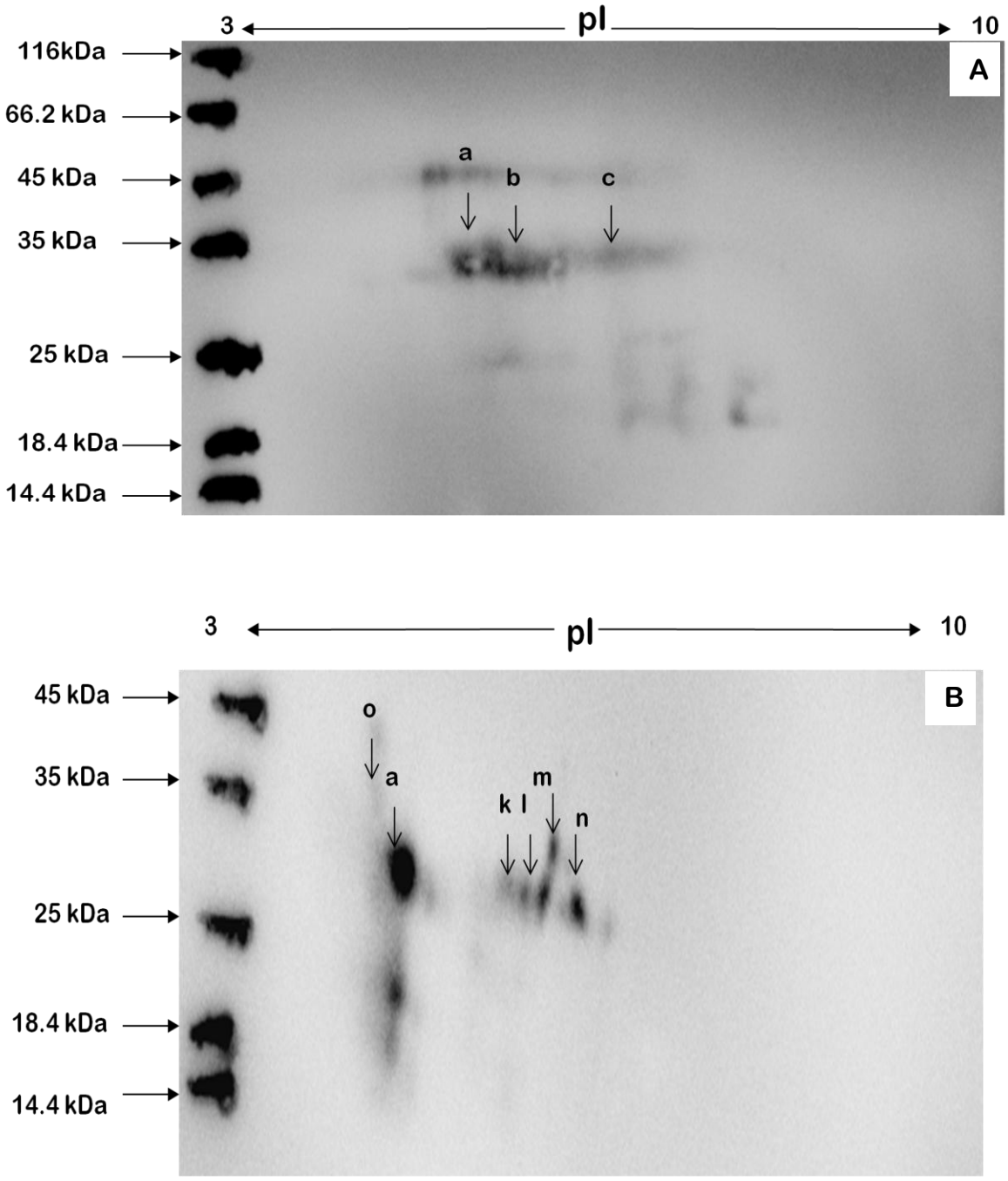

Figure 28. Probing of DCG-04 labelled extracts from leaves of a 6 week old soybean plant (A) and 10 week old soybean plant (B) after separation by 2D-PAGE.

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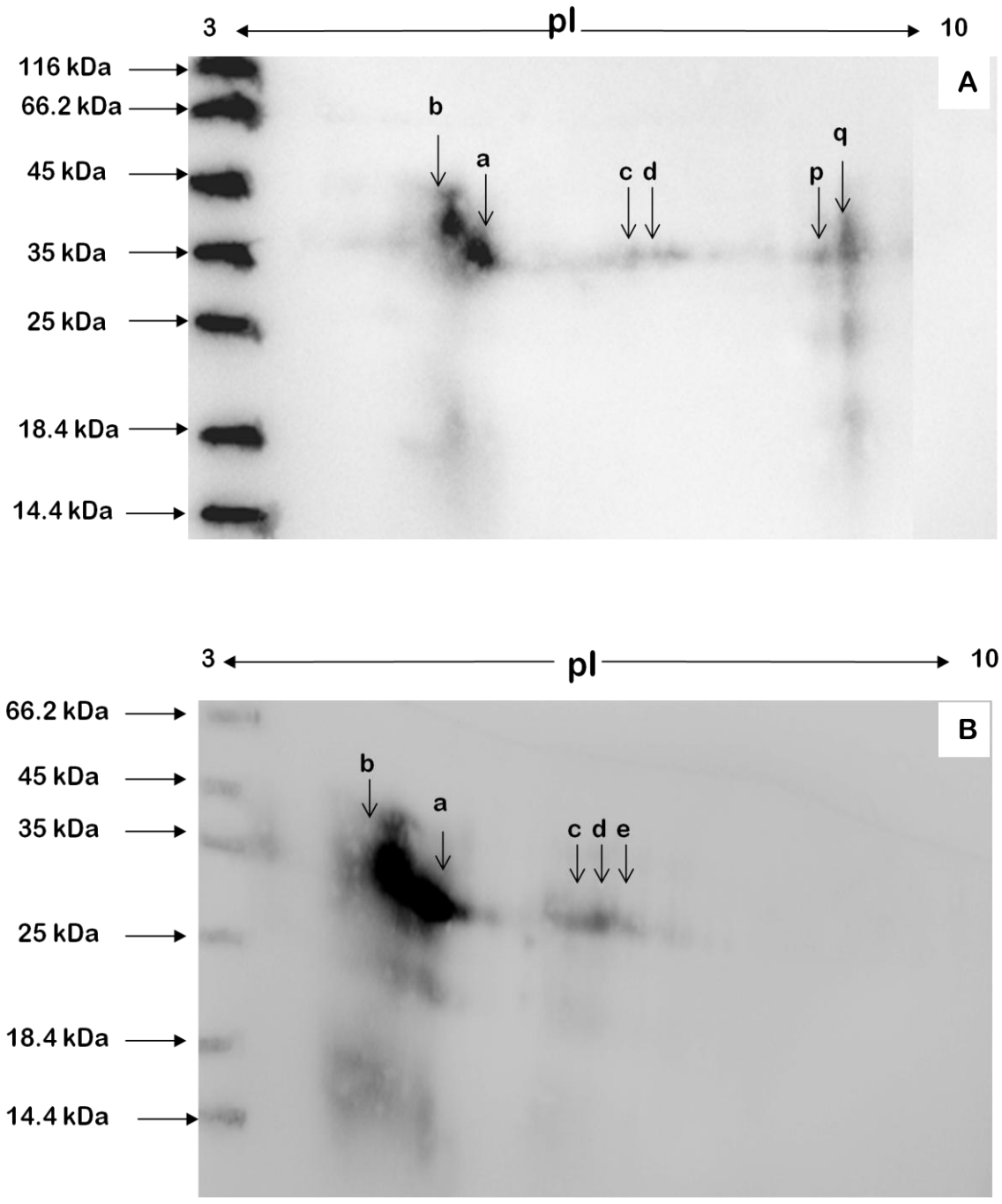

Figure 29. Probing of DCG-04 labelled extracts from leaves of a 12 week old soybean plant (A) and 16 week old soybean plant (B) after separation by 2D-PAGE.

4.7 Isolation of cysteine proteases

4.7.1 Affinity precipitation

Affinity precipitation was carried out to isolate specifically, the labelled cysteine proteases from extracts described in section 3.2. The attempt to isolate cysteine proteases from the soybean extracts by affinity precipitation yielded a strong band with the expected size of between 26 and 30 kDa observed on a coomassie stained acrylamide gel (Figure 30). Weak bands of high molecular weight were also observed. When the protein bands from a duplicate gel were transferred to nitrocellulose membrane and detected using streptavidin and chemiluminescence, a strong signal was obtained only at about 26 kDa (Figure 30). In the control experiments were E-64 was added in excess and some of the soybean extracts were not labelled with DCG-04, probing of the extracts yielded no signal (Figure 31).

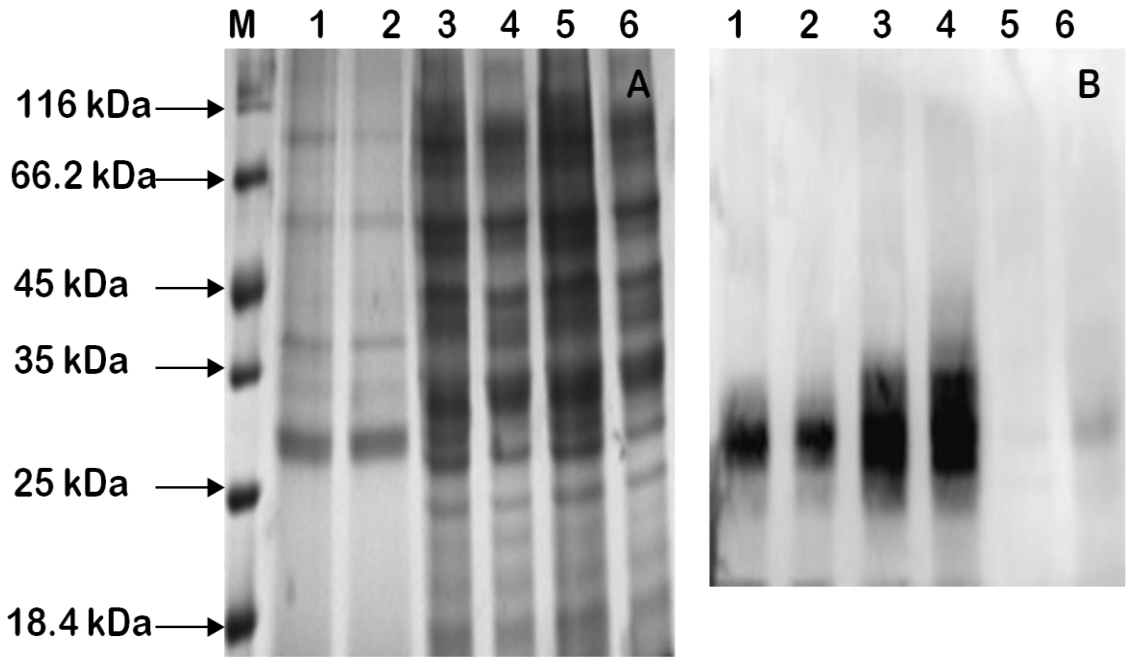

Figure 30. SDS-PAGE analysis (A) and probing of DCG-04 labelled (B) 4 and 16 week soybean supernatants after affinity precipitation of cysteine proteases. Lane M: Molecular weight marker; lane 1 and 2: bound fractions eluted from streptavidin magnetic beads incubated with extracts from 4 weeks and 16 weeks old nodules respectively: bound fraction eluted from streptavidin magnetic beads incubated with extracts from old nodule extracts; lane 3 and 4: crude extracts from the 4 weeks and 16 weeks old nodules respectively; lane 5 and 6: supernatants after affinity precipitation of cysteine proteases in 4 week and 16 week old nodules respectively.

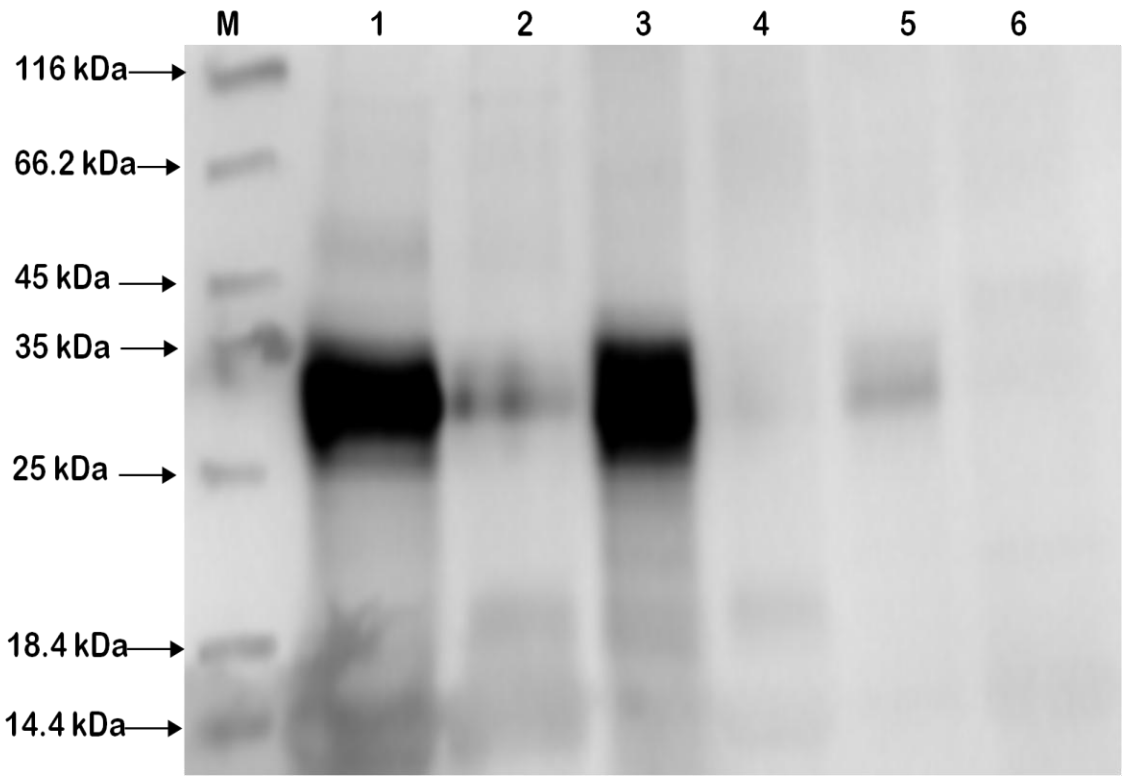

Figure 31. Probing of DCG-04 tagged soybean nodule extracts. Lane M: Molecular weight marker; lane 1: 4 week eluted labelled fraction; lane 2: 4 week eluted unlabeled fraction; lane 3: 16 week eluted labelled fraction; lane 4: 16 week eluted unlabeled fraction; lane 5: 4 week eluted labelled fraction with E-64 and lane 6: 16 week eluted labelled fraction with E-64.

CHAPTER 5

5 DISCUSSION

5.1 Developmental changes and changes in total protein of the leaves and nodules

As demonstrated by the BCA assay (Figure 10), a decrease in total soluble protein of the soybean nodule extracts as plants grew older was observed. This trend was also demonstrated in SDS-PAGE analysis in which most protein bands present in the younger nodule extracts disappeared in the 12 and 16 weeks nodule extracts. This could be a result of the developmental changes that take place in the nodules as they mature. As most nodules develop, they consist of a gradient of developmental zones which include the persistent apical meristern, an infection zone and a fixation zone. In mature nodules, a senescence zone is established proximal to the fixation zone (Van de Velde *et al*., 2006). At 4, 6 and 10 weeks of nodule development, the nodules are pinkish-reddish in colour due to leg-haemoglobin indicating nitrogen fixation is taking place (Figure 7). Because the nitrogenase enzyme involved in nitrogen fixation, is very sensitive to oxygen, leghemoglobin buffers the amount of oxygen that is free in the infection zone of the nodules. The high soluble protein content in the young nodules can also be attributed to the presence of the heme protein, leghemoglobin which disappears in the older nodules. The nodules were green at 12 and 16 weeks which signifies that the nodules were no longer fixing nitrogen and the symbiotic relationship between the *rhizobia* and nodules had been lost. Loss of the *rhizobium*-soybean symbioses has been reported to coincide with nodule senescence (Puppo *et al*., 2004). These changes explain the drop in total soluble protein as the plant grew. Nodule senescence is also characterised by mass degradation of proteins (Van de Velde *et al*., 2006).

5. 2 Proteolytic activity of the plant and nodule extracts

Azocasein is a well known substrate for measuring proteolytic activity. Fernandez and Pomilio, (2003) have used azocasein to measure total proteolytic activity in callus culture of pineapple in order to determine the catalytic type of the peptidase complex from callus culture of pineapple (*Ananas comosus*). The decrease in the total proteolytic activity of the nodule extracts as the plants grew older (Figure 12) was not expected and in contrast to that of the leaf extracts which increased with the age of the plants (Figure 13). An increase in the proteolytic activity in the leaf extracts as the plants grew older is as expected because the older leaves are senescing and a lot of protein degradation is taking place hence the high proteolytic activity (Figure 13). Proteolysis during senescence has been reported to be a result of the release of active proteases from the vacuole into the cytosol and *de novo* synthesis of proteases during senescence (Azeez *et al*., 2006). It has also been reported that senescence of nodules and leaves is directly associated with elevated proteolytic activity and decreased soluble protein content including the disappearance of leg-haemoglobin in legumes (Malik *et al*., 1981). The decrease in proteolytic activity of the nodules as the plants grew older (Figure 12) is however unexpected but could have been due to the fact that the older nodules (12 and 16 weeks old) collected were very much degraded as nitrogen fixation had ceased and therefore the nodules were about to be discarded by the plant. The high total proteolytic activity observed for 10 weeks nodule extract could be signifying the onset of nodule senescence (Figure 12).

Cleavage of the Z-Arg-Arg-AMC substrate by the nodule and leaf extracts indicated presence of cysteine proteases in the soybean plant extracts. Z-Arg-Arg-AMC is a well-known substrate specific to cysteine proteases. For example, isolated cysteine proteases in *Phaseolus vulgaris* leaves have been reported to be highly active towards Z-Arg-Arg-AMC (Popovic *et al*., 1998). The decrease in the cysteine protease activity of the 4 to 16 weeks soybean nodule extracts from 100% to 43% (Figure 14) is however unexpected but probably suggests that there were fewer active cysteine proteases in the 12 and 16 week nodules or possibly high content of endogenous cysteine protease inhibitors or cystatins

which control the activity of cysteine proteases at 12 and 16 weeks of nodule development. Cysteine protease activity is expected to increase in all senescing tissues as the plants grow older as mass degradation of proteins takes place in older tissues. In this study, this was not the case with nodules but an increase in cysteine protease activity in the leaf extracts as the plants grew older was observed from 30% to 100% (Figure 15). The increase in cysteine protease activity is related to what has been reported in a similar investigation using Z-Phe-Arg-AMC in senescing alfalfa leaves (Nieri *et al*., 1998). In the soybean nodules, the highest cysteine protease activity detected in the 4 week nodule extract could have been that of cysteine proteases involved in a number of roles in the young soybean plants. Cysteine proteases have been reported to be involved in diverse aspects of plant physiology and development including protein storage and mobilization (Grudkoswa and Zagdanska, 2004). In the current study, there was a positive correlation of the cysteine protease activity and total soluble protein content of the soybean leaf and nodule extracts. In the soybean nodules, as the cysteine proteases activity decreases, the total soluble protein content also decreases which suggests that autolysis might have been taking place, which is degradation of proteins by other proteases other than cysteine proteases. The cysteine proteases may also be compartmentalised and therefore not released into the cytoplasm but function in the vacuole. Cysteine protease activity in the 16 week old leaves which was the highest was 13×10^5 fluorescence units whereas that of the 16 week nodules was 18×10^6 fluorescence units. There could be a possibility that in the nodules, these are levels high enough to degrade proteins and therefore be responsible for senescence as is the case in the leaves. Z-Arg-Arg-AMC has also been used in a similar approach to investigate cysteine protease activity in mature and senescent tobacco leaves. The cysteine protease activity was significantly higher in senescent leaves than in mature green leaves (Beyene *et al*., 2006)

Zymography of one dimensional gel systems has been extensively employed as convenient technique for identification and characterisation of the activity of proteases (Saitoh *et al*., 2005 and Simona-Stoilova *et al*., 2009). Clear zones observed in protease zymograms indicated protease activity in all the leaf (Figure 17) and nodule (Figure 18) extracts. The proteolytic activity of the leaf extracts increased as the plants grew older.

This was evidenced by the increase in the intensity of the clear bands which is as expected as the triggering of a wide range of proteolytic activities that causes large scale protein degradation is a distinctive characteristic of senescence (Van de Velde *et al*., 2006). This was consistent with expectations and findings from a similar study by Beyene *et al*. (2006) wherein tobacco senescent leaves has higher proteolytic activity than mature green leaves when also assayed by the gelatin SDS-PAGE method. The proteolytic activity of the nodule extracts was however in contrast with that of the leaves in which the protease activity decreased with an increase in the age of the nodules as evidenced by the decrease in the intensity of the clear bands. The same trend was also demonstrated by azocasein assay in measuring the total proteolytic activity of the nodule and leaf extracts (Figure 12 and 13 respectively). The finding however suggests that nodule and leaf senescence do not occur concurrently and may involve different proteases.

The reduction in the intensity of the band P3 in nodule (Figure 18) and leaf (Figure 17) extracts pre-incubated with E-64 suggests that these are cysteine proteases that were largely inhibited by E-64. Complete inhibition was not observed probably because of the concentration of E-64 used or competition with other endogenous molecules binding to the active site of cysteine proteases. The presence of other clear bands in the leaf and nodule extracts labelled P1, P2, P4 and P5 (Figure 17 and 18 respectively) which were not inhibited by E-64 could be evidence suggesting the presence of other proteases other than cysteine proteases. In broccoli, three classes of proteases were reported to be involved in senescence of broccoli florets which include cysteine, aspartate and serine proteases using a similar approach (Wang *et al*., 2004). In gel assays were also done on senescing gladiolus flower which revealed the presence of active serine proteases as the protease activity was inhibited by PMSF, a known serine protease inhibitor (Azeez *et al*., 2006). In protease reverse zymography performed in the current study (Figure 19), the protein bands observed in the 4 and 6 week nodules could be proteins that have evolved resistance to proteolytic attack or protease inhibitors mainly expressed in the young nodules. In the young nodules a high proteolytic activity was observed which can be correlated to the high expression of proteins with a high resistance to papain. Protease inhibitors produced by plants have also been reported to have different roles in protection

of the plants against insect damage, microbes and viruses (Jongsma and Beekwilder, 2008). The main class of protease inhibitors which have been characterised and isolated are cystatins which are tight binding inhibitors of papain-like cysteine proteases widespread in plants and in animals with the main function of regulating protein turnover and defence against pathogens as well as the host-parasite immune relationship (Zhou *et al*., 2009).

5.3 Activity profiling of Cysteine protease in the nodule extracts

A cysteine protease activity profile from soybean nodule extracts was obtained using DCG-04 which is a biotinylated label of E-64. DCG-04 was used as a label because it has high efficiency in specifically tagging cysteine proteases as was observed in van der Hoorn *et al.* (2004) in an investigation on *Arabidopsis thaliana*. Transcriptomic analysis has pointed out the involvement of cysteine proteases in the senescence of a variety of food crops which include wheat (Martinez *et al*., 2006), soybean (Cheng *et al*., 1998), rice and barley, (van der Hoorn *et al.*, 2004) and in various plants such as *Arabidopsis thaliana* (van der Hoorn *et al.*, 2004) and coriander (Weibo *et al*., 2002). However, these studies only reveal the mRNA transcripts expressed in a particular tissue which might not necessarily be the active protein in the tissue. Use of a mechanism based probe in active site labelling of cysteine proteases and detection by streptavidin blotting has not been done on soybean. The rationale behind the design of such a functional proteomics approach is to assess the activity profiles of protease species in complex biological samples rather than merely their presence or absence. Identification and activity measurement can be performed without the need for purification of the individual enzymes under study. In a 1-D probing of DCG-04 tagged nodule extracts (Figure 20), the broad band observed at a size approximately ranging from 25-30 kDa was evidence of the presence of active cysteine proteases in the soybean root nodules because when the extracts where pre-incubated with E-64, a highly reduced signal was observed (Figure 21). This was as expected because E-64 is a competitive inhibitor for cysteine proteases and therefore if added in excess, it competes with the affinity tag, DCG-04 to bind to the

active site of the enzyme and therefore disabling the tagging of cysteine proteases with E-64.

Cysteine proteases that are expressed in the nodules have also been detected in pea (Groten *et al*., 2006), alfalfa (Nieri *et al*., 1998), and *Medicago truncatula* (Shoekand *et al*., 2005) using molecular techniques. The researchers suggested that activity of cysteine proteases increased as the nodules aged. However, in the current study, for the nodules, the intensity of the cysteine protease band with a size ranging from approximately 25-30 kDa appeared to decrease from 100% to about 30% reduction with an increase in the age of the nodules which indicates a decrease in the expression of active cysteine proteases (Figure 20). The same trend was observed in the fluorometric assay whereby there was higher cysteine protease activity in the younger nodules (4 and 6 weeks old) compared to the 12 and 16 week old nodules. This is however unexpected but as suggested by Alesandrini *et al*. (2003), a down regulation in cysteine proteases as the plant develops could imply that exchanges between the symbiotic partners is reduced hence the cysteine proteases are not very active. The higher activity of cysteine proteases in the younger nodules (Figure 20) could also imply the involvement of these cysteine proteases in other roles other than nodule senescence as nodule senescence understood to start at about 10 weeks of nodule development (Mashamba *et al*., 2000). In this study the soybean nodule were senescing at around 60 days after inoculation. The lower expression of cysteine proteases in the 12 and 16 week old nodules could be a result of that the nodules were almost dead and about to be discarded by the plant. For the leaf extracts the intensity of the cysteine protease band increased with an increase in the age of the leaves which implies that cysteine proteases were more expressed in the older leaves compared to the younger ones (Figure 21). This was as expected as several reports have pointed out involvement of cysteine proteases in leaf senescence which takes place at about 12 to 16 weeks of leaf development in plants.

Probing of DCG-04 labelled soybean proteomes separated using 2D electrophoresis was used to profile cysteine protease isoforms expressed in the soybean nodule and leaf extracts as two-dimensional (2-D) electrophoresis has the ability to separate and resolve

complex mixtures of thousands of proteins in a single gel (Choe and Lee, 2000). A comparison of the 1-D and 2-D blots clearly indicated the presence of multiple (6) cysteine proteases in the younger nodules (4, 6 and 10 week old) and the older leaves, 10, 12 and 16 week old (6). About 6 low pI isoforms were observed in the nodules and the leaves which explained why the band with molecular size ranging from 25-30 kDa appeared as a broad band which was a result of multiple bands (Figure 23 and 24). Twelve week and 16 week nodule extracts only showed one main spot which correlates with the very low signal intensity observed in a 1-D blot. Presence of one spot in the 12 and 16 week old soybean root nodules may imply that it is the main cysteine protease responsible for senescence (Figure 25) and the other spots expressed in the younger nodules are cysteine proteases performing other functions in the development of the soybean plant.

Using a different approach, Schoekand and Brewin (2003) carried out a study on analyzing gene expression and functionality using transgenic lines of *Medicago truncatula* during seed germination, vegetative growth and nodule development and observed that the cysteine proteases gene Cyp 15a was strongly expressed in cotyledonary leaves, senescent leaves and root nodules. The researchers suggested that in the development and function of legume root nodules, cysteine proteases could be involved in several important processes which include defence responses to root invasion by microorganisms, protein turnover required during the formation of new tissue, cellular homeostasis and metabolism, adaptation of host cells to physiological stresses and control of nodule senescence. This should explain the variation in cysteine proteases expressed in the soybean root nodules at different stages of development. It was expected that cysteine proteases would be highly expressed in the senescing nodules as was observed in the leaves (Figure 29). The function of cysteine proteases in the nodules might be to activate other proteases involved in nodule senescence (Dubey and Jaqannadham, 2003). Serine proteases involvement in floral senescence has been reported in Gladiolus flower also known as corn lily (Azeez *et al*., 2006). Of the total protease activity of the Gladiolus flower, serine proteases have accounted for 60-70% of the activity while cysteine proteases accounted for 23-25%. Expression of other proteases labelled P1, P2, P4 and P5

were also observed in this study using zymography in the leaf and nodule extracts (Figure 17 and 18 respectively). These could be proteases other than cysteine proteases because they were not inhibited by E-64.

5.4 Isolation of cysteine proteases

Pure cysteine protease had to be obtained from soybean root nodule extracts as it was desirable to obtain the pure cysteine proteases and get an insight into the amino acid sequence information using liquid chromatography- mass spectrometry (LC-MS). Therefore in this study, affinity precipitation was employed using streptavidin magnetic beads and probed soybean nodule extracts. The nodule extracts were tagged with DCG-04, a biotinylated cysteine protease inhibitor E-64 The experiment resulted in a low yield of purification as there were two main bands with molecular size of about 26 kDa which could be cysteine proteases and three weak bands with sizes of about 40, 58 and 85 kDa after SDS-PAGE analysis (Figure 26). This may have been due to low efficacy of the streptavidin magnetic beads to bind with the labelled cysteine proteases.

Jinka *et al*. (2009) isolated a cysteine protease from germinating cotyledons of horse gram with a molecular weight of 30 kDa and two cysteine proteases were also isolated from germinating cotyledons of soybean with the sizes of 26,178 and 26,429 Da measured by MALDI-TOF mass spectrometry (Asano *et al*., 1999) which are in the same range as the bands observed in this study. Because most plant cysteine proteases are proteins of molecular mass of about 21-30 kDa (Vierstra, 2003), further analysis was done to see if the three weak bands were also cysteine proteases. After SDS-PAGE analysis, the bands were transferred to a nitrocellulose membrane and were detected by streptavidin and chemiluminescence. No signals were obtained for the three weak bands but only for the stronger bands with the size of about 26 kDa which can imply that they were just “sticky” proteins resulting from unspecific binding (Figure 27).

CHAPTER 6

6 CONCLUSIONS

Soybean plants were successfully grown in a growth chamber and the proteolyitic activity of the collected nodules and leaves, measured using flourimetric assay and azocasein assay. Profiles of cysteine proteases in the different tissues were also obtained. Expression of cysteine proteases in soybean nodules decreases with age whereas that of the leaves increases. There is therefore no correlation between senescence and total cysteine proteases activity per unit weight of nodules. The role of cysteine proteases which have more isoforms indicating a larger variety of cysteine proteases in the young nodules is still not clear. Cysteine proteases may not be the main proteases involved in the degradation of proteins during nodule senescence. Other proteases and protease systems such as the ubiquitin-proteasome system may also be responsible. The function of cysteine proteases expressed in the nodules may be to activate other proteases. The profiling of proteases in leaves provided a comparison of trends between the nodules and the leaves as cysteine protease activity during leaf senescence is well understood. The findings from this study therefore disprove the original hypothesis that cysteine proteases are the main proteases responsible for protein degradation during nodule senescence of the soybean plant.

CHAPTER 7

7 RECOMMENDATIONS

Work that needs to be done in future includes:

1. Identifying the cysteine proteases and searching for their DNA sequences in the soybean genome database to produce these proteases in vitro for biochemical characterisation.

2. Determining the amino acid sequence the main cysteine protease expressed in the soybean nodule extracts during development, maturation and senescence of the soybean plant using LC-MS and MALDI-TOF MS, and comparison of the profile with already known plant cysteine proteases.

3. Perform Real time-PCR to profile expression of cysteine proteases in the root nodules during development, maturation and senescence of the soybean plant so as to confirm the trends at tracription level.

4. Use cystatin as an affinity ligand to purify cysteine proteases expressed in the young nodules.

5. Use DCG-04 as an affinity tag to profile expression of cysteine proteases in soybean stem during development, maturation and senescence of the soybean plant.

CHAPTER 8

8. REFERENCES

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