

Characterisation of dark chilling effects on the functional longevity of soybean root nodules

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Preface

There is a growing interest in soybean products in South Africa because of the health benefits associated with soybean. Soybean consumption in the country is estimated at 32% for oil and oilcake, 60% for animal feed (especially in the broiler and egg industries) and 8% for human consumption. In the past ten years, the area under soybean cultivation in South Africa more than doubled from 134,000 hectares in 2001/02 to 311,000 hectares in 2010/2011. The increase in soybean production is expected to continue because of the availability of genetically modified soybean seeds in South Africa. However, soybean is sensitive to low night temperatures (dark chilling), thereby limiting its yield and making it difficult to successfully cultivate over a broad geographic range within South Africa. The reduction of soybean yield by dark chilling in high-altitude areas is a major agricultural problem because sustainable protein production is central to the nutritional and economic well-being of the population of South Africa and Africa as a whole.

Main reasons for the poor performance of soybean under chilling stress conditions are the negative effects on vegetative and reproductive development as well as on key metabolic processes such as photosynthesis and symbiotic nitrogen fixation (SNF). Optimal SNF in soybean root nodules during the growth season is crucial in ensuring high yields and high seed protein content. Symbiotic nitrogen fixation is rapidly and severely inhibited by chilling and various other environmental constraints. Any perturbation in SNF during chilling stress could potentially trigger the onset of premature nodule senescence. Once nodule senescence is initiated, the gradually increasing loss of SNF capacity leads to nitrogen limitation within the plant with associated reductions in crop yield. The specific nature and sequence of events involved in nodule senescence has not yet been clarified. The need for improved crop plants with greatly enhanced stress tolerance is real and urgent. Stress tolerance is a major trait target of legume breeding programs, but relatively little of this effort is directed at delayed nodule senescence, mainly because of a lack of knowledge regarding the biochemical and molecular processes linking the perception of stress within the plant to the causative effects of nodule senescence.

A key aim of legume improvement programmes is to develop more stress tolerant genotypes with superior SNF capacity under field conditions. One important option in achieving this aim is to find ways to delay nodule senescence during environmental stress, thereby increasing nodule sustainability under field conditions. In order to achieve success in the case of soybean, it is crucial to unravel the specific processes involved in premature nodule senescence and to identify targets for future genetic manipulation.

The research exemplified in this thesis involved establishing the baseline and change over time for key parameters involved in SNF. Furthermore, induced dark chilling effects on nodule function, to determine the alterations in key parameters of SNF, was examined and finally, to determine if premature nodule senescence was triggered, the recovery following an extended dark chilling period was monitored. This study provided novel information regarding dark chilling effects on soybean nodules that could be exploited in further undertakings directed at developing chilling tolerant soybean genotypes for agricultural use.

The experimental work discussed in this thesis was conducted during the period of January 2006 to August 2010 in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, Potchefstroom, South Africa.

The research conducted and presented in this thesis represents original work undertaken by the author and has not been previously submitted for degree purposes to any university. Where use have been made of the work of other researchers, it is duly acknowledged in the text.

The reference style used in this thesis is according to the specification given by the Council of Biology Editors (CBE) Scientific style using the name-year system ([http:// http://writing.colostate.edu/guides/guide.cfm](http://writing.colostate.edu/guides/guide.cfm)).

Any opinion, findings and conclusions or recommendations expressed in this material are those of the author and therefore the National Research Foundation (NRF, South Africa) does not accept any liability in regards thereto.

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Don't only practice your art, but force your way into its secrets, for it and knowledge can raise men to the divine – Ludwig van Beethoven

List of abbreviations

ATP	Adenosine tri-phosphate
ADP	Adenosine di-phosphate
Ar	Argon
C	Carbon
C	Control
CaCl ₂	Calcium chloride
Chl <i>a</i>	Chlorophyll <i>a</i>
Chl <i>b</i>	Chlorophyll <i>b</i>
CO ₂	Carbon dioxide
°C	Degree celcius
Δ	Delta
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediamine-tetraacetic acid
FBP	Fructose-1,6-bisphosphate
FBPase	Chloroplast fructose-1,6-bisphosphatase
Fe	Iron
Fe-EDTA	Iron ethylenediamine-tetraacetic acid
Fru	Fructose
G	gram
G-6-P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GOGAT	Glutamate-oxoglutarate amino transferase
Glu-6-P	Glucose-6-phosphate
GS	Glutamate synthetase
Hepes	(N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
H ₂ O	Water
H ₂ O ₂	Peroxide
HCL	Hydrochloric acid
HK	Hexokinase
HPLC	Hewlett Packard liquid chromatograph
HSD	Honest significant difference
μ	micro
μg	micro gram
IC	Inner cortex
IRGA	Infra red gas analyser
IZ	Infected zone
KCl	Potassium chloride
kDa	Kilo dalton
K ₂ FeCN	Potassium ferricyanide

KH ₂ PO ₄	Potassium phosphate monobasic
KOH	Potassium hydroxide
LCOs	Lipid chitooligosaccharides
L _n	Number of trifoliolate leaves longer than 25mm
L _{n+1}	Number of trifoliolate leaves shorter than 25mm
L _{ref}	Leaflet reference length
M	Molar
m	metre
m ⁻²	square metre
MC	Middle cortex
mg	milligram
MgSO ₄	Magnesium sulphate
mmol	millimolar
ms	milliseconds
MoFe	Ironmolybdenum
Mops	3-Morpholinopropane-1-sulfonic acid
N	Nitrogen
N ₂	Atmospheric nitrogen
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADP-MDH	NADP-dependent malate dehydrogenase
NADP	β-Nicotinamide adenine phosphate
NADPH	β-Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NH ₃	Ammonia
NH ₄	Ammonium
NO ₃	Nitrate
NR	Nitrate reductase
O ₂	Oxygen
OH	Hydroxyl
OsO ₄	Osmium tetroxide
PEP	Phosphoenolpyruvate
PGI	Phosphoglucosomerase
Pheo	Pheophytin
<i>PI</i>	Plastochron index
PLC	Programmable logic control
PI	Performance index
PS I	Photosystem I
PS II	Photosystem II
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RuBP	Ribulose biphosphate

s	second
SBPase	sedoheptulose-1,7-bisphosphatase
SC	Shoot chilling
SE	Standard error
Suc	Sucrose
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNF	Symbiotic nitrogen fixation
SPS	Sucrose phosphate synthetase
SS	Sucrose synthase
TEMED	Tetramethylethylenediamine
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride
UDP-Glu	Uridine di-phosphate glucose
UDPG	Uridine diphosphoglucose
UV	Ultra violet
v/v	Volume per volume
WPC	Whole plant chilling
w/v	Weight per volume

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Abstract

Characterisation of dark chilling effects on the functional longevity of soybean root nodules

A large proportion of the world's nitrogen needs is derived from symbiotic nitrogen fixation (SNF), which contributes substantially to agricultural sustainability. The partnership between legumes and rhizobia result in the formation of specialised structures called root nodules. Within these nodules SNF is supported by the sucrose transported from the leaves to the nodules for respiration. The end products of SNF in soybean (*Glycine max* (L.) Merr.) root nodules, namely ureides, are transported to the upper parts of the plant to supply nitrogen. Symbiotic nitrogen fixation provides a vital advantage for the production of soybean compared with most grain crops in that soybean fixes the nitrogen required for its growth and for the production of the high-protein content in seed and oil.

The process of SNF is dramatically affected by drought, salt, cold and heavy metal stresses. Since SNF is such an important yield-determining factor, a lack in understanding these complexes inevitably delays progress towards the genetic improvement of soybean genotypes and also complicates decisions with regard to the suitability of certain genotypes for the various soybean producing areas in South Africa. The largest soybean producing areas in South Africa are situated at high altitudes, with minimum daily temperatures which can be critically low and impeding the production of soybean. Soybean is chilling sensitive, with growth, development and yield being affected negatively at temperatures below 15°C. Dark chilling (low night temperature) stress has proved to be one of the most important restraints to soybean production in South Africa.

Among the symptoms documented in dark chilling sensitive soybean genotypes are reduced growth rates, loss of photosynthetic capacity and pigment content, as well as premature leaf senescence and severely inhibited SNF. Existing knowledge about stress-induced nodule senescence is based on fragmented information in the literature obtained in numerous, and often diverse, legume species. The precise nature and sequence of events participating in nodule senescence has not yet been fully explained.

The main objectives of this investigation were to characterise the natural senescence process in soybean nodules under optimal growth conditions and to characterise the alteration of the key processes of SNF in a chilling sensitive soybean genotype during dark chilling. Moreover, to establish whether recovery in nodule functionality following a long term dark chilling period occurred, or whether nodule senescence was triggered, and if sensitive biochemical markers of premature nodule senescence could be identified.

A known chilling sensitive soybean genotype, PAN809, was grown under controlled growth conditions in a glasshouse. To determine the baseline and change over time for key parameters involved in SNF, a study was conducted under optimal growing conditions over a period of 6 weeks commencing 4 weeks after sowing. The cluster of crown nodules were monitored weekly and analysis included nitrogenase activity, ureide content, respiration rate, leghemoglobin content, sucrose synthase (SS) activity and sucrose content. Further investigations focused on induced dark chilling effects on nodule function to determine the alterations in key parameters of SNF. Plants were subjected to dark chilling (6°C) for 12

consecutive nights and kept at normal day temperatures (26°C). The induced dark chilling was either only shoot (SC) exposure or whole plant chilling (WPC). These treatments were selected since, in some areas in South Africa cold nights result not only in shoot chilling (SC) but also in low soil temperatures causing direct chilling of both roots and shoots. To determine if premature nodule senescence was triggered, the recovery following 12 consecutive nights of chilling treatment was monitored for another 4 weeks.

It was established that the phase of optimum nitrogenase activity under optimal growing conditions occurred during 4 to 6 weeks after sowing where after a gradual decline commenced. This decline was associated with a decline in nitrogenase protein content and an increase in ureide content. The stability of SS activity and nodule respiration showed that carbon-dependent metabolic processes were stable for a longer period than previously mentioned parameters. The negative correlation that was observed between nitrogenase activity and nodule ureide content pointed towards the possible presence of a feedback inhibition trigger on nitrogenase activity.

A direct effect of dark chilling on nitrogenase activity and nodule respiration rate led to a decline in nodule ureide content that occurred without any limitations on the carbon flux of the nodules (i.e. stable sucrose synthase activity and nodule sucrose content). The effect on SC plants was much less evident but did indicate that currently unknown shoot-derived factors could be involved in the minor inhibition of SNF. It was concluded that the repressed rates of respiration might have led to increased O₂ concentrations in the nodule, thereby inhibiting the nitrogenase protein and so the production of ureides.

It was found that long term chilling severely disrupted nitrogenase activity and ureide synthesis in nodules. Full recovery in all treatments occurred after 2 weeks of suspension of dark chilling, however, this only occurred when control nodules already commenced senescence. This points toward reversible activation of the nitrogenase protein with no evidence in support of premature nodule senescence. An increase in intercellular air space area was induced by long term dark chilling in nodules, specifically by the direct chilling of nodules (WPC treatment). The delayed diminishment of intercellular air space area back to control levels following dark chilling may be an important factor involved in the recovery of nitrogenase activity because enlarged air spaces would have favoured gaseous diffusion, and hence deactivation of nitrogenase, in an elevated O₂ environment (due to suppressed nodule respiration rates). These findings revealed that dark chilling did not close the diffusion barrier, as in the case of drought and other stress factors, but instead opened it due to an increase in air space areas in all regions of the nodule.

In conclusion, this study established that dark chilling did not initiate premature nodule senescence and that SNF demonstrated resilience, with full recovery possible following even an extended dark chilling period involving low soil temperatures.

KEY WORDS: Dark chilling, intercellular air spaces, nitrogenase activity, nodule longevity, nodule respiration, nodule senescence, recovery, symbiotic nitrogen fixation (SNF), soybean.

Chapter 1 - Literature review

1.1 Importance of soybean on a global scale

The use of soybean for agricultural practices dates back 7000 years as recorded in Chinese medical compilations, from there its use spread to South-east Asia and reached Europe at the end of the eighteenth century (El Agroudy et al., 2011). Until the 1920s China produced about 80% of the world's soybean, but since then, soybean has revolutionised the agricultural economies of many countries as it has immense potential for food, feed and industrial uses (Bisaliah, 1986). Soybean is one of nature's most versatile plants, producing an abundant supply of protein and oil. In weight, the protein yield of soybean is about twice that of meat and of most beans and nuts (Smith and Huysler, 1987). The economic viability of soybean production is determined by the commercial utilisation of its sub-products, meal and oil. Soy oil and meal are consumed worldwide as food and animal feedstuff respectively (Thoenes, 2006). After palm oil, soy oil is the most important vegetable oil, and accounts for 25% of the global vegetable/animal oil and fat consumption. Soybean supplies two-thirds of the world's protein concentrate animal feeds and three-quarters of the world trade in high protein meals (Keyser and Li, 1992). Products made from soybeans are so numerous and diverse that it has been called the "miracle crop" (Lee et al., 2007). Nearly all soybeans are processed for their oil, but after processing the high-protein fiber that remains is toasted and processed into animal feed for poultry, pork, cattle, other farm animals and pets. Soybean products can also be found in building material which uses soy-based wood adhesives, and in home and commercial products such as carpets, auto upholstery, crayons, margarine, etc. Soybean oil can be processed into biodiesel, which is low in carbon dioxide emissions and has great potential as an eco-friendly replacement for fossil fuel. Soybeans are unique among legumes as they contain phyto-estrogens, antioxidants and other compounds that have the potential to decrease cholesterol, heart disease, osteoporosis, menopause, breast-, prostate- and colon cancer (Jooyandeh, 2011; Messina 1999; Zeisel 2000). Soybean plays an important role in the traditional diets of many regions throughout the world. In many countries with rapid growing populations, these crops enhance the nutritional value of the local diets (Hume et al., 1985) and may be a key-factor when considering strategies for alleviating world hunger.

Soybean cultivation is mainly focused in the United States of America, Brazil, Argentina and China, accounting for almost 90% of the world's soybean production. It was projected that for the 2010 production year USA, Brazil and Argentina planted 66.8, 25.4 and 22.9 million hectares to soybean, respectively (Pocket K16, 2011).

1.2 Soybean production in South Africa

The South African Bureau for Food and Agricultural Policy (BFAP, 2010) stated that the expansion of soybean production in South Africa will be driven by growth in the livestock and poultry sectors, as rising incomes will increase demand for animal protein. More than 90% of the soybean meal consumed in South Africa is imported (USDA, 2010).

In South Africa production takes place on 250 000 hectares, delivering about 500 000 tons of soybean per annum. In the 2010/2011 season, South Africa produced 561 000 tons of soybean, for the first time exceeding the production of sunflower. The increase in soybean production was driven by the increase in area planted as well as an increase in yield per unit area (Soybean Market Value profile, 2010-2011). The main growing region for soybean is Mpumalanga, which produces about 262 000 tons of the country's crop followed by the Free State and KwaZulu-Natal with 99 000 and 75 600 tons respectively. Since 2005 the Mpumalanga Province has been the top producer of soybeans followed by the Free State, Kwazulu-Natal, Limpopo and North-West provinces. The Western and Eastern Cape provinces of South Africa have been the lowest producers of soybean with the Western Cape going out of production between 2007 and 2009.

South Africa is a net importer of soybean and unable to satisfy local demand. The majority of imported soybean is from the America's including USA, Brazil and Argentina. With favorable logistics and location, Argentina is the country of choice for importing soybean into South Africa (Soybean Market Value Chain Profile 2010-2011). Factors that increase the demand for meal and soybean oil includes rising incomes and populations, leading to higher demand for livestock products as food consumption increases. Thereupon the demand for animal feed is stimulated as the production of livestock is increased to meet rising food demand (Bruisma, 2003).

1.3 Environmental factors adversely affecting soybean production

Worldwide agricultural production is governed by the combination of climate, soil tilth, technology, genetic resources and farm management decisions such as tillage, manure and fertilizer applications and variety selection (Duvick and Cassman, 1999). As sessile organisms, plants must adapt to their environment. Climate change influences crop yield and casts a shadow on food production (Matsumura and Sugimoto, 2011). Samach and Wigge (2005) cited that plants demonstrate extensive physiological and biochemical adaptation to large geographical differences in temperature.

Legumes, including soybean contain specialised bacteria in their roots which utilises nitrogen (N) from the air, compared to nitrate which is taken up directly from the soil by the roots (Harper, 1987). Soybean forms a N-fixing symbiosis with *Bradyrhizobium japonicum* (Keyser and Li, 1992) in specialised structures called root nodules. This partnership can fix approximately 300kg N ha⁻¹ under normal conditions but also contributes significantly to the quantity of N to the soil in which the plants are cultivated (Bergersen, 1997). Soybean is therefore a crop that can be cultivated without the extensive application of N fertiliser and also reduces input costs in crop rotation practices with other crops such as maize. Global warming caused by increased carbon dioxide levels, which causes a rise in temperatures and changes in distribution of precipitation, will alter plant growth, biomass and plant community composition. Increasing atmospheric CO₂ along with other components of climate change has the potential to exert a severe influence on the productivity of N fixing bacteria and the amounts of N contributed by these organisms to natural and agricultural systems (Thomas et al., 2006).

Drought stress occurring during flowering and early pod development, significantly increases the rate of pod abortion thus decreasing final soybean seed yield (Westgate and Peterson, 1993). Pod expansion is a critical stage in soybean reproductive development which requires active cell division in the young ovules and this process is known to be very sensitive to soil water deficits (Peterson et al., 1992). Drought stress affects rhizobial survival and growth as well as population structure in the soil. The lack of water can cause major effects on nodulation and lead to low N fixation. Severe drought may lead to irreversible cessation of N fixation (Sprent, 1971; Vincent, 1980; Walker and Miller, 1986; Venkateswarlu et al, 1989; Guerin et al., 1991).

Hungria and Vargas (2000) cited that indirect effects of high temperatures on the metabolism of the host plant and direct effects on N fixation have been recognised for a long time. High temperatures have been shown to negatively affect the bacterial infection and SNF of soybean, which is optimum between temperatures of 30 and 33°C (Pankhurst and Sprent, 1976; Munevar and Wollum, 1981; Piha and Munns, 1987). Exchange of molecular signals between the host plant and the rhizobia are also influenced by temperatures higher than 39°C, while the release of nod-gene inducers was decreased at temperatures of 39°C (Hungria, 1995; Hungria and Stacey, 1997). Because an increase in temperature decreases rhizobial survival, repeated inoculation of legumes and a higher rate of inoculum application may be needed to sustain N fixation resulting in higher input costs.

Gbetibouo and Hassan (2005) cited that evidence exists that suggests that the agricultural sector in the Southern African region is highly sensitive to future climatic shifts and increased climate variability. This variability includes changes in extreme events such as increases in extreme high and low temperatures, and increases in intense precipitation events (Easterling et al., 2000). In legumes, low temperatures can lead to delayed plant development and root nodule formation, the site of SNF (see below for more detail) (Legros and Smith, 1994). Reproductive abortion can occur at the early stage of embryo development after fertilisation and is brought about by a deficit in water as a result of low temperature exposure (Kato, 1964, Westgate and Peterson, 1993; Gass et al., 1996). Short term exposure of plants to low temperature can inhibit net photosynthesis due to accumulation of soluble sugars (Ebrahim et al., 1998; Chaumont et al., 1995). Chilling also damages roots by reducing the absorption of water as changes in membranes and root ion pumps occur (Markhart et al., 1979). In certain regions of the world, including parts of South Africa, plants are subjected to normal day temperatures, with low temperatures only occurring at night (dark chilling) (Strauss and Van Heerden, 2011). Frequently soil temperatures do not cool down to the same extent as air temperatures but there are incidences where soil cooling can be severe (Walsh and Layzell, 1986; Legros and Smith, 1994; Zhang and Smith, 1994). As far back as 1727, Stephen Hales conducted experiments showing that seedlings wilt in cold soils because chilling impedes water absorption by the roots. It has been suggested that overall balance between photosynthesis and respiration determine levels of N fixation. Low soil temperature affects the growth of plants and this growth is dependent on N fixation much more so than plants receiving mineral N (Abberton et al., 1998). One major aspect influenced by low soil temperature is a delay in nodule initiation (Pan and Smith, 1998). A recent study by Van Heerden et al. (2008) showed the sensitivity of N fixation to dark chilling in a chilling sensitive soybean genotype.

Nitrogen is among the most abundant elements on Earth but it is a critical limiting factor for growth of most plants due to its unavailability (Smil, 1999; Socolow, 1999; Graham and Vance, 2000). Vance (2001) cited that plants acquire N from two principal sources; firstly, the soil, through commercial fertiliser, manure and/or mineralisation of organic matter and secondly, the atmosphere through symbiotic N₂ fixation. Availability of N fertiliser for extensive agriculture as practiced in the developing world causes a conundrum. Due to weak infrastructure, poor transportation and high cost, N fertiliser is often unavailable for subsistence farmers, leaving N from intercropping legumes and other species capable of symbiotic N₂ fixation as the only source of N (Vance, 2001).

1.4 Nitrogen cycle

Soybean as a legume is an agronomically and economically important crop because of its ability to assimilate atmospheric N (N₂). The importance of all legume crops is anticipated to increase with the ongoing drive towards more environmentally sustainable agricultural practices (Serraj et al., 1999). N is a major element and it accounts for approximately 6.25% of the dry mass of all organisms and about 78% of the atmosphere's composition. The atmosphere contains approximately 10¹⁵ tons of N₂ gas and the N cycle involves the transformation of some 3 X 10⁹ tons of N₂ per year (Sprent and Sprent, 1990). Of this, lightning is responsible for 10% of the world's supply of fixed N (Sprent and Sprent, 1990). The use of fertiliser also provides important quantities of chemically fixed N. Globally, fixed N from dinitrogen for chemical fertilisers accounts for 25% of newly fixed N₂ and biological processes for about 60%. Biological N fixation or SNF is the process by which organisms fix N through the conversion of stable N gas in the atmosphere into a biologically useful form (Dixon and Wheeler, 1986).

Nitrogen undergoes a variety of oxidations and reductions forming components such as nitrate, nitrite and ammonium. Among others, these components make up the N cycle and all reactions are performed by bacteria, archaea and some specialised fungi (Downie, 1994; Spanning et al., 2007). Various free living organisms and bacteria induce the formation of specialised root organs. These bacteria are from the genera *Rhizobium*, *Azorhizobium*, *Photrhizobium*, *Sinorhizobium* or *Bradyrhizobium*, collectively known as rhizobia (Stacey, 2007; Downie, 1994; Schultze and Kondorosi, 1995). These specialised organs, called root nodules, are the sites of primary N assimilation, comparable in rate of metabolic activity to that of the leaf, the organ of primary C assimilation (Walsh et al., 1989).

1.5 Nodule morphology

1.5.1 Nodule structure

Nodule development is controlled by the plant genome and determines gross nodule morphology, anatomy and type of N₂ product exported (Gresshoff, 1993). The development pattern of nodules can either be determinate or indeterminate. Determinate nodules form on the roots of species like soybean and common bean, whereas indeterminate nodules form on the roots of species like clover and alfalfa. Determinate nodules are initiated from cortical cell divisions but largely grows through cell expansion and results in a

globular nodule structure. These nodules have peripheral vascular tissue and since development is in a radial pattern, distinct zones are difficult to distinguish. Indeterminate nodules appear as modified lateral roots with lateral vascular tissue and terminal apical meristem. Since these nodules form new cells from their tip, the full development of the nodule can be seen in cross section (Stacey, 2007). Both determinate and indeterminate nodules consist of three important tissues; a central infection zone, an inner cortex which includes vascular bundles and an outer cortex. The inner cortex can be divided into several zones, expanding outwards from the infected zone is a distribution zone which is small cells often with large intercellular spaces, followed by a boundary layer of tightly packed cells with little room for intercellular spaces. The middle cortex is found between the boundary layer and the endodermis or scleroid layer which are large cells with thickened cell walls and large intracellular spaces. Outside of the endodermis layer is a zone of large, loosely packed cells with large intercellular spaces called the outer cortex (Witty et al., 1987; Parsons and Day, 1990; Brown and Walsh, 1994) (Figure 1.1). The interior of both determinate and indeterminate nodules contains very low oxygen (O_2) levels. Within the outer cortex of the nodule a physical barrier to O_2 exists. Leghemoglobin, specifically produced in nodules, binds O_2 . The low O_2 concentration is essential as nitrogenase (enzyme involved with N fixation under strict anaerobic conditions) is inactivated by O_2 .

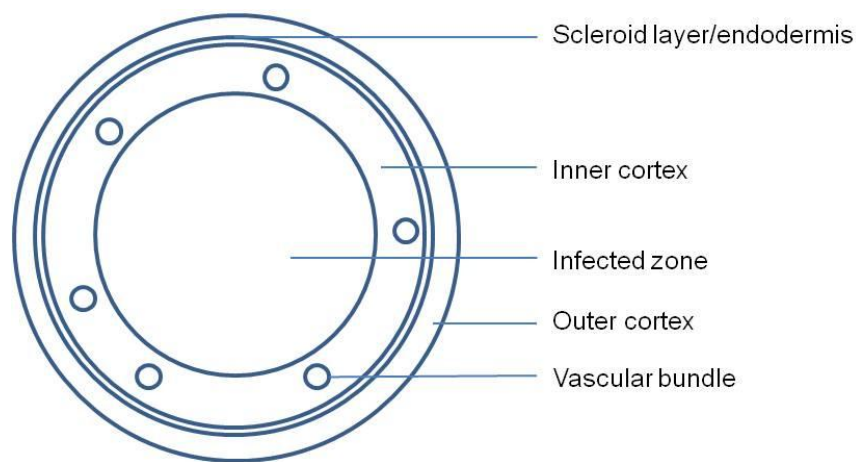


Figure 1.1: Line diagram representing different zones of determinate root nodule.

1.5.2 Nodule initiation and development

The establishment of the symbiosis requires extensive recognition and signaling from both the plant (host) and bacterium (Long, 2001). Nodulation is a highly host-specific interaction in which specific rhizobial strains infect a limited range of plant hosts. Plants secrete flavonoids or isoflavonoids that are recognised by compatible bacteria, resulting in the induction of nodulation genes (Stougaard, 2000; Esseling and Emons, 2004; Riely et al., 2004; Geurts et al., 2005; Mulder et al., 2005; Oldroyd et al., 2005).

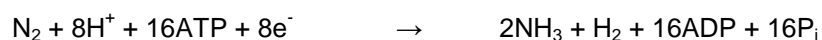
Rhizobia produce nodulation (*nod*) factors that are essential signal molecules which play an important role during the initiation of nodule development and bacterial invasion (Broughton et al., 2000; Perret et al., 2000). *Nod* factors consist of an oligomeric backbone of β -1,4-linked *N*-acetyl-D-glucosaminyl residues, *N*-acylated

at the nonreducing chitooligosaccharides (LCOs) (Denarie et al., 1996; Kamst et al., 1998). The synthesis of *nod* factors depends on the expression of several *nod* genes, including *nod*, *nol* and *noe* genes. Responses like the formation and deformation of root hairs, intra- and extracellular alkalisation, membrane potential depolarisation, changes in ion fluxes, induction of early nodulin gene expression and formation of nodule primordial is triggered by these *nod*-factors (Broughton et al., 2000; Perret et al., 2000). The *nod* genes also encode enzymes that synthesise a specific *nod* signal, which activates many of the early events in the root hair infection process (Esseling and Emons, 2004; Geurts et al., 2005; Oldroyd et al., 2005; Riely et al., 2004; Mulder et al., 2005).

The bacteria enter the plant via the root epidermis during the infection process and induce the changing of the root cortical cell development and formation of the nodule. This infection occurs through the root hairs. The process of infection can be categorised into several steps, beginning with a) curling of the root hair thereby enclosing the bacteria within the root hair curl where the plant cell wall is degraded, b) the cell membrane becomes invaginated and an intercellular tubular structure namely an infection thread is established, c) The bacteria enter the root hair and eventually ramify into the root cortex within this infection thread, d) *nod* factors modify the plant hormone balance as to stimulate mitosis and permit development of the symbiosome that will house the bacteria within the plant (Ferguson and Mathesius, 2003). The symbiosome or peribacteroid is formed when the release of the bacteria into individual cells by endocytosis occurs. Enclosing the bacteria within a plant membrane isolates the bacteria from the host cytoplasm and allows essential structural, metabolic roles and controls the exchange of metabolites and signals (Colebatch et al., 2004; Goodchild and Bergersen, 1966). The host provides a unique micro-aerobic low O₂ environment for the bacteria within the symbiosome that controls the expression of the bacterial N fixation genes as well as cytochromes that work optimally in these conditions (Long, 2001; Puppo et al., 2004).

1.6 Nitrogenase (EC1.18.6.1)

Symbiotic N fixation is catalysed by the enzyme nitrogenase. Rainbird et al. (1984), found that during SNF, nodule maintenance consumed 22% of total respiratory energy while the functioning of nitrogenase consumed a further 52%. nitrogenase is a nucleotide-utilising enzyme coupling the energy of the nucleotide binding and hydrolysis to electron-transfer reactions within a macromolecular complex (Howard and Rees, 1996). nitrogenase catalyses the reduction of N₂ to NH₃ in a reaction illustrated below:



Nitrogenase is composed of two component proteins called the iron (Fe) protein and the iron-molybdenum cofactor (MoFe) protein. The Fe protein is a homodimer of approximately 60 -kDa and serves as a specific ATP-binding site, whereas the site for substrate binding and reduction is located upon the MoFe protein. Nucleotides bind to the homodimeric Fe protein, which contains a single [4Fe-4S] cluster, which bridge the Fe and MoFe protein and which is probably the electron donor. ATP binding and hydrolysis is to regulate the electron transfer of an electron from the [4Fe-4S] cluster inside the Fe protein to the MoFe protein. The ultimate acceptor of electrons in the MoFe protein is a mixed cluster called FeMo cofactor, where substrates

bind and are reduced (Shah and Brill, 1977). During catalysis, the Fe protein binds to two ATP molecules, which are reduced and then associates with the MoFe protein thereby donating a single electron to the MoFe protein in a reaction coupled to ATP hydrolysis and protein dissociation. After each electron-transfer, the Fe protein dissociates from the MoFe protein and oxidised Fe protein is reduced concomitant with the replacement of the ADP molecules with ATP. The hydrolysis of ATP to ADP seems to regulate the affinity for association between the Fe protein and the MoFe protein. All substrate reduction reactions catalysed by nitrogenase require two or more electrons making the association between the two proteins essential (Hageman and Burris, 1978). Dean and Jacobson (1992), cited that since multiple electrons are required for N₂ reduction, several cycles of component protein association and dissociation is needed for nitrogenase turnover. Genes encoding products that participate in N₂-fixation-specific electron transport are known as *fixABCX* (Earl et al., 1987). Whereas genes that encode the nitrogenase structural components are *nifH* – Fe protein subunit, *nifD* – MoFe protein α -subunit and *nifK* – MoFe protein β -subunit (Scott et al., 1981; Sundaresan and Ausubel, 1981). The trigger for expression of these *fix* and *nif* genes, appears to be low O₂ which results from low O₂ permeability of the nodule cortex (Fisher, 1994; Hennecke, 1998; Soupene et al., 1995).

The product of nitrogenase exists in two forms within the cell, ammonium (NH₄⁺) and ammonia (NH₃), which are easily inter-convertible. Ammonia cannot diffuse across membranes but it is thought that it can diffuse freely out of the bacteroid. Thereafter the ammonia may either diffuse or be transported across the symbiosome membrane (Bisseling et al., 1979), where assimilation by the host plant takes place forming glutamate by the enzyme glutamine synthetase and glutamate synthase. Glutamate serves as the central N metabolite in the plant nodule cells for the synthesis of the other amino acids, nucleic acids and any other N containing compounds (Day et al., 1990; Karr and Emerich, 1988; Karr et al., 1990; Katinakis et al., 1988).

1.7 Oxygen barrier

N fixation is a high energy demanding process which requires respiration rates in the nodules to be very high thereby providing sufficient ATP and reducing power. The activity of nitrogenase is both O₂ sensitive and O₂ demanding, which implies that a balance of O₂ supply and consumption must be maintained in the root nodules at all times (Bergersen, 1982). Oxygen supply to the central infected cells in nodules is regulated by the resistance to O₂ diffusion present in the nodule cortex (Sheehy et al., 1983; Witty et al., 1984, 1986; Layzell and Hunt, 1990). Variable nodule O₂ permeability exists and is maintained at low concentration in the infected cells to prevent nitrogenase inhibition (Witty et al., 1984; Layzell et al., 1990; Minchin, 1997) as exposure to O₂ may lead to a conformational change in nitrogenase or a decreased transfer of electrons to nitrogenase (Robson and Postgate, 1980). The synthesis of leghemoglobin, which gives the nodule its pinkish color, is necessary to support a high O₂ flux under micro-aerobic conditions as well as to scavenge O₂ to protect the highly O₂-sensitive nitrogenase from O₂-induced damage (Hirsch, 1992). Oxygen is a non-reversible inhibitor of nitrogenase but it is also required by bacteroids to drive respiration and therefore nitrogenase activity. Consequently, nodules must maintain a high O₂ flux at low O₂ concentration. Hartwig (1998) cited that nodule permeability operates over and above the persistent diffusion restriction which is the

product of nodule morphogenesis and this is essential to create the micro-aerobic environment within nodules, O₂ protection of nitrogenase and the distinct gene expression.

It was suggested by Parsons and Day (1990) that a layer of interlocking cells without air spaces in the inner cortex may be of importance thereby forming a boundary layer. Layers of cells, internal to the boundary layer and immediately adjacent to the infected cells, contain large interconnecting air spaces thereby facilitating rapid gas exchange within the infected tissue.

The involvement of a rapid osmotic mechanism in operation within the diffusion barrier has been suggested. Cells of the inner cortex are also osmo-contractile and may collapse thereby reducing the size of the intercellular spaces and also cells can expel water from or into these air spaces (Purcell and Sinclair, 1994; Serraj et al., 1995). Other evidence in the form of micro-electrode measurements and X-ray micro-analysis of O₂-induced membrane depolarisations and disturbances in shifts of Ca²⁺ and K⁺ ions in the inner cortex, also suggest the operation of an osmotic mechanism within the inner cortex (Witty et al., 1987; Denison and Kinraide, 1995; Minchin et al., 1995). Intercellular spaces of certain nodules may contain glycoprotein molecules that can enhance resistance of the cortex to O₂ diffusion by blocking a significant number of spaces that would otherwise be utilised as air passageways to the infected zone (Van den Bosch et al., 1989; James et al., 1991).

1.8 Nitrogen fixation

As previously mentioned, the nitrogenase enzyme is very sensitive to O₂, however, it's activity depends on large quantities of ATP which is produced by oxidative phosphorylation within the infected cells (Robson and Postgate, 1980). Ammonium (NH₄⁺) produced from the initial step during SNF is exported from the symbiosome into the infected cell cytosol where it is assimilated into glutamine via the combined action of glutamate synthetase (GS) and glutamate-oxoglutarate amino transferase (GOGAT) (Figure 1.2). Glutamine is subsequently converted into the amino acid asparagine and exported from the infected cell to the xylem for transport. Glutamine may also be exported from the cytosol into the plastids where it can be utilised by glutamate synthase to produce two molecules of glutamate as depicted in the reaction below. One of these is used in amide and ureide biosynthesis in the uninfected cells, while the other glutamate is recycled to glutamine synthetase for NH₃ assimilation (Day et al., 2001).

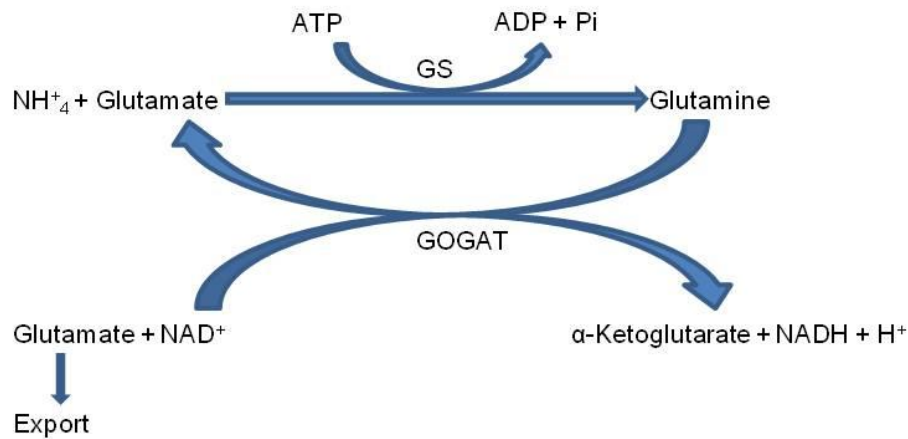


Figure 1.2: Schematic representation of the glutamate synthase cycle (Adapted from Hopkins and Hüner, 2004).

Waters et al. (1998) mentioned that glutamate serves as the central N metabolite in nodule cells for the synthesis of other amino acids, nucleic acids and other N-containing compounds. Organic N compounds formed during SNF can be exported to the upper parts of the plant as either amides (asparagine and glutamine) or as ureides (allantoin and allantoic acid) (Schubert, 1986). Ureides are the principal N compounds exported from the nodules to the shoots and leaves of N₂-fixing soybeans. Ureides are formed by the condensation of urea with a two-carbon compound (glyoxylate) and are products from purine oxidative catabolism. Purines for ureide biogenesis may arise from turnover of nucleic acids or by de novo synthesis (Schubert, 1986; Atkins and Smith, 2000).

1.9 The link between N and carbon metabolism

As mentioned previously, SNF is an energy demanding process, which requires high respiration rates in the nodules. As such, N metabolism is intimately linked to carbon metabolism. Nodule function depends on photosynthates supplied by the plant, which is used by nitrogenase as a source of energy and reducing power to fix N₂ (Larrainazar et al., 2002; Aranjuelo et al., 2011; Kaschuk et al., 2012). This linkage causes nitrogenase activity to be regulated by photosynthesis (carbon supply), N availability and N demand within the whole plant (Aranjuelo et al., 2011). Hartwig (1998) stated that biochemical modulation of N containing metabolites may occur through the regulation of the activity of key enzymes involved in C and N metabolism in the nodule.

1.9.1 Photosynthesis

Photosynthesis is an extremely efficient energy conversion process and can be divided into three stages (i) the photochemical stage, (ii) electron transfer to which is coupled the formation of ATP and (iii) the biochemical reactions involving the incorporation of CO₂ into carbohydrates (Figure 1.3) (Nobel, 1991). Gas exchange between the leaf and the surrounding air containing CO₂ is dependent upon diffusion which is controlled by the stomata. The absorption of light causes excitation of photosynthetic pigments leading to the photochemical events during which electrons are donated by chlorophyll. The electrons are transferred along

a series of molecules leading to the reduction of NADP^+ to NADPH . ATP formation is coupled to these electron transfer steps. The biochemical reactions of photosynthesis require 3 moles of ATP and 2 moles of NADPH per mole CO_2 fixed into carbohydrates (Nobel, 1991).

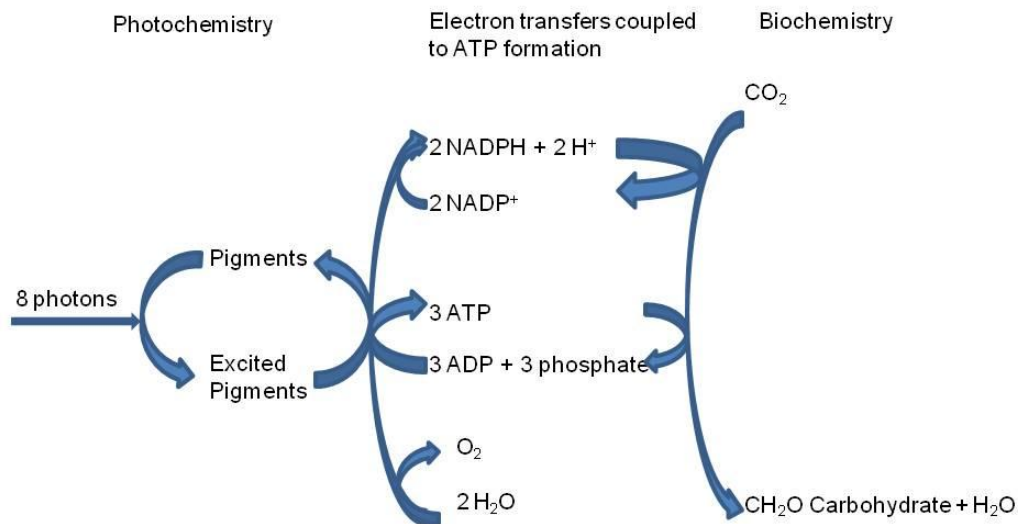


Figure 1.3: Schematic representation of the process of photosynthesis divided into 3 three stages (i) photochemistry, (ii) electron transfer and (iii) biochemistry (Adapted from Nobel, 1994).

1.9.2 Carbohydrate transport and metabolism in the nodules

In general the lower leaves supply the carbon assimilates needed by the roots and nodules via the phloem (Pate 1966; Layzell et al., 1981). Sucrose produced during photosynthesis, is the primary carbon source to the nodule (Stacey, 2007). Sucrose is delivered to the nodule through the nodular vascular system and is translocated apoplastically and/or symplastically into the cells (Day and Copeland, 1991). Sucrose is rapidly respired inside the nodules and converted into dicarboxylic acids (malate and succinate) which are the carbon sources used by the bacteroids (Walsh, 1990). Nodule metabolism is limited by the ability of the phloem to supply carbohydrate to the nodule rather than the carbohydrate status of the plant. Walsh et al. (1987) determined that nodules were not only limited by phloem supply but also by the ability to utilise the available photosynthate.

Nodules are primarily dependent on the import and metabolism of sucrose (Suc) to provide the energy and C skeletons for biological N fixation (Gordon et al., 1999), the assimilation of ammonia and the export of nitrogenous fixation products from the nodules. Sucrose metabolism occurs in the uninfected cells of the nodule cortex because the low O_2 tension in infected cells prevents mitochondrial respiration from supplying carbon to bacteroids at a sufficient rate (Day and Copeland, 1991). Sucrose is metabolised by one of two enzymes, sucrose synthase (EC 2.4.1.13) or alkaline invertase (EC 3.2.1.26) (Figure 1.4). These reactions produce uridine di-phosphate glucose (UDP-Glc) and free hexoses, which enter the glycolytic or oxidative pentose phosphate pathway after phosphorylation by hexokinases to produce phosphoenol pyruvate (PEP). PEP is converted to oxaloacetic acid and then to L-malate by PEP carboxylase (EC 4.1.1.31) and malate

dehydrogenase (EC 1.1.1.37). The first step of sucrose hydrolysis, predominately by sucrose synthase, is a key step in N fixation (Day and Copeland, 1991). White et al., (2007) cited that a number of studies showed that dicarboxylates stimulate bacteroid N fixation *in vitro*, indicating their participation as the carbon source for bacteroid metabolism *in planta* (Poole and Allaway, 2000; Lodwig and Poole, 2003). The oxaloacetate produced through the action of PEP carboxylase could be used either as a substrate for the synthesis of malate or as a source of carbon skeletons for the synthesis of amino acids, amides or ureides (Chollet et al., 1996).

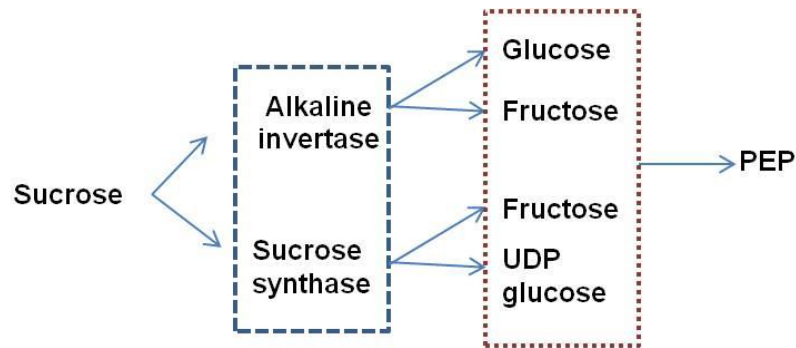


Figure 1.4: Scheme illustrating the cleavage of sucrose to form uridine di-phosphate glucose (UDP-Glucose) and free hexoses that will produce phosphoenol pyruvate PEP following phosphorylation by hexokinases (Adapted from Koch, 2004).

1.9.3 Ureide synthesis

Fixed N requires carbon skeletons for assimilation (Atkins, 1991). Organic N compounds formed by N_2 fixation can be transported to the upper parts of the plant either as amides or ureides (allantoin and allantoic acid) (Figure 1.5).

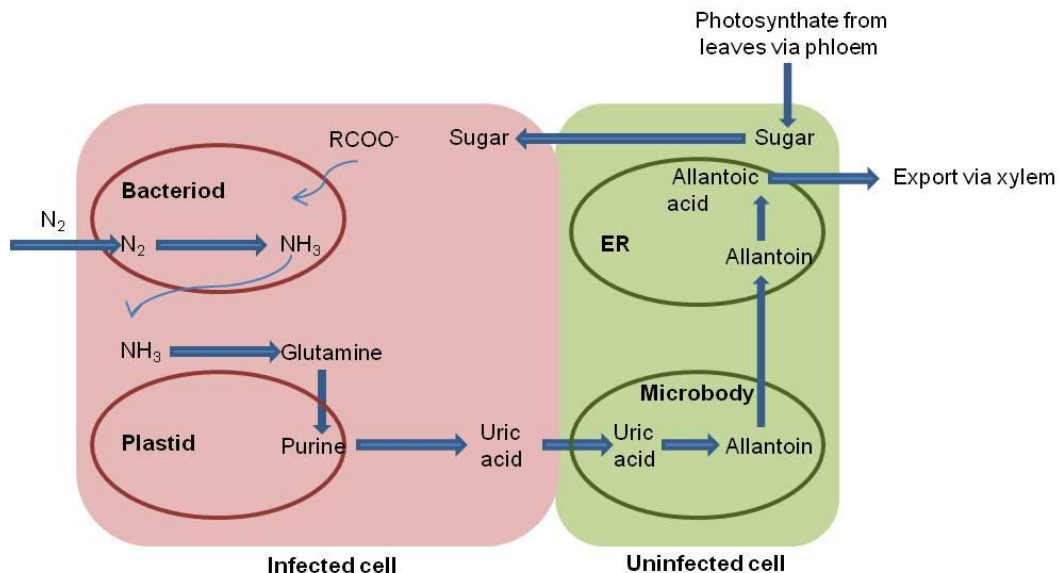


Figure 1.5: Schematic illustration of ureide synthesis during the assimilation of fixed N.

Uric acid derived from xanthine dehydrogenase action in infected cells in the nodules moves to the uninfected cells where it is oxidised by uricase and catalase in the peroxisomes eventually forming allantoin (Smith and Atkins, 2002). Ureides are products from purine oxidative catabolism through the condensation of urea with a two-carbon compound, glyoxylate. The ureides, allantoin and allantoic acid are the final products of N₂ fixation that are exported from soybean nodules to the shoot (McClure and Israel, 1979) where they are catabolised.

1.10 Effects of chilling and dark chilling on specific plant developmental stages

1.10.1 Plant growth and development

Plants demonstrate a maximum rate of growth and development at a favorable temperature or over a diurnal range of temperatures (Fitter and Hay, 1981; Levitt, 1972). Crop yields are constrained by what have been termed thermal thresholds for optimal growth (Greaves, 1996). The imposition of a temperature stress on a plant leads to the modification of metabolism in one of two ways. Firstly, cellular metabolism will adjust to the change in temperature and its effect on metabolic processes and metabolism overall due to the change in structure, catalytic properties and function of enzymes (Kubien et al., 2003). Secondly, a change in temperature would be linked with enhanced tolerance metabolism or stress tolerance and include alterations in soluble sugars, amino acids, organic acids, polyamines and lipids (Guy, 1990; Levitt, 1972). Guy et al. (2008), cited that at lower temperatures, inducible enhanced stress tolerance mediated by exposure to reduced temperature is known as chilling acclimation. Chilling tolerance is the ability of a plant to tolerate low temperatures in the range of 0-15°C without injury or damage (Lyons and Wheaton, 1964; Somerville, 1995).

Soybean like cucumber, tomato and maize is sensitive to suboptimal temperatures. The ability to acclimate to chilling temperatures is severely influenced by the stage of plant development at the given time, since certain growth phases are more sensitive than others (Hällgren and Öquist, 1990). The sensitivity of soybean to night temperatures below 15°C also known as dark chilling (Strauss et al., 2006, 2007; Van Heerden et al., 2003a,b,c), is observed in fluctuations in metabolism, growth, development and yield (Musser et al., 1983, 1984; Van Heerden et al., 2003a, b, c). The reduction of production potential is caused by the inhibition of key processes namely growth, photosynthesis and symbiotic N fixation (Caulfield and Bunce, 1988; Zhang et al., 1995).

Dark chilling has adverse effects not only during vegetative growth but also during reproductive growth as was determined by Van Heerden and Krüger (2004). Low temperature is detrimental to flowering and pod development during the reproductive phase which will be delayed and can also cause floral abortion, poor pollen germination, and impaired ovule development, failure in pod set and reduction in seed filling (Singh et al., 1993, 1996, Srinivasan et al., 1998; Nayyar et al., 2005). Hume and Jackson (1981) found that a single night of dark chilling, with minimum temperatures of 8°C, will inhibit pod formation. A study by Gass et al. (1996), revealed that flower abscission in soybean was induced by chilling.

1.10.2 Effects on photosynthesis

1.10.2.1 Stomatal limitation

Photosynthesis is one of the first processes affected by chilling (Flexas et al., 1999) which leads to sub-optimum CO₂ assimilation (Kirschbaum, 1994). Photosynthesis can be reduced via a decrease in canopy leaf area, stomatal closure (stomatal limitation) or a decrease in the efficiency of carbon assimilation (mesophyll limitation) of individual leaves (Kramer, 1983; Frederick et al., 1989). Stomatal closure protects the plant against water loss but this also limits the availability of CO₂ for carbon assimilation. Short-term exposure to chilling night or day temperatures causes reductions in CO₂ assimilation and stomatal conductance. Several studies have shown that chilling stress cause inhibition of CO₂ assimilation through reduced stomatal conductance (Melkonian et al., 2004; Singh et al., 2005; Koscielniak and Biesaga-Koscielniak, 2006; Van Heerden et al., 2003a; Strauss and Van Heerden, 2011).

1.10.2.2 Mesophyll limitation

Mesophyll limitation as a result of stress factors includes decreases in photosystem I and II activities (Mayoral et al., 1981), inhibition of CO₂ assimilation pathways and reductions in photophosphorylation activity (Sharkey and Badger, 1982). Strauss et al. (2007), found that following a dark chilling period of the shoots of PAN809, a chilling sensitive soybean genotype, inhibition of CO₂ assimilation in conjunction with changes in chlorophyll *a* fluorescence was found, pointing towards direct effects of low temperature stress on photosynthesis and the onset of N limitation within the leaves. Chilling temperatures affect the photosynthetic carbon reduction cycle (Calvin-Benson) reactions more than the light reactions (Sassenrath et al., 1990). The Calvin-Benson cycle provides precursors of carbohydrates and may affect the activity of enzymes such as sucrose-phosphate synthase (SPS) which have been shown to be sensitive to temperature shifts (Labate and Leegood, 1990). Two important players in RUBP regeneration for photosynthesis are the chloroplastic bisphosphatases FBPase and SBPase. A recent study revealed the superior dark chilling sensitivity of chloroplastic fructose-1,6-biphosphatase in soybean (Strauss and Van Heerden, 2011). Reduced activity of Rubisco and the reduced capacity for electron transport are other key sites for the inhibition of CO₂ assimilation at low temperatures (Farquhar and Sharkey, 1982).

The major form of sugar transported via the phloem is sucrose that is synthesised in the cytosol and catalysed by SPS and sucrose synthase (SS) (Noël and Pontis, 2000). Chilling temperatures lead to increased levels of sucrose and fructans in some species (Guy et al., 1992; Miao et al., 2006). The accumulation of sugars under extreme environmental conditions such as cold serves as a mechanism of protection and attempts to explain the hardiness especially in some chilling resistant species (Tognetti et al., 1990; Signora et al., 1998; Strand et al., 2003). The fore mentioned accumulation of sugar is known to be in conjunction with increased SPS activity (Guy et al., 1992; Zuniga-Feest et al., 2005).

1.10.3 Effect on symbiotic nitrogen fixation

Various environmental constraints (e.g. drought and chilling) can have severe effects on N₂ fixation in root nodules (Finn and Brun, 1980; Walsh and Layzell, 1986; Kuzma and Layzell, 1994; Gordon et al., 1997). Temperature changes can cause delays in the appearance of nodules, and decreases in nodule number, nodule size and nodule growth rate (Lira-Junior et al., 2005). It is hypothesised that the inhibition of SNF during drought stress occurs due to the operation of the O₂ diffusion barrier that is unable to regulate the O₂ flux into the nodules which results in the inactivation of nitrogenase (Hunt and Layzell, 1993). A decrease in the permeability of gas diffusion causes a decrease in O₂ concentration in the infected cells resulting in O₂ limitation of nitrogenase-linked respiration (Weisz and Sinclair, 1988; Earnshaw, 1981). Studies with soybean nodules have shown that even under normal conditions the O₂ diffusion barrier in the inner nodule cortex regulate the O₂ concentration within the infected cell thereby limiting nitrogenase (Diaz del Castillo et al., 1994; Schubert et al., 1995). However, the mechanism by which legume nodules adapt to O₂ status appears to be different under temperatures changes than other environmental treatments such as phloem deprivation and nodule detachment (Kuzma and Layzell, 1994). During low temperature the increase in O₂ limitation measured had only minor effects even though the magnitude of nitrogenase inhibition by low temperatures was similar to that observed after stem phloem deprivation, nodule detachment, stem girdling and NO₃⁻ fertilisation (Kuzma and Layzell, 1994).

Besides nitrogenase activity, the export of ureides from the nodules can also be inhibited by chilling. An inhibition of ureide export from the nodules after a period of dark chilling is likely as a result of the solubility of ureides that decrease sharply at low temperatures (Legros and Smith, 1994). The accumulation of ureides in the petioles and leaves of soybean is caused by stress effects like drought (Vadez and Sinclair, 2000). This accumulation is probably as a result of the decreased catabolism of ureides in leaves in conjunction with negatively affected nodule activity. Some investigators have hypothesised that an accumulation of ureides and certain amino acids in leaves is most likely a main cause of lower nodule activity by acting as a feedback mechanism (Serraj and Sinclair, 1996). Symbiotic N fixation seems to be more sensitive to low soil temperatures than growth as was seen by Legros and Smith (1994), who demonstrated decreased levels of N in soybean when exposed to low soil temperatures. The activity of nitrogenase has been found to play an important role when soybean plants are exposed to chilling stress (Van Heerden et al., 2008). It was established that the loss of SNF was as a result of the inhibition of nodule respiration and the inefficient regulation of the O₂ diffusion barrier which lead to the inactivation of nitrogenase by elevated O₂ concentration (Van Heerden et al., 2008). The previously mentioned study compared a chilling sensitive genotype (PAN809) against a chilling tolerant genotype (Highveld Top) and found that unlike the chilling sensitive genotype, Highveld Top acclimated to the chilling temperatures by exerting more control over the O₂ diffusion barrier and this mechanism protected nitrogenase from inactivation (Van Heerden et al., 2008).

1.11 Senescence

Adverse environmental conditions affect plant growth and development and trigger a wide range of responses which include altered gene expression and modifications in cellular metabolism that result in changes in growth rate and crop yields. Evans et al. (1999) noted that soybean nodule senescence is the sequence of biochemical and physiological events during the final stage of development from the mature, fully developed state until death. There are several models that propose to define the threshold of senescence. One such model, namely the decay model proposes that a point of no return exists and involves a breakdown in built-in processes which defend against auto destruction by free radicals. All aerobic organisms, while having the ability to utilise O_2 , have the associated risk of oxidative stress caused by reactive O_2 species. Much of the injury to plants during environmental stress is associated with oxidative damage at the cellular level (Allen, 1995). Reactive O_2 species are closely related to the cell cycle and can influence natural nodule senescence. Senescence is characterised by the breakdown of proteins in senescing organs and nutrient remobilisation to other developing parts of the plant (Callis, 1995; Nooden, 1988). Oxidative stress, arising from an imbalance in the generation and removal of reactive O_2 species (ROS), is likely involved in plant responses to drought, temperature changes, excess excitation energy, ultraviolet (UV) irradiation and ozone (Desikan et al., 2001).

Nodule senescence can be triggered prematurely by various types of stress (Gogorcena et al., 1997; Gonzalez et al., 1998; Matamoros et al., 1999). Stress-induced nodule senescence progresses much more rapidly than developmental (natural) senescence and presents features of oxidative stress and plant cell death much like developmental senescence (Puppo et al., 2005). Natural senescence follows progression in an age dependant manner whereas premature senescence is triggered by biochemical or physiological responses due to a stressor. Groten et al., (2006) cited that nodule senescence is a highly organised process carried out in an age-dependant manner, much like leaf senescence. Features of nodule senescence include loss of leghemoglobin and N_2 fixation capacity as well as enhanced proteolytic activities. Senescence occurs on multiple levels, (i) structural, (ii) N fixation capacity and (iii) proteinase activity changes occur. Visible structural changes can be noticed by a change in nodule tissue color (red to green) due to an alteration in the leghemoglobin protein associated with the heme group (Roponen, 1970; Swaraj and Bishnoi, 1996). Ultra-structural changes in the symbiosome and cellular organelles are observed, for instance, the cytoplasm becomes less electron dense (Andreeva et al., 1998) and vesicles and ghost membranes appear due to disintegration of the host and symbiosome membrane (Timmers et al., 2000). Nodule senescence involves the triggering of a wide range of proteolytic activities that cause large scale protein degradation (Pladys and Vance, 1993). The initial effects of stress lead to the decrease in nitrogenase activity since the O_2 concentration reaching the infected cells and bacteroids declines (Witty et al., 1986; Carroll et al., 1987; Layzell et al., 1990). Results by Van Heerden et al. (2008), however, suggests that O_2 levels will increase during dark chilling in soybean nodules. Most of the O_2 consumed during respiration in bacteroids and mitochondria is reduced to water although activated O_2 species, including the superoxide radical (O_2^-) and hydrogen peroxide are always formed as by products. Elevated O_2 levels during dark chilling will increase the likelihood of ROS formation. Cellular redox regulation is very important, because it plays a key role in the

regulation of diverse signaling pathways that is used when a plant cell respond to developmental and environmental changes (Foyer and Noctor, 2005a,b). Prolonged stress results in enhanced production of oxidants and the lowering of antioxidant defenses (Escuredo et al., 1996; Gogorcena et al., 1997). Foyer and Noctor (2005a; 2005b) found that reactive O_2 species do not trigger cell death or senescence by causing physiochemical damage to the cell but instead these metabolites act as signals that activate genetically programmed pathways of gene expression that lead to regulated cell suicide events.

Nodule senescence results in the loss of SNF which leads to diminishing N fixation ability and correlates with decreases in total nodule protein (Klucas, 1974; Sutton, 1983). Stress-induced nodule senescence causes an early decrease in the capacity for SNF, which in turn leads to N limitation within the plant with associated reductions in crop yield. Elevated O_2 levels in the nodules will be present as a result of reduced respiration rates during chilling which may influence the oxidative metabolism during senescence (Van Heerden et al., 2008). A change in the gradient of O_2 across the cortex is less efficient in senescing nodules which will allow an increased flux of O_2 to reach central tissue and enhance ROS production (Witty et al., 1987).

Several processes contribute to the high ROS levels in nodules. Symbiotic N fixation requires high reducing conditions as many electron transfer components, such as ferredoxin, uricase and hydrogenase are susceptible to auto-oxidation resulting in superoxide formation (Dalton et al., 1991). The high oxy-leghemoglobin concentrations can also produce superoxide radicals (Puppo et al., 1991). The reaction between leghemoglobin and H_2O_2 can generate highly oxidizing molecules such as ferryl-haem proteins and protein radicals (Davies and Puppo, 1992; Moreau et al., 1996). Also, environmental conditions such as drought or xenobiotics may give rise to an excess production of ROS resulting in oxidative damage (Apel and Hirt, 2004). The auto-oxidation of oxygenated leghemoglobin to ferric leghemoglobin yields O_2^- which is favored by the pH of nodules which becomes more acidic during senescence (Pladys et al., 1988). Oxidation of key proteins in bacteroids through the radical O_2^- can dismutate to H_2O_2 which attack leghemoglobin releasing free Fe and thereby producing the very devastating hydroxyl radical (OH) by means of Fenton chemistry (Puppo and Halliwell, 1988). Marino et al. (2006) cited that nodules contain an impressive array of antioxidant metabolites and enzymes that scavenge ROS thereby protecting cells from oxidative damage.

Normal developmental senescence has been proposed to result from a decrease in the ascorbate-gluthatione antioxidant pool coupled to decreasing carbon to N ratios inside the nodule via the abscisic acid mediated signaling pathway mobilising proteolytic activities (Puppo et al., 2005). Stress induced senescence is reversible if plants are returned to normal growing conditions (Pfeiffer et al., 1983). Recovery of determinate nodules from induced senescence is surprising as these nodules lack defined meristems when mature and therefore the bacteroids must be more stable than expected which has been seen with both induced and natural senescence (Paau and Cowles, 1979; Pfeiffer et al., 1983; Cohen et al., 1986). Puppo et al. (2005) described that senescence in determinate nodules does not occur homogenously throughout the nodule and changes in the organisation of cellular structures are also observed. The cytoplasm of senescent soybean nodule cells becomes less electron-dense and numerous vesicles appear. Furthermore, it has also been observed that the size and shape of the symbiosomes change during senescence.

1.12 Problem statement

The reduction of soybean yield by dark chilling is a major agricultural problem because sustainable protein production is central to the nutritional and economic well-being of the population of South Africa and Africa as a whole. In South Africa, regions of maximum soybean production potential are located at high altitudes where yields are adversely affected by dark chilling (Smith, 1994). Upon exposure to dark chilling reduced growth rates, loss of photosynthetic capacity and pigment content, as well as premature leaf senescence and severely inhibited SNF are among the symptoms documented in chilling sensitive soybean genotypes (Strauss et al., 2006, 2007; Van Heerden et al., 2008; Strauss and Van Heerden 2011).

Current knowledge about stress-induced nodule senescence is based on fragmented information in the literature obtained in numerous, and often diverse, legume species. The exact nature and sequence of events participating in nodule senescence has not yet been explained fully in any of these legumes. The need for improved crop plants with greatly enhanced stress tolerance is real and urgent. Stress tolerance is a major trait target of legume breeding programs, but relatively little of this effort is directed at delayed nodule senescence, mainly because of a lack of knowledge regarding the biochemical and molecular processes linking the perception of stress within the plant to the causative effects of nodule senescence.

1.13 Hypothesis

It is hypothesised that dark chilling will lead to premature nodule senescence. Dark chilling effects on nodule function in either shoot chilled (SC) or whole chilled (WPC) plants will give insight into specific alterations and initiations of stress response in nodules and/or premature nodule senescence. Investigating the recovery process following a long-term dark chilling exposure of nodules will lead to a better understanding regarding the progression of premature nodules senescence.

1.14 Objectives of the study:

The main objectives of this investigation were:

- 1) to characterise the natural senescence process in soybean nodules under optimal growth conditions;
- 2) to characterise the alteration of the key processes of SNF in a chilling sensitive soybean genotype during dark chilling;
- 3) to establish whether recovery in nodule functionality following a long term dark chilling period occurs or whether premature nodule senescence is triggered;
- 4) To identify sensitive biochemical markers of premature nodule senescence.

Chapter 2 - Materials and Methods

2.1 Plant material and growth conditions

2.1.1 Soybean genotype selection

A screening of 30 South African soybean genotypes for dark chilling tolerance by means of chlorophyll a fluorescence ranked the genotype PAN809 as highly chilling sensitive (Strauss et al., 2006). In response to dark chilling this genotype gradually develops a chlorotic phenotype, presumably because of N-limitation caused by inhibition of symbiotic N fixation (SNF) (Strauss et al., 2007). Van Heerden et al., (2008) used PAN809 together with a chilling tolerant genotype to evaluate the effects of short term (three nights) dark chilling on symbiotic N fixation and found severely inhibited levels of nitrogenase activity in PAN809 but not in the tolerant genotype. These results supported the previous findings of Strauss et al., (2006, 2007) and emphasised the chilling sensitivity of PAN809 further. Because of its chilling sensitivity, PAN809 was used in all experiments conducted in the present study.

2.1.2 Plant growth conditions

A Conviron PGW 36 growth chamber (Controlled Environments Ltd., Winnipeg, MB, Canada, R3H 0R9) and glasshouse situated on top of the J.S. van der Merwe building, North-West University, Potchefstroom, South Africa (26°41'358"S, 27°05'437"E) was used to evaluate growth, development and nodule longevity of PAN809 under controlled and dark chilling growth conditions. The evaluation of PAN809 was conducted to give insight into the functional and structural changes that occur in root nodules during chilling stress. Growth chamber conditions included a light intensity of $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a 26°C/20°C and 15 h/9 h day/night cycle. Throughout the diurnal growth cycle plants were subjected to a $350 \mu\text{mol mol}^{-1} \text{CO}_2$ concentration.

Experiments conducted in the glasshouse were done under a 26°C/20°C and 15 h/9 h day/night regime which was controlled with a digital programmable logic control system (PLC) (AGE Technologies (Pty) Limited, 50 Charl Cilliers Street, Alberton, North Gauteng, South Africa, PO Box 136873, Alberton North, 1456). The diurnal temperature pattern in the glasshouse for a continuous period of 7 days is showed in Figure 2.1, note that a fluctuation in temperature of less than 2% occurred during either dark or light period. The photo period was controlled using fluoro lamps (Osram L/17, w58) (Osram, 15th Street, Klerksdorp, 2571) linked to a time switch.

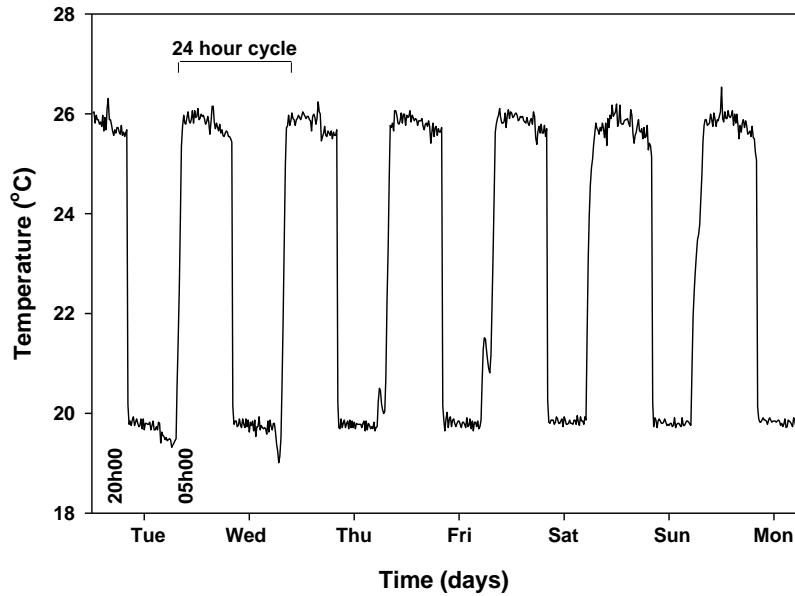


Figure 2.1: A representation of the diurnal temperature pattern in the glasshouse for a period of 7 days.

2.1.3 Plant cultivation

Fine vermiculite was used to sow seeds of the genotype PAN809 in 1.5 dm³ pots. Inoculation occurred during sowing; using *Bradyrhizobium japonicum* inoculant of the bacterial strains WB74 to guarantee optimum root nodule formation and SNF. Seedlings received N-free Hoagland's nutrient solution (Hoagland and Arnon, 1950) twice weekly and distilled water daily. The nutrient solution (pH 6.8) consisted of 2 mM MgSO₄, 1 mM KH₂PO₄, 90 µM Fe-EDTA, 5 mM CaCl₂, 5 mM KCl, along with the necessary trace elements.

2.1.4 Plastochron index

The vegetative development of plants was monitored by measuring the plastochron index (Erickson and Michelini, 1957) twice weekly. The time interval between initiation of consecutive leaves on a plant is termed the "plastochron". The plastochron index (PI) is considered a very sensitive indicator of plant development and has been used extensively for various physiological investigations, especially in legumes under controlled environmental conditions (Snyder and Bunce, 1983, Yourstone and Wallace, 1990, Jamadagni et al., 1994). Among its applications, the PI enables precise quantification of shoot growth rates (Snyder and Bunce 1983). From three weeks after sowing, the PI of selected plants were measured and repeated twice weekly on the same set of plants until a PI of 5 was reached. All trifoliate leaves with central leaflets exceeding a reference length of 25 mm (L_{ref}) were counted. The length of the youngest central leaflet longer than or equal to 25 mm, as well as the length of the central leaflet (shorter than 25 mm) on the next trifoliate leaf was measured. The PI of each plant was calculated using the following formula:

$$PI = n + (\log L_n - \log L_{ref}) / (\log L_n - \log L_{n+1})$$

where n = number of trifoliolate leaves with central leaflets longer than the reference length of 25 mm (L_{ref}), L_n = length of the central leaflet on trifoliolate leaf L_n (which by definition is longer than or equal to L_{ref}) and L_{n+1} = length of the central leaflet on trifoliolate leaf L_{n+1} (which by definition is shorter than L_{ref}). The PI was used to evaluate and monitor all experiments to ensure that the commencement of the chilling treatment occurred at the same vegetative development stage ($PI = 5$).

2.2 Chilling stress treatments

A refrigerated chamber (T+S chilling, Klerksdorp, South Africa) was used to simulate low night temperatures (dark chilling). Plants were transferred to the refrigerated chamber during the night period where temperatures were controlled at 6°C. Control plants remained in the glasshouse or growth chamber at normal night conditions of 20°C. Dark chilling treatments consisted of two different sets of chilling treatments, either whole plant chilling (WPC) or shoot chilling (SC). A separation between these two treatments was important as low root temperatures are far less common in the field than low shoot temperatures (Allen and Ort, 2001). In several soybean producing areas plants may be exposed to normal day temperatures but with low night temperatures and although soil temperatures do not cool down as much as air temperature it can still be problematic (Walsh and Layzell, 1986; Legros and Smith, 1994, Zhang and Smith, 1994), especially considering that some clusters of root nodules (crown nodules) are located close to the soil surface.

In the WPC treatment the entire plant (roots and shoots) were subjected to 6°C night temperatures. In the SC treatment the roots of the potted plants were protected from low temperatures and maintained close to 20°C, by circulating warm air around pots inside custom designed pot-incubators (Analytical Scientific Instruments, PO Box 5832, Weltevreden Park, 1715, South Africa). Pot incubators were box-shaped plastic containers measuring 230 cm in length, 38 cm in width and 24 cm in height, with 10 circular openings in which to place the 1.5 dm³ pots. Extra protective measures were also taken to ensure SC treated plants did not experience any root chilling, by covering pot surfaces with polystyrene disks. Before onset of the next light period plants were moved back to the glasshouse or growth chamber containing the control plants for the day at an air temperature of 26°C. Dark chilling exposure periods varied from a single dark period to 12 consecutive dark periods, depending on the focus of the specific experiment. Several experiments (detail provided below) were conducted not only to evaluate the short term effect of chilling treatments on nodule longevity but also long term effects where the capability to recover nodule function was evaluated.

These experiments included:

- i. Dark chilling effects during a 24 hour diurnal period,
- ii. Dark chilling effects during 12 consecutive nights of chilling treatment, and
- iii. Recovery following 12 consecutive nights of chilling treatment.

The crown nodules investigated throughout the study were situated directly on the tap root, only slightly (± 2 cm) below the surface of the vermiculite. Therefore if root zone temperatures were not accurately controlled the difference between SC and WPC would not have been pronounced and causing artifacts in data interpretation. For this purpose the difference in root zone temperatures between SC and WPC plants were tested by placing thermometers at a level of 2 cm and 6 cm from the top into the vermiculite for the duration of the dark period as well as in pots of control plants as a reference point for the SC treated plants (Table 2.1).

Table 2.1: Root zone temperature difference between treatments (C - control, SC - shoot chilled and WPC - whole plant chilled) at crown nodule (2 cm) and middle (6 cm) depth. All values indicate the mean of 4 replicates \pm SE. Comparisons between treatment means at each soil depth followed by different superscript letters are statistically different ($p < 0.05$).

Treatment	Temperature ($^{\circ}$ C) at 2 cm	Temperature ($^{\circ}$ C) at 6 cm
C	22 ± 1.256^a	21 ± 1.921^a
SC	19 ± 1.056^a	20 ± 1.476^a
WPC	6 ± 0.429^b	6 ± 0.0821^b

The root zone temperature for both the C and SC (± 1.921 and ± 1.476 , respectively) was relatively stable. This confirms that the SC treatment was not influenced by a decrease in the root zone temperature and that any physiological changes could be attributed solely to exposure of the shoots to dark chilling. The root zone temperature for the WPC treatment was 6° C at a depth of 2 cm and 6 cm, making these plants ideal for investigating dark chilling effects of both shoot and root chilling.

2.3 Nodule function

2.3.1 Preparation of samples

All analyses were standardised to include only the crown nodules (McDermott and Graham, 1989). These nodules form a cluster approximately 2 cm from the soil surface directly on the tap root (Figure 2.2). Since the evaluation of changes in nodule function and structure over time was monitored for very long periods in some experiments, standardisation was deemed necessary. The cluster of crown nodules was best suited for this purpose, being of the same age and easily recognisable.



Figure 2.2: A photo illustrating the cluster of crown nodules of uniform age and size situated directly on the tap root (indicated by the red circle) making them ideal when monitoring changes in nodule function over time.

Root systems were carefully rinsed to remove the vermiculite particles and blotted dry. The sections of taproot carrying the crown nodules were excised by cutting these sections from the root systems and sealing them in gas-tight glass tubes. Nitrogenase activity was measured by the acetylene reduction method (Bergersen, 1980; Turner and Gibson, 1980) with some modifications and prior optimisation as described below.

2.3.2 Optimisation of nitrogenase (EC1.18.6.1) activity assay

Nodules were exposed to an acetylene-argon gas mixture (Seibod Gas, Sasolburg, South Africa) of two different concentrations (1% and 10% v/v) and incubated in the gas-tight glass tubes for different periods of time (0 – 60 min) at room temperature. Air samples (1 ml) were collected with a 1001TL syringe (Separations, Randburg, South Africa) and ethylene and acetylene were separated with a gas chromatograph having a hydrogen ionisation detector at an oven temperature of 60°C. A column (Hewlett Packard, AI203, 19091P-S15) of 50m x 0.32mm x 8µm was used for separation.

2.3.3 Acetylene concentration

The acetylene reduction method is a simple, inexpensive and sensitive measure of nitrogenase activity but has been met with some criticism (Minchin et al., 1983). Minchin et al. (1986), demonstrated that nitrogenase may be inhibited by acetylene due to the interruption to NH₃ production within the nodule. An incubation atmosphere of 10% acetylene is commonly used to assess maximum nitrogenase activity, but acetylene might inhibit the enzyme at that concentration. Van Heerden et al. (2008), demonstrated that a 1% (v/v) acetylene concentration resulted in higher nitrogenase activities than a 10% (v/v) concentration. A comparison between these two concentrations was also performed under the current experimental conditions (Figure 2.3). Crown nodules collected from control and dark chilled (WPC for a single night period) plants was

used to evaluate the concentration effect of acetylene on nitrogenase activity. Consistent with the finding by Van Heerden et al., (2008), it was determined that the 1% acetylene concentration resulted in much higher rates of nitrogenase activity compared to the commonly used 10% concentration after 10 minutes of incubation. Furthermore the percentage difference in nitrogenase activity between control and dark chilled plants with the use of 1% or 10% acetylene, was on average 62% in both cases indicating that the use of a 10 times diluted concentration did not compromise the measurements because the relative differences in nitrogenase activity between control and dark chilled plants were maintained.

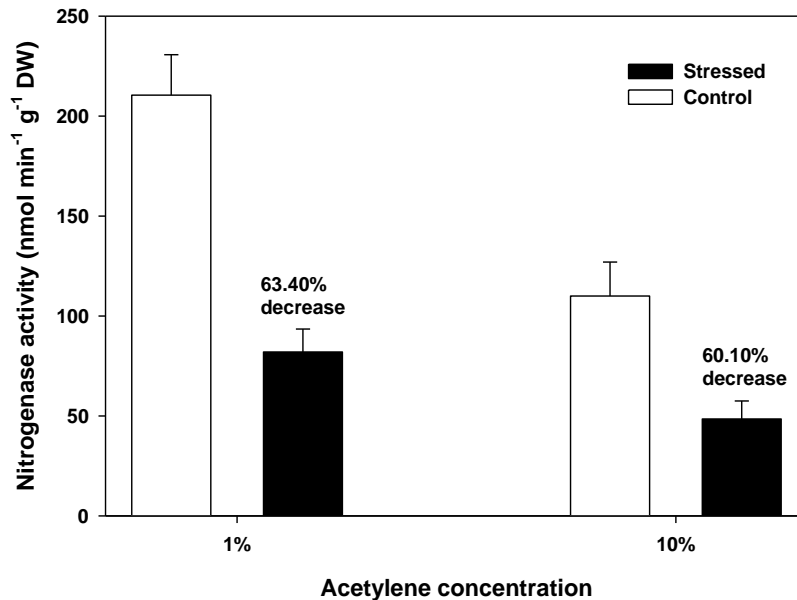


Figure 2.3: A comparison of nitrogenase activity in the presence of 1% acetylene mixture versus the commonly used 10% mixture. White and black bars represent control and dark chilled samples, respectively. Each data point represents the mean of four replicates \pm SE.

2.3.4 Incubation period

After optimisation of the acetylene concentration, determining the optimum period of sample incubation was necessary. The incubation period allows for the conversion of acetylene to ethylene by nitrogenase. This conversion should be linear over time. It is important to affirm this period of linearity as there have been speculations that removing nodules from the intact root system would increase levels of endogenous ethylene production by wounding (Abeles, 1982; Glick, 2005). The duration of linearity of ethylene production was investigated and it was found that linearity was maintained for a period of up to 60 minutes in the presence of 1% acetylene mixture with an r^2 -value of 0.988 (Figure 2.4). An incubation period of 10 minutes was selected and was used in all subsequent experiments.

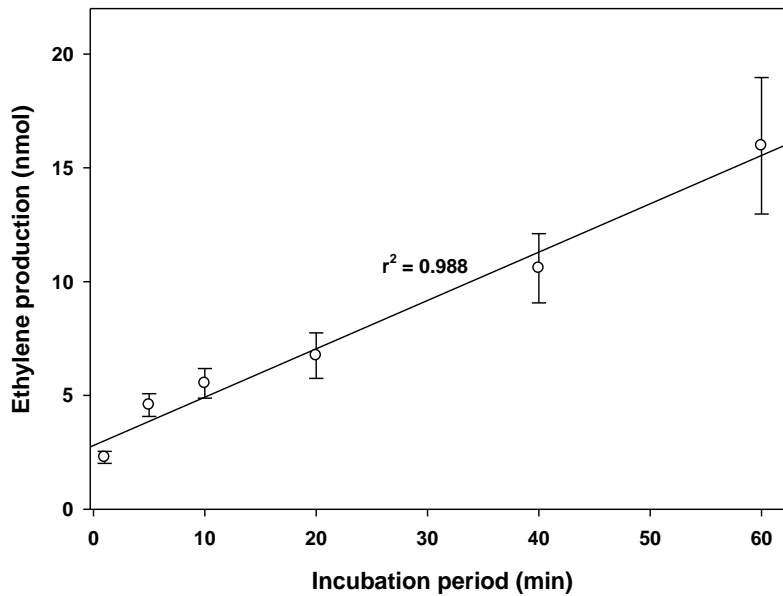


Figure 2.4: Linear production of ethylene during acetylene reduction to determine nitrogenase activity. Each data point represents the mean of four replicates \pm SE.

2.3.4 Growth environment

Nitrogenase activity was determined for plants growing in the growth chamber and glasshouse to clarify whether different growth conditions had an effect on nodule function and which growth situation would be more suited and applicable to field conditions. It was found that glasshouse grown plants had a 48% higher nitrogenase activity than growth chamber grown plants (Figure 2.5). As this study focused on nodule function and longevity it was decided that all future experiments would be conducted in the glasshouse facilities.

2.3.5 Sampling time

The nitrogenase activity during one diurnal (24-hour) cycle was monitored and it was found that nitrogenase activity peaked at around 05h00 in the morning with lowest activity from midday (12h00) until the start of the following dark period (Figure 2.6). To standardise all analyses, sampling occurred at 05h00 – 06h00 to ensure nitrogenase activity was always at its maximum and that observed treatment effects was not confounded by the normal fluctuations in diurnal activity.

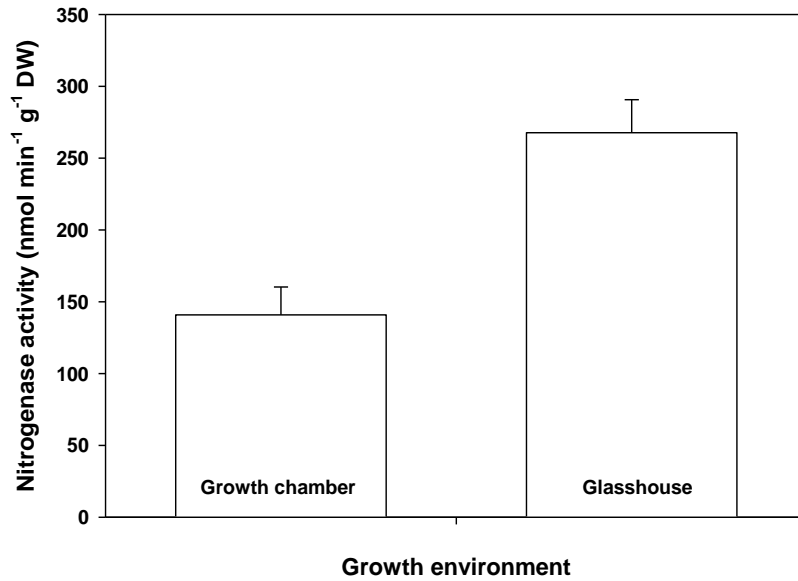


Figure 2.5: Difference between nitrogenase activity in plants grown in the growth chamber and glasshouse. All samples were collected at midday. Each data point represents the mean of four replicates \pm SE.

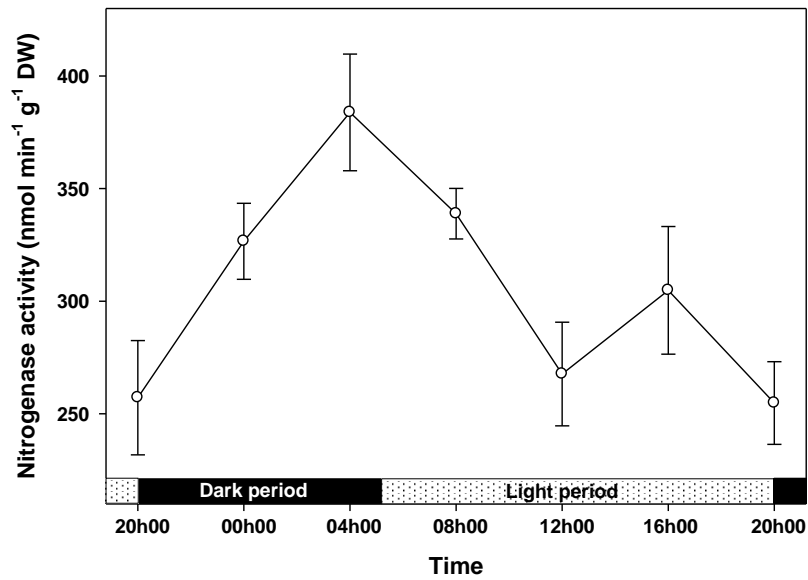


Figure 2.6: Nitrogenase activity monitored over a 24-hour cycle. Plants were grown in a glass house and each data point represents the mean of four replicates \pm SE.

2.4 Biochemical analysis

2.4.1 Sampling procedure

Crown nodules from four different plants were collected and immediately frozen in liquid N to ensure the integrity of the samples. Time intervals between sampling points varied depending upon the focus of each specific experiment, for example, every 4 h during diurnal studies, every 3 days during a 12 night chilling period or weekly after a 12 night chilling period to assess recovery. Samples were stored at -80°C until analysis could be performed.

2.4.2 Extraction method

Frozen nodules (300 mg) were extracted with 1.5 ml ice cold extraction buffer containing 50 mM Mops (pH 7), 4 mM MgCl₂, 20 mM KCl, 200 mM sorbitol and 10 mM dithiothreitol (DTT) in a pre-cooled mortar. A protease inhibitor cocktail for plant cell extracts (P9599, Sigma) was added to the extraction buffer during extraction. The crude extract was centrifuged for 30 min at 20 000 X g at 4°C. The supernatant was desalted using Sephadex G-25 superfine gel packed in disposable 2 ml polystyrene columns. The columns were pre-equilibrated with 6 ml buffer containing 50 mM Mops (pH 7) and 4 mM MgCl₂ and used to desalt the supernatants by centrifugation at 700 X g and 4°C for 2 min (Helmhorst and Stokes, 1980).

2.4.3 Sucrose synthase (SS, EC 2.4.1.13) activity

Sucrose synthase activity in aliquots (35 µl) of desalted supernatant was determined in the sucrose synthesis direction in assay buffer (35 µl) containing 50 mM imidazole (pH 8.5), 5 mM MgCl₂, 20 mM Fru, 80 mM Glc-6-P and 20 mM uridine diphosphoglucose (UDPG) as describe by Van Heerden et al (2008). Reactions (10 min at 25°C) were terminated by the addition of 70 µl of 30% (w/v) KOH. Sucrose formation was determined with the resorcinol method (Huber and Israel, 1982). Resorcinol reagent was prepared by dissolving 0.125 g Resorcinol in 12.5 ml ethanol and 37.5 ml 30% HCl. The reagent is light sensitive and was therefore kept in the dark for a maximum period of three days. To each tube 1 ml of resorcinol reagent was added and incubated at 80°C for 10 min. Tubes were briefly cooled on ice and the absorbance was measured at 520 nm with a spectrophotometer (Camspec, Camspec Ltd., Sawston, Cambridge, UK). Sucrose synthase activity was calculated following the completion of a standard curve.

2.4.4 Nodule sucrose content

Soluble sugars were extracted from nodules in hot ethanol (80% v/v) to denature any degrading enzymes. Samples were ground with liquid N, after which a volume of 1 ml hot ethanol (80°C) was added to the fine powder in a microfuge tube. After 15 sec the tube was closed, mixed vigorously using a vortex mixer and then incubated at 80°C for 20 min. The samples were then cooled on ice and centrifuged at 10000 X g for 10 min. The supernatant was stored at -20°C for enzyme-based determination of sucrose content as described by Jones et al. (1977).

For the sucrose content assay, 2.5 µl of supernatant was added to 142.5 µl reaction mixture containing 100 mM Imidazole-HCl (pH 6.9) and 10 mM MgCl₂, as well as 1.1 mM ATP, 0.5 mM NAD, 1.4 U glucose-6-phosphate dehydrogenase (G6PDH), 0.75 U hexokinase (HK) and 0.35 U phosphoglucosomerase (PGI). Absorbance was determined at 340 nm in a micro plate reader (Power Wave Select, Bio-Tek Instruments, Inc. Highland Park, USA) until a stable value was obtained. To start the reaction, 250 U Invertase was added and the total increase in absorbance determined.

2.4.5 Determination of leghemoglobin content

Nodule material was extracted and desalted as explained in section 2.4.2. A volume of 400 µl desalted supernatant was mixed with 600 µl distilled water and used for the determination of leghemoglobin content. A volume of 33.8 ml of pyridine was added to 0.2 M NaOH in water (pH 7.4) to obtain a final pyridine concentration of 4.2 M. An amount of 1ml of pyridine solution was added to the diluted supernatant. The diluted extract (2ml) was equally aliquoted between two 1 ml quartz cuvettes which either contained a few crystals of potassium ferricyanide (K₂FeCN) or sodium dithionite. The content in each cuvettes were stirred and the absorbance in the range of 530 to 570 nm determined spectrophotometrically. The absorbance values at 539 and 556nm was used to calculate leghemoglobin content according to the method of Appleby and Bergersen, 1980.

$$\text{Leghemoglobin content} = \frac{(A_{556\text{nm}} - A_{539\text{nm}}) * 5}{\Delta \epsilon}$$

ϵ – (Molar absorptivities for leghemoglobin) $\Delta \epsilon = 23.4 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Bergersen et al., 1973)

2.4.6 Nodule ureide content

Nodules were dried at 60°C in an oven prior to ureide (allantoin and allantoic acid) extraction. Ureides were extracted from nodules with 1 ml 0.2 M NaOH. Extracts were boiled for 20 min and after cooling on ice, centrifuged at 10 000 x g for 10 min. The ureide concentrations of the clarified supernatants were measured spectrophotometrically as the phenylhydrazone derivate of glyoxylate (Young and Conway, 1942). The ureide content was calculated following the completion of an allantoin standard curve.

2.4.7 Nodule respiration rate

Nodule respiration was monitored by using a Ciras-1 Infra-red-gas analyser (IRGA) (PP Systems International, Haverhill Road, Suite 301, Amesbury, Massachusetts, U.S.A) connected to a modified conifer-type photosynthetic leaf chamber (PP Systems International, Haverhill Road, Suite 301, Amesbury, Massachusetts, U.S.A). The nodules under investigation was left attached to the roots and placed within the leaf chamber. A flow rate of 600 ml min⁻¹ was maintained through the leaf chamber and temperature (28°C) and humidity (ambient) were controlled throughout analysis. Upon opening and closing the chamber, a stabilisation period of 5 min was allowed after which the measurement was taken. Firstly, total (root and nodule) respiration rates were determined followed by measurements of root respiration rates which were after the removal of the nodules. Nodule respiration rate was estimated as the difference between the two rates.

2.4.8 Detection of proteins by Western Blot Analysis

2.4.8.1 Extraction method

Host (plant) proteins were extracted by grounding nodules with 1.5 ml ice cold extraction buffer containing 50 mM Mops (pH 7), 4 mM MgCl₂, 20 mM KCl, 200 mM sorbitol and 10 mM dithiothreitol (DTT) in a cooled mortar. A protease inhibitor cocktail (P9599, Sigma) was added to the mortar during extraction and crude extract was centrifuged for 30 min at 20 000 X g at 4°C. The soluble host protein content within the supernatant was determined according to the method of Bradford (1976).

Bacterial proteins were extracted from the pellet by re-suspending the pellet 3 times in extraction buffer and centrifuging at 20 000 X g for 30 min at 4°C. The washed pellet was re-suspended a fourth time in a buffer solution without the addition of sorbitol. This suspension was placed into a sonication bath for two-30s pulses at 0°C. The supernatant obtained after a further centrifugation step (20 000 X g for 30min at 4°C) contains the bacterial proteins and was used to determine soluble bacterial protein content.

2.4.8.2 Sample preparation

A volume of 750 µl of supernatant was used and mixed with 250 µl of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer consisting of 250 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 0.287 mM mercaptoethanol, 0.015 mM bromophenol blue and 40% (v/v) glycerol. The sample was boiled for 5 min and stored at -20°C until further analysis could be performed.

2.4.8.3 Protein separation

Proteins were separated by SDS-PAGE with a mini-gel system (Mini-PROTEAN 3 Electrophoresis Module, Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California, USA). The running gel solution contained a 2.5 ml 40% acrylamide/bisacrylamide mixture (Bio-Rad Laboratories) and 2.5 ml 1.5 M Tris-HCl (pH 8.8). The gel was cast between the glass plates after addition of 2.5 ml distilled water, 0.1 ml 10% (w/v) SDS, 0.1 ml 10% ammonium persulphate and 10 µl TEMED. The stacking gel solution contained 0.2 ml 40%

acrylamide/bisacrylamide mixture and 0.5ml 0.5M Tris-HCl (pH 6.8). The gel was casted on top of the polymerized running gel after the addition of 20 μ l 10% (w/v) SDS, 25 μ l 10% (w/v) ammonium persulphate and 5 μ l TEMED. A plastic comb was immediately inserted into the stacking gel solution and allowed to set for 1 h, thereby forming a 10-sample well placement in which protein extractions and marker proteins were loaded. Sample wells were rinsed thoroughly with SDS running buffer consisting of 192 mM glycine, 25 mM Tris and 0.5% SDS. An amount of 15 μ g total soluble protein was loaded in the sample wells, together with 10 μ l of a broad range pre-stained SDS-PAGE protein standard (Bio-Rad Laboratories). Proteins were separated for 120 min at 75 V at room temperature.

2.4.8.4 Protein transfer

After separation with SDS-PAGE, proteins were transferred from the running gel to a Hybond C-extra nitrocellulose membrane (Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Bucks HP 79NA, UK). The membrane, two layers of filter paper (Whatmann #1) and two sheets of blotting foam, were covered with transfer buffer consisting of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol (HPLC-grade) and 0.1% SDS for 30 min. The running gel was carefully removed from the glass plates and equilibrated for 1 min in transfer buffer. The membrane was positioned on top of a double layer of filter paper on a wet blotter (Bio-Rad Laboratories). The gel was then placed on top of the membrane and covered with a double layer of filter paper. Any air bubbles between the different layers were removed carefully. Protein transfer from the gel to the membrane was conducted at 100 V for 25 to 60 min, depending on the size of the protein under investigation.

2.4.8.5 Labeling of proteins with primary and secondary antibodies and subsequent detection

The Hybond C-extra nitrocellulose membrane containing the transferred proteins was incubated in blocking buffer solution containing 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA and 1% (w/v) skimmed milk powder for about 3 h. The membrane was probed overnight with primary anti-rabbit antibody according to recommended dilution ratios for a particular antibody, being 1:1000 for Nase and sucrose synthase and 1:2000 for leghemoglobin (anti-bodies received from AJ Gordon at The Institute of Grassland and Environmental Research, UK). The following day the membrane was washed 3 times for a period of 10 min with blocking solution and probed with anti-rabbit IgG secondary Horse Radish Peroxidase linked antibody for 2 h (1:1000 dilution). The membrane was washed again 3 times with blocking buffer solution and procedure repeated with ultrapure water.

Protein detection occurred by incubating the nitrocellulose membrane in a detection reagent consisting of 100 ml 50 mM Tris-HCl (pH 7.6), 40 mg chloronaphtol (dissolved in 500 μ l 100% ethanol) and 50 μ l 30% H₂O₂ for 15 min. The development of the membrane was stopped by replacing the detection reagent with ultrapure water.

2.5 Ultra structural studies

2.5.1 Light microscopy

Nodule samples were taken pre-dawn and cut into 1 mm² pieces with a very sharp blade and placed in Todd's fixative (Todd, 1986). The fixation schedule was conducted at room temperature. After 24 h the nodule pieces were rinsed three times for 15 min in a 0.05 M cacodylate buffer (pH 6.7), followed by post-fixation with 1% aqueous osmium tetroxide (OsO₄) for 1 h. The nodule pieces were then rinsed 3 times for 15 min in distilled water and stained for 30 min with 2% (w/v) uranyl acetate dissolved in distilled water.

All samples were dehydrated for 15 min in 50%, 70%, 90% ethanol series, respectively and twice in 100% ethanol. During this process the samples were not exposed to air. Infiltration was conducted in a 1:1 mixture of acetone and resin (Spurr, 1969) for 3 h. The acetone-resin mixture was replaced with fresh mixture and left overnight. After 24 h, the samples were placed in a fresh mixture of 100% resin for 2 h to ensure that the exchange of resin and ethanol was complete. Each sample was then embedded in fresh 100% LR white (medium grade) resin in an empty capsule and labeled. Polymerisation took place at 60°C for 12 h.

The embedded sample blocks were trimmed and ultra-thin cross sections ($\pm 0.5 \mu\text{m}$) were made with a microtome (Reichert Jung Ultracut) using a glass knife. Sections were placed on a drop of distilled water on a microscope slide, dried on a hotplate, stained with 0.5% toluidine blue at moderate heat and rinsed. Slides were stained for a second time using 0.05% neufuchsin, dried and viewed. Slides were examined with a Nikon Eclipse 80e light microscope and micrographs captured digitally.

Intercellular spaces were measured in the infected zone, inner cortex and middle cortex using Nikon NIS-Elements version 2.3 image-analysis software. Areas of 50 or more intercellular spaces in each tissue type were measured.

2.6 Statistical analysis

Statistical analysis was conducted with the software package Statistica for Windows version 10 (StaSoft, Inc. 2300 East 14th Street, Tulsa OK 74104, USA). Normal distribution of data was determined using the Shapiro-Wilk W test (Shapiro et al., 1968). In data sets with parametric distribution, breakdown and one-way ANOVA were performed and the Tukey's honest significant difference (HSD) test was used to determine significant differences between treatment means. In the case of nonparametric data, the Kruskal-Wallis ANOVA and the Median test were applied to determine statistically significant differences between the samples.

Chapter 3 – Characterisation of nodule lifespan under optimal growth conditions

3.1 Introduction

Symbiotic nitrogen fixation by legumes via their association with root nodule bacteria plays a critical role in agricultural systems throughout the world (Herridge et al., 2008; Unkovich et al., 2009; Melino et al., 2012). A large proportion of the world's nitrogen needs are derived from SNF and contributes to agricultural sustainability, therefore improving SNF can have considerable economic impacts (Schulze, 2004). Nitrogen fixation provides an important advantage for the production of soybean compared with most grain crops in that soybean fixes the nitrogen required for its growth and for the production of the high-protein content in seed and oil (Purcell et al., 2000).

The establishment of a nitrogen-fixing symbiosis between *Bradyrhizobium* bacteria and the soybean plant is a complex developmental process during which legumes responds to bacterial signals, resulting in the formation of specialised structures called root nodules (Sen et al., 1986). Both partners benefit from the symbiosis as the host requirements for nitrogen are largely covered by the rhizobia, which in turn receive organic carbon from the host produced by photosynthesis (Vance et al., 1997). Fotelli et al., (2011) reported that the accessibility of photoassimilates has been found to be vital in controlling nodule growth (Viosin et al., 2003a) and nitrogen fixation (Viosin et al., 2003b). Sucrose from the shoot is the principal source of carbon and energy for nodule development and metabolism. Sucrose is transported to the nodules via the plant vascular system and distributed to nodule cells where it is catabolised for SNF, assimilation of ammonia and export of amino acids and other nitrogenous compounds (Gordon et al., 1995; Vance et al., 1997). Nodule development and SNF require substantial amounts of fixed carbon, making the nodules a strong sink for carbon (Minchin and Pate, 1973; Silsbury, 1977). The process of SNF comes at a high respiratory cost (Rainbird et al., 1984; Ryle et al., 1984) with concomitant loss of CO₂ (Pate et al., 1979), which accounts for more than 60% of carbon allocated to the nodules.

Environmental constraints such as drought and chilling severely inhibit SNF (Gordon et al. 1997). The symbiotic balance between the host plant and nitrogen-fixing bacteria in the root nodules is susceptible to early senescence (ageing) in the presence of environmental stress (Puppo et al., 2005). Once nodule senescence is initiated, the gradually increasing loss of SNF capacity leads to nitrogen limitation within the plant with associated reductions in crop yield. Depending on the legume species, the process of nodule senescence is typically characterised by one or more of the following symptoms: loss of nitrogenase activity, alterations in nodule protein profiles (Dalton et al., 1986; Lahiri et al., 1993), changes in proteinase activity, shifts in the redox homeostasis of the nodules (Hernandez-Jimenez et al., 2002; Vandenabeele et al., 2003) and changes in the levels and ratios of phytohormones.

Main reasons for the poor performance of soybean under chilling stress conditions are the negative effects on key metabolic processes such as SNF (Layzell et al., 1984). Optimal SNF during the growth season is crucial in ensuring high yields and high seed protein content.

In South Africa the largest soybean producing areas are situated at high altitudes, with minimum daily temperatures which can be critically low and hinders the production of soybean. The demand for soybean oil cake has exceeded the supply, creating the incentive for increased production (Smit, 1998). Since soybean is chilling sensitive, with growth, development and yield being affected negatively at temperatures below 15°C (Gass et al., 1996), chilling stress proves to be one of the most important constraints to soybean production in South Africa. McKersie and Leshem (1994) showed that even a brief chilling event could lead to adverse symptoms gradually appearing after a plant has been returned to optimal growing conditions. Van Heerden et al., (2008) showed that chilling causes biochemical, physiological and anatomical changes inside soybean root nodules which severely inhibit SNF.

This chapter focused on characterising the functioning and gradual senescence of soybean root nodules under normal (optimal) growing conditions. Monitoring the alteration in key parameters involved in SNF and senescence would give insight into normal changes that occur within the lifespan of soybean nodules. Chilling stress might result in deviations in nodule function and the natural senescence pattern either anatomically or physiological and will be discussed in later chapters. The exclusion of chilling stress for this particular phase of the study was pertinent to establish a time-dependent baseline for main processes involved in SNF. This will enable the accurate quantification of chilling stress effects on these processes which will provide insights into the effects of chilling stress on SNF (Chapter 4) and premature senescence or recovery (Chapter 5).

3.2 Results and discussion

The typical growth, development and senescence of soybean crown nodules are illustrated in Figure 3.1. The photographic series shows the visual appearance of the crown nodules on the taproot between four and ten weeks after sowing. It was notable that the size of crown nodules increased from 4 until 7 weeks. Internally root nodules changed color, from pinkish to almost red (results not shown). The pink-reddish color can be attributed to the presence of leghemoglobin, which supplies the bacteria with a controlled oxygen environment. A change in external color was visible after 7 weeks. Inside the nodules the color changed from pink to green caused by bilirubin accumulation during leghemoglobin breakdown (Lehtovaara and Perttilä, 1978) (results not shown). This change in internal color coincided with the external discoloration of the nodules from white to brown seen from week 7 onwards.

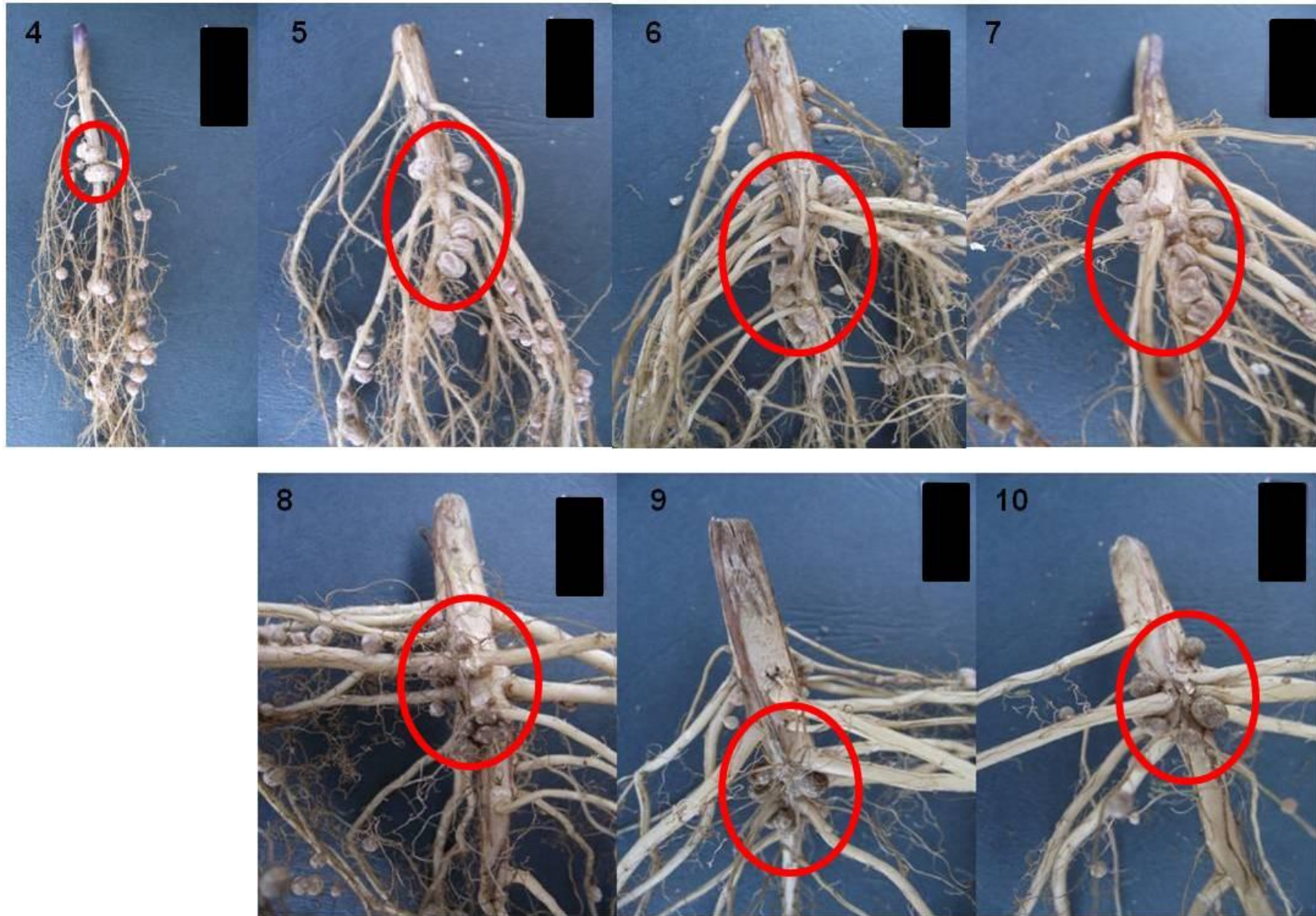


Figure 3.1 A photo series illustrating the growth, development and senescence of soybean crown nodules over a 6 week period, starting 4 weeks after sowing. The black bar represents a scale of 1.3cm long and 0.6cm wide and red circles indicate crown nodules.

The functionality of nodules is determined by the activity of nitrogenase which determines the proficiency of SNF. Nitrogenase activity was monitored from 4 weeks after sowing for a six week period to evaluate the physiological changes that occur during normal growth, development and senescence of the crown nodules. It was found that nitrogenase activity was at its highest 4 – 5 weeks after sowing where after the activity steadily decreased to a minimum 10 weeks after sowing (Figure 3.2). A similar trend was observed in the presence of the Mo-Fe nitrogenase protein (58kD) with the highest protein presence seen 4 - 5 weeks after sowing followed by a steady decline to very low levels 10 weeks after sowing (Figure 3.2). Groten et al., (2005) observed similar results when evaluating the development and senescence of a pea variety (*Pisum sativum* cv. Phoenix). In that study nitrogenase activity began to decline from 3 weeks after sowing until undetectable levels 11 weeks after sowing. The changes in protein presence matched the changes in nitrogenase activity during the development of diminishing capacity for SNF.

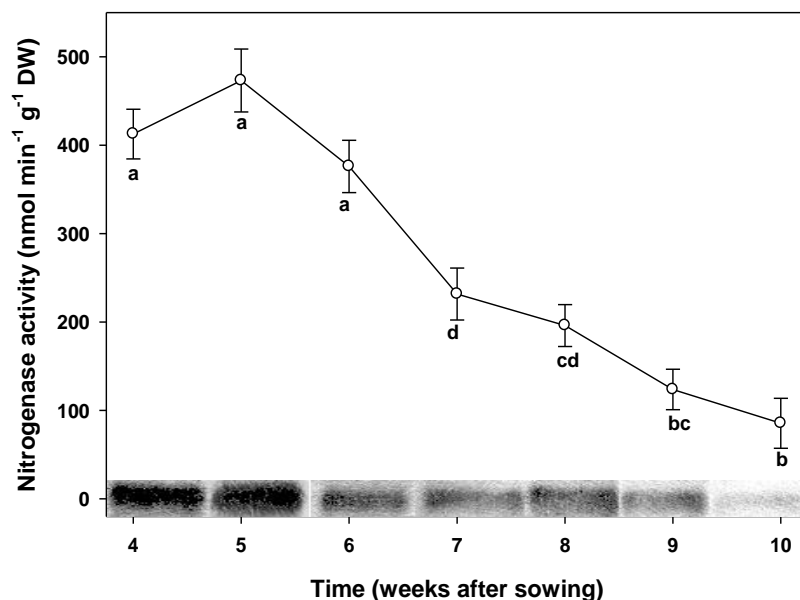


Figure 3.2: Nitrogenase activity (nmol min⁻¹ g⁻¹ DW) monitored for a 6 week period from 4 weeks after sowing. Each data point represents the average of four replicates with standard error. Significant statistical differences over time is shown by the use of letters. Western blots of nitrogenase Mo-Fe protein (58kD) abundance at each time point is shown on the x-axis label.

The product of SNF in the nodules, namely ureides, was determined over the same time period (Figure 3.3). In contrast with the declining trend of nitrogenase activity over time (Figure 3.2), nodule ureide content was found to gradually increase from 5 weeks after sowing until a maximum content was reached 7 weeks after sowing. There after it remained constant for the remainder of the investigation period. It is known that the solubility of ureides is relatively low (Schubert and Boland, 1990), therefore when senescence of nodules starts, desiccation occurs, which could result in decreased export and resultant accumulation of ureides in the nodules with ageing. This accumulation of ureides is known to be an inhibitor of nitrogenase activity. Vadez et al. (2000), found that decreases in SNF were closely tied to feedback inhibition of nitrogenase activity by nitrogenous compounds including ureides. The relationship between nodule ureide content and nitrogenase activity is shown in Figure

3.4. The close negative correlation that existed between nitrogenase activity and nodule ureide content during the investigation period with a R^2 value of 0.786 lends support towards a possible feedback inhibition mechanisms that have been found in other studies (Vadez et al., 2000 ; Ladera et al., 2007). Furthermore when nodule senescence and leaf senescence is compared similarities exists. An analysis of *Medicago truncatula* leaf and nodule senescence as compared to *Arabidopsis* leaf senescence by De Michele et al., (2008) revealed that 7% of genes overlapped for these two organs when in the phase of senescence. The allocation of nutrients such as nitrogen and phosphorous and energy reserves once the leaf is no longer photosynthetically functional, could serve as model to explain the increase in ureide content within the nodules. Another possibility is, that as a result of the repression of the protein UPS1, accumulation of ureides will occur and decreased nitrogen partitioning to roots and shoot will be subsequent. This UPS1 protein is responsible for the transport of allantoin and allantoic acid out of the nodule, and when this protein is repressed, nitrogenase activity is rapidly down-regulated in soybean (Collier and Tegeder, 2012). A key feature of leaf senescence is a temporal high level of soluble sugars (Navabpour et al., 2003). Considering the previous statement an increase in ureide content (Figure 3.3) over the experimental period could point towards a symptom of nodule senescence.

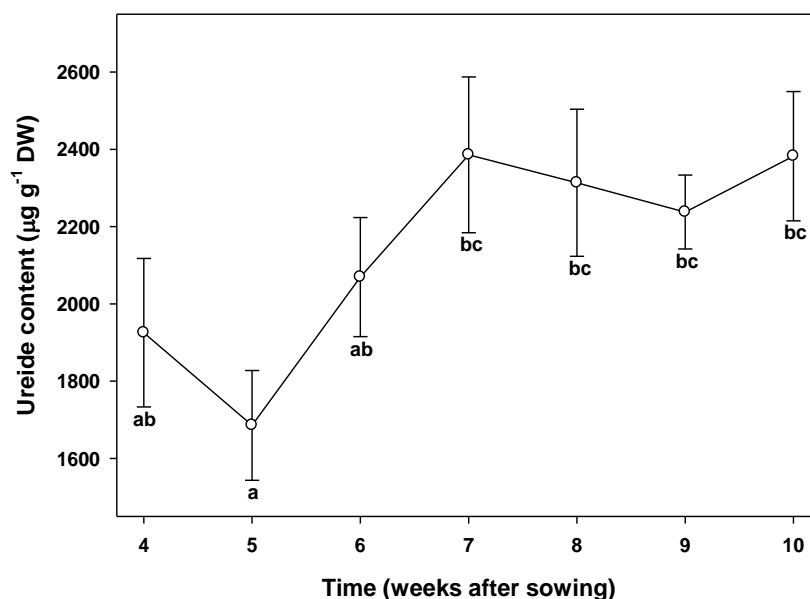


Figure 3.3: Nodule ureide content ($\mu\text{g g}^{-1}$ DW) monitored for a 6 week period from 4 weeks after sowing. Significant statistical differences over time is shown by the use of letters. Each data point represents the average of four replicates with standard error.

Leghemoglobin content measured within the crown nodules indicated a period of increase up to 7 weeks after sowing where after a sharp decline was observed (Figure 3.5). The mechanism responsible for the degradation of leghemoglobin is still relatively unknown but its conversion to biliverdin and bilirubin is either catalysed by heme oxygenase (Brown et al., 1990; Baudouin et al., 2004) or nonenzymatically at a pH of 7.5. This biliverdin-like pigment is associated with a decrease in SNF activity and leghemoglobin content in senescent nodules

(Virtanen and Laine, 1964; Roponen 1970). The decrease in leghemoglobin content was preceded by the decrease in nitrogenase activity (Figure 3.2) indicating lower nodule function *per se* and not through indirect effects induced by breakdown of the O₂ diffusion barrier. The decrease in SNF has been ascribed to a direct effect on nitrogenase (Burns et al., 1985) or an indirect effect through decreases in leghemoglobin content, respiration rate and malate concentration (Delgado et al., 1993, 1994). The increase in ureide content in contrast to the decrease in both leghemoglobin content and nitrogenase activity could point towards a change in the transport of ureides from the nodules. Two mechanisms could be involved here, direct degradation of nitrogenase protein during senescence (as supported by the western blots – Figure 3.2B) and product feedback inhibition by ureide accumulation. The possibility that ureide accumulation with resultant feedback inhibition could act as the trigger for the nitrogenase protein degradation to start should be investigated further.

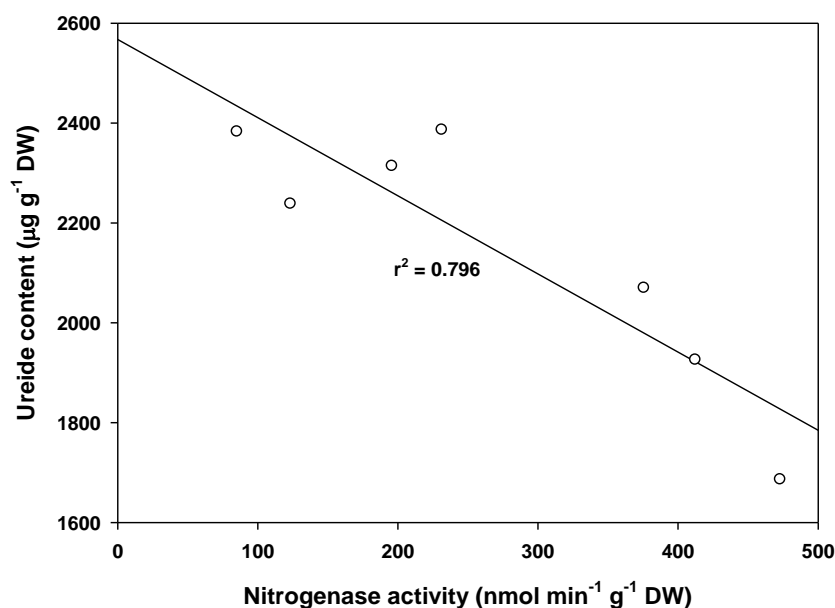


Figure 3.4 : Correlation between nitrogenase activity (nmol min⁻¹ g⁻¹ DW) and nodule ureide content (µg g⁻¹ DW) monitored over a 6 week period from 4 weeks after sowing

The linkage of the carbon cycle to the nitrogen cycle during SNF is very important; the hydrolysis of sucrose by sucrose synthase (SS) for respiration supplies the energy necessary for the N fixation process. The SS activity measured for the 6 week period (Figure 3.6) remained relatively stable with no statistically significant changes in activity observed over time. The abundance of the SS protein shown in Figure 3.6 corresponded with the unchanged function of this enzyme and indicated its stability throughout the experimental period. A similar trend was observed over the same period when the nodule respiration rate was investigated with a stable level throughout the period of investigation (Figure 3.7). For optimum SNF and the production of ureides the conditions should be ideal, these include adequate abundance of leghemoglobin for optimum nitrogenase function and sucrose as energy source made available by sucrose synthase for maintenance of high respiration

rates that drives SNF. These parameters interlock with one another to establish the processes involved and necessary for SNF under normal growth, development and senescence.

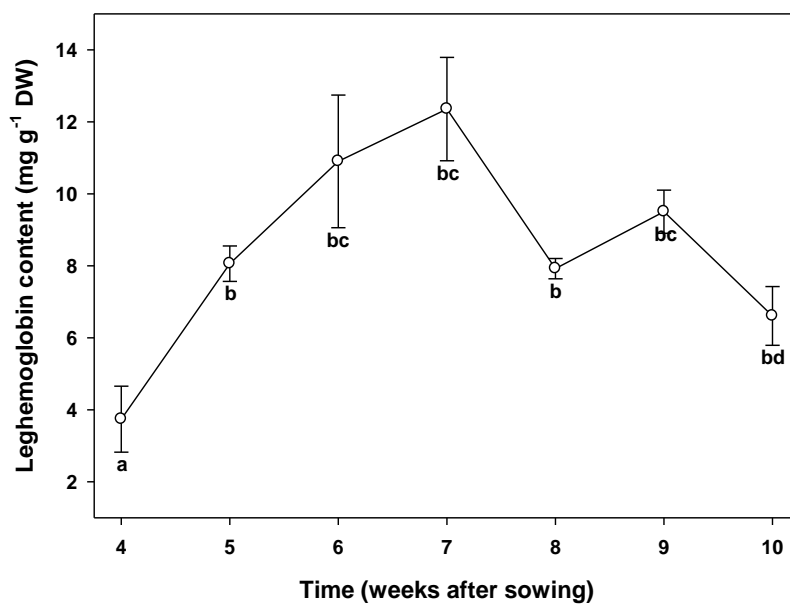


Figure 3.5: Leghemoglobin content (mg g⁻¹ DW) measured over a 6 week period from 4 weeks after sowing. Significant statistical differences over time is shown by the use of letters. Each data point represents the average of four replicates with standard error.

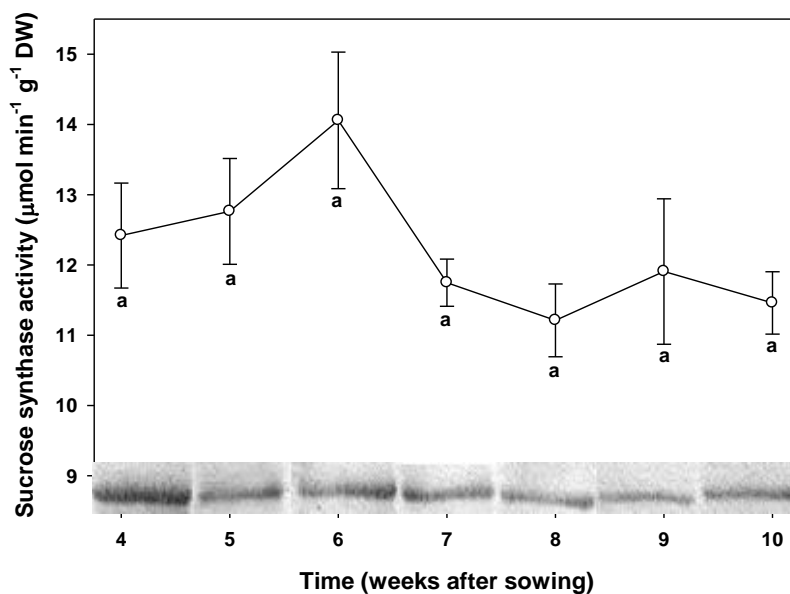


Figure 3.6: Sucrose synthase activity expressed per dry weight (μmol min⁻¹ g⁻¹ DW) measured over 7 weeks; Western blots of sucrose synthase protein (64kD) abundance at each time point is shown on the x-axis label. Significant statistical differences over time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

Nodule sucrose content remained at relatively constant levels during the first 7 weeks of growth as corroborated by the absence of any statistical significance differences over time. After 7 weeks, however, a steep decline in nodule sucrose content occurred (Figure 3.8). The decline in nodule sucrose content could have been caused possibly by lower import of sucrose into the nodules. Following flowering of the soybean plant, photosynthesis will be down regulated to limit energy spent which will rather be allocated to pod formation and filling thereby decreasing carbohydrate production and translocation to nodules. With the result that less sucrose will be available for cleavage by SS in senescing nodules.

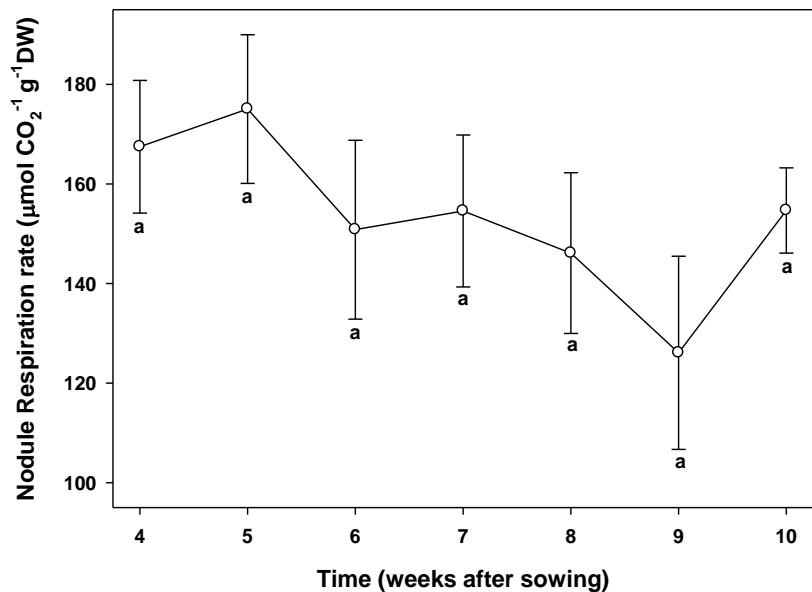


Figure 3.7: Nodule respiration rate expressed per dry weight ($\mu\text{mol CO}_2 \text{ g}^{-1} \text{ DW}$) measured over a 6 week period from 4 weeks after sowing. Significant statistical differences over time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

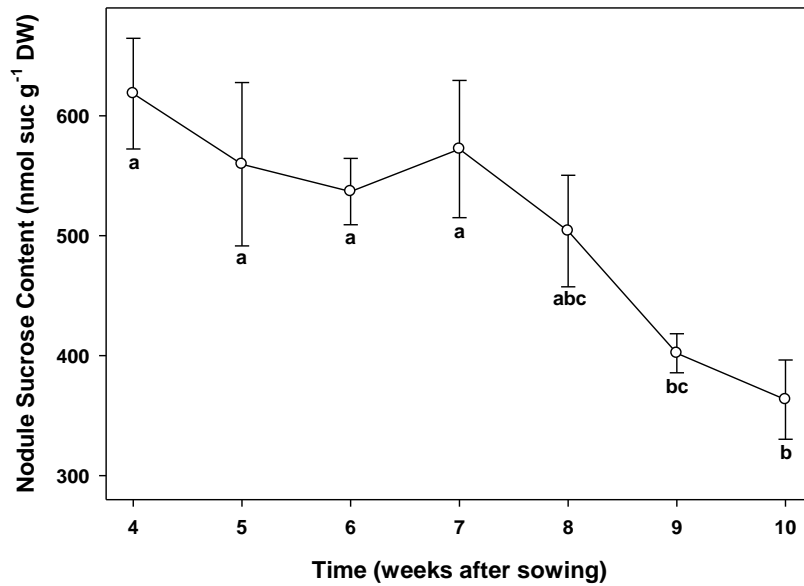


Figure 3.8 Sucrose content (nmol suc g⁻¹ DW) measured over a 6 week period from 4 weeks after sowing. Significant statistical differences over time is shown by the use of letters. Each data point represents the average of four replicates with standard error.

3.3 Conclusions

Optimal nitrogenase activity in the crown nodules occurred during 4 to 6 weeks after sowing then gradual decline commenced from 6 weeks onwards. The decline in activity was associated with the decline in nitrogenase content and the increase in ureide content within the ageing nodules. The negative correlation between nitrogenase activity and ureide content could point towards a possible feedback inhibition trigger. The stability of SS activity and nodule respiration indicated that carbon-dependent metabolic processes were maintained for much longer. The results reported in this chapter on the development and senescence of nodules established a baseline for these key parameters under optimal growth conditions. These baselines will be tested in Chapter 4 to evaluate induced dark chilling effects on nodule function. Furthermore, the induction of premature senescence or capacity for recovery following a long term chilling stress period will be investigated in Chapter 5.

Chapter 4 – Dark chilling effects on nodule function

4.1 Introduction

Soybean (*Glycine max* L. Merrill) yield is often severely reduced due to its natural sensitivity towards various stress factors including drought and sub-optimal growth temperatures (chilling). There are several reasons for the poor performance of soybean under chilling stress conditions which include chilling induced alterations in key metabolic processes such as symbiotic nitrogen fixation (SNF) (Layzell et al., 1984). Optimal SNF is crucial for ensuring high yields and high seed protein content (Keyser and Li, 1992). A fluctuation in SNF during or as a result of stress could potentially trigger the onset of premature nodule senescence. The symbiotic balance between host plant and the nitrogen-fixing bacteria in the root nodules is susceptible to early senescence in the presence of environmental stress (Puppo et al., 2004). Stress-induced nodule senescence causes an early decrease in the capacity for SNF, which in turn leads to nitrogen limitation within the plant with associated reductions in crop yield (Bordeleau and Prévost, 1994; Hungria and Vargas, 2000).

Symbiotic nitrogen fixation is rapidly and severely inhibited by drought, chilling and various other environmental constraints (Gordon et al., 1997). Several researchers have found that during chilling stress, SNF can be inhibited before other key physiological processes that include photosynthesis (Durand et al., 1987) and the resultant biomass accumulation (Sinclair et al., 1987). Even though chilling causes severe effects on SNF, the mechanisms involved in the inhibition are complex and still not clear. There are several ways by which dark chilling can inhibit SNF, which include the following 1) Severe dark chilling that lead to sub-optimal root zone temperatures could directly inhibit the symbiosis in the root nodules by a decrease in the permeability of nodule membranes to gas diffusion or reductions in nodule metabolic potential (Vance and Heichel, 1991; Gordon et al., 1997); 2) It is possible that the export process from nodules could be restricted during chilling stress, which might result in the accumulation of N compounds within the nodules (Walsh, 1990). Legros and Smith (1994) indicated that the solubility of ureides and hydraulic conductivity decrease sharply during chilling stress. The resultant accumulation of ureides in the nodules could lead to product (feedback) inhibition of nitrogenase activity (Streeter, 1993; Gordon et al., 1997); 3) A reduction in shoot growth during dark chilling stress could decrease the demand for ureides in the sink tissues, such as young developing leaves. Accumulation of ureides, or other nitrogenous compounds, in nodules via phloem import could therefore also result in product inhibition of nitrogenase activity (Parsons et al., 1993); 4) Dark chilling could induce premature nodule senescence leading to a decrease in the capacity for SNF and ultimately nodule death (Puppo et al., 2004).

In a South African context, variation in the chilling stress response of local genotypes has only recently been characterised (Strauss et al., 2006). Since SNF is such an important yield-determining factor, a lack in understanding these complexities inevitably delays progress towards the genetic improvement of soybean cultivars/varieties/strains/genotypes and also complicates decisions with regard to the suitability of certain genotypes for the various soybean-producing areas in South Africa. This often results in lower than potential yields and inferior seed protein content in areas that experiences low night temperatures. Consequently, more often than not, the result of any decision is that yield potentials are rarely, if ever realised.

Our knowledge about stress-induced nodule senescence is based on fragmented information in the literature obtained in numerous and often diverse legume species. The need to improve crop plants with enhanced stress tolerance in the light of food security is real and urgent. Stress tolerance is a major trait target of legume breeding programs (Reddy et al., 2012), but relatively little of this effort is directed at delayed nodule senescence, mainly because of a lack of knowledge regarding the biochemical and molecular basis underlying this process.

This chapter will focus on the alteration in key parameters of SNF (those studied under optimal growth conditions in Chapter 3) when dark chilling was applied. The induced dark chilling was specified as either only shoot (SC) exposure or whole plant chilling (WPC). The SC treated plants allowed for the more-frequent situation to be studied, where cold nights often do not result in direct chilling of roots and nodules. However, in some areas around the world (including South Africa) dark chilling may result in cooling of soil and subsequently root chilling under certain conditions. The monitoring throughout a long term chilling period will give insight into specific alterations and initiations of stress response in soybean nodules. This will give particular information regarding the sequence of events during a stress response and the recovery processes following the suspension of chilling will confirm if premature nodule senescence was initiated or if recovery ensued (Chapter 5).

4.2 Results and Discussion

Metabolic and environmental constraints including defoliation, water shortage, long-term darkness, nitrate fertilisation and chilling has a strong negative effect on SNF (Duke et al., 1979; Walsh and Layzell 1986; Matamoros et al., 1999). The changes in nitrogenase activity caused by a long term (12-night) dark chilling period in control (C), shoot chilled (SC) and whole plant chilled (WPC) treatments are presented in Figure 4.1 A. The period of chilling started after 4 weeks of normal growth following sowing. Day 6 of the dark chilling period therefore coincides with approximately 5 weeks after sowing and day 12 of chilling with approximately 6 weeks after sowing. The weeks after sowing are indicated at the top of each figure and by vertical dotted lines inside the figures. After 3 nights of chilling the nitrogenase activity of SC treated plants did not differ from the control plants ($p = 0.428$), opposed to the WPC treated plants of which the nitrogenase activity was inhibited significantly ($p = 0.045$) by 67%. The nitrogenase activity in the control plants was similar to the values measured in the previous experiments (Chapter 3; Figure 3.1) in nodules of the same age and a comparable trend of increase in activity from week 4 to week 5 following sowing was also observed. Following 6 nights of consecutive chilling it was observed that the significant difference between control and SC plants was brought about more by the progressive increase in nitrogenase activity in the control, and not by a decline in SC treatment *per se*. However, in the WPC treatment a constant decrease remained until the end of the experimental period where a maximum inhibition of 82% was documented. The drastic decline of nitrogenase activity induced by WPC was not found in SC treated plants. This point towards a much more pronounced effect, when nodules experience direct chilling in comparison with indirect chilling experienced by only the above-ground plant parts in the SC treatment. Considering nitrogenase protein abundance, there was no consistent effect of any of the chilling treatments over time (Figure 4.1B). Results following a short term chilling (3 days) experiment by Van Heerden et al. (2008), revealed an inhibition of approximately 80% in nitrogenase activity and

unaffected nitrogenase protein abundance for PAN809. This supports the results shown in Figure 4.1A and B.

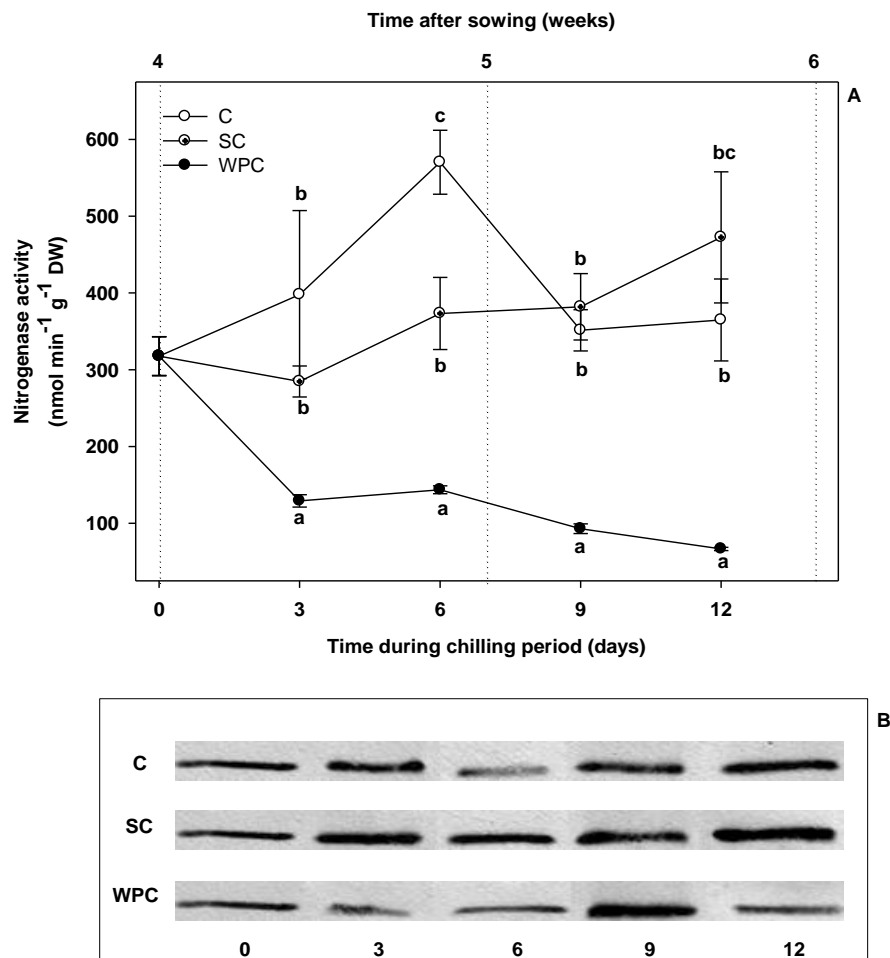


Figure 4.1: Nitrogenase activity ($\text{nmol min}^{-1} \text{g}^{-1} \text{DW}$) monitored for a 12 night chilling period. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants (A). Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error. Western blots of nitrogenase Mo-Fe protein (58kD) abundance at each time point (B).

Since organic nitrogenous compounds formed by SNF are transported to upper parts of the plant either as amides, glutamine or ureides (Schubert, 1986), and since ureides are the compound of choice in soybean, the ureide content of nodules was determined. The ureide content in crown nodules and the change over a 12 night chilling period are depicted in Figure 4.2. A comparable trend for C and SC plants was visible with an initial increase and slight plateau in ureide content over time. The control plants experienced a more pronounced increase in ureide content over 12 nights for the approximate period 4 to 6 weeks after sowing, with a resultant increase of 69%. The degree of reduction at the end of the experimental period as compared to the control plants varied with a 25% and 69% reduction for SC and WPC treated plants, respectively. A reduction of approximately 50% in ureide content for PAN809 was determined after 3 nights of WPC chilling by Van Heerden et al. (2008). Similar results were documented after a period of 9 nights of WPC chilling, which resulted in a 70% and 60% inhibition of nodule ureide content for the soybean genotypes Fiskeby V and Maple Arrow, respectively (Van Heerden and Krüger, 2004). Strauss et al. (2007), showed that chilling effects on nodules and the transport of ureides from the

roots to the leaves are vital in the responses of photosynthesis. Predominantly, when the whole plant was exposed to dark chilling, the diminished leaf ureide content led to severe chlorosis which is a known symptom of nitrogen deprivation within the plant. Other studies have also shown that exposure to chilling might limit transport of nitrogen metabolites resulting in chlorosis of leaves (Castle et al., 2006). Van Heerden et al. (2004), demonstrated that the primary target for chilling-induced inhibition was SNF and under long-term N deprivation, all leaves turned chlorotic. The decrease in ureide content observed in this study, together with studies cited; highlight the integral role of SNF and the importance of optimal nitrogen supply to the shoot for maintenance of the photosynthetic machinery in the leaves.

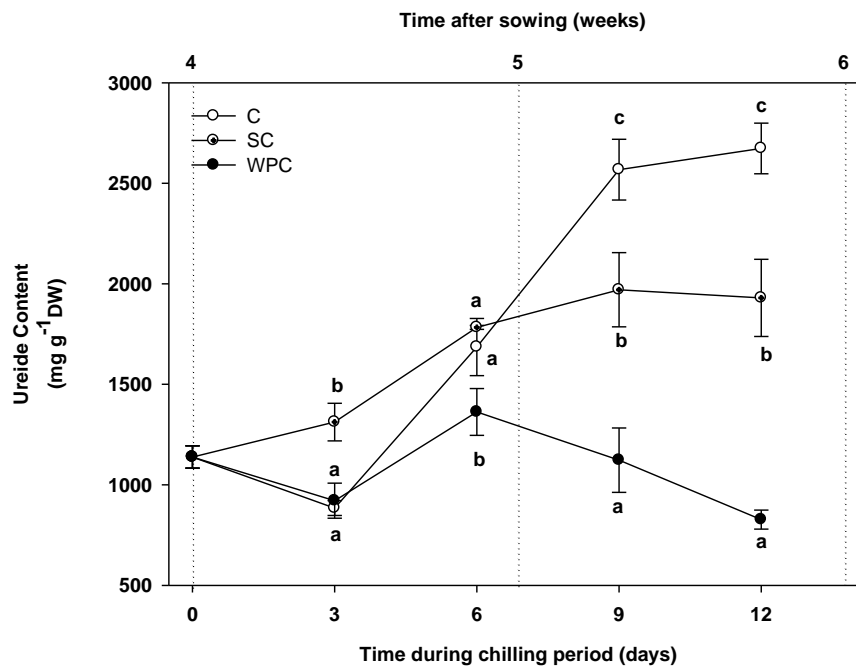


Figure 4.2: Nodule ureide content (mg g^{-1} DW) monitored for a 12 night period. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants. Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

Energy derived from respiration is required to support nitrogen-fixing bacteria and the process of SNF (Ryle et al., 1984). Various factors influence respiration, for example, differences between species (Poorter et al., 1991; Lambers et al., 1996a), plant age (Poorter and Pothmann, 1992), soil moisture and temperature (Maier and Kress, 2000). Under normal growing conditions plant respiration increases exponentially as a function of temperature (Salisbury and Ross, 1996). The rate of nodule respiration during the experimental period (12 nights) for the treatments C, SC and WPC is shown in Figure 4.3. Respiration rates measured before the chilling period started (4 weeks after sowing) was very similar to the values shown in Figure 3.7, indicating the reproducibility and accuracy of measurements. For the first 6 nights of dark chilling WPC and SC plants had statistically lower respiration rates compared to the C plants and also differed from each other (significantly on day 6) with WPC plants affected more severely. On completion of the chilling period nodule respiration rates in WPC still remained significantly ($p = 0.0005$) inhibited, approximately 44% lower than the respiration rates in C plants. Notably, the decreased nodule respiration rates experienced from day 6 to 9 in C plants corresponded with a decrease in nitrogenase activity (Figure 4.1A).

A correlation for treatments C, SC and WPC, between nitrogenase activity and nodule respiration rate (Figure 4.4) revealed that nitrogenase activity was directly affected by nodule respiration rate as indicated by the R^2 values of 0.966, 0.646 and 0.896 in C, SC and WPC plants, respectively. This accentuates the fact that regardless of treatment, nitrogenase activity and respiration rate was closely linked but also that drastic differences occurred between the treatments SC and WPC in terms of the degree of inhibition of these two parameters.

The severe inhibition of nitrogenase activity in WPC treated plants indicated that chilling had a direct effect on nitrogenase activity, since the whole plant, including the root nodules, were chilled. A study on short term dark chilling by Van Heerden et al. (2008), found severely depressed nodule respiration rates, which could have caused elevated O_2 concentration inside the nodule thereby inhibiting nitrogenase activity.

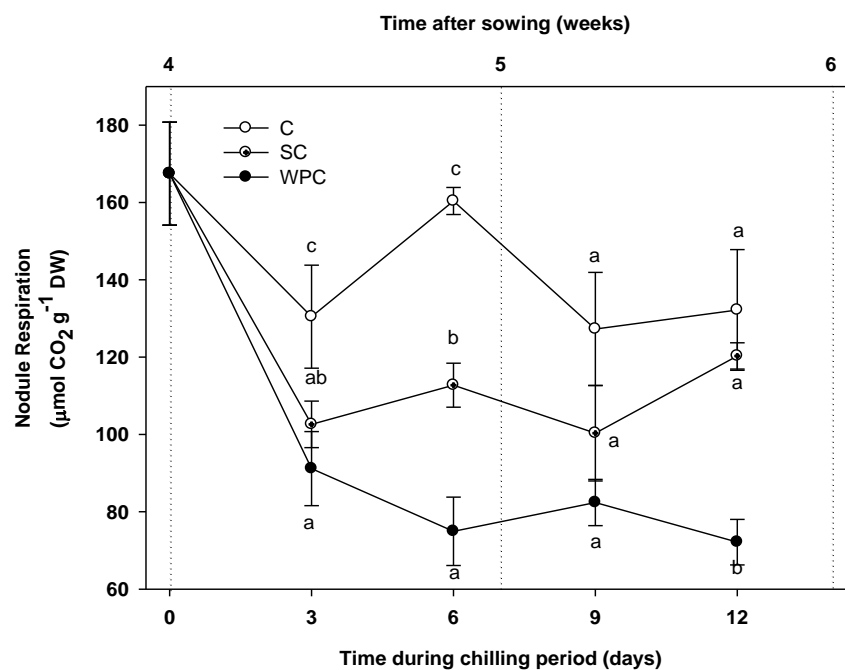


Figure 4.3: Nodule respiration rate ($\mu\text{mol CO}_2 \text{ g}^{-1} \text{ DW}$) monitored for a 12 night period. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants. Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

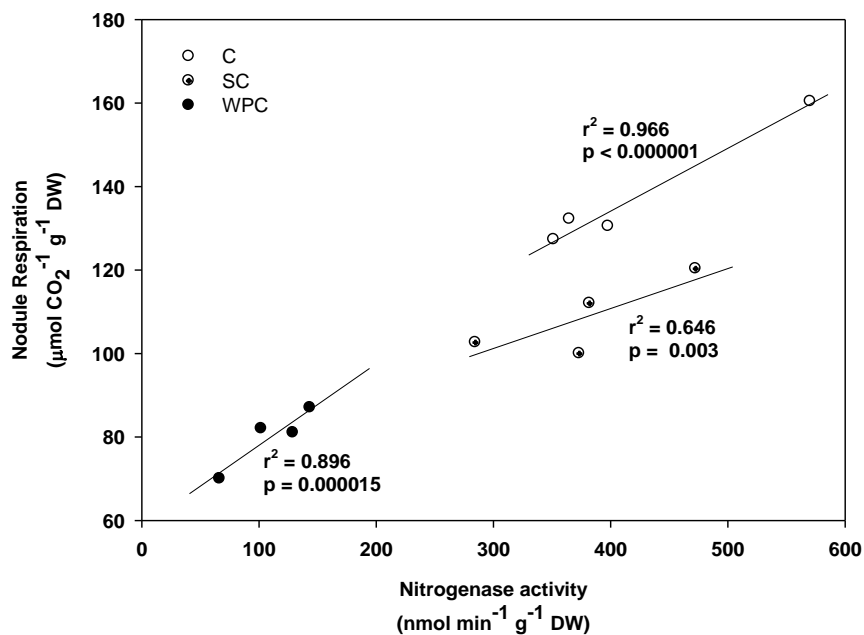


Figure 4.4: The relationship between nodule nitrogenase activity ($\text{nmol min}^{-1} \text{g}^{-1} \text{DW}$) and respiration rate expressed per dry weight ($\text{mmol CO}_2 \text{g}^{-1} \text{DW}$) monitored for a 12 night period. Control plants (C), shoot chilled (SC) and whole plant chilled (WPC).

Nodules show high rates of oxygen consumption (Rawsthorne and La Rue. 1986b; Day and Mannix, 1988; Puppo et al., 1987). Leghemoglobin content has a regulatory function (Appleby, 1984) as it delivers O_2 directly to the bacteroids and allows free O_2 concentration at a very low level throughout the infected cells. Nodule leghemoglobin content was monitored for a 12 night period and results are shown in Figure 4.5A. A comparable trend for all treatments was observed with an increase on day 6 where after a decrease followed, although not statistically significant. Chilling seemingly had no effect on the leghemoglobin content and no differences occurred between treatments throughout the experimental period, indicating that the leghemoglobin protein (Figure 4.5B) was not a target for chilling induced changes. Similar findings, showing no decrease in leghemoglobin abundance following a 3 night chilling period was observed by Van Heerden et al. (2008). This is contradictory with other studies which found a reduction in leghemoglobin content and inhibition of respiration in pea, common bean and soybean nodules following salt stress (Delgado et al., 1994) and drought stress in soybean nodules (Ruiz-Lozano et al., 2001). The above mentioned differences point towards different effects induced by different stress factors.

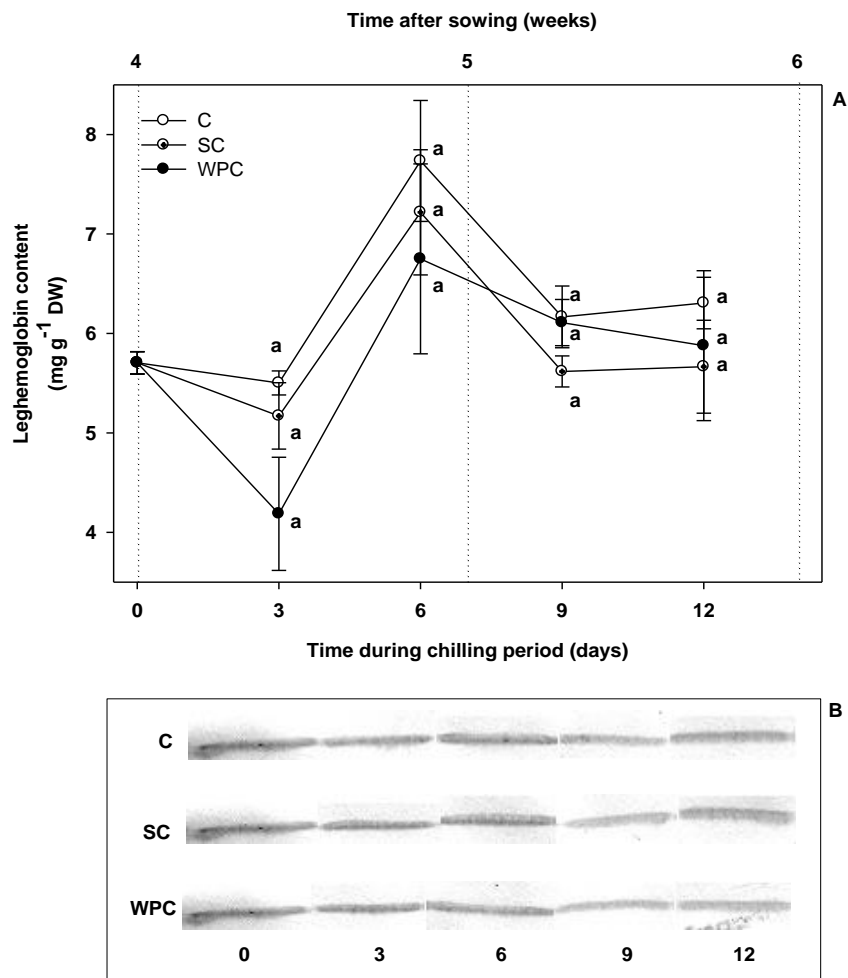


Figure 4.5: Leghemoglobin content (mg g⁻¹ DW) for a 12 night period. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants (A). Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error. Western blots of leghemoglobin protein (15kD) abundance at each time point (B).

The activity of the enzyme responsible for the cleavage of sucrose for respiration, sucrose synthase, was monitored for a 12 night period and results are presented in Figure 4.6A. Sucrose synthase activity was not negatively affected by the SC and WPC treatments during the experimental period and remained at relative constant levels. Unaffected sucrose synthase protein abundance in a chilling sensitive soybean genotype in response to short-term chilling was showed by Van Heerden et al. (2008), and corresponds with results obtained after a 3 times longer chilling period (Figure 4.6B). Sucrose synthase is responsible for the hydrolysis of sucrose and is a key role player in maintaining optimal nitrogenase activity. No degree of statistical difference between treatments was observed. These results imply that the ability to hydrolyse sucrose for nitrogenase-linked nodule respiration was not a limiting factor during dark chilling and is contrary to published reports on other stress factors such as drought. Galvez et al. (2005), indicated that pea plants experiencing drought stress had a declined SS activity along with nitrogenase activity. It was also speculated that a likely limitation of carbon flux might be involved in the decline of nitrogenase activity.

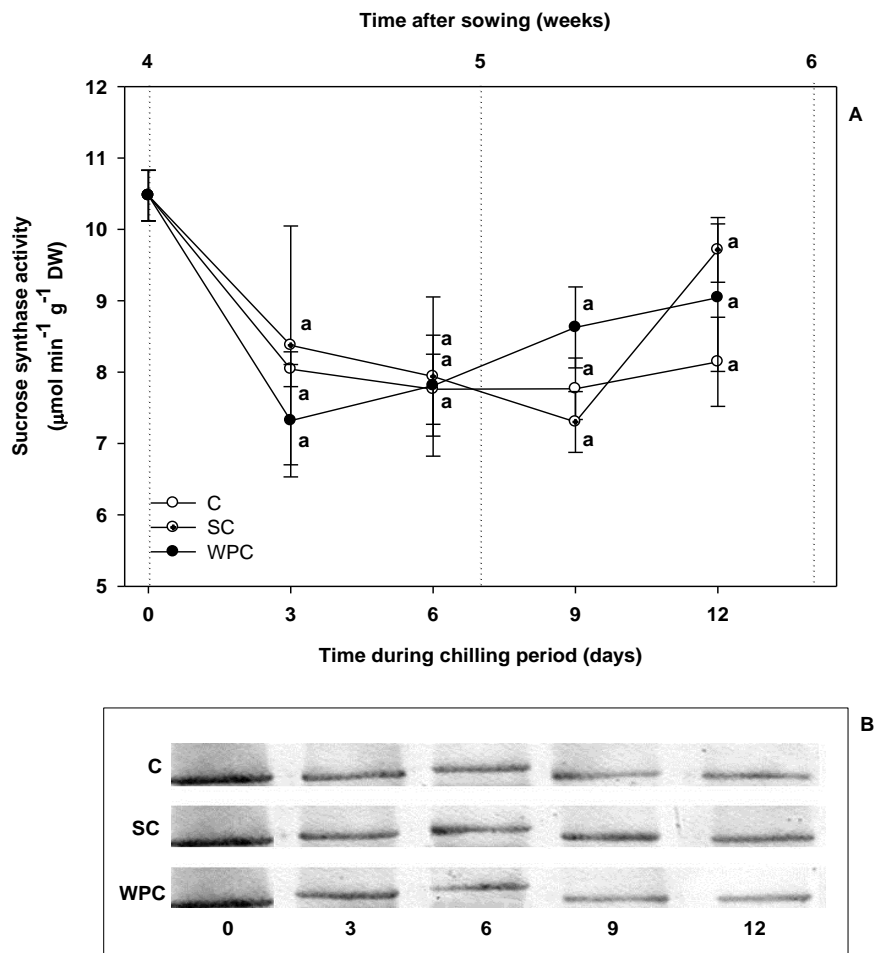


Figure 4.6: Sucrose synthase activity expressed per dry weight ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{DW}$) for a 12 night period. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants (A). Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

Sucrose synthase was shown to be essential for nitrogen fixation in soybean (Gordon et al., 1999). However, different stress responses have different effects on the carbon flux (SS and sucrose content). Drought stress caused major decreases in SS mRNA levels in soybean nodules (Gordon et al., 1993, 1997) and subsequently nitrogenase activity, whereas dark stress decreased nitrogen fixation and caused rapid depletion of sucrose rather than changes in the enzyme capacity to metabolise sucrose.

It has long been shown that SNF relies on photosynthate supply (Hardy and Havelka, 1976; Sprent et al. 1988). Therefore, the sucrose content was determined for all treatments for a 12 night period and is portrayed in Figure 4.7. On completion of the chilling period it was found that the nodule sucrose content showed no decreases in response to WPC and SC treatments, confirming that the inhibition of nitrogenase activity in both WPC and SC-treated plants cannot be attributed to a decline in carbon availability or flux as observed during drought stress (Ladrera et al., 2007). In fact, there was a tendency of increased sucrose content in chilled nodules compared to the control for at least the first 6 days of chilling. The same result was also found following a short-term chilling period experienced by soybean nodules and supports the observed sucrose accumulation (Van Heerden et al., 2008). The fact that

sucrose concentrations in the nodule increased suggested that the nodules were not deprived of photosynthates. The availability of photoassimilates has been found to be an important factor controlling nodule growth (Voisin et al., 2003b) and nitrogen fixation (Voisin et al., 2003c). Strauss and Van Heerden (2011) evaluated the effects on both roots and shoots of soybean during dark chilling and concluded that chilling stress effects on the shoots included reduced photosynthesis capacity, inhibition of PSII and lower fructose-1,6-bisphosphatase (cFBPase) and sucrose phosphate-synthase (SPS) activity. Thus the decrease in sucrose content in WPC following the initial accumulation during the first 6 nights of chilling exposure may be attributed to the less than optimal conditions for photosynthesis thereby decreasing the production of carbohydrates and the transport of sucrose to the nodules.

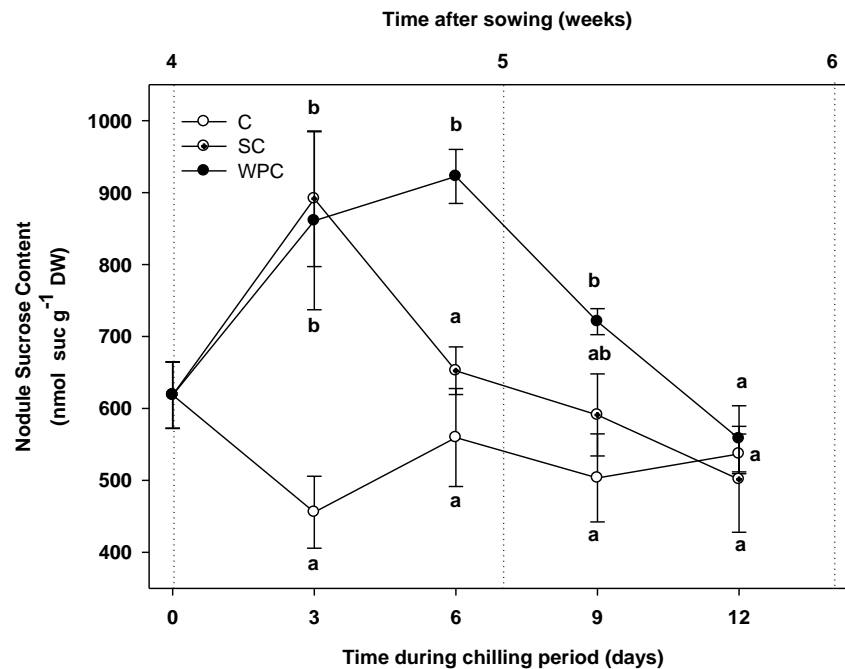


Figure 4.7 Sucrose content (nmol suc g⁻¹ DW) for a 12 night period. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants (A). Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

4.3 Conclusions

The results reported in this chapter supports a direct effect of dark chilling on nitrogenase activity (Figure 4.1A) and nodule respiration rate (Figure 4.3), which leads to a decline in nodule ureide content (Figure 4.2). This occurs in the absence of any limitations on carbon flux (i.e. reduced sucrose synthase activity and sucrose content – Figure 4.6 and 4.7), which would inhibit nitrogenase activity indirectly through limitations on nitrogenase-linked respiration. The fact that nitrogenase activity, ureide content, nodule respiration rate and nodule sucrose content was affected in SC plants, albeit to a much lesser extent than in WPC plants, provides some indication of some shoot-derived factor(s) also involved in the minor inhibition of SNF.

King and Purcell (2005) investigated SNF during drought stress and found that ureide content and total free amino acids increased in nodules and leaves which coincided with the decline in SNF. These authors concluded that SNF was reduced through a feedback mechanism involving accumulation of amino acids

in the leaves. On the other hand, Ladrera et al. (2007) found that a combination of both reduced carbon flux and ureide accumulation in nodules appears to be involved in the inhibition of SNF. Our results do not support a similar response within nodules during dark chilling stress since nodule ureide content actually decreased and carbon flux was not limiting (sucrose synthase activity and sucrose content). The depressed rates of respiration due to chilling might have led to elevated O₂ concentrations in the nodules inhibiting nitrogenase, which in turn would result in decreased ureide content within these nodules. The alteration in key parameters involved in SNF as a result of chilling induced changes, might trigger premature nodule senescence as less than ideal conditions prevailed during the chilling period, in particular in the WPC plants. The exact nature and sequence of events contributing to nodule senescence is still unknown. In the following chapter the processes in nodules following the long-term dark chilling exposure period will be investigated and will lead to information regarding the possible premature progression of nodule senescence or recovery.

Chapter 5 – Recovery of nodule function following dark chilling

5.1 Introduction

Environmental triggers that affect plant growth ultimately alter the demand for nutrients such as nitrogen (Ingestad, 1982). The long-term performance and survival of legumes under a changing environment depends on the physiological adaptability of plants, rhizobia and the symbiotic system (Hartwig, 1998). Van Heerden et al. (2008), cited that plants vary greatly in their ability to tolerate low growth temperatures and there is much inter and intra-specific variation within the capacity to maintain optimal metabolism under suboptimal growth temperatures. Nodule longevity is presumed to be plant controlled, much as nodule numbers and the extent of symbiotic root nodule formation through signaling as well as cross talk between metabolite and hormone pathways are controlled (Puppo et al., 2004). Nodule senescence can be triggered prematurely by various types of stress (Gogorcena et al., 1997; Gonzalez et al., 1998; Matamoros et al., 1999). Natural senescence follows progression in an age dependent manner whereas premature senescence is triggered by biochemical or physiological responses due to a stressor. Groten et al. (2006), indicated that nodule senescence is a highly organised process and concur in some ways with leaf senescence. Features of nodule senescence include loss of leghemoglobin and N₂ fixation capacity as well as enhanced proteolytic activities.

Escuredo et al. 1996, characterised the first stage of a stress-induced scenario by a rapid fall in nitrogenase activity and nodule O₂ permeability, where no oxidative damage was observed and the decrease in nitrogenase activity was to some extent reversible. In contrast, the second stage (after 4 days of nitrate exposure) was characterised by an important lowering in antioxidant defenses. The decrease in antioxidant content and the ensuing oxidative stress were not responsible for the initial decline of nitrogenase activity, which was probably due to the limited O₂ supply to the bacteroids as a result of an increased resistance to O₂ diffusion. The close link between nitrogen fixation and respiration makes it critical for stringent O₂ control to maintain optimal conditions for both respiration and nitrogenase activity (Hunt et al., 1987).

A comparison between dark stress-induced and natural senescence by Pfeiffer et al. (1983), revealed a similar response regarding nitrogen fixation, carbohydrate reserves, soluble protein and leghemoglobin. In addition, there was degeneration of nodule tissue caused by dark stress treatment, but this was a reversible process. Reduced nitrogenase activity may be the product of morphological changes in the cortex of the nodules, through the alteration in the proportion of liquid versus gas-filled intercellular spaces or through changes in the size of intercellular spaces (Hartwig, 1998). Studies on lupin and soybean nodules suggested that morphological changes in the cortex were probably associated with occluding glycoproteins in the intercellular spaces of the inner cortex along with a change in the size of intercellular spaces (James et al., 1991; De Lorenzo et al., 1993; Iannetta et al., 1993a,b; Iannetta et al., 1995).

During an experiment where dark stress treatment of the legume species *Medicago truncatula* plants resulted in induced (premature) nodules senescence (Guerra et al., 2010), the bacteroid content condensed, the peribacteroid space increased and individual symbiosomes became fused. In this study, it was also found that the peribacteroid membrane remained intact, even when most bacteroid content had disappeared, which

contrasts strongly to normal developmental senescence during which dissolution of the symbiosome occurred (Guerra et al., 2010). Therefore, membrane dismantling and fatty acid degradation were identified as features of developmental (natural) senescence, in contrast to premature senescence, which is more reminiscent of a general stress response with rapid death of both the microsymbiont and the host cell as a consequence.

The objective of this chapter was to establish whether premature nodule senescence was induced as a result of prior plant exposure to long-term dark chilling and if the response differed depending on SC and WPC treatment. The results obtained in Chapter 3 regarding the progression of natural nodule senescence and alteration in key parameters concerning SNF, provided a baseline against which to identify the occurrence of premature nodule senescence. The previous chapter (Chapter 4) illustrated the effects of dark chilling on these key parameters and results obtained in this chapter will elucidate aspects regarding the recovery of nodule function following dark chilling.

5.2 Results and Discussion

The potential for SNF recovery following a 12 night chilling period was evaluated and it was found that nitrogenase activity of previously dark chilled plants remained at a lower level than in control plants for a two-week period after the chilling stress ended (Figure 5.1). The nitrogenase activity following the long-term exposure period to dark chilling was comparable with results obtained in Chapter 4 (Figure 4.1A). A highly statistically significant ($p = 0.00009$ and $p = 0.001$) inhibition of nitrogenase activity directly after and 1 week following the suspension of chilling, respectively, was only observed in WPC treated plants, indicating the severity of direct chilling effects on the nodules. A noteworthy finding was that following 2 weeks of recovery, both the WPC and SC treated plants showed no difference compared to control nodules. Rapid recovery of nitrogenase activity after partial inhibition induced by increased O_2 concentration has been demonstrated by Hunt et al. (1989) and King et al., 1988. Walsh (1995) provided evidence that individual nodules can adapt anatomically and physiologically to a given stress over a period of days. James et al. (1993) detailed a recovery in nodule structure and function following a two week exposure to 50mM NaCl at low (ca. $300 \mu\text{mol m}^{-2} \text{s}^{-1}$) and high (ca. $600 \mu\text{mol m}^{-2} \text{s}^{-1}$) light intensities. It is worth mentioning that in this investigation nitrogenase activity (Figure 5.1) in previously chilled nodules only reached control levels at a point in time when nodule senescence already commenced in control nodules as indicated by the progressive decline in nitrogenase activity over time which was also comparable to nitrogenase activity (Figure 3.1A) measured without dark chilling stress.

The recovery in nitrogenase activity was further corroborated by the observed changes in ureide content, where a trend of gradual increase from 6 to 9 weeks after sowing in SC and WPC was observed to levels similar to those of the control (Figure 5.2). During the first 2 weeks after chilling ended there was a significant difference between SC and WPC plants reflecting the much more severe effect of WPC treatment on nodule ureide content. Nodule ureide content in all treatments following 10 weeks of sowing was similar to the ureide content of nodules measured after 10 weeks of growth under optimal conditions (Chapter 3 - Figure 3.3). These results support the progressive accumulation of ureides observed in nodules during natural

senescence (Chapter 3) and further demonstrate that dark chilling does not alter this response once recovery has been achieved.

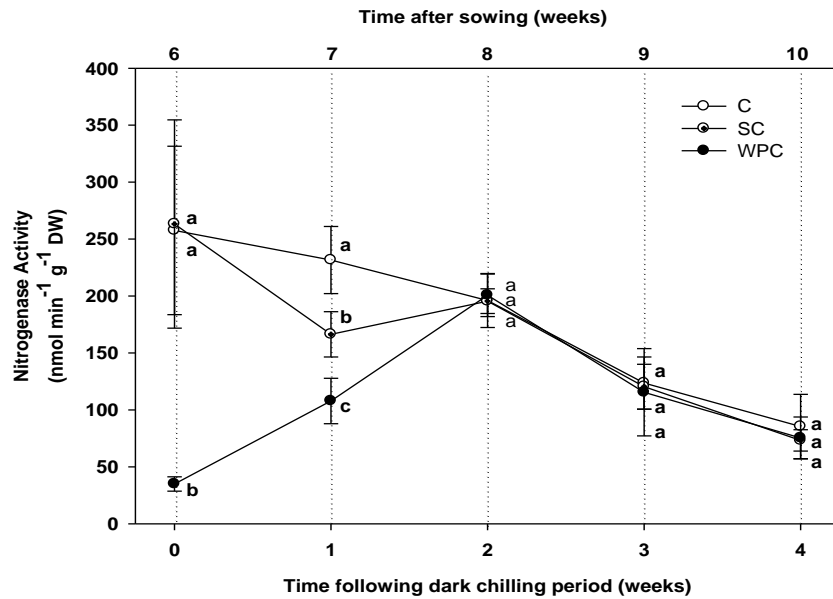


Figure 5.1: Nitrogenase activity (nmol min⁻¹ g⁻¹ DW) monitored following a 12 night chilling period for a further 4 week period under normal growth temperatures. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants. Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

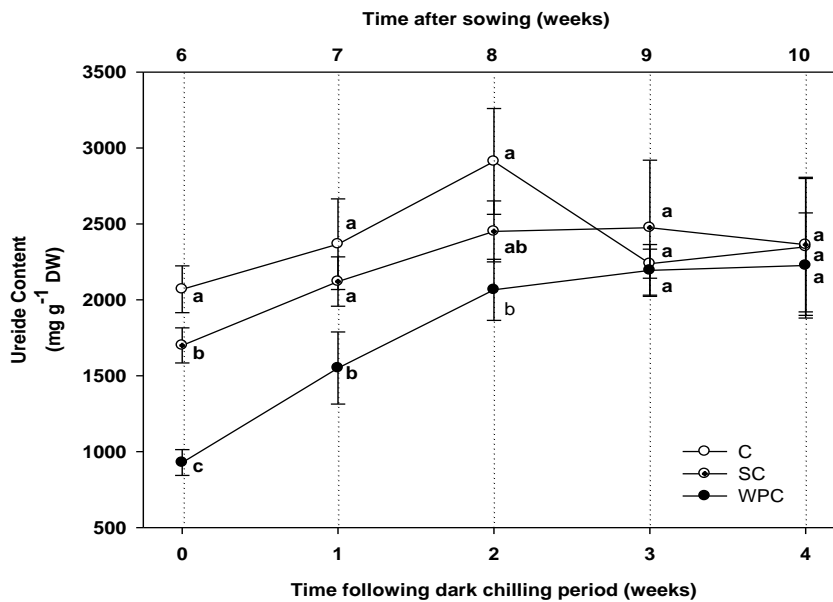


Figure 5.2: Nodule ureide content (mg g⁻¹ DW) monitored following a 12 night chilling period for a further 4 week period under normal growth temperatures. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants. Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

It has been suggested that any reduction of nodule activity will result in a loss of respiratory protection for nitrogenase protein, placing the enzyme at risk of O₂ inactivation. It was noted that only WPC treated plants following a long-term chilling period had repressed respiration rates compared to control plants. Furthermore, a 2 week recovery period was necessary to reach respiration rates comparable to those in control plants (8 weeks after sowing). This increase and recovery of nodule respiration rates coincided with the point in time at which nitrogenase activity in previously WPC treated plants also recovered to control values, further supporting the good correlation between these two parameters reported in chapter 4 (Figure 4.4). As discussed in chapter 4, the SC treatment had little effect on nodule respiration rate and is further supported by the data shown in Figure 5.3. A study on short term dark chilling by Van Heerden et al. (2008) suggested that severely depressed nodule respiration rates, which could have caused elevated O₂ content in the nodules, was the main reason for the deactivation of nitrogenase in PAN809. Denison et al. (1992a) noted that the mechanism of nitrogenase inhibition may differ between transient and prolonged O₂ exposures. Dalton et al. (1998) mentioned that respiratory O₂ consumption could be an important component of the diffusion barrier that operates in conjunction with changes in intercellular spaces that provide for maximum regulation of O₂. Studies by Kuzma and Layzell (1994) determined that unlike other environmental treatments, including phloem deprivation, nodule detachment, nitrate fertilisation or Ar:O₂ exposure, temperature has a different effect on nodule mechanisms to adapt their O₂ status and that O₂ limitation played only a minor role in the regulation of nitrogenase activity during changes in temperature.

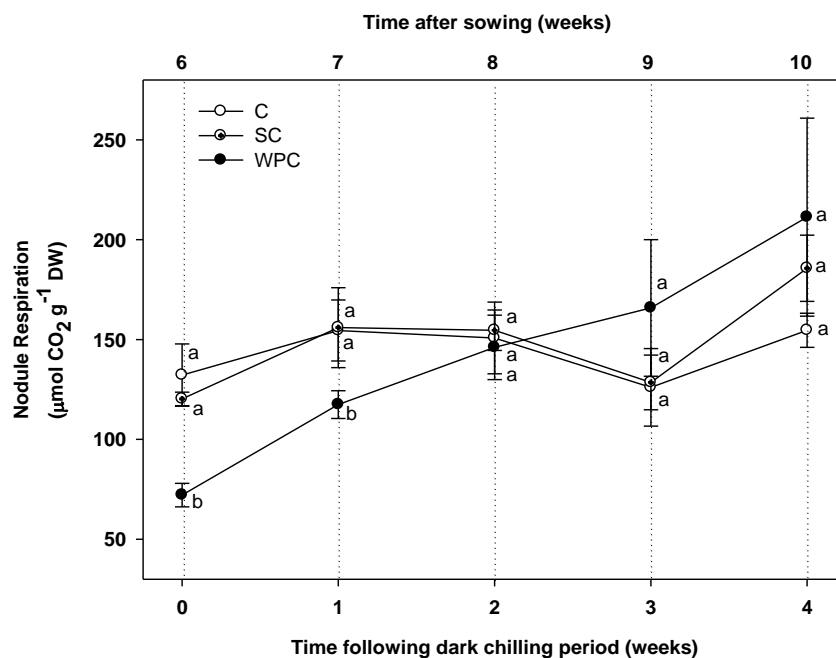


Figure 5.3: Nodule respiration rate ($\mu\text{mol CO}_2 \text{g}^{-1} \text{DW}$) monitored following a 12 night chilling period for a further 4 week period under normal growth temperatures. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants (A). Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error

Nodule metabolism is limited by carbon supply (Lawn and Brun, 1974). Photosynthate from the leaf moves into the phloem primarily as sucrose (Clauss et al., 1964) and reaches the nodule predominately in this form (Reibach and Streeter, 1983) and is then respired by the nodules for SNF. The change in nodule sucrose content following a long-term chilling period is depicted in Figure 5.4. A similar trend was observed in all treatments over the 4 week recovery period, possibly indicating a decline in sucrose content as time progresses without any influence by the previous exposure to WPC and SC treatments.

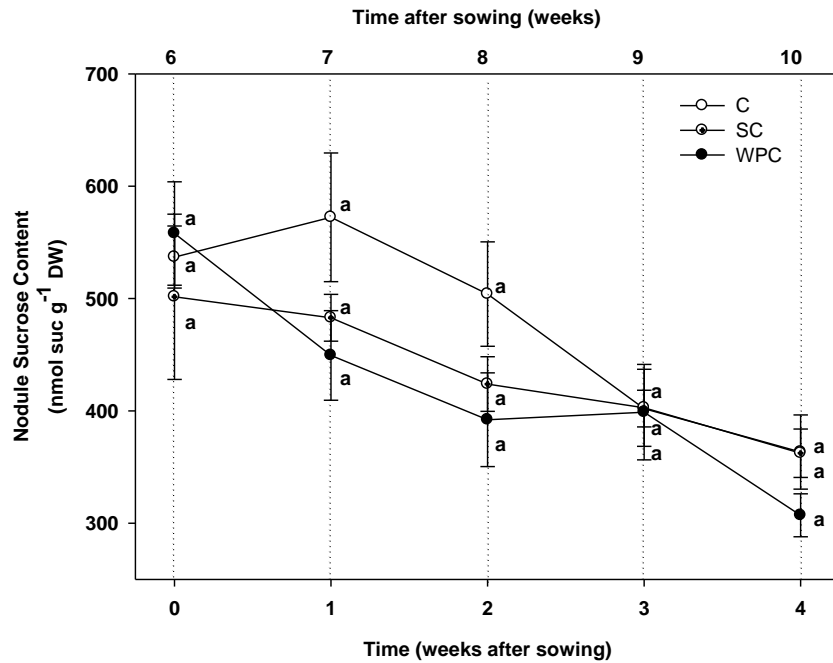


Figure 5.4 Nodule sucrose content (nmol suc g⁻¹ DW) monitored following a 12 night chilling period for a further 4 week period under normal growth temperatures. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants. Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of four replicates with standard error.

The control of O₂ concentration within the nodule is critical for optimum nitrogenase activity and SNF (Hunt and Layzell, 1993). Consequently, the metabolism of legume nodules is thought to be limited by the supply of O₂ at all times. By controlling permeability to O₂ diffusion, nodules are able to reduce further the supply of O₂ to the infected cells and thereby down-regulate metabolism (Hartwig et al., 1987; Vessey et al., 1988; Denison et al., 1992a; de Lima et al., 1994). Since the permeability of O₂ in root nodules is influenced by several factors, which include, alterations in the distribution and size of intercellular air spaces, rapid water movements into or out of these air spaces and the formation of glycoprotein occlusions, the intercellular air space areas after 12 consecutive nights of dark chilling was therefore determined. It was found that intercellular air space areas in all regions of the nodules relative to the control drastically increased (Figure 5.5A, B and C). Following the chilling period the enhanced intercellular spaces in the inner cortex was significantly ($p = 0.02$) different between WPC and SC treatments.

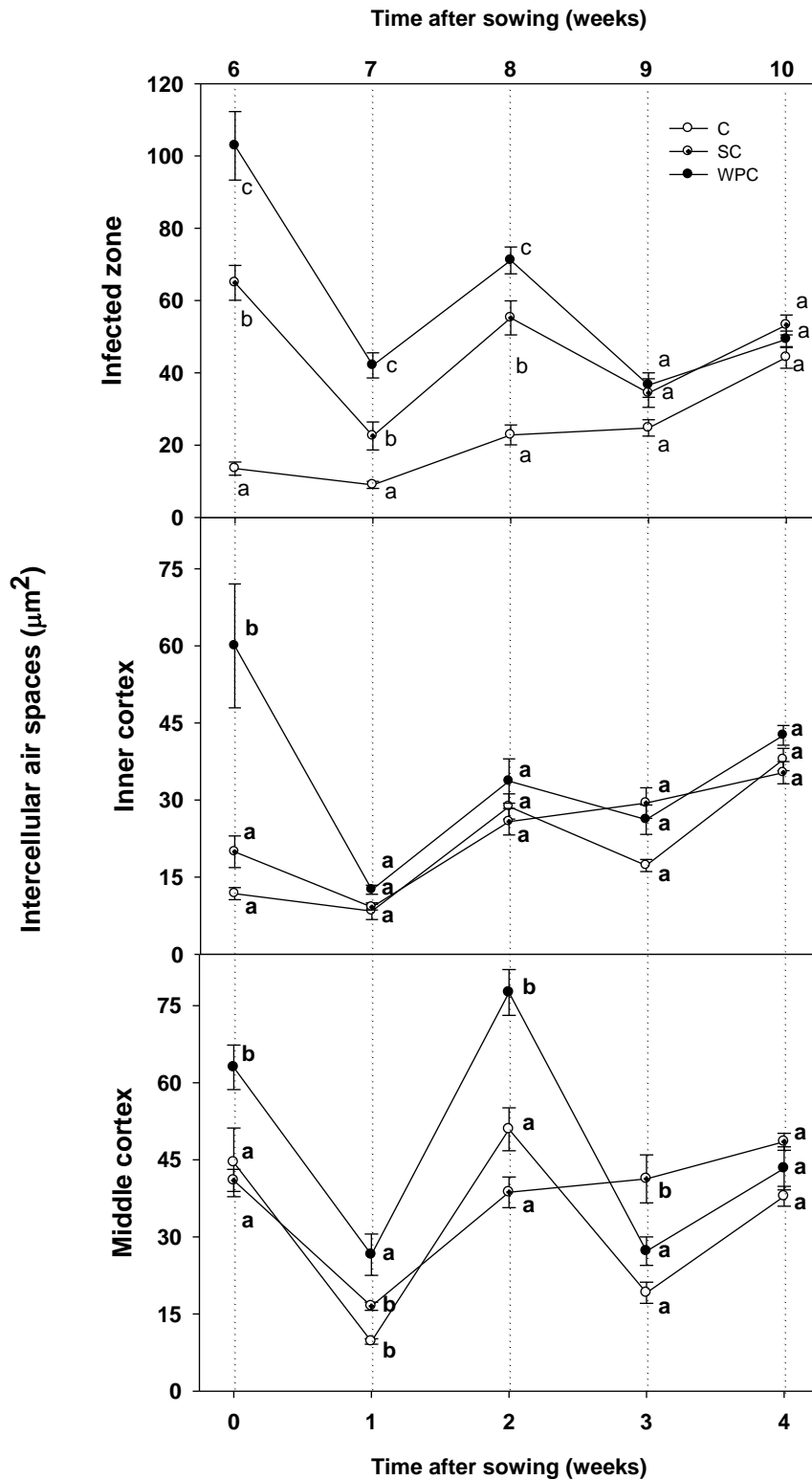


Figure 5.5 Intercellular air space area (μm^2) in the infected zone (IZ), inner cortex (IC) and middle cortex (MC) of nodules monitored following a 12 night chilling period for a further 4 week period under normal growth temperatures. Treatments included control (C), shoot chilled (SC) and whole chilled (WPC) plants. Statistical differences between treatments for each point in time are shown by the use of letters. Each data point represents the average of measurements done on 40 air spaces with standard error.

Due to the severely depressed nodule respiration rates in previously WPC-treated plants, elevated O_2 concentration are expected inside nodule tissues (Van Heerden et al., 2008). Thus larger intercellular air space areas will enhance the diffusion of O_2 inside the nodules, potentially aggravating deactivation of nitrogenase by high O_2 . After one week of recovery, intercellular air space areas partially diminished within nodules of previously dark chilled plants (SC and WPC) and after two weeks of recovery the dark chilled plants (SC and WPC) showed that the intercellular spaces in the inner cortex, where a barrier exists which controls the O_2 permeability (Witty et al., 1986), was similar in area to control plants. The infected zone only showed full recovery 3 weeks after exposure to dark chilling was terminated. An investigation by Van Cauwenberghe et al. (1994) described that a critical step in the regulation of nodule permeability to O_2 may be localised in the infected zone and involve changes in the ratio of the surface area of intercellular space to volume of the infected cell. All regions were fully comparable with the control plants 10 weeks after sowing. The general reduction in intercellular air space areas corresponded with the recovery of nitrogenase activity following dark chilling. Interestingly, no evidence of oxidative damage was observed, for example no membrane damage were seen (Figure 5.6, green and red arrows), even at the hypothesised high O_2 levels during dark chilling. Transmission electron micrographs indicated a different response in the physical change in intercellular air space areas in the middle and inner cortex (MC and IC) as well as the infected zone (IZ) in C and WPC treatments. (Figure 5.6, green and red circles). The distribution of air spaces in the inner and outer cortex was different across the treatments, increasing in area in both regions of the nodule when exposed to WPC treatment. An investigation on cowpea by Dakora and Atkins (1990) and soybean nodules by Parsons and Day, 1990 revealed the development of large intercellular spaces in the inner cortex and a proliferation of lenticels when exposed to low rhizosphere O_2 , while at high O_2 , the inner cortex developed many layers of small, tightly packed cells. Our findings differ from Parsons and Day (1990) indicating a clear difference in stress responses, as dark chilling resulted in an increase in intercellular air space areas. Another disparity with the work of Parsons and Day (1990) was the absence of occluded spaces in either the inner or outer cortex, or infected zone. Occlusion of intercellular spaces occurred in the chilling tolerant soybean genotype, Highveld Top, under WPC treatment but was not observed in PAN809 (Van Heerden et al., 2008), which corresponds with the findings reported in this chapter.

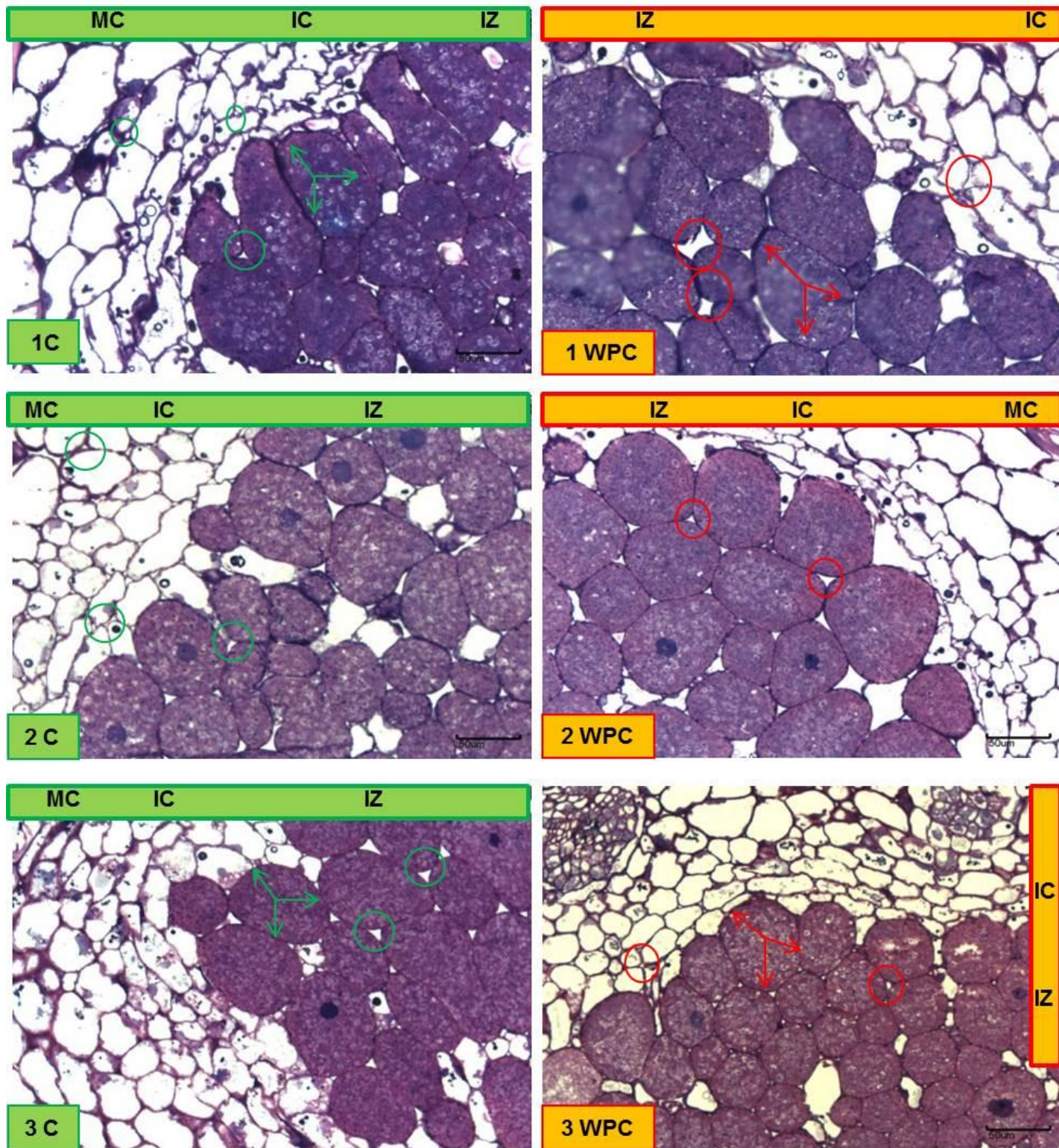


Figure 5.6 Micrographs of cross sections through control (C) and whole plant chilled (WPC) nodules showing the infected zone (IZ), inner cortex (IC) and middle cortex (MC) directly after long-term chilling (1), as well as one week (2) and two weeks (3) following the suspension of chilling. Green (C) and red (WPC) arrows indicate intact membranes and Green (C) and red (WPC) circles indicate individual intercellular air spaces. Scale bars - 50µm.

5.3 Conclusions

Long term dark chilling severely disrupted nitrogenase activity and ureide synthesis in nodules. Furthermore, recovery of nitrogenase activity following dark chilling was slow and only occurred after control nodules started to senesce. The observed recovery in nitrogenase activity in all treatments point towards reversible activation of nitrogenase following long term dark chilling and there was no evidence for induction of premature nodule senescence. A study by Pfeiffer et al. (1983), concluded that stress-induced senescence is reversible and in this study it was also confirmed that there was no stress-induced senescence, rather severe inhibition by dark chilling stress followed by gradual, but complete recovery when plants were returned to normal growth conditions. The increase in ureide content that corresponded with the increase in nitrogenase activity speaks to the functioning of mechanisms involved with SNF after the suspension of dark chilling. Additionally, the increase in nodule respiration of WPC plants indicated the re-establishment of energy production necessary for SNF. An increase in intercellular air space volume was induced by long term dark chilling in nodules, specifically by the direct chilling of nodules (WPC treatment). The delayed diminishment of intercellular air spaces volume back to control levels following dark chilling, may be an important factor involved in the lag period required for recovery of nitrogenase activity.

Remarkably, there was also no indication of oxidative stress, which was surprising considering the increased O₂ levels which could be expected due to chill-induced inhibition of nodule respiration rates. Some studies have reported that the operation of the diffusion barrier in soybean nodules during water stress or temperature change appear to be affected via changes in respiration rates (Diaz del Castillo et al., 1994; Kuzma and Layzell, 1994; Diaz del Castillo and Layzell, 1995; Kuzma et al., 1995; Purcell and Sinclair, 1995). Our findings revealed that clearly, dark chilling did not close the diffusion barrier but instead opened it due to an increase in air space areas in all regions of the nodule.

Chapter 6 – General discussion and recommendations

The main aims of this study were, firstly, to characterise the natural senescence process related to SNF in root nodules of the chilling sensitive soybean genotype PAN809 under optimal growing conditions; secondly, to characterise the alteration of the key physiological/biochemical processes of SNF during a long-term dark chilling period in this genotype; thirdly, to establish whether recovery in nodule functionality following dark chilling occurs or whether premature nodule senescence is triggered; and finally, to identify sensitive biochemical markers of premature nodule senescence.

It was hypothesised that dark chilling would cause alterations in key physiological/biochemical processes of SNF, and that these alterations would then trigger the onset of premature nodule senescence. The treatments, designed to allow either exposure of only the shoots (SC treatment) or the whole plant (WPC treatment) to dark chilling, clarified whether shoot-derived or whole plant-derived responses to dark chilling were responsible for alterations in SNF and the possible triggering of premature nodule senescence. The nature and sequence of events underpinning nodule senescence has not yet been characterised in soybean and this knowledge is required for a better understanding of whole-plant response to dark chilling. Strauss et al. (2006) and Van Heerden et al. (2008) found that the chlorotic phenotype, resulting from N-limitation caused by inhibition of SNF, contributed significantly to the pronounced dark chilling sensitivity of the soybean genotype PAN809. For this reason PAN809 was selected for this study, since any effects of dark chilling on nodule function would be highlighted by its innate chilling sensitivity.

Characterising the natural senescence of root nodules was achieved by monitoring the change in key physiological/biochemical processes involved in SNF. This gave insight into normal deviations that occur during the lifespan of soybean nodules. The exclusion of chilling stress for this particular phase of the study was pertinent to establish a time-dependent baseline for the key physiological/biochemical processes involved in SNF. Based on this evaluation it was concluded that nitrogenase activity (Figure 3.2) decrease gradually from a point of maximum activity 6 weeks after sowing. The decline in nitrogenase activity was accompanied by an increase in nodule ureide content (Figure 3.3), whilst sucrose synthase activity (Figure 3.6) and nodule respiration rates (Figure 3.7) were maintained, which pointed towards stability of carbon dependent metabolic processes for SNF throughout the experimental period. The accumulation of ureides during natural nodule senescence might constitute a feedback inhibition trigger as indicated by the negative correlation that was observed between nitrogenase activity and nodule ureide content (Figure 3.4).

The alteration in key parameters of SNF when dark chilling was applied was scrutinised by either induced dark chilling, specific as either only, shoot chilling (SC) or whole plant chilling (WPC). Realistically, cold nights rarely result in direct chilling of roots and nodules, rather, mostly only shoots will be affected. However, in some areas around the world, South Africa included, dark chilling may result in cooling of soil and subsequently root chilling under certain conditions. The monitoring throughout a long term chilling period gave insight into specific alterations and initiations of stress response in soybean nodules. A direct effect of dark chilling on nitrogenase activity (Figure 4.1A) and nodule respiration rate (Figure 4.3), which led to a

decline in nodule ureide content (Figure 4.2), was observed. The inhibition of nitrogenase activity occurred in the absence of any restrictions on carbon flux (i.e. stable sucrose synthase activity and sucrose content – Figure 4.6 and 4.7), which would have otherwise inhibited nitrogenase activity indirectly through limitations on nitrogenase-linked respiration. Nitrogenase activity, ureide content and nodule respiration rate was affected much less by SC than WPC treatment. However, the minor inhibition of SNF that did occur under SC conditions points towards some involvement by currently unknown shoot-derived factors. The depressed rates of respiration due to direct effects of low soil temperature might have led to elevated O₂ concentrations in the nodules thereby inhibiting nitrogenase, which in turn would have resulted in the observed decrease in nodule ureide content. These alterations in key parameters involved in SNF due to dark chilling, might have triggered premature nodule senescence as less than ideal conditions prevailed during the chilling period, in particular in the WPC plants.

Finally, defining whether premature nodule senescence was induced as a result of plant exposure to long-term dark chilling and if the response differed depending on SC and WPC treatment gave insight into the recovery process of SNF. The results obtained in Chapter 3 provided a baseline against which to identify the incidence of premature nodule senescence. It was established that long term dark chilling severely disrupted nitrogenase activity and ureide synthesis in nodules. In addition, recovery of nitrogenase activity following dark chilling was slow and occurred only after control nodules already started to senesce. However, the observed recovery in nitrogenase activity in all treatments point towards reversible activation of nitrogenase following long term dark chilling and in addition, there was no evidence for induction of premature nodule senescence. The increase in ureide content in previously chilled nodules matched the recovery of nitrogenase activity after the suspension of dark chilling. Furthermore, the increase in nodule respiration of WPC plants indicated the re-establishment of energy production necessary for SNF. An increase in intercellular air space area was induced by long term dark chilling in nodules, specifically by the direct chilling of nodules (WPC treatment). The delayed diminishment of intercellular air spaces area back to control levels following dark chilling might have been an important factor involved in the lag period required for recovery of nitrogenase activity, because enlarged air spaces would have favoured gaseous diffusion in an elevated O₂ environment (due to suppressed nodule respiration rates). These findings revealed that dark chilling did not close the diffusion barrier, as in the case of drought and other stress factors, but instead opened it due to an increase in air space areas in all regions of the nodule. There were no signs of oxidative stress, which was unexpected considering the likely elevated O₂ environment.

The novelty of this study was the discovery that dark chilling did not trigger premature nodule senescence and that SNF proved to be resilient, with full recovery possible, following even a prolonged dark chilling period involving low soil temperatures. Apart from the crown nodules investigated in this study, younger nodules situated deeper in the soil would not have been exposed to chilling under field conditions and would therefore still function and supply N to the plant.

Considering that the inhibition of SNF was much less affected by SC than WPC treatment and that the inhibition was fully reversible under both scenarios, it is concluded that physiological processes in the shoots, rather than SNF in the nodules, should be targeted by breeding programs directed at improving chilling tolerance in soybean. The mechanisms involved in the dark chilling-induced inhibition of photosynthesis in soybean were elucidated in detail by Strauss et al. (2006, 2007) and Strauss and Van Heerden (2011). These previous studies found reduced CO₂ assimilation capacity, inhibition of photosystem II function and lower chloroplast fructose-1,6-bisphosphatase (cFBPase) and sucrose-phosphate-synthase (SPS) activity. When nodules experienced direct chilling, additional restraints steadily developed. These previous studies indicated that cFBPase was specifically targeted during dark chilling resulting in severe inhibition of CO₂ assimilation capacity. This supports the recommendation to focus on enhancing the photosynthetic apparatus in achieving better dark chilling tolerance in soybean genotypes. A possible approach to improve the resilience of photosynthetic carbon metabolism in the shoots of soybean plants could be to focus on the over-expression of cFBPase or expression of protease inhibitors aimed at suppressing the proteases involved in cFBPase degradation during dark chilling stress.

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