THE IMPACT OF DARK CHILLING FOLLOWED BY EXPOSURE TO HIGH LIGHT INTENSITIES ON ULTRASTRUCTURE AND SELECTED BIOCHEMICAL REACTIONS OF PHOTOSYNTHESIS IN *GLYCINE MAX* (L.) MERRILL.

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"SOLI DEO GLORIA"

Dedicated to my parents
Thank you for being such good role models to me

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ABBREVIATIONS

A	CO_2 assimilation rate at ambient CO_2 concentration
A_{max}	Maximum CO_2 assimilation rate at saturating CO_2 concentration
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BNF	Biological nitrogen fixation
c_a	Atmospheric CO_2 concentration
CE	Carboxylation efficiency
c_i	Intercellular CO_2 concentration
CI	Chilling injury
CR	Chilling resistant
CS	Chilling sensitive
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N,N,N,N'-tetraacetic acid
FBP	Fructose-1,6-bisphosphate
FBPase	Stromal fructose-1,6-bisphosphatase
F6P	Fructose-6-phosphate
FK	'Fiskeby V'
g_s	Stomatal conductance
G6P	Glucose-6-phosphate
Hepes	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
MA	'Maple Arrow'
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
LSU	Large subunit of Rubisco
ℓ	Relative stomatal limitation of photosynthesis
NADP-MDH	NADP-dependent malate dehydrogenase
OAA	Oxaloacetate
PCR	Photosynthetic carbon reduction
P_i	Inorganic phosphate

PMSF	Phenylmethylsulfonyl fluoride
PS I	Photosystem I
PS II	Photosystem II
Γ	CO_2 compensation concentration
ROI	Reactive oxygen intermediate
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
RZT	Root zone temperature
SBPase	Sedoheptulose-1,7-bisphosphatase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPS	Sucrose-phosphate-synthase
SSU	Small subunit of Rubisco
Tris	Tris(hydroxymethyl)-aminomethan
TBS	Tris buffered saline
TBST	Tris buffered saline- Tween
TCA	Tricarboxylic acid

PREFACE

This study constitutes an analysis of the impact of dark chilling followed by high light intensity on the physiological, biochemical and ultrastructural response of *Glycine max* (L.) Merrill (soybean).

I praise and thank God for providing me with the ability, faith and opportunity to do this study.

To all those who have given advice, showed interest, encouraged or in some or other way contributed in the completion of this thesis, I give my sincere thanks.

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I hereby declare that this study presented for the degree Magister Scientiae at the Potchefstroom University for Christian Higher Education, consists exclusively of my own original research and has not previously been presented for a degree at any other university.

SUMMARY

THE IMPACT OF DARK CHILLING FOLLOWED BY EXPOSURE TO HIGH LIGHT INTENSITIES ON ULTRASTRUCTURE AND SELECTED BIOCHEMICAL REACTIONS OF PHOTOSYNTHESIS IN *GLYCINE MAX* (L.) MERR.

The impact of dark chilling on ultrastructure and key reactions of photosynthesis was assessed in two soybean genotypes, 'Maple Arrow' and 'Fiskeby V'. Both these genotypes are regarded as chilling tolerant (albeit differentially tolerant). These genotypes were subjected to three nights of dark chilling followed by exposure to high light intensities. Half of the plants were subjected to whole plant chilling, while the other half were only shoot-chilled. The shoot-chilled plants were grown in the presence/absence of nitrate supplementation. The aim of this study was to increase the current understanding of the physiological, biochemical and ultrastructural basis for the limitation of photosynthesis by dark chilling in soybean genotypes. The photosynthetic response to dark chilling in the presence or absence of root chilling was also compared and the ameliorating effect of nitrate treatment on photosynthetic limitation during dark chilling was evaluated.

Non-intrusive analysis conducted to assess the physiological and biochemical impact of dark chilling included photosynthetic gas exchange measurements. Intrusive analysis included ultrastructural and anatomical studies, measurement of the activities of key enzymes involved in photosynthesis and Western Blot analysis.

The results demonstrated the existence of genotypic differences in the response of photosynthesis in two chilling tolerant soybean genotypes to dark chilling. Photosynthesis was inhibited to a lesser extent in 'Maple Arrow' than in 'Fiskeby V'. It appeared that not only mesophyll limitation, but also stomatal limitation, was much more evident in 'Fiskeby V'.

Novel evidence is provided for the existence of an inverse relationship between loss of FBPase activity and loss of photosynthetic capacity in the two chilling tolerant soybean

genotypes. The results presented suggest that 'Maple Arrow' is capable of "sensing" dark chilling stress much better than 'Fiskeby V'.

No severe ultrastructural disruption was visible in the plants investigated. The changes observed in ultrastructure corroborate the changes in photosynthetic capacity. No genotypic differences in leaf anatomy could be seen in untreated plants.

The comparative investigation regarding the effects of dark chilling, in the presence or absence of root chilling, on the photosynthetic response in 'Maple Arrow' and 'Fiskeby V' confirmed that whole-plant dark chilling caused a greater decrease in photosynthetic capacity than shoot chilling alone. In the absence of root chilling it was not possible to evaluate the ameliorating effect of nitrogen on the inhibition of photosynthesis by dark chilling because photosynthesis was not inhibited in the two genotypes under these conditions. Although dark chilling of only the shoots did not induce a significant decrease in photosynthesis, the trend did suggest that 'Maple Arrow' was more responsive to the change in temperature.

In conclusion, the results presented strongly suggest a more dark chilling tolerant physiological and biochemical make-up in 'Maple Arrow' compared to 'Fiskeby V'. However, this effect was only visible during whole plant chilling. Genotypic differences in the effectiveness of the host – Bradyrhizobium interaction could have contributed towards the chilling sensitivity of 'Fiskeby V'. When roots remained at higher temperatures during chilling, the differential chilling sensitivity between the genotypes were not revealed, suggesting that 'Fiskeby V' is more sensitive to root chilling than 'Maple Arrow'.

KEY WORDS: Chilling stress; chilling tolerance; dark chilling; Fructose-1,6-bisphosphatase; *Glycine max* (L.) Merr.; photosynthetic gas exchange; NADP-dependent malate dehydrogenase; Ribulose-1,5-bisphosphate carboxylase/oxygenase; Sedoheptulose-1,7-bisphosphatase; ultrastructure.

OPSOMMING

DIE EFFEK VAN LAE NAGTEMPERATUUR EN DAAROPVOLGENDE BLOOTSTELLING AAN HOË LIGINTENSITEIT OP ULTRASTRUKTUUR EN GESELEKTEERDE BIOCHEMIESE REAKSIES VAN FOTOSINTESE IN *GLYCINE MAX* (L.) MERR.

Die invloed van lae nagtemperatuur op ultrastruktuur en sleutelreaksies van fotosintese is in twee sojaboongenotipes, 'Maple Arrow' en 'Fiskeby V', ondersoek. Beide die genotipes word as kouetolerant (alhoewel differensieel tolerant), beskou. Die genotipes is aan drie nagte van kouebehandeling, gevolg deur hoë ligintensiteit, blootgestel. Helfte van die plante is aan heelplant-verkoeling blootgestel, terwyl die ander helfte slegs bogronds verkoel is. Genoduleerde, bogrond-verkoelde sojaboonplante is in die teenwoordigheid of afwesigheid van nitraattoevoeging gegroei.

Die doel van die studie was om die huidige begrip van die fisiologiese, biochemiese en ultrastrukturele basis van die beperking van fotosintese deur lae nagtemperatuur te verbeter. Die reaksie van fotosintese op lae nagtemperatuur in die teenwoordigheid of afwesigheid van wortelverkoeling is vergelyk. Verder is die moontlike vermindering van die negatiewe effekte van lae nagtemperatuur deur nitraattoevoeging geëvalueer.

Nie-destruktiewe metings, wat fotosintetiese gaswisselingsanalise ingesluit het, is uitgevoer om the fisiologiese en biochemiese impak van lae nagtemperatuur te bepaal. Destruktiewe analises het ultrastrukturele en anatomiese studies, bepaling van die aktiwiteit van sleutelensieme betrokke by fotosintese en 'Western Blot' analise ingesluit.

Resultate het aangetoon dat daar genotipiese verskille in die reaksie van fotosintese na kouebehandeling in die twee genotipes voorgekom het. Fotosintese was minder geaffekteer in 'Maple Arrow' as in die geval van 'Fiskeby V', en dit het verder geblyk dat nie net mesofilbeperking nie, maar ook stomatale beperking in 'Fiskeby V' oorheers het.

Die studie het nuwe bewys gelewer vir die bestaan van 'n omgekeerde verband tussen die afname in FBPase aktiwiteit en die afname in fotosintetiese kapasiteit in die twee kouetolerante genotipes. Die resultate dui daarop dat 'Maple Arrow' 'n beter vermoë as 'Fiskeby V' openbaar om kouestres waar te neem.

Geen ernstige ultrastrukturele skade is in die genotipes waargeneem nie. Die ultrastrukturele veranderinge wat wel voorgekom het, ondersteun die veranderinge in fotosintetiese kapasiteit. Verder is geen genotipiese verskille in blaaranatomie in kontroleplante van die twee genotipes waargeneem nie.

Die vergelykende ondersoek rakende die invloed van lae nagtemperatuur, in die teenwoordigheid of afwesigheid van wortelverkoeling, het bevestig dat heelplant-verkoeling 'n groter afname in die fotosintetiese kapasiteit veroorsaak het as slegs bogrond-verkoeling. Dit was nie moontlik om die effek van stikstof op die inhibisie van fotosintese gedurende kouestres in die afwesigheid van wortelverkoeling te evalueer nie, omdat fotosintese nie in die twee genotypes onder hierdie omstandighede geïnhibeer was nie. Geen gevolgtrekking kon gemaak word t.o.v. die verwantskap tussen kouetoleransie en nitraattoevoeging nie. Alhoewel bogrond-verkoeling nie 'n afname in fotosintesetempo veroorsaak het nie, is bevind dat 'Maple Arrow' beter gereageer het op veranderinge in temperatuur.

'n Meer kouetolerante fisiologiese en biochemiese samestelling is in 'Maple Arrow', in vergelyking met 'Fiskeby V', aangetoon Die effek was egter net sigbaar tydens heelplant-verkoeling. Tydens bogrond-verkoeling is die verskil in sensitiwiteit tussen die genotipes opgehef. Dit dui daarop dat 'Fiskeby V' meer sensitief vir wortelverkoeling is as 'Maple Arrow'. Genotipiese verskille in simbiotiese assosiasie tussen die twee genotipes en die entstofras kon bygedra het tot 'Fiskeby V' se groter kouesensitiwiteit.

SLEUTELTERME: fotosintetiese gaswisseling; Fruktose-1,6-bisfosfatase; *Glycine max* (L.) Merr.; kouestres; kouetoleransie; lae nagtemperatuur; NADP-afhanklike malaat

dehidrogenase; Ribulose-1,5-bisfosfaat karboksilase/oksigenase; Sedoheptulose-1,7-bisfosfatase; ultrastruktuur.

1. Literature review

1.1 The economic importance of soybean

Soybean (*Glycine max* [L.] Merr.) combines in one crop both the dominant world supply of edible vegetable oil and high-protein feed supplements for livestock (Keyser & Li 1992). In addition, soybean has substantial economic importance in a wide range of industrial, food, pharmaceutical and agricultural products (Smith & Huyser 1987). Some of these products include bread, margarine, food drinks, antibiotics, livestock feed, adhesives, textiles, soap, paper, fertiliser, paint and many others (Scott & Aldrich 1983). The approximate nutritional composition of soybean seed is 40% protein, 21% oil and 34% carbohydrate (Scott & Aldrich 1983). In countries with rapidly increasing populations, soybean is viewed as a crop that enhances nutritional value of the local diets, and alleviates national shortages of vegetable oil (Hume *et al.* 1985).

1.2 The sensitivity of soybean to low night temperature (dark chilling)

One of the most important factors that limits the distribution and productivity of important agricultural crops is dark chilling (Allen & Ort 2001). Soybean metabolism, growth, development and yield are affected by chilling temperatures of about 15°C or less (Holmberg 1973; Schmid & Keller 1980; Hume & Jackson 1981; Musser *et al.* 1983, 1984; Seddigh & Jolliff 1984; Cabané *et al.* 1992; Gass *et al.* 1996). Photosynthetic metabolism is among the most chilling-sensitive processes in these plants and thus limits the geographical range and contributes to the annual variation in yield (Boyer 1982). Dark chilling affects a wide range of physiological processes including photosynthesis (Gourdon & Planchon 1982; Purcell *et al.* 1987; Caulfield & Bunce 1988; Van Heerden & Krüger 2000; Van Heerden *et al.* 2002, 2003) and symbiotic nitrogen fixation (Walsh & Layzell 1986; Lynch & Smith 1993).

According to Smit (1998), the demand for soybean oil cake in South Africa exceeds the local supply, which creates encouragement for soybean production by South African farmers. As a result, soybean production in South Africa increased from 10 000 ton to more than 100 000 ton during the last 20-30 years (Smit 1998). Due to the high altitude of the areas of soybean production in South Africa, the night temperatures can be very low. The low night temperatures that are often encountered in temperate climates and in

high altitude regions limit the agricultural distribution of soybean (Van Heerden *et al.* 2003). The potential to extend the cultivation of soybean and many other crops into these areas is limited by their chilling sensitivity (Cabané *et al.* 1992). Selection pressures to cope with low temperature do not exist in chilling sensitive plant species, especially those from tropical or sub-tropical origin (Tucker & Ort 2002). Besides the direct effects of chilling on plant metabolism, chilling can also impair water uptake and transport in plants under certain conditions, thereby inducing physiological drought stress (Crookston *et al.* 1974). Sustainable agricultural systems will have to intensify yield per unit area, while often facing adverse environmental conditions (Larson 1986). Under South African field conditions, a combination of drought and chilling are often encountered simultaneously.

Sustainable agriculture is the only solution to feed an ever-increasing population in an environment that is constantly being degraded by human interference (Hume *et al.* 1985). The vitality of each organism in the ecosystem strongly depends on its adaptability to certain environmental conditions (Srivastava & Strasser 1999).

Relatively little is known about the molecular and metabolic mechanisms that contribute to the chilling sensitivity of soybean (Van Heerden *et al.* 2003). Information concerning the physiological and biochemical mechanisms of chilling and drought tolerance in soybean is of utmost importance in ensuring that production meets the growing demand for soybean in South Africa (and Africa). Genetic manipulation, with the aim to increase soybean production, is dependent on a better understanding of the physiological and biochemical mechanisms of chilling response and tolerance (Shabala & Newman 1997). The identification and development of soybean genotypes with greater tolerance against unfavorable environmental conditions offer considerable promises for higher soybean production in South Africa by supplying important information in the long term goal of agriculture. The lack of rapid and accurate selection methods is often limiting in breeding programs aimed at increasing chilling tolerance, with available methods often being subjective and unpractical. Techniques must be developed which supply fast, accurate and quantitative information with regard to the injury caused by chilling (Shabala & Newman 1997). The identification of chilling tolerant genotypes is important

to ensure that soybean production in South Africa satisfy the growing demand. Genetic engineering may supply solutions to the problem of establishing sustainable food production by producing more stress tolerant crop plants. Looking to the future, it seems that some environmental stresses will inevitably intensify, and that these, coupled with practices such as using land marginally suited for agriculture and cultivating crop plants in climates for which the plants are ill-adapted, are likely to create an increasing need for new stress-tolerant genotypes (Van Rensburg & Krüger 1993).

1.3 The stress concept

Stress is defined as an external factor that has a negative influence on a plant (Taiz & Zeiger 1991). The literal meaning of the word "stress" (derived from the Latin *stringere*) is a constraining or impelling force (Larcher 1995). But what is stress? Larcher (1995) defined stress as a significant deviation from the optimal conditions of life that elicits changes and responses at all functional levels of the organism, which, although at first reversible, may become permanent. Even if the stressful event is temporary, the vitality of the plant is progressively reduced the longer the stress is maintained. From a botanical viewpoint, stress can be described as a state where increasing demands on the plant, can lead to initial destabilisation of functions (elastic changes), followed by adaptation and increase in resistance. If the resistance barrier and adaptation capacity is exceeded, permanent damage (plastic changes) or death occurs. Stress thus contains constructive and destructive elements and can be regarded as a selection factor as well as a driving force for improving resistance and adaptation.

According to Lichtenthaler (1988), plants have optimum conditions where growth and development as well as individual metabolic processes such as photosynthesis take place. The optimum conditions for plant growth and cell metabolism are influenced by a variety of environmental factors, as well as endogenous factors that differ between plants and are dependent of the habitat and genetic make-up of the plant. For example, plants growing in temperate environments have a much lower temperature optimum for photosynthesis and respiration than plants growing in tropical environments (Lichtenthaler 1988).

According to Lichtenthaler (1988), a distinction must be made between moderate and intense stress effects within plants. Moderate stress may activate cell metabolism and increase the physiological activity of the plant, but have no harmful effect even over the long term. These moderate stress effects are beneficial to the plant and are known as "Eu-stress". Inhibitors of plant growth and cell metabolism are examples of this. At low concentrations these inhibitors have a beneficial effect, which is positive and stimulating to the plant. At higher concentrations, these inhibitors (eg. herbicides) can suppress plant metabolism including photosynthesis and growth processes. Under these conditions the physiological activity of the plant decreases, which lead to accelerated senescence and death. These harmful and inhibiting stress conditions can be viewed as "Di-stress". The transition between "Eu-stress" and "Di-stress" may be sequential.

Larcher (1987) distinguished between the following four phases of stress:

i) ***Reactional phase:***

Also known as the alarm reaction when stress commences and is characterised by:

-Deviation from the functional norm

-Decline in vitality

-Catabolic processes exceed the anabolic processes

ii) ***Restitutional phase (sustained stress):***

The stress resistance of the plant increases and is characterised by:

-Adaptation

-Convalescent (recovery) processes

-Hardening (reactivation).

iii) ***End phase (long-term stress):***

It is a state of overstraining after long-term stress, where the stress intensity is too high and leads to overcharging of the adaptational ability of the plant

iv) ***Regenerative phase:***

Partial or full recovery of the physiological function of the plant when the stress situation is removed

The stress concept can be described in terms of the thermodynamics of the plant (Strasser 1988). According to this, three concepts can be described:

i) Stress: A state in which a plant occurs as a result of an applied force (e.g. chilling);

ii) Strain: The reaction of the plant to stress. It can thus be seen as the expression of stress, before damage occurs (e.g. reduced photosynthesis);

iii) Damage: The result of stress that exceeds the boundary of elasticity, and for which the plant cannot compensate.

On a daily basis, plants are exposed to a variety of environmental conditions, where internal changes must take place to reach a state of adaptation to the new set of environmental conditions. Each external change in the environment can thus be seen as a stressful occurrence, in which it leads to a deviation of the non-stress situation, independent of the ability of the plant to adapt. When a thermodynamic optimum state is reached, the plant is viewed as a system in harmony with its environment. Stress can be seen as changes in environmental factors, which lead to a sub-optimal state and force adaptation of the plant (Strasser 1988). According to Strasser (1988) and Tsimilli-Michael *et al.* (1996), plant systems undergo continuous stress-adaptation processes in search of harmony with the environment.

1.4 Chilling injury

A large number of crop species from tropical origin begin to show deleterious responses at air temperatures below 20°C (Musser *et al.* 1984). It is referred to as 'chilling injury' (CI) if there is physiological and structural damage (George & Lyons 1979; Lyons *et al.* 1979; Levitt 1980). According to Parkin *et al.* (1988), CI is a physiological defect of plants and their products that results in reduced quality and loss of product utilisation following exposure to low but non-freezing temperatures.

Although the nature of CI has been studied since the turn of the previous century, and hypotheses have been advanced to explain its cause, no general agreement on the cause or nature of CI or the primary events triggering it, exists (Raison & Lyons 1986; Parkin *et*

al. 1988). Most authors conclude that the process of CI should be considered in two stages, first a primary stage, occurring instantaneously at some threshold temperature, followed by a secondary stage, which is time and temperature dependent and lead to visible symptoms of CI (Raison & Lyons 1986).

Chilling temperatures are one of the most important environmental factors that limit the productivity and geographical distribution of plants in large areas of the world, including important agricultural crops (Kömer & Larcher 1988; Allan & Ort 2001). The survival capacity of a plant species or genotype is determined by the extent to which its metabolic processes continue to function during chilling stress and by its chilling tolerance (Kömer & Larcher 1988). Certain stages in the life cycle of a plant are more sensitive to chilling than other stages of development (Nishida & Murata 1996). Generally, adult plants are more resistant than juveniles and seedlings, with dormancy generally representing the most tolerant stage (Lyons 1973; Levitt 1980).

In the case of soybean, metabolism, growth, development and yield are affected by chilling temperatures of 15°C or lower (Holmberg 1973; Schmid & Keller 1980; Hume & Jackson 1981; Musser *et al.* 1983, 1984; Seddigh & Joliff 1984). Short term chilling for several days also affects soybean metabolism, but the effects are often reversible after return to normal growth conditions (Musser *et al.* 1983, 1984).

Typically, CI can be identified at multiple sites of metabolism (Öquist & Martin 1986). The difficulty in defining the biochemical events leading to CI can be attributed to the variety of symptoms and responses that is induced (Parkin *et al.* 1988). Although many workers have tried to identify the mechanism of CI, the diversity of tissue responses to this defect provides the rationale to suggest that no universal mechanism exists (Parkin *et al.* 1988), and several hypotheses have been advanced to account for the nature of CI.

Lyons (1973) provided the first contemporary and cohesive theory regarding the nature of CI in plants. The basis of this theory was that membrane lipid phase transitions occurred at a certain critical temperature, resulting in the formation of gel phase lipids. This leads

to increased permeability or leakiness of cellular membranes, bringing about loss of regulatory control. Ultimately, an irreversible metabolic imbalance arose, leading to physiological dysfunction and death. Much of the early evidence in support of this theory came from studies on plant mitochondria. Lyons *et al.* (1964) speculated about the relationship between the physical nature of cellular membranes and chilling sensitivity in plants after detecting a greater degree of lipid unsaturation in mitochondrial membranes of chilling resistant (CR) compared to chilling sensitive (CS) plant species. The need to correlate perturbations in biochemical function with those occurring at the biophysical level in membranes was apparently satisfied by further studies. Spin labeling studies of mitochondrial membranes of CS plants detected the presence of "phase changes" taking place at low, nonfreezing temperatures (Lyons *et al.* 1979).

Mitochondria are not the only organelles alleged to be sensitive to chilling-induced dysfunction. Arrhenius discontinuities were also observed for $NADP^+$ reduction at approximately 12°C in chloroplasts isolated from CS tomato and bean leaves, whereas none were found for these organelles from CR lettuce and pea leaves for a range of temperatures between 0 and 25°C (Shneyour *et al.* 1973).

According to Parkin *et al.* (1988), most investigations support the idea of lipid phase changes in cellular membranes as being a primary response to chilling, disposing the susceptible tissue to the development of secondary disorders. There is good evidence that the phase transition hypothesis, in its original form, is appropriate to explain chilling-induced dysfunctions in thermophilic cyanobacteria (Murata 1989), but it has been seriously questioned for higher plants (Martin 1987). Currently, the general consensus seems to be that CI in CS plants does not involve phase transition of bulk membrane lipids, although there is still a distinct possibility that some minor lipid fractions do undergo transitions at chilling temperatures (Quinn 1988). According to Parkin *et al.* (1988), loss of membrane integrity can lead to reduced regulatory control of cellular energy generation, membrane semi-permeability, and metabolite and ion compartmentation. Physical perturbations in membranes can influence the activity of enzymes as well. Due to the important role of calcium as a secondary messenger for

several processes, loss of membrane semi-permeability, and the consequent inability to maintain calcium gradients, may increase the loss of metabolic control. Only when the mechanism(s) of CI has been defined can efforts to alleviate this problem be expected to be successful. Current technological practices are not particularly effective in alleviating CI. Breeding programs and plant biotechnology efforts will likely have a greater impact on producing plants with greater resistance to chilling stress (Parkin *et al.* 1988).

Although it remains a controversial issue, many investigators believe that a universal mechanism for CI is shared by CS plant species (Parkin *et al.* 1988). The search for primary events in CI, regardless of universality, is still necessary to develop an understanding of the mechanisms involved. Identification of the primary sites of CI may also lead to a better understanding of the origin of the secondary plant responses to chilling stress (Parkin *et al.* 1988).

1.5 Ultrastructural features of leaves after dark chilling

1.5.1 *Ultrastructure*

Chilling stress has been recognized as a unique environmental impact on crop plant physiology for over 70 years (Kratsch & Wise 2000). As early as 1926, Faris reported the presence of chlorotic bands that correlated to tissue damage caused by cold nighttime temperatures in maize. However, the phenomenon of chilling stress was not systematically investigated until the 1970s and 1980s when studies by Van Hasselt in the Netherlands on cucumber (Van Hasselt 1974a), Taylor and colleagues in Australia on maize (Taylor & Graig 1971), and Gemel and colleagues in Poland on tomato (Gemel *et al.* 1986) were conducted. These pioneers investigated the impact of low temperatures on both the physiology and the ultrastructure of photosynthetic tissues. Chloroplasts are frequently affected first (and in different ways), while other organelles show injury later. Factors such as a species' inherent sensitivity to chilling, concomitant irradiance, duration of chilling, plant water status, and acclimation may interact to exacerbate or mitigate the injury. What remains much less understood is the precise physiological mechanism(s) underlying a particular ultrastructural lesion (Kratsch & Wise 2000).

The inherent chilling sensitivity of a plant, as well as the ability of some species to acclimatise to chilling, influences the timing and appearance of ultrastructural injury with the resulting outcome being mild, moderate, or severe (Kratsch & Wise, 2000). There are many factors such as light intensity, relative humidity and the inherent sensitivity of the plant to chilling that interact and may either enhance the effect or act as protection against injury (Kratsch & Wise, 2000). The more sensitive a plant is to chilling, the sooner and more extensive are the ultrastructural changes (Kimball & Salisbury 1973; Nessler & Wernsman 1980; Wise & Naylor 1987a; Ma *et al.* 1990).

Graham and Patterson (1982) proposed that changes in enzyme levels, kinetic properties or the property of cold lability of proteins could account for the disturbances brought about by low temperature exposure. Quinn and Williams (1978) had previously postulated that the mechanism of low temperature dysfunctioning of respiratory and photosynthetic processes might lie in unfavorable physical changes in protein structure. Since both processes involve a series of electron-transfer steps, subtle changes in the functioning of one or more of these steps may impair the entire process (Parkin *et al.* 1988).

Unlike other environmental stresses such as drought, excessive light or air pollution, the imposition of chilling represents a large, instantaneous, and simultaneous change in the thermodynamic microclimate of every molecule in every cell of every plant (Kratsch & Wise 2000). Enzymatic reactions will instantly slow down due to decreases in substrate diffusion rates. Membrane properties will be immediately altered, although the hypothesis that a membrane phase change might be the overriding 'primary sensor' of chilling temperatures (Wolfe 1978; Lyons *et al.* 1979) has not survived in its simplest form (Martin 1986). Finally, lowered temperatures will also interrupt transport processes across membranes. Any ultrastructural damage therefore, is likely to be a direct or indirect consequence of the primary effects of decreases in substrate diffusion rates, membrane phase changes and interrupted transport processes.

Although there are a number of variables affecting the severity and time course of CI, general ultrastructural symptoms are largely similar across species. They include swelling and disorganisation of both chloroplasts and mitochondria, reduced size and number of starch granules, dilation of thylakoids and unstacking of grana, formation of small vesicles of chloroplast peripheral reticulum, lipid droplet accumulation in chloroplasts and condensation of chromatin in the nucleus (Jagels 1970; Taylor & Craig 1971; Nessler & Wernsman 1980; Murphy & Wilson 1981; Leddet & Geneves 1982; Wise *et al.* 1983; Musser *et al.* 1984, Gemel *et al.* 1986; Ma *et al.* 1990; Ishikawa 1996; Yun *et al.* 1996).

Yun *et al.* (1996) reported rapid (within 3 s) ultrastructural changes in the palisade cells of the CS plant *Saintpaulia ionantha* under conditions of low temperature and high humidity. These cells exhibited a disordered arrangement of the thylakoid intergranal lamellae, shrinkage of the cytoplasm away from the cell wall and condensation of the chromatin in the nucleus. Physiologically the cells remained active, although a reduction in chlorophyll fluorescence and decreased photosynthetic activity was observed.

Older studies of winter rye leaf development at low growth temperatures reported increases in cell size and cytoplasmic content, smaller vacuoles, and multivacuolated cells (Huner *et al.* 1981, 1984; Griffith *et al.* 1985).

Soybean is grouped by Kratsch & Wise (2000) as a 'less chilling sensitive species'. Several studies (Taylor & Craig 1971; Van Hasselt 1974a; Wise *et al.* 1983; Gemel *et al.* 1986) indicated that soybean exhibited a delayed response to chilling, such as chloroplast swelling, thylakoid dilation, randomly tilted granal stacks, formation of peripheral reticulum, serpentine-like thylakoids and accumulation of lipid droplets in the stroma and chloroplasts that disintegrate with prolonged chilling.

Ultrastructural CI develops progressively with time (Wise *et al.* 1983; Ma *et al.* 1990). The longer a plant is exposed to chilling conditions, the more extensive and irreversible the injury. Different stages of chilling induced injury may thus be recognized. The

earliest stage is often characterised by swelling and distortion of chloroplast thylakoid membranes, decreased number and size of starch granules, and formation of small vesicles in the chloroplast peripheral reticulum (Van Hasselt 1974a; Wise *et al.* 1983). Ishikawa (1996) also observed whorls of rough endoplasmic reticulum (RER) surrounding clear regions of cytoplasm, markedly rough vacuolar membranes, plastids and mitochondria with large vesicles, enlargement of Golgi vesicles and dilation of the RER in chilled cells of *Vigna radiata* L. The next stage involves a continued disintegration of the envelope and accumulation of lipid droplets with increased staining of the stroma (Wise *et al.* 1983). More advanced stages of chilling result in unstacking of grana and marked dilation of thylakoid intraspaces (Taylor & Craig 1971; Van Hasselt 1974a; Wu *et al.* 1997). According to Lee *et al.* (2002), marked changes in the ultrastructure of cortical cells of *Cucumis sativus* L. were visible after only 15 min of exposure to chilling stress (8°C), and greater parts of the cortex were affected with longer periods of exposure. The sequence of morphological changes in cell components, however, was similar to that found in the roots exposed to chilling stress for 15 min. These included alterations in cell walls, nuclei, ER, mitochondria, plastids and ribosomes, although the extent varied greatly among cells.

1.5.2 Effect of light

Irradiance during chilling greatly exacerbates the injury due to chilling stress alone (Wise *et al.* 1983). Thylakoid rearrangement did not occur in dark-chilled bean plastids but did in the light, thus light must somehow affect the change. The nature of the light receptor and the source of energy used in reorganising the thylakoid system are questions remaining to be answered (Wise *et al.* 1983). Ristic & Ashworth (1993) have shown that *Arabidopsis thaliana* L. leaf parenchyma cells also undergo ultrastructural changes after transfer to low temperatures in continuous light, leading to multiple invaginations of the plasma membrane, and the accumulation of microvesicles associated with the plasma membrane, tonoplast, chloroplast envelope and mitochondrial envelope. Past work has also associated lipid peroxidation with photooxidative losses in photosynthetic activity when CS plants were exposed to low temperatures in the light (Van Hassett & Van Berlo 1980). Musser *et al.* (1984) reported that after 24 h of chilling soybean plants in the light,

few leaf chloroplasts had visible intact outer membranes and most chloroplasts had a very dark stroma. Characteristically, there was a rearrangement of the thylakoid membranes leading to almost complete unstacking of the grana (Musser *et al.* 1984).

1.5.3 Relative humidity

Chilling sensitivity is markedly influenced by relative humidity (Wright & Simon 1973). High relative humidity (100%) was found to be protective to chloroplasts in both cotton (CS) and bean (CR) leaf tissue during chilling, and the protective effect was enhanced by treatment in the dark (Wise *et al.* 1983). Injury was still possible, however, in a highly CS species such as the tropical *Episcia reptans* Mart., even in a saturated atmosphere (Murphy & Wilson 1981). According to studies performed by Wise *et al.* (1983) on three different CS species, least CI occurred in all three species in the dark at high humidity.

1.5.4 Membranes

Temperature has a marked effect on the structure of water, changing its solvent properties and hence affecting the concentration of different solutes in the immediate proximity of the membrane. These would profoundly affect the biophysical properties of membranes (Martin 1985).

That cellular membranes are the primary sites for CI is suggested by the present understanding of biological adaptations to reduce temperatures. It has long been established that plants grown at reduced temperatures tend to compensate by synthesising elevated amounts of unsaturated fatty acids (Parkin *et al.* 1988). However, according to Parkin *et al.* (1988), few scientists remain convinced that bulk membrane lipid phase transitions are responsible for CI, and according to Lyons *et al.* (1979), it is no longer regarded as a tenable hypothesis. It appears that the unsaturation of lipids in thylakoid membranes protects the photosynthetic machinery from photoinhibition at low temperature (Nishida & Murata 1996). In trying to delineate between CS and CR plants, the balance between the degree of membrane perturbation brought about by chilling and the rate at which the tissue can adapt by the retailoring process may dictate the degree and irreversibility of chilling sensitivity.

Studies of ultrastructural changes associated with the development of CI also implicated cellular membranes as primary sites of damage (Parkin *et al.* 1988). Loss of membrane semi-permeability in tomato seedling cotyledons following exposure to reduced temperature has also been documented by ultrastructural analysis (Ilker *et al.* 1976). Lyons (1973) and Raison (1973) first proposed the hypothesis that the thermotropic phase transition of membrane lipids might play an initiative role in the chilling sensitivity of plants. Low temperature induces a number of changes in cellular metabolism. These changes include increases in the extent of unsaturation of fatty acids and phospholipid content, changes in protein composition, and induction of chilling inducible genes (Nishida & Murata 1996). It is important for living organisms to maintain the fluidity of their cell membranes at a certain level, and this requirement is achieved by changes in the levels of unsaturated fatty acids in membrane lipids that are catalysed by fatty acid desaturases (Russel 1984; Cossins 1994). Temperature reduction initiates a physical state change in the hydrophobic matrix of the membrane and produces semi-crystalline to crystalline lamellae, thus increasing the susceptibility of the membrane to stress (Lyons 1973). With exposure to chilling, the phase-separated biomembranes become incapable of maintaining ionic gradients and cellular metabolism becomes disrupted, resulting in the death of plant cells (Murata 1989).

Quinn and Williams (1978) argued that temperature-dependent changes in protein interactions rather than alterations in lipid fluidity were responsible for Arhenius breaks for cytochrome oxidase activity in mitochondrial respiration. Bligny *et al.* (1985) postulated that intrinsic thermotropic changes in the membrane proteins might be responsible for their temperature dependency. Several studies indicated that ion permeability in tissue of some chilling sensitive plants actually declined with lowered temperature and marked increases in permeability were observed only when they were transferred to warmer temperatures after prolonged chilling (Lyons *et al.* 1979).

With the advent of more sensitive techniques to determine phase transitions in membranes and lipids it was found that only low levels, i.e., less than 5%, (Raison 1985; Raison & Orr 1986) or no detectable amounts (O'Neill & Leopold 1982) of membrane lipids in CS plants

underwent actual phase transitions at physiological temperatures. According to Parkin *et al.* (1988), what may be considered a refinement of the lipid phase transition theory, is that discrete lipid domains in the membrane may undergo a temperature dependent increase in ordering or phase transition (separation) below a critical temperature. The successful genetic manipulation of enzymes responsible for the biosynthesis (Slabas *et al.* 1993) and desaturation (Murata & Wada 1995) of fatty acids has been achieved, as well as the isolation of mutants with defective enzymes (Somerville & Browse 1991; Browse & Somerville 1994).

Alterations in other membrane structures, such as the plasma membrane, take place after a longer induction period (Norman *et al.* 1985). Using ultrastructural analysis, Niki *et al.* (1978) has speculated that the loss of integrity of the tonoplast membrane is responsible for the irreversibility of chilling induction. The ER is the principal site for the retailoring process, since changes in phospholipid molecular species take place here, prior to being observed at other cellular locations (Norman *et al.* 1985; Thompson 1986).

Lowering the root temperature may directly affect the structure and function of the plasma membrane transport enzymes. Changes in membrane structure and dynamics may limit the ability of integral transport proteins to adopt configurations necessary for normal function. Changes in lipid composition, carrier activity, permeability and ultrastructure have been shown to occur at the membrane level (Kuiper 1974; Clarkson *et al.* 1980; Palta *et al.* 1982).

1.5.5 Chloroplasts

Given the central role of photosynthesis in plant physiology, and its sensitivity to chilling stress (Taylor & Rowley 1971), it is not surprising that the chloroplast is commonly the earliest visible site of ultrastructural CI in the plant cell (Kimball & Salisbury 1973; Levitt 1980). Chloroplasts are the first and most severely impacted organelle (Klein 1960; Ballantine & Forde 1970; Taylor & Craig 1971; Wise *et al.* 1983; Kratsch & Wise 2000). Under permissive temperatures, chloroplasts have numerous starch granules and

well-developed granal stacks interconnected by stromal thylakoids, and the two membranes of the chloroplast envelope are intact (Kratsch & Wise 2000).

Typically, the first manifestations of CI are chloroplast swelling, a distortion and swelling of thylakoids, a reduction in the size and number of starch granules (Jagels 1970; Taylor & Craig 1971; Van Hasselt 1974a; Murphy & Wilson 1981; Wise *et al.* 1983; Musser *et al.* 1984; Kratsch & Wise 2000) and the formation of small vesicles of the chloroplast peripheral reticulum (Wise *et al.* 1983). Prolonged chilling leads to accumulation of lipid droplets, a darkening of the stroma, unstacking of grana and disintegration of the chloroplast envelope, the stromal contents mixing with the cytoplasm. Starch granules continue to diminish with time and, in plants that are CS, disappear completely (Jagels 1970; Taylor & Craig 1971; Van Hasselt 1974a; Murphy & Wilson 1981; Musser *et al.* 1984). Severe chloroplast disintegration may result from prolonged chilling (Kratsch & Wise 2000).

Chloroplast swelling is almost universally reported as a symptom in studies of the effects of chilling temperature on cellular ultrastructure (Kratsch & Wise 2000). The enzymes responsible for the breakdown of starch are found in the chloroplast stroma (Slack *et al.* 1969) and remain relatively active at low temperatures (Tishel & Mazelis 1966). There are several lines of evidence implicating starch degradation products as being the osmotically active agents involved in chloroplast swelling. Transport of photosynthates out of the chloroplast might be reduced by chilling temperatures. Protein uptake by chloroplasts, an energy-requiring process, is inhibited by chilling due to the lack of sufficient trans-envelope proton motive force (Leheny & Theg 1994). It is therefore probable that photosynthate export, another energy-requiring process, from the chloroplast may likewise be inhibited.

Chloroplast swelling could be due to the starch degrading enzymes remaining active when the membrane-bound triose-phosphate translocator (Heldt & Rapley 1970), responsible for removing the osmotically active degradation products, is inactivated or reduced in activity. This would lower the water potential of the stroma and cause an

influx of water from the cytoplasm. Thus the possible build-up of soluble photosynthates, in addition to causing chloroplast swelling, may have other physiological consequences (Wise *et al.* 1983). Leaf soluble sugars tend to increase during chilling (Strand *et al.* 1997). This leads to the conclusion that an increase in stromal osmolytes accompanies chilling stress (Kratsch & Wise 2000).

The extent of chloroplast swelling and thylakoid dilation is dependent upon chilling sensitivity (Jagels 1970; Wise *et al.* 1983). Other studies have reported a disorientation of grana with respect to one another and warping and bending of granal stacks followed by the disruption and subsequent disappearance of stromal lamellae (Nessler & Wernsman 1980; Wise *et al.* 1983; Musser *et al.* 1984; Gemel *et al.* 1986; Yun *et al.* 1996)

Thylakoid dilation is a common feature of CI (Taylor & Craig 1971; Forde *et al.* 1975; Nessler & Wernsman 1980). Whether or not a specific component of the photosynthetic electron transfer chain is affected by chilling seems to depend on how the component is investigated and considerable controversy exists concerning the actual site of dysfunction (Wise *et al.* 1983).

During chilling acclimation, chloroplast thylakoid membranes are not characterised by any qualitative modifications in composition. Mainly quantitative changes occur in chloroplast membranes. Chilling acclimation generally seems to cause accumulation of chlorophyll and carotenoids (Linder 1972; Huner *et al.* 1984)

It appears that chloroplast thylakoids undergo significant structural changes at a number of sites when developing at low temperatures (Hällgren & Öquist 1990). However, it is equally evident that these alterations are not reflected in major changes in the basic components of the thylakoids. Furthermore, it is well established that despite the observed changes in thylakoid organisation during cold acclimation, there are no effects on the efficiency by which thylakoids converts light energy to chemical energy. This has

been shown in studies of the quantum yield of CO_2 assimilation in *Pinus sylvestris* (Öquist *et al.* 1980; Öquist & Strand 1986) and *Secale cereale* (Huner *et al.* 1986).

The question remains whether the organisational modifications of chloroplast thylakoids are (1) directly related to increased chilling tolerance or (2) indirectly related through either adjustments to maintain functional efficiency and integrity of photosynthesis at chilling temperatures or when the osmolarity of the stroma increases during accumulation of cryoprotective compounds.

1.5.6 Peripheral reticulum

After exposure to chilling, a peripheral reticulum (vesicles arising from the inner membrane of the chloroplast envelope) appears (Musser *et al.* 1984; Kratsch & Wise 2000). It is proposed that the peripheral reticulum increases surface area of the transport-limiting membrane (chloroplast inner membrane) in response to the chilling induced reduction in metabolite transport (Kratsch & Wise 2000). Morré *et al.* (1991) came to an alternative proposal from their study of chilling stress in leaf discs of soybean. They proposed that the peripheral reticulum (called the 'stromal low temperature compartment' in their study) serves to increase transport of lipids and thylakoid constituents from the envelope to the thylakoid during acclimation to chilling temperatures. Since the peripheral reticulum results from invaginations of the transport-controlling membrane, the increase in surface area it provides may allow for the chloroplast to survive the osmotic increases apparently associated with chilling (Wise *et al.* 1983).

1.5.7 Organelles

According to Kratsch & Wise (2000), organellar development and ontogeny may also be disrupted by chilling temperatures, but mitochondria, nuclei and other organelles are less susceptible to CI than the thylakoids of chloroplasts.

Effects of chilling temperatures on the nucleus are not widely reported in the literature, either because visible changes rarely occur or they are not pronounced (Kratsch & Wise 2000). However, condensation of chromatin was observed in the nucleus of chilled

tissues of *S. ionantha* (Yun *et al.* 1996) as were changes in the appearance of the nucleolus in *Triticum aestivum* (Gazeau 1985). Ishikawa (1996) reported swollen nuclei with fragmented chromatin and nucleoli, and bundles of microfilaments and other dense material in the nucleus and cytoplasm of cultured cells of chilled *V. radiata*. Enlargement of Golgi vesicles and dilation of the endoplasmic reticulum were also observed just prior to chilling-induced swelling of membranes (Kratsch & Wise 2000).

In contrast to chloroplasts, mitochondria appear to be more resistant to chilling temperatures (Kratsch & Wise 2000). The ultrastructure of mitochondria in leaves of *Nicotiana tabacum* L. was not noticeably affected by exposure to chilling temperatures, even during advanced stages of chloroplast degeneration (Nessler & Wernsman 1980). The same was true of cucumber (Wise & Naylor 1987a) and maize (Taylor & Craig 1971) mitochondria during chilling. On the other hand, Murphy & Wilson (1981) reported swelling and disorganisation of mitochondria in *Episcia reptans*, an extremely CS plant, after chilling for 6 h at 5°C. Leddet & Geneves (1982) also observed dilated mitochondria in *Ephedra vulgaris* (Richt.) after chilling for 24 h at 2°C. Additionally, vacuolation and enlarged cristae were noted by Ishikawa (1996) in mitochondria of chilled cells of *V. radiata*. It would appear that only mitochondria in plants that are hypersensitive to low temperature are visibly affected by chilling (Kratsch & Wise 2000).

1.5.8 Lipids

Lipid accumulation as a result of chilling stress has been reported in the intercellular spaces (Ilker *et al.* 1976), between the cell wall and the plasmalemma (Platt-Aloia & Thomson 1976), in the cytoplasm (Ilker *et al.* 1976; Platt-Aloia & Thomson 1976) and in the plastid itself (Slack *et al.* 1974). The presence of lipid droplets is considered indirect evidence for partial chemical or enzymatic degradation of membranes and their persistence suggests that further degradation does not occur. Harris & Arnott (1973) and Platt-Aloia & Thomson (1976) concluded that lipid extrusions might be a part of a general degradative or senescence process.

1.5.9 Anatomy

Examination of sections of maize leaves grown at 25°C and 14°C with both the light and the electron microscope indicated that reduction in growth temperature does not result in major changes in leaf anatomy (Robertson *et al.* 1993). Leaf thickness and chloroplast size were slightly greater in the 14°C-grown leaves but no marked differences in the proportion of mesophyll to bundle sheath cells or to epidermal cells in leaves grown at the two temperatures were evident (Robertson *et al.* 1993). In some cells of leaves grown at low temperature, the chloroplast profile diameter was increased by up to 30% and the chloroplasts were more rounded (Robertson *et al.* 1993).

1.6 Adaptation to chilling temperatures

Chilling temperatures have a significant impact on the geographical distribution of plants (Alscher & Cumming 1990). This is because chilling temperatures affect the rates of individual biochemical processes to a different extent, inducing imbalances between partial processes in metabolic pathways (Alscher & Cumming 1990). Adjustments to buffer the negative effects of chilling temperatures are seen in most plant processes including growth and development, photosynthesis, respiration and reproduction (Kömer & Larcher 1988). The term adaptation is often used to describe genotypic adjustments to a prevailing temperature regime, whereas the term acclimation is used to describe phenotypic adjustments to short-term changes in temperature (Alscher & Cumming 1990). According to Ougham & Howarth (1988) and Chen *et al.* (1983) there is a requirement for protein synthesis for induction of cold hardiness.

It is difficult to relate molecular changes at enzyme and membrane levels to functional properties of physiology at the cell and organism level and a causal relationship is seldom established (Alscher & Cumming 1990). According to Alscher & Cumming (1990), the establishment of causal relationships is the ultimate goal of all stress and adaptation research (e.g. Quinn & Williams (1978), had previously postulated that the mechanism of low temperature dysfunction of respiratory and photosynthetic processes, might lie in unfavorable physical changes in protein structure).

Genetic variability for chilling tolerance of photosynthesis has been observed within and between closely related species (Berry & Björkman 1980; Öquist 1983). For example, in the genus *Lycopersicon*, some populations of the wild tomato (*Lycopersicon hirsutum*) found in Andean regions at elevations of 3000 m, are considerably more chilling tolerant than the cultivated tomato (*L. esculentum*) (Vallejos 1979).

When leaves of cold-hardy herbaceous plants develop at low growth temperatures, they show a remarkable recovery of photosynthetic capacity (Strand *et al.* 1999). Even in the short term, low temperatures lead to an increase in the activation state of PCR cycle enzymes in chilling tolerant C_3 plants such as spinach, winter rye and winter oilseed rape (Holaday *et al.* 1992; Hurry *et al.* 1994, 1995). In chilling sensitive species the opposite are often found (Strand *et al.* 1999).

1.7 Acclimation to chilling temperatures

Acclimation of chilling sensitive plants to tolerate chilling temperatures is very limited, and when it occurs, it seems to be related mainly to improved water conservation (Öquist & Martin 1986). An increase in the volume of the cytoplasm may provide an important mechanism for increasing the enzymes and metabolites in chilling-acclimated leaves (Strand *et al.* 1999).

Levitt (1980) proposed that metabolites accumulate during cold acclimation and carbon assimilation proceeds efficiently with a decrease in temperature. The increases in photosynthetic capacity that result from acclimation to lower growth temperatures could be the result of a number of factors. For example, plants acclimating to low temperature show increases in soluble protein content, the rate of electron transport and in the activities of enzymes such as Rubisco and stromal fructose-l,6-bisphosphatase (FBPase), which parallel the increase in photosynthetic capacity (Berry & Björkman 1980; Badger *et al.* 1982).

Cold acclimation studies are frequently carried out in controlled environment chambers by lowering the chamber temperature and exposing both roots and shoots to low

temperature (Fennell *et al.* 1990). Under these conditions it is unclear if acclimation is a direct effect of chilling on the shoot or an indirect effect of water deficit caused by decreased water movement through the chilled roots. Exposure of roots to chilling temperatures reduces hydraulic conductivity (Kramer 1942; Babalola *et al.* 1968; Anderson & McNaughton 1973; McKenzie *et al.* 1974; Kaufmann 1975, 1977; Tranquillini 1982; Smit-Spinks *et al.* 1984). Under these conditions, chill-induced drought stress develops in the shoots, which in turn could enhance or promote the cold acclimation process.

1.8 Cross-tolerance

According to Bowler & Fluhr (2000), the existence of a common defense system to combat stress was first inferred from simple observations that, plants resistant to one stress, are often also resistant to others. This phenomenon is defined as cross-tolerance. Cross-tolerance is extremely important for agriculture because plants can be selectively bred that are tolerant to more than one stress factor (Bowler & Fluhr 2000). Numerous studies have shown that calcium (Ca) and reactive oxygen intermediates (ROI) fulfill important signaling functions in responses to both biotic and abiotic stresses. These findings imply that Ca and ROI might be central components controlling cross-tolerance, at least at the cellular level, and that cytosolic Ca might act at a convergence point for integrating different stress signals (Bowler & Fluhr 2000).

1.9 The effect of chilling on vegetative growth and development

It has been shown that low growth temperatures have an adverse effect on the vegetative growth and development of the cultivated tomato *L. esculentum*, while high-altitude populations of *L. hirsutum* were significantly less affected (Vallejos *et al.* 1983).

Growth, as measured by the plastochron index, also showed a capacity for acclimation in *L. hirsutum* that was absent in *L. esculentum* (Vallejos *et al.* 1983). Thus growth appears to be more sensitive to a temperature decrease than photosynthesis, even after acclimation (Vallejos *et al.* 1983). Nevertheless, there is a close correlation between the capacity for acclimation of photosynthesis and growth in *L. hirsutum*, and the lack thereof in *L.*

esculentum. This suggests that the factors responsible for the acclimation of both processes are similar, or that selection has acted in a parallel manner on both (Vallejos *et al*. 1983).

1.10 The effects of chilling temperatures on biological nitrogen fixation

Biological nitrogen fixation (BNF) is important from the viewpoint of limiting the use of inorganic nitrogen fertiliser and reducing the cost of crop production (Luna & Planchon 1995). The symbiotic association of *Bradyrhizobium japonicum* with roots of soybean provides one N input system to the plant, while nitrate taken up by soybean roots from the soil, provides the second N input system (Harper 1987). These inputs are interdependent (Harper 1987). Photosynthetic carbon assimilation is an important factor in determining plant growth (Kao & Forseth 1993). The positive linear relationship between N content of leaves and light saturated rates of photosynthesis indicates that N nutrition contributes substantially to plant growth (Evans 1989). Biological nitrogen fixation is an energy-demanding process, utilizing about 25% to 50% of photosynthetic carbon gain (Phillips 1980; Minchin *et al*. 1981).

Inorganic nitrogen-fed soybean plants, grown under field conditions, have rarely been shown to yield more than soybean plants acquiring nitrogen through BNF (Welch *et al*. 1973; Peterson & Varvel 1989). It is noteworthy that small amounts of starter N are desirable and actually stimulate BNF over that found in plants grown in the complete absence of any external N (Harper 1974; Eaglesham *et al*. 1983).

Kuo & Boersma (1971) reported that at about 17°C, the cell membranes that water must pass to enter the plant are altered, leading to drought stress that may have a direct effect on the rate of BNF. According to Legros & Smith (1994), internal N concentration decreased in plants with a decrease in root temperature. It may be that the N concentration in the root has to be above a critical level before export to the shoot can take place. Biological nitrogen fixation was more inhibited by low root zone temperature (RZT) than was overall plant growth and the accumulation and partitioning of N by N_2-fixing soybean was more affected by low RZT than were those of nitrate-fed soybean

(Legros & Smith 1994). They also argued against the hypothesis that a decrease in the solubility of ureides at low temperatures cause N_2-fixing soybean to be more sensitive to low RZT than N_2-fixing lupin. This implies that the export of N from nodules is not the main cause of the low RZT sensitivity of N_2-fixing soybean plants. According to Harper (1987), low temperatures during the night, as frequently encountered under field conditions, resulted in decreased acetylene (C_2H_2) reduction, which is an *in vitro* measure of BNF activity. Soybean plants acquiring N through BNF, appeared more sensitive to low (10°C) (Matthews & Hayes 1982) and high (40°C) (Munevar & Wollum 1981) temperatures than nitrate-fed plants. This led Matthews & Hayes (1982) to conclude that soybean sown under cool environmental conditions would benefit from applied N at the time of sowing.

The physiological mechanisms underlying the inhibition of BNF by environmental stress factors in legume nodules are not yet clearly understood (Serraj *et al.* 1999a). Most investigations trying to elucidate these mechanisms have focused on the effects of drought stress on BNF. It is however not unrealistic to assume that some of these mechanisms might be similar in the case of chilling stress, especially in the presence of chill-induced drought stress. The effects of drought stress on BNF usually have been perceived as a consequence of straightforward physiological responses acting on nitrogenase activity (NA) and involving exclusively one of three mechanisms: carbon shortage, oxygen limitation or feedback limitation due to accumulation of nitrogenous compounds (ureides or amides) (Serraj *et al.* 1999b).

Carbohydrates and other metabolites are delivered to the nodule infection zone via diffusion from the phloem into the nodule cortex (Serraj *et al.* 1999b). Ureides (allantoin and allantoic acid) are the major nitrogenous compounds synthesised in soybean nodules (Serraj *et al.* 1999b), from where the are exported to the shoot via the xylem and degraded (Winkler *et al.* 1987; Vadez *et al.* 2000). More than 80% of nitrogenous compounds exported out of the nodules in soybean is in the form of ureides (Serraj *et al.* 1999a).

Because there appears to be little water transport into the nodule from any source other than the phloem (Walsh 1995), the export of N products from the nodules through the xylem is dependent on the water influx to the nodules from the phloem (Serraj *et al.* 1999b). Although BNF activity is drastically decreased under water deficit, ureides accumulate in shoots (De Silva *et al.* 1996; Serraj & Sinclair 1996a), probably as a result of decreased ureide degradation (catabolism), even though at the same time nodule activity is very much decreased (Vadez *et al.* 2000). Other research also demonstrated that ureides accumulated in soybean petioles in response to developing water deficits (de Silva *et al.* 1996; Serraj & Sinclair 1996a; Purcell *et al.* 1998). Purcell *et al.* (1998) found that the increase in petiole ureide concentration during water deficit occurred prior to any effect on ureide concentration in xylem sap, from which they concluded that the primary effect of water deficit was on unloading or catabolism of ureides in leaves rather than a direct effect on ureide synthesis in the nodules.

It has been hypothesised that ureide accumulation in the shoot could be responsible for a feedback inhibition of nodule activity (Serraj & Sinclair 1996b), in agreement with the hypothesis of a down regulation of NA by excess nitrogenous compounds (Parsons *et al.* 1993; Hartwig *et al.* 1994; Neo & Layzell 1997; Serraj *et al.* 1999a).

Although no mechanism has yet been established, the basic principle would be that nitrogenous compounds would feedback to the nodule in the case of excess nitrogen in the shoot, inducing a decrease in NA (Heim *et al.* 1993; Parsons *et al.* 1993; Streeter 1993; Hartwig *et al.* 1994; Serraj *et al.* 1999b). Since genotypes with tolerant BNF, accumulate less ureides in the shoot under water deficit than sensitive ones (Serraj & Sinclair 1996a; Purcell *et al.* 1998), ureide degradation is potentially important in understanding the nitrogen fixation response to water deficit (Vadez *et al.* 2000), and possibly also chilling stress.

Ureides may also accumulate in the nodules in response to water deficit (Purcell & Sinclair 1995; de Silva *et al.* 1996; Gordon *et al.* 1997; Serraj *et al.* 1999a, 1999b). It is hypothesised that ureides accumulate in nodules because phloem water flow coming to

nodules is decreased under water deficit so that there is insufficient water to flush ureides from the nodules into the xylem (Walsh *et al.* 1989; Streeter & Salminen 1993; Serraj *et al.* 1999b). This hypothesis results from the apparent dependence of xylem water flow from the nodule on the inflow of water from the phloem (Serraj *et al.* 1999a). Therefore, factors that decrease phloem flow to the nodules will decrease the export rate from the nodules, and consequently, ureides will accumulate (Serraj *et al.* 1999a). For stressors other than drought, substantial increases in nodule ureide levels have also been found (Walsh *et al.* 1989; Streeter & Salminen 1993).

There are two initial steps in the degradation of ureides (Vadez *et al.* 2000). Allantion (Aln) is first degraded into allantoic acid (Alac) by allantoinase (Vadez *et al.* 2000). The subsequent step of Alac degradation in soybean has been attributed to one of two enzymatic pathways: allantoate amidinohydrolase (Shelp *et al.* 1984) and allantoate amidohydrolase (Winkler *et al.* 1987; Stebbins & Polacco 1995). A critical factor involved in ureide accumulation is likely to be the rate of ureide degradation in the leaves (Vadez *et al.* 2000).

Sinclair & Serraj (1995) compared the sensitivity of BNF to drought among nine grain legume species, and found that species that transported ureides from the nodules were more drought sensitive than species transporting amides. They concluded that differences in the solubility of ureides and amides could have been responsible for this observation.

1.11 The influence of dark chilling on photosynthesis

A number of sites in photosynthesis appear to be affected by low temperatures in CS plants, but there is still no consensus concerning the primary site of damage (Hällgren & Öquist 1990). Chilling disrupts many cellular processes in chilling-sensitive species ranging from nutrient transport to photosynthesis (Martin *et al.* 1981; Ort *et al.* 1989; Monroy *et al.* 1997). Of all physiological processes, photosynthesis appears to be the most extensively investigated with respect to physiological and molecular adaptation to low temperature (Hällgren & Öquist 1990). When CS plants are exposed to low temperatures, photosynthesis is one of the first processes to be affected (Long 1983;

Öquist & Martin 1986; Baker *et al.* 1988; Labate & Leegood 1988). The time of exposure and the developmental history of the leaves, as well as genotypic and species differences, are factors that influence the response of plants to low temperatures (Huner *et al.* 1993). Photosynthetic acclimation of C_3 plants in response to low growth temperatures (typically 10-15°C) has been studied extensively in herbaceous plants (Treharne & Eagles 1970; Regehr & Bazzaz 1976; Holaday *et al.* 1992). Allen & Ort (2001) showed that the effects of concurrent light are greater than that of chilling alone and likely to mask those induced by chilling *per se*. Reports indicate that chilling can disrupt essentially all major components of photosynthesis including thylakoid electron transport, the PCR cycle and control of stomatal conductance (Allen & Ort 2001; Van Heerden *et al.* 2002, 2003).

Identifying the primary effects that underlie the *in vivo* dysfunction within this highly interactive and regulated system is one of the important challenges to research in this field. For example, stomatal closure following a chilling episode could be a direct low temperature effect on guard cell function or an indirect response to a rising intercellular CO_2 concentration (c_i) within the leaf caused by a chill-induced loss of Rubisco activity (Allen & Ort 2001). Plants in natural and agricultural habitats generally experience the lowest temperatures at night and thus it is important to study the effects of chilling in the dark on photosynthesis (Allen & Ort 2001). At temperatures below 17°C, the potential capacity for photosynthesis is reduced as a result of a reduced amount of photosynthetic apparatus per unit leaf area (Nie & Baker 1991; Nie *et al.* 1992). According to Makino *et al.* (1994), some key components of the photosynthetic apparatus seem to be affected more than others during short-term (minutes to hours) temperature change. For example, whereas low temperature had only a slight effect on the rate of CO_2-limited photosynthesis, it strongly influenced the rate of CO_2-saturated photosynthesis in barley leaves (Labate & Leegood 1988).

Van Heerden *et al.* (2003) showed that dark chilling inhibited photosynthesis in two soybean genotypes ('Fiskeby V' and 'Maple Arrow') that are generally regarded as chilling tolerant. They also showed that these genotypes experienced differential chilling

sensitivity in response to dark chilling. They showed that the inhibition of CO_2 assimilation was characterised by a simultaneous decrease in stomatal conductance (g_s) and intercellular CO_2 concentration (c_i) in 'Maple Arrow', whereas a similar decrease in g_s in 'Fiskeby V' occurred without any change in c_i.

Studies by Holaday *et al.* (1992) on bean showed that rates of photosynthesis were very low after exposure to low temperature (Holaday *et al.* 1992). In most studies, a shift of CS plants to low temperatures resulted in a decrease of photosynthesis (Hurry *et al.* 1994; Perera *et al.* 1995). This decrease appears to be associated with the inactivation of regulatory thioredoxin-activated enzymes of the PCR cycle (Sassenrath & Ort 1990; Sassenrath *et al.* 1991; Holaday *et al.* 1992; Brüggemann *et al.* 1994; Van Heerden *et al.* 2003).

Control can be shared between several enzymes or processes in a metabolic pathway and, depending on the conditions, the distribution of control may vary (Stitt & Schulze 1994). Several investigators have proposed that there are other factors limiting the overall rate of photosynthesis. For example, the maximum rate of photosynthesis can be partly restricted by stomatal conductance (Farquhar & Wong 1984; Jones 1985; Woodrow *et al.* 1990) or resistance to CO_2 diffusion through the aqueous phase of the leaf (Evans & Terashima 1988). At moderately low temperature, the light saturated rate of photosynthesis may also be restricted by the rates of sucrose and starch synthesis (Leegood & Furbank 1986; Sage & Sharkey 1987) or high CO_2 concentration (Sharkey 1989; Stitt & Quick 1989). Photosynthesis following a dark chill may be primarily restricted through interference with carbohydrate metabolism, inhibition of Rubisco activity and stomatal closure (Allen & Ort 2001).

Disruption of metabolism by chilling stress has been proposed to take place through several possible mechanisms (Hariyadi & Parkin 1993), involving membrane lipid phase transitions (Lyons 1973; Murata 1983), a redistribution of cellular Ca (Minorski 1985) and a direct relationship between low temperature and enzyme dysfunction (Graham & Patterson 1982).

1.11.1 Stomatal function

CO_2 has to diffuse from the atmosphere into the leaf to the site of carboxylation before photosynthesis can take place (Krüger 2002). There are a few aspects that give resistance to the diffusion of CO_2 into the leaf as seen below in figure 1.1:

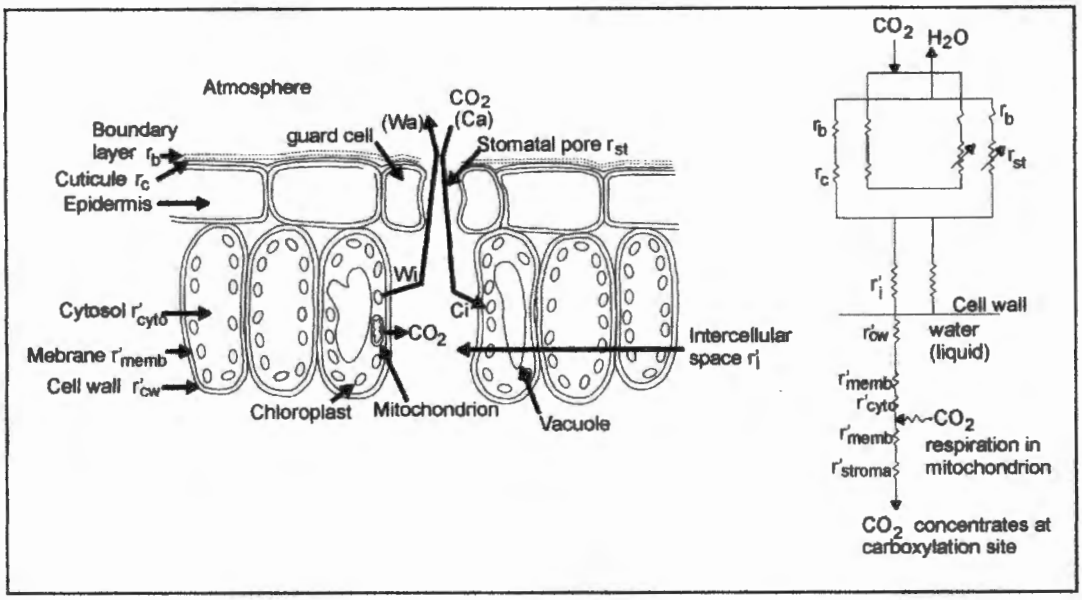


Figure 1.1: The pathway of water vapour loss and CO_2 uptake by a leaf in the light – also indicated as an electrical analog with resistance indicating the areas in the leaf where diffusion is limited (From: Krüger 2002).

- The water vapour boundary layer that surrounds the leaf (boundary layer resistance).
- The stomatal opening (stomatal resistance).
- Resistance against the movement of CO_2 through the intercellular air spaces.
- Resistance that develops at the level of separation between air and the cell wall where CO_2 is dissolved in water and moves as a liquid into the chloroplast (mesophyll resistance).

The stomatal opening is where the largest amount of resistance against CO_2 diffusion to the chloroplast occurs (Krüger 2002).

In many CS species such as cucumber, tomato, bean, cotton and soybean, low temperatures can cause stomata to appear locked open and unable to respond normally to leaf water deficit causing this species to wilt quickly (Martin *et al.* 1981; Eamus 1987; Guye & Wilson 1987). Stomatal closure can nevertheless be observed when more realistic, near-saturation conditions with warm roots are used (Allen & Ort 2001).

According to Allen & Ort (2001), two potential causes of chill-induced stomatal closure exist. First, a direct inhibition of mesophyll photosynthesis could lead to a rise in c_i, which in turn induces stomatal closure. Alternatively, stomata themselves might be the primary target of the chill and their closure could lead to a reduction in c_i, leading to a decline in photosynthesis. Such observations frequently attribute the chill-induced inhibition of CO_2 assimilation to a combination of stomatal and non-stomatal effects (Martin *et al.* 1981; Bongi & Long 1987; Flexas *et al.* 1999). Identification of the extent to which stomata limit photosynthesis remains problematic because interactions between components of photosynthesis appear to be mediated by c_i (Allen & Ort 2001).

According to Van Heerden *et al.* (2003), stomatal limitations on photosynthesis result from dark chilling in two soybean genotypes, particular in 'Maple Arrow'. They suggested a much greater involvement of non-stomatal limitations on photosynthesis in 'Fiskeby V' than in 'Maple Arrow'.

1.11.2 The photosynthetic carbon reduction cycle (PCR cycle)

The photosynthetic carbon reduction (PCR) cycle is located in the chloroplast stroma and is the primary pathway of carbon fixation in C_3 plants (Fig. 1.2). A wealth of information exists on the functioning of this pathway, and on the enzymes which catalyse the individual reactions (Buchanan 1980; Robinson & Walker 1981; Leegood *et al.* 1985; Woodrow & Berry 1988). The PCR cycle involves eleven different enzymes and can be divided into three distinct phases: carboxylation of RuBP, reduction of 3-phosphoglycerate and regeneration of RuBP (Ölçer *et al.* 2001).

According to Scheibe (1987), the light-modulated PCR enzymes catalyse irreversible steps that determine the flux through the pathway. The activity of the key enzymes reflects the potential metabolite flux through the respective steps and the subsequent reversible reactions (Scheibe 1987).

Little is known about how growth temperature affects the *in vivo* balance between the capacities of the respective photosynthetic limiting processes, in spite of the understanding of the short-term response of photosynthesis to temperature (Makino *et al.* 1994). Changes in activities of Rubisco, other PCR enzymes and electron transport in response to growth temperature have been frequently reported (Holaday *et al.* 1992). For example, a factor contributing to the poor performance of french bean to low temperature, is the inability to fully activate certain PCR enzymes at low temperatures (Holaday *et al.* 1992).

a) Rubisco activity and changes in protein structure

Rubisco is by far the most extensively studied plant enzyme and its properties have been extensively reviewed (Andrews & Lorimer 1987; Gutteridge 1990). According to Raines *et al.* (1991), Rubisco is a bifunctional enzyme with opposing carboxylase and oxygenase activities. The efficiency of CO_2 assimilation is determined by the ratio between these competing reactions and the importance of this in determining plant productivity has made Rubisco a target for genetic manipulation (Ellis & Gatenby 1984).

Rubisco is a hexadecameric protein, and in higher plants and cyanobacteria, consists of two types of subunits, referred to as the large (LSU) and small (SSU) subunits (Raines *et al.* 1991). The holoenzyme have a molecular weight of about 536 kDa and is in a L_8S_8 configuration, consisting of eight LSU with a molecular weight of about 52 kDa and eight SSU with a molecular weight of about 15 kDa, (Raines *et al.* 1991). The active site is situated between the LSU dimer (Andersson *et al.* 1989; Knight *et al.* 1989). The SSU is essential for full catalytic activity of Rubisco, although the mechanism of action is incompletely understood (Andrews 1988).

The holoenzymes in different plant species differ appreciably in their carboxylation/oxygenation ratios, despite the significant sequence conservation between LSU from various sources (Andrews & Lorimer 1987). Both Andrews & Lorimer (1985) and Incharoensakdi *et al.* (1986) found that the ratio of carboxylation to oxygenation is determined exclusively by the LSU.

Portis (1992) made it clear that the understanding of the regulation of Rubisco has emerged as an important subject of photosynthesis research because several environmental factors, particularly light intensity, have been shown to modulate the activity of the enzyme. The regulation of Rubisco is thus an important component in the general concept that a coordinated regulation of RuBP utilisation during carboxylation/oxygenation, light absorption, and use of the captured light energy for RuBP regeneration is essential for efficient photosynthesis in dynamic natural environments (Woodrow & Berry 1988).

Portis (1992) suggested that since Rubsico exists in multiple forms, the activity of the enzyme can vary independent of substrate levels and inhibitors. The enzyme exists in the simplest case either in the form with catalytic potential, ECM, or as inactive forms, E and EC. Lorimer *et al.* (1976) found that the relative amounts of these forms are determined by pH, CO_2 and Mg^{2+}. According to Jensen & Bahr (1977), light regulates Rubisco activity.

The assimilation of CO_2 into the PCR cycle is catalysed by Rubisco, and it is thought that this step limit the maximum rate of light-saturated photosynthesis (Andrews & Lorimer 1987; Woodrow & Berry 1988). According to Andrews & Lorimer (1987), Rubisco has a low rate of catalysis (K_{cat}), and the *in vivo* rate of carboxylation is further reduced by its poor affinity for CO_2 and the competing reaction with O_2. However, large amounts of Rubisco protein are found in the leaf (20-25% of total leaf protein), which compensate for this inefficiency (Evans 1989).

During conditions where Rubisco does not limit photosynthesis (e.g. low irradiance), catalysis would be out of step with the remainder of the photosynthetic apparatus if *in vivo* Rubisco activity is not reduced (Stitt & Schulze 1994). Andrews & Lorimer (1987) suggests that Rubisco activity is decreased by the deactivation of the enzyme via a unique mechanism of post-translational regulation, which involves decarbamylation of a lysine residue in the active site. According to Portis (1990), the carbamylation of the lysine residue is catalysed by Rubisco activase, which requires ATP, and is inhibited by ADP.

Investigations indicated that in several species of monocots and dicots, Rubisco activity increases in the cold (Treharne & Eagles 1970; Chabot *et al.* 1972; Holaday *et al.* 1992; Hurry *et al.* 1994, 1995).

The initial part of the A:c_i response curve, known as the demand function, is a linear correlation (where CO_2-assimilation is limited by c_i and saturated with RuBP) from which the carboxylation efficiency (CE) can be determined from the slope, $\delta A/\delta c_i$ (Von Caemmerer & Farquhar 1981). According to Von Caemmerer & Farquhar (1981), CE provides an *in vivo* measure of the Rubisco activity within the leaf. Ferrar *et al.* (1989) used the initial slope of the demand function to estimate Rubisco activity in *Nerium oleander* and a range *of Eucalyptus* species acclimated to low temperature. They found that cold-acclimation of these plants resulted in a higher CE than in warm-acclimated plants, indicating parallel changes in Rubisco activity and photosynthetic capacity in the cold-acclimated plants.

According to Labate & Leegood (1988), there is a lack of information about the effect of temperature on the activation state of Rubisco. Makino *et al.* (1994) found that the activation state and catalytic turnover rate of Rubisco was not affected by growth temperature. However, Brüggemann *et al.* (1992b) found in tomato that chill-induced reduction in photosynthesis is associated with irreversible inactivation of Rubisco.

Experiments by Van Heerden *et al.* (2003) indicate that dark chilling had little effect on maximum catalytic Rubisco activity, Rubisco protein content or Rubisco activation state in the subsequent light period following a dark chill in two chilling tolerant soybean genotypes, 'Maple Arrow' and 'Fiskeby V'. They concluded that limitations on Rubisco cannot explain the greater chilling sensitivity of photosynthesis in 'Fiskeby V'.

Chilling resulted in the loss of Rubisco protein content in tomato (Brüggemann *et al.* 1992b; Holaday *et al.* 1992). In various studies it was found that a loss of Rubisco activity was responsible for declines in photosynthesis after a chill in both the dark and the light (Kingston-Smith *et al.* 1997). It has been suggested that Rubisco protein structure might be damaged by chilling (Kingston-Smith *et al.* 1997). On the other hand, Raines *et al.* (1991) suggested that the recently discovered redox regulation of the larger Rubisco activase isoform (Zhang & Portis 1999) might be affected by chilling, as was observed for SBPase and FBPase. Rubisco activase has been found in all higher plants investigated so far and is essential for activation of Rubisco (Raines *et al.* 1991).

2-Carboxyarabinitol-1-phosphate (CA1P) is a naturally occurring inhibitor of Rubisco and is synthesised in leaves in response to periods of darkness or reduced light intensity (Salvucci 1989; Seemann *et al.* 1990; Servaites 1990). This inhibitor binds with high affinity at the active site to the carbamylated form of Rubisco and thus directly reduces its catalytic activity (Portis 1992). According to Portis (1992), CA1P plays an important role in the regulation of Rubisco activity during photosynthesis. In addition to its regulatory function, Khan *et al.* (1999) also suggested a protective role for CA1P. According to these authors, nocturnal binding of CA1P to Rubisco, protects the enzyme against attack by protease enzymes. Van Heerden *et al.* (2003) determined that the nocturnal abundance of CA1P is reduced by dark chilling in soybean and suggested that this might render Rubisco more susceptible to proteolytic attack.

b) The effect of chilling on NADP-malate dehydrogenase

NADP-malate dehydrogenase (NADP-MDH) is one of the most extensively studied chloroplast enzymes activated by the light-driven ferredoxin-thioredoxin system

(Buchanan 1980; Crawford *et al.* 1989) and is a perfect example of an all-or-nothing type of functioning (Miginiac-Maslow *et al.* 2000).

In both C_3 and C_4 plants, the activity of NADP-MDH is regulated by light intensity (Ashton *et al.* 2000). In darkness, the activity of the enzyme can be less than 1% of the maximal activity found at high light intensities, and upon illumination, the NADP-MDH activity increases about 100 fold to a new steady state within 2 min (Ashton *et al.* 2000). If the light is turned off, the activity declines within about 4 min to the original low level (Leegood *et al.* 1982; Miginiac-Maslow *et al.* 1985; Scheibe *et al.* 1989; Ashton *et al.* 2000).

NADP-malate dehydrogenase is reduced in the light by photosynthetic electron flow via a redox cascade whose last steps consist of thiol disulfide interchanges between reduced ferredoxin-thioredoxin reductase (FTR) and thioredoxin, and finally between reduced thioredoxin and NADP-MDH (Scheibe & Anderson 1981; Miginiac-Maslow *et al.* 2000). In C_3 plants this enzyme is not directly involved in CO_2 assimilation (Scheibe 1987). $NADP^+$ strongly inhibits the activation of NADP-MDH, whereas it has no effect on the activity of the fully activated enzyme (Miginiac-Maslow *et al.* 2000). According to Miginiac-Maslow *et al.* (2000), NADP-MDH functions as a 'valve' (Fridlyand *et al.* 1998), feeding malate to the dicarboxylic acid shuttle in the chloroplast envelope and thus exporting excess reducing equivalents not required for the operation of the PCR cycle. Chloroplast NADP-MDH is characterised by two additional terminal peptides, which make the enzyme receptive to regulation by light-modulation (Scheibe 1987).

According to Scheibe (1987), the activation state of NADP-MDH is very sensitive to the stromal $NADPH/NADP^+$ ratio and is regulated by this parameter more than the thioredoxin redox state *per se*. The activity of NADP-MDH will be adjusted to the required activity as sensed by the $NADPH/NADP^+$ ratio, depending on the nature and availability of substrate to be reduced by photosynthetic electron flow (Scheibe 1987). The complex redox regulation of NADP-MDH is very strictly controlled by the reducing power of the chloroplast. The enzyme can thus respond precisely to variations in the

photosynthetic electron flow, and variations in light intensity (Miginiac-Maslow *et al.* 2000). This regulatory mechanism is obviously designed to prevent the enzyme from functioning when the electron flow decreases (Miginiac-Maslow *et al.* 2000). In C_3 plants, it prevents the leakage of reducing equivalents outside the chloroplast. NADP-MDH activity *in vivo* is obviously regulated much more by the proportion of active enzyme than by the total amount of enzyme present. The former regulation is much more rapid and precise than the latter, quickly adapting enzyme activity to current metabolic needs (Miginiac-Maslow *et al.* 2000).

Under conditions of stress, when there is often an increase in the stromal redox state, the increase in the NADPH/$NADP^+$ ratio, leads to an increase in the activation state of NADP-MDH. The dark chilling-induced decrease in the activation state of NADP-MDH, observed by Van Heerden *et al.* (2003) in the soybean genotype 'Maple Arrow', may indicate a decrease in the stromal NADPH/$NADP^+$ ratio (Scheibe 1987; Harbinson *et al.* 1990).

c) Functioning and activation of FBPase and SBPase

According to Scheibe (1987) and Labate & Leegood (1988), there is a lack of information about the effect of low temperature on the light-activated enzymes of the PCR cycle. Since the two stromal bisphosphatases, FBPase and SBPase, work in tandem in the RuBP regeneration phase of the PCR cycle, it is sensible that their activity be coherently regulated (Hutchison *et al.* 2000). SBPase and FBPase are activated by the ferredoxin-thioredoxin system and their activity is tightly coupled to the redox state of the stroma under optimum conditions (Scheibe 1987). In tomato, a decrease in the activity of these two enzymes is one of the main restrictions imposed on photosynthesis by a light chill and is apparently caused by impairment in their reductive activation (Sassenrath *et al.* 1991; Hutchison *et al.* 2000).

Fructose-1,6-bisphosphatase (FBPase)

According to Raines *et al.* (1988), FBPase is a homotetramer of about 160 kDa in size and appears to be highly conserved between species. According to Ocón *et al.* (2001),

FBPase catalyzes the Mg^{2+}-dependent hydrolysis of fructose-1,6-bisphosphate (FBP) and is widely distributed in nature. In higher plants, it is present in the cytosol and chloroplast. Cytosolic FBPase is regulated by metabolites and takes part in gluconeogenesis (Zimmermann *et al.* 1978; Marcus 1981; Prado *et al.* 1991). In contrast, stromal FBPase participates in the regeneration of RuBP in the PCR cycle and in starch synthesis, and is activated by light (Zimmermann *et al.* 1976; Buchanan 1980).

According to Buchanan (1980, 1991), the activation of stromal FBPase by light is mediated through the ferredoxin-thioredoxin system. In the presence of light, the reducing equivalents originating from the photosynthetic electron transport chain act as a cellular signal, activating FBPase (Ocón *et al.* 2001). Electron transport reduces ferredoxin and in turn, this protein reduces FTR, which reduces thioredoxin *f*, a small ubiquitous protein very efficient in thiol-disulfide interchanges with a high number of protein disulfides (Ocón *et al.* 2001).

Stromal FBPase activates slowly, even under high illumination (Leegood *et al.* 1982; Miginiac-Maslow *et al.* 1985; Scheibe *et al.* 1989). The interaction between stromal FBPase and thioredoxin *f* is mainly of an electrostatic nature (Hermoso *et al.* 1996; Reche *et al.* 1997; Sahrawy *et al.* 1997; Hermoso *et al.* 1999), possibly involving carboxyl groups of FBPase and amino groups of thioredoxin *f* (Ocón *et al.* 2001).

Van Heerden *et al.* (2003) indicated in soybean that dark chilling decreased the maximum extractable activity and activation state of stromal FBPase, which occurred in parallel with the inhibition of photosynthesis. The inhibition of FBPase activity occurred after two consecutive nights of chilling in 'Maple Arrow' and after one night in 'Fiskeby V'. Van Heerden *et al.* (2003) suggested that of all the enzymes studied in soybean, stromal FBPase appeared to be the most sensitive to dark chilling and it is thus appropriate to consider the mechanisms involved in determining its chilling sensitivity. Stromal FBPase is subject to regulation involving hysteresis, whereby the enzyme is locked in its active form by its substrate via fructose 1,6-bisphosphatase followed by activation with thioredoxin *f*. Van Heerden *et al.* (2003) proposed that it is possible that

this type of enzyme regulation is more chilling sensitive than the thioredoxin reduction process *per se*.

Sassenrath *et al.* (1991) suggested that chilling disrupts the normal thioredoxin-dependent activation of the stromal bisphosphatases by showing that the light dependent reductive activation of FBPase was restricted by chilling in tomato. Similar findings were also obtained in chilled bean plants, where only weak activation of FBPase was observed (Holaday *et al.* 1992).

Sedoheptulose-1,7-bisphosphatase (SBPase)

Sedoheptulose-1,7-bisphosphatase (SBPase) is found only in chloroplasts and has no cytosolic counterpart (Raines *et al.* 1991). In higher plants the enzyme is a homodimer with a subunit molecular weight of between 35 – 38 kDa (Nishizawa & Buchanan 1981; Cadet *et al.* 1987). Its relationship to stromal FBPase is of particular interest because their substrates are very similar (Raines *et al.* 1991). Both are activated by reduced thioredoxin (Nishizawa & Buchanan 1981) and they are immunologically related (Cadet & Meunier 1988). According to Raines *et al.* (1991), the main structural difference between the two enzymes appears to be that the SBPase holoenzyme is a homodimer whereas FBPase is a homotetramer.

According to Raines *et al.* (2000), SBPase is unique to the PCR cycle where it catalyses the dephosphorylation of sedoheptulose-1,7-bisphosphate (SBP). The SBPase gene is located in the nuclear genome (Raines *et al.* 1992; Willingham *et al.* 1994; Hahn *et al.* 1998). Light, development and levels of hexose sugars regulates SBPase gene expression (Willingham *et al.* 1994; Jones *et al.* 1996). Sedoheptulose-1,7-bisphosphatase, together with FBPase, operates in the regeneration phase of the PCR cycle where the CO_2 acceptor molecule (RuBP) is regenerated from triose phosphates through a series of sugar condensation and carbon rearrangement reactions (Woodrow & Berry 1988; Geiger & Servaites 1995).

Similar to stromal FBPase, the activity of SBPase is regulated by light (Raines *et al.* 2000). As a result of light-modulated activation by thioredoxin *f*, the catalytic activity of SBPase increases more than 10 fold in response to a dark-light transition (Breazeale *et al.* 1978; Laing *et al.* 1981; Wirtz *et al.* 1982). Thioredoxin *f* reduces the regulatory disulphide bond by binding in a stable complex to the inactive SBPase enzyme (Geck *et al.* 1996; Jaramillo *et al.* 1997). According to Brandes *et al.* (1996), this activation mechanism involves the formation of protein-protein disulphide bonds, followed by the release of the reduced and active SBPase protein and the formation of oxidized thioredoxin *f*. Reduction of the disulphide bond changes the conformation of the active site, resulting in activation of SBPase (Raines *et al.* 2000).

Many investigations revealed that light-induced changes in stromal Mg^{2+} levels and pH also regulate SBPase activity (Portis *et al.* 1977; Purczeld *et al.* 1978; Nishizawa & Buchanan 1981; Woodrow & Walker 1982; Woodrow *et al.* 1984b). According to Suss *et al.* (1993), an additional level of regulation may result from the association of SBPase into complexes with other PCR cycle enzymes, which improves the efficiency of the cycle by facilitating the flux of intermediates between enzymes. Petterson & Ryde-Petterson (1989) and Poolman *et al.* (2000) have suggested that SBPase may play an important role in the control of carbon flux through the PCR cycle. In addition, the location of SBPase at the branch point between regeneration of RuBP and biosynthesis of starch and sucrose could potentially influence the distribution of carbon between these three competing pathways (Ölçer *et al.* 2001). Decreased SBPase activity often cause reductions in carbohydrate levels, particularly in starch, but the change in carbohydrate content was also dependent on the developmental status of the leaf (Ölçer *et al.* 2001). Carbohydrate levels tend to decline the most in plants with the greatest reductions in SBPase activity (Ölçer *et al.* 2001).

Numerous studies indicated that the control of carbon assimilation is shared between Rubisco, SBPase and aldolase (Hudson *et al.* 1992; Stitt & Schulze 1994; Harrison *et al.* 1998; Haake *et al.* 1998, 1999). According to Raines *et al.* (2000), the distribution of control between these three enzymes is not constant and can vary depending on

environmental conditions. Several researchers (Koβmann *et al.* 1994; Paul *et al.* 1995; Price *et al.* 1995) found that several of the other highly regulated enzymes in the PCR cycle, such as glyceraldehyde-3-phosphate dehydrogenase, FBPase and phosphoribulokinase, made only modest contributions towards the control of carbon assimilation. The primary effects of a short chill in the light and the dark on photosynthesis are illustrated in Fig. 1.3.

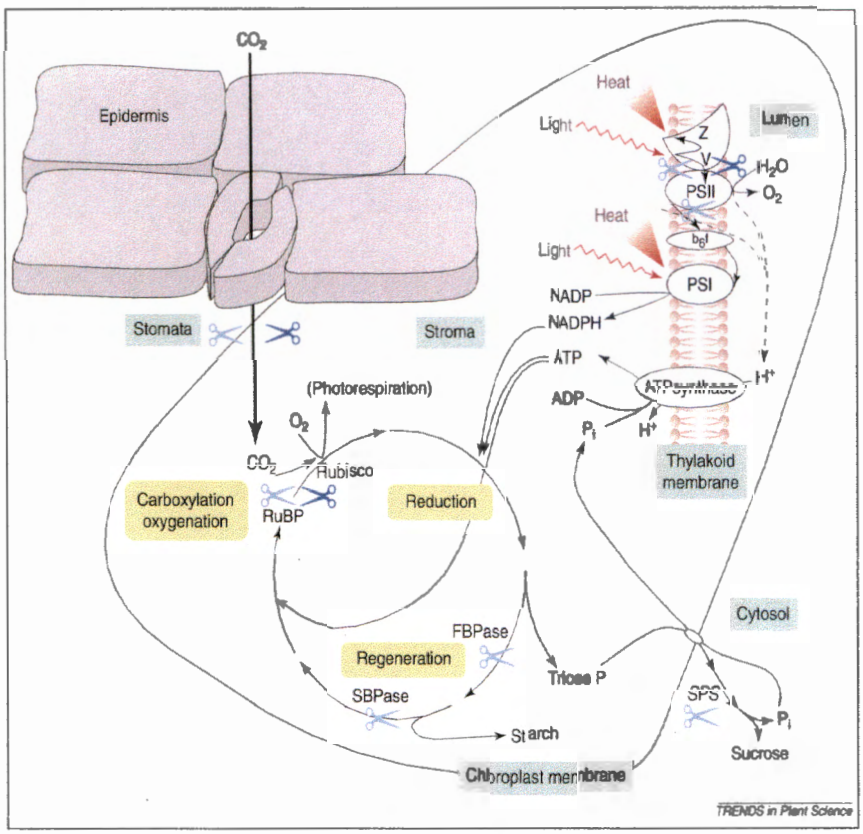


Figure 1.3. Primary effects of a short chill in the light and the dark on photosynthesis in thermophilic plants. Chilling effects are apparent within the processes of photophosphorylation in the thylakoid membrane, the carbon reduction cycle in the stroma, carbohydrate use in the cytosol and the CO_2 supply to the chloroplast through the stomata. Abbreviations: ATPsynthase, chloroplast ATP synthase; b_6f, cytochrome b_6f complex; FBPase, stromal fructose 1,6-bisphosphatase; P_i, inorganic phosphate; PS I, photosystem-I complex; PS II, photosystem-II complex; RuBP, ribulose 1,5-bisphosphate; SBPase, sedoheptulose 1,7-bisphosphatase; V, violaxanthin; Z, zeaxanthin and antheraxanthin; light gray scissors represent the primary impact of a light chill; black scissors represent the primary impact of a dark chill (From: Allen & Ort 2001).

The effect of chilling on the protein content of PCR cycle enzymes

According to Hutchison *et al.* (2000), Western Blot analysis revealed that there was no loss in the steady state protein content of FBPase in tomato plants following a light chill.

Makino *et al.* (1994) also found that there was no chilling-induced change of Rubisco content in rice.

In contrast, Strand *et al.* (1999) reported that the protein content of *Arabidopsis* (Heynh.) plants increased 2.5-fold in chilled leaves and that this increase was responsible for the increased activity of Rubisco and other PCR cycle enzymes. In contrast, Van Heerden *et al.* (2003) reported a transient decrease of Rubisco protein content in dark chilled soybean, which correlated with the decrease in maximum catalytic Rubisco activity. Experiments by Brüggemann & Dauborn (1993) indicate a gradual decline in total soluble protein and Rubisco content in young tomato plants during chilling.

1.11.3 End-product synthesis

According to Labate & Leegood (1988), there is a lack of information about the effect of low temperature on sucrose synthesis. Carbohydrate metabolism has a greater instantaneous low temperature sensitivity than other components of photosynthesis (Leegood & Edwards 1996). According to Strand *et al.* (1999), a rapid shift from warm growth conditions to chilling temperatures leads to a pronounced inhibition of photosynthetic carbon assimilation. Chilling inhibits phloem export, leading to a rapid accumulation of soluble sugars and repressing photosynthetic gene expression (Paul *et al.* 1992; Krapp & Stitt 1995; Strand *et al.* 1997). Development under low temperature growth conditions allows plants to acclimate carbon metabolism by shifting partitioning towards soluble sugar synthesis (Strand *et al.* 1999). According to Huner *et al.* (1993), it is possible that low temperature limitation of photosynthesis occurs at the level of the triose-phosphate translocator, rather than sucrose synthesis *per se*.

Inhibition of end-product synthesis at low temperatures results in the accumulation of phosphorylated metabolites in the cytosol and phosphate limitation of photosynthesis in the chloroplast (Leegood & Furbank 1986; Sharkey *et al.* 1986). This may be partly due to the temperature-dependent changes in the affinity of cytosolic FBPase for its effectors, especially AMP (Stitt & Grosse 1988).

It is suggested that the accumulation of phosphorylated intermediates contribute towards the feedback down-regulation of photosynthesis, because the cytosolic and chloroplastic pools of Pi become depleted to the point where photophosphorylation is Pi limited (Herold 1980; Mächler *et al.* 1984; Leegood & Furbank 1986; Labate & Leegood 1988; Stitt & Grosse 1988). Prolonged exposure to low temperature-induced Pi deficiency might, however, be buffered by the release of Pi from the vacuole (Woodrow *et al.* 1984a; Mimura *et al.* 1990).

It is further suggested that Pi limitation of photosynthesis occurs because sucrose synthesis proceeds too slowly at low temperatures (Leegood 1985; Sharkey 1985a, 1985b; Sharkey *et al.* 1986; Stitt *et al.* 1987). Leegood (1985) suggests that this may be due to the fact that optimal rates of photosynthesis at low temperature require higher Pi concentrations than photosynthesis at optimal temperature. With a decrease of leaf temperature in spinach and wheat, the PGA/Pi ratio, which reflects the phosphorylation potential of the chloroplast, increases (Kobza & Edwards 1987; Stitt & Grosse 1988; Labate & Leegood 1990). According to Stitt *et al.* (1987), an increase in this ratio will favor starch synthesis within the chloroplast over sucrose synthesis in the cytosol. A decrease in phosphorylation potential would cause a significant limitation on CO_2 assimilation (Huner *et al.* 1993). Van Heerden *et al.* (2003), found that short-term dark chilling had no effect on leaf sucrose and starch contents in soybean leaves and suggested that there was no evidence to suggest that dark chilling impaired the capacity for sucrose or starch biosynthesis.

The relationship between low temperature and the inhibition of photosynthetic metabolism is not so clear when CS species are chilled at night (Tucker & Ort 2002). According to Jones *et al.* (1998), it has not been possible to assign the cause of dark chilling inhibition of photosynthesis to individual reactions. Inhibition is only partly accounted for by changes in stomatal conductance (Martin *et al.* 1981). Rates of electron transfer cannot always account for the decline in net photosynthesis, because they are generally sufficient to support the rate of photosynthesis (Kee *et al.* 1986). Nor are there significant changes in the affinity of Rubisco for CO_2 under chilling conditions that could

explain the chill-induced dysfunction seen at the whole plant level (Martin *et al.* 1981). According to Jones *et al.* (1998), the underlying cause of the inhibition of net photosynthesis by dark chilling in plant species such as tomato and cucumber, are likely to involve disruption of coordination among the component reactions of photosynthesis, rather than direct inhibition of the individual reactions *per se*.

According to Tucker & Ort (2002), a more promising clue concerning potential factors underlying altered photosynthetic metabolism due to low temperatures at night was the discovery that endogenous circadian rhythms in CS species are disrupted by exposure to dark chilling. The abundance of many transcripts of nuclear genes encoding photosynthetic proteins start to increase before dawn and is greatest in the morning when the rate of photosynthesis is at a maximum (Van Heerden *et al.* 2003). The circadian regulation of photosynthesis depends on the coordinated light-regulated transcription of nuclear and plastid genes. An intriguing effect of low temperature on the circadian regulation of transcription (Martino-Catt & Ort 1992) indicates that the chilling-induced inhibition of photosynthesis in tomato may result from the loss of coordination in the expression of critical enzymes controlling photosynthetic metabolism (Jones *et al.* 1998). Catalase, chlorophyll *a/b* binding protein (*cab*), phosphoenolpyruvate carboxylase (PEPcase) and Rubisco activase (*rca*) all exhibit endogenous rhythms in transcript levels (Carter *et al.* 1991; Martino-Catt & Ort 1992; Zhong *et al.* 1994). By exposure to dark chilling conditions, oscillations of *cab* and *rca* transcripts are suspended in tomato, causing the rhythms to be delayed when optimal temperatures are restored (Martino-Catt & Ort 1992). According to Jones *et al.* (1998), the endogenous clock mechanism that regulate gene expression and enzyme activity stalls for the duration of the dark chilling treatment and resumes upon rewarming. The affected rhythms are then out of phase with the actual time of day. This transient mistiming of rhythms in CS plants does not necessarily lead to detectable changes in cellular protein levels. For example, because *cab* and *rca* are exceedingly abundant and stable proteins (Cooper & Ort 1988), mistiming cannot be expected to cause the observed severe inhibition of net photosynthesis (Jones *et al.* 1998). However, if the affected enzymes were critical control points of cellular carbon or nitrogen metabolism, mistiming would likely have a severe negative effect on

photosynthetic performance (Jones *et al.* 1998).

Recently, our understanding of such effects has broadened to encompass changes in the activity rhythm of sucrose-phosphate-synthase (SPS) (Tucker & Ort 2002). Sucrose-phosphate-synthase is a central enzyme in photosynthetic metabolism because it catalyses a rate-limiting step in sucrose synthesis (Jones *et al.* 1998). Jones & Ort (1997) showed that SPS activity exhibits a diurnal and circadian rhythm in tomato, which is the result of corresponding oscillations in the phosphorylation state of SPS. An endogenous ultradian rhythm in SPS activity, with a period of about 12 h, has also been reported in soybean (Kerr *et al.* 1985). In the case of tomato, dark chilling stalls the circadian rhythm of SPS activity, probably through interference with the circadian transcriptional regulation of a type 2A protein phosphatase, which is responsible for the dephosphorylation of SPS (Jones *et al.* 1998). Unpublished results by our laboratory *(M.M. Viljoen, M.Sc. project, PU for CHE, 2002)*, indicate that the ultradian rhythm of SPS activity in soybean is also disrupted by dark chilling in a chilling sensitive genotype, whereas the rhythm in a chilling tolerant genotype appears to be unaffected.

Taken together, these recent findings suggests that the low-temperature inhibition of photosynthesis in CS plants is to a large extent caused by low-temperature-induced mistiming of the normal diurnal activity pattern of key enzymes, thereby disrupting photosynthetic and cellular metabolism.

1.11.4 Photochemical reactions

Photosynthetic control is the mechanism by which plants maintain a balance between energy conversion through electron transport and energy consumption by carbon assimilation (Foyer *et al.* 1990; Fisahn *et al.* 1995). Photosynthetic organisms are constantly confronted with reconciling excessive energy supply with the demand of the PCR cycle for the products of electron transport, ATP and NADPH (Huner *et al.* 1993). This control is required in order to protect photochemistry from excessive excitation and potential photoinhibition, and at the same time, ensuring that the rate of ATP and NADPH synthesis is sufficient for the regeneration of PCR cycle intermediates necessary

to maintain optimal rates of CO_2 assimilation and triose phosphate export for sucrose synthesis (Huner *et al.* 1993). Exposure of plants to a combination of high light intensities and either a sudden, short-term shift from high to low temperature or prolonged growth and development at low temperature will disturb the balance between energy conversion and energy consumption (Huner *et al.* 1993).

The sensitivity of photosynthesis to low temperature is generally associated with the reduction of the maximum quantum yield of photosystem II (PS II) (Long 1983; Baker & Nie 1993; Massacci *et al.* 1995). The marked decrease in the maximum quantum efficiency of PS II photochemistry is more likely a dynamic down-regulation of PS II function than actual damage to PS II *per se* (Krause 1994). This allows the plant to dissipate excessive excitation energy that cannot be utilised for photosynthesis because of the low temperature induced reduction in CO_2 assimilation capacity. Down regulation of PS II function has been proposed to be associated with the deactivation of PS II reaction centers (Krause 1994).

Dark chilling of tomato leaves caused a significant inhibition of light-saturated, whole-chain electron transport measured at room temperature (Kee *et al.* 1986), whereas PS I-mediated electron transport was not inhibited. In contrast to PS II, chilling resulted in the stimulation of light saturated PS I activity in isolated thylakoid membranes. According to Brüggeman *et al.* (1992a), factors other than the photochemical efficiency of PS II are responsible for the chilling-induced sustained depression in photosynthetic performance in tomato.

1.11.5 Photoinhibition

Moderate to high light intensities, in combination with chilling temperatures, cause deactivation of FBPase and SBPase (Sassenrath *et al.* 1991; Holaday *et al.* 1992), alterations in photochemistry and non-photochemical energy dissipation during a process commonly known as photoinhibition (Bowler *et al.* 1992). It has been suggested that the inability to cope with excessive light, rather than an inherent intolerance to low temperature, is responsible for the high susceptibility of CS plants to low temperature-dependent photoinhibition (Smillie *et al.* 1988). Photoinhibition originates as a result of

the fact that some enzymes of the PCR cycle are more sensitive to low temperature than the photochemical reactions. Under these conditions, reducing equivalents (NADPH) are produced in excess of what the PCR cycle can use, which causes an over-reduction of the photosynthetic apparatus. Plants have adapted to tolerate the variations in light quantity and quality via several dissipative energy processes (Demig-Adams & Adams 1992; Long *et al.* 1994; Schindler & Lichtenthaler 1994; Srivastava & Strasser 1997).

Over-reduction of the chloroplast stroma leads to excess light energy being channeled to oxygen because of a shortage of $NADP^+$. Under these conditions, formation of reactive oxygen intermediates (ROI) is promoted which can cause severe damage to membranes. Photoinhibition is rarely observed immediately following chilling of even the most extreme CS species, provided that the low temperatures were experienced in the dark (Martin *et al.* 1981; Flexas *et al.* 1999). However, when CS plants are exposed to low temperatures in the presence of light, photoinhibition and ROI formation are common occurrences that often result in severe lipid peroxidation within membranes (Van Hasselt 1974b; Kaniuga & Michalski 1978; Wise & Naylor 1987a, 1987b; Senaratna *et al.* 1989). However, it is not clear if lipid peroxidation also occurs during dark chilling and, if so, what impact this may have on tissue viability (Hariyadi & Parkin 1993).

There is a general consensus that light-induced damage to the photosynthetic machinery is particularly serious in PS II (Powles 1984; Aro *et al.* 1993). The higher the light intensity during the day following dark chilling, the greater the probability of photoinhibition. Dynamic processes that operate within the chlorophyll antennae array of PS II can rapidly dissipate a large portion of the absorbed light as heat, thereby greatly diminishing the threat of ROI production (Owens 1996).

Oxygen is reduced in the Mehler reaction when normal pathways and acceptors of photosynthetic electron transport are restricted, providing a pathway for the removal of excess electrochemical energy from thylakoid membranes (Marsho *et al.* 1979; Furbank & Badger 1983; Furbank *et al.* 1983). While the reduction of oxygen is an effective strategy to sustain electron flow, it nevertheless carries the hazards inherent in the

formation of ROI (Asada 1996). During the Mehler reaction, molecular oxygen is reduced to the superoxide radical anion (O_2^-) (Asada *et al.* 1974; Badger 1985; Robinson 1988) and subsequently to hydrogen peroxide (H_2O_2). Since O_2^- and H_2O_2 are both capable of initiating damaging reactions (Elstner 1982; Robinson 1988), the production of O_2^- and H_2O_2 could play a key role in the chilling-induced inhibition of photosynthesis (Hodgson & Raison 1991).

Although it appears that, in most plants, the PS II reaction center is the most sensitive component of the photochemical apparatus, PS I is also sensitive to photoinhibition (Sonoike 1996; Ivanov *et al.* 1998; Terashima *et al.* 1998).

Plants possess several mechanisms to protect themselves against photooxidative damage. The most important among them are antioxidant enzymes and ROI scavenging compounds such as superoxide dimutases, catalases, ascorbate, glutathione, α-tocopherol and carotenoids (Foyer & Mullineaux 1994). Since the activities of antioxidant enzymes has been shown to be reduced at low temperature in CS plants (Jahnke *et al.* 1991), the capacity of non-enzymatic protective systems to limit the formation and/or scavenging of ROI appears to be of particular importance (Haldimann 1997).

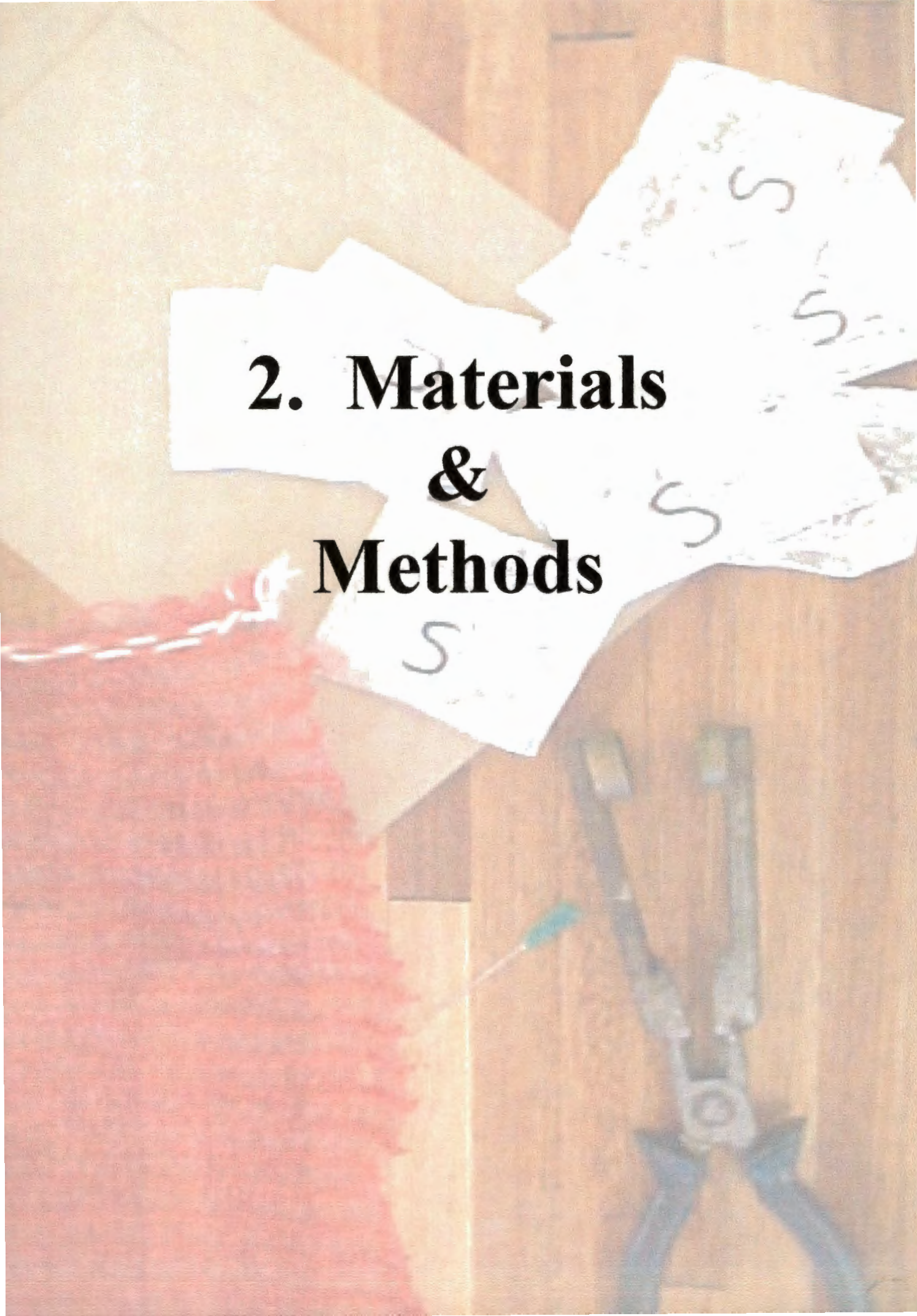
1.12 Research objectives

The objectives of this investigation were to:

1. Increase the current understanding of the physiological, biochemical and ultrastructural basis for the limitation of photosynthesis by dark chilling in soybean genotypes;
2. Compare the photosynthetic response to dark chilling in the presence or absence of root chilling;
3. Evaluate the ameliorating effects of nitrate treatment on photosynthetic limitation during dark chilling stress;

1.13 Research hypothesis

Since it is known that the chilling tolerance of soybean genotypes from temperate origin, based on agronomic criteria, is heterogeneous, it is hypothesised that a heterogeneous physiological and biochemical response to dark chilling must also exist. The project is based on the belief that genotypic differences in the response of photosynthesis to dark chilling would be significant enough to contribute towards a better understanding of the physiological and biochemical basis of dark chilling tolerance.



2. Materials & Methods

2.1 Plant material

Two soybean [*Glycine max* (L.) Merr] genotypes were used in this study. The genotypes were 'Maple Arrow' and 'Fiskeby V'. Seeds of both genotypes were obtained from the USDA-ARS Soybean germplasm collection (Urbana, IL 61801, USA). The choice of genotypes was based on the fact that both originate from temperate climates, exhibit comparable early maturity (maturity groups 00 and 000 respectively) under favorable conditions and are genetically related (Seddigh *et al.* 1988). A soybean genotype is placed in a maturity group according to its photoperiodic response and area of cultivation. These groups are 000, 00, 0 and I – VIII. Genotypes in maturity groups 000, 00 and 0 (early maturity groups) are cultivated in areas with short summer periods and long summer days such as southern Canada and northern USA. Genotypes in the late maturity groups such as VII and VIII are cultivated in tropical and sub-tropical climates. Genotypes in the early maturity groups are chilling tolerant compared to maturity group VII or VIII genotypes. Both these genotypes are regarded as chilling tolerant on the basis of pod formation, flower development and other reproductive traits (Hume & Jackson 1981; Seddigh *et al.* 1988; Gass *et al.* 1996). However, when these genotypes are exposed to a number of consecutive nights of dark chilling followed by exposure to relative low light intensities, evidence strongly suggests that the photosynthetic capacity of 'Maple Arrow' are less affected than in the case of 'Fiskeby V' (Van Heerden *et al.* 2002, 2003).

2.2 Growth conditions

Plants of both genotypes were cultivated in a computerised Conviron PGW 36 growth chamber (Controlled Environments Ltd., Winnipeg, MB. Canada R3H0R9). The pots were placed on metal racks, allowing water and nutrient solution to flow freely through the pots (Fig. 2.1). This ensured adequate drainage and also lowered the risk of pathogens. Plants were rotated on a daily basis to ensure that all plants were subjected to equal illumination during the growth period.

For all chilling stress experiments, seeds of both genotypes were planted in 1.5 dm^3 plastic pots with drainage holes in the bottom of the pots. The pots were filled with

sterile river sand after the bottom of each pot was lined with material filters to prevent the sand from leaking through the drainage holes. The seeds were inoculated with *Bradyrhizobium japonicum* (bacterial strain WB 74) at the time of sowing to ensure optimal root nodule development and biological nitrogen fixation (BNF). Four seeds were sowed in each pot. Plants were watered daily with distilled water to field capacity.

Illumination was supplied by a mixture of fluorescent (Sylvania Cool White VHO, 215W) and incandescent (General Electric, Neodymium R80, 100W) lamps. The light intensity inside the chamber was measured as 1000 µmol m^{-2} s^{-1} at the height of the pots. Seed germination and plant growth were conducted under a 15 h day period at 23°C and a 9 h night period at 20°C. The CO_2 concentration inside the growth chamber was controlled at 350 µmol mol^{-1}.

Figure 2.1. *Soybean plants were cultivated in a computerised growth chamber under high illumination.*

Plants were fertilised with two Hoagland nutrient solutions (Hoagland & Arnon 1950) of different nutrient composition. The one nutrient solution contained nitrate (+N solution) at the normal full strength concentration, while the other nutrient solution contained no mineral nitrogen (-N solution). The +N solution contained 2 mM $MgSO_4$, 1 mM KH_2PO_4, 90 µM Fe-EDTA, 5 mM $Ca(NO_3)_2$, 5 mM KNO_3 and micro nutrients, consisting of 46 µM H_2BO_3, 9 µM $MnCl_2 \cdot 4H_2O$, 0.81 µM ZnCl2, 0.293 µM $CuCl_2 \cdot 2H_2O$, 0.1 µM

$Na_2MoO_4 \cdot 2H_2O$. The –N solution contained 2 mM $MgSO_4$, 1 mM KH_2PO_4, 90 µM Fe-EDTA, 5 mM $CaCl_2$, 5 mM KCl and the recommended amount of micro nutrients. Half of the plants received the –N solution and the other half the +N solution. One hundred milliliters of nutrient solution were applied three times a week. On all remaining days, plants were watered with distilled water.

The contrasting N-levels were used to determine if supplementation of N_2-fixing soybean with nitrate had any beneficial effect on the photosynthetic capacity of the plant during and after exposure to chilling stress. It is known that nitrogen-fixing bacteria on the root system of leguminous plants like soybean are particularly sensitive to chilling stress and evidence suggests that addition of nitrate might help the plant to cope better under these conditions (Legros & Smith 1994).

2.3 Dark chilling treatments

2.3.1 Whole plant chilling

Plants of both genotypes were grown under the growth conditions described above (section 3.2) until the onset of the V3 growth stage (Fehr & Caviness 1977). At the V3 growth stage of a soybean plant, there is a mature trifoliate leaf situated at the second node above the unifoliate leaves. The trifoliate leaf at the third node is unfolded to such an extent that the edges of the pinnae do not touch. At the end of one of the light periods, half of the plants were transferred to a refrigerated chamber controlled at 8°C for one entire dark period (9 h), while the control plants were kept at 20°C. Fifteen minutes before the end of this dark period, the chilled plants were returned to the control growth chamber for the entire light period (15 h) at 23°C. This dark chilling treatment was repeated for up to four consecutive nights on the same set of plants.

2.3.2 Roots not subjected to chilling (shoot chilling)

In another set of experiments, plants were transferred to a refrigerated chamber controlled at 8°C for one entire dark period. However, in these experiments the pots (after sealing the drainage holes with rubber stoppers) were placed in a circulating water bath controlled at 20°C. By employing this strategy, only the leaves and stems of the plants

were dark chilled. The roots were not chilled in order to prevent the possibility of chill-induced drought stress during the day following the chilling episode. Chilling of roots decrease hydraulic conductivity which may result in leaf wilting upon rewarming (Markhart *et al.* 1979). The control plants were kept at 20°C for the night. Fifteen minutes before the end of this dark period, the shoot-chilled plants were returned to the control growth chamber for the entire light period at 23°C. The dark chilling treatment was repeated for up to four consecutive nights on the same set of plants.

2.4 Non-intrusive measurements

2.4.1 Quantification of vegetative development

Vegetative development was quantified by the plastochron index (PI), which is a rapid and non-intrusive method. The PI was developed by Erickson & Michelini (1957). A brief explanation of the PI is provided below:

The shoots of certain higher plants, including soybean, produce leaves at a constant rate. The time interval between the initiation of consecutive leaves is termed the "plastochron". The initial rate of leaf elongation is exponential, so when the natural logarithm of the length of successive leaves is plotted against time, a series of straight, parallel, and equally spaced lines are revealed. The spacing between these lines along the x-axis is constant and represents the plastochron, which in turn can be used as a measure of vegetative development (Fig. 2.2).

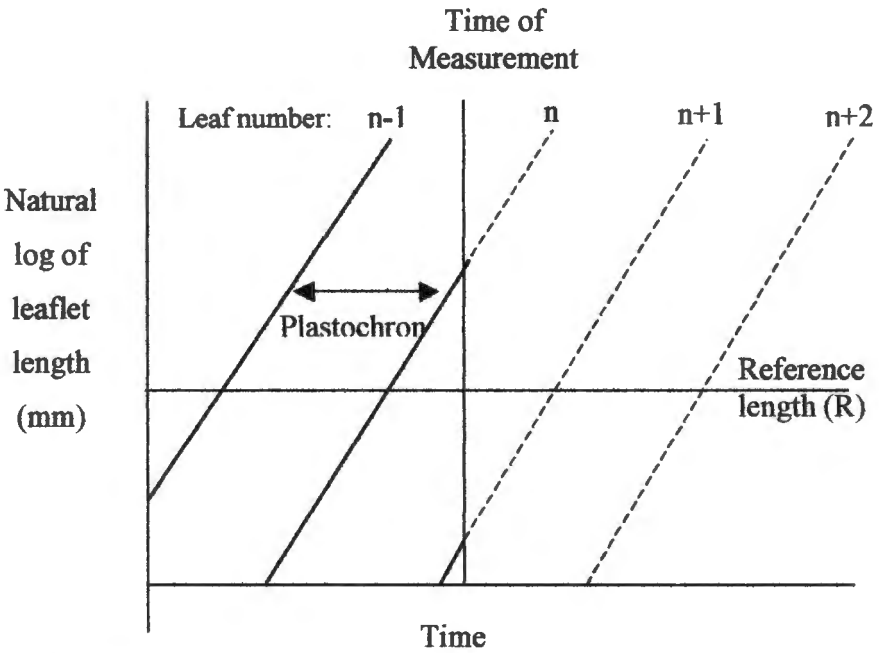


Figure 2.2. *Idealised representation of the natural logarithm of leaflet length of successive leaves over time (From Yourstone & Wallace 1990).*

The plastochron index can be calculated very easily (Yourstone & Wallace 1990). A leaf length, within the exponential phase of elongation that can be measured accurately, is chosen as a reference length (25 mm for most species). Leaves are assigned a serial number which corresponds to their order of appearance (L_1, L_2, L_3,.. . . L_n). The plastochron index of a plant is "n" when the "index leaf" (L_n) reaches the reference length of 25 mm. However, when the index leaf (L_n) is longer than the reference length and the next leaf (L_{n+1}) is shorter than the reference length, the plastochron index of the plant can be calculated according to the following formula:

$$\mathrm{PI} = n + \frac{\ln L_n - \ln REF}{\ln L_n - L_{n-1}}$$

where n is the serial number of leaf L_n and ln REF, ln L_n, and ln L_{n+1} is the natural logarithm of the reference length (25 mm), the length of the index leaf, and the length of the next smaller leaf, respectively. If the plastochron is constant, a plot of PI versus time

yields a straight line. Changes in the plastochron, as affected by factors such as temperature, can be monitored using PI versus time plots. The PI is considered a sensitive indicator of vegetative development and has been used extensively for various physiological studies (Lamoreaux *et al.* 1978). In the case of soybean, Handa & Yong Son (1974) determined that the central leaflet of a trifoliate leaf was easier to measure and produced less error than measuring the whole leaf. Therefore, each day, the central leaflet of the youngest trifoliate leaf 25 mm or longer and the central leaflet of the next leaf shorter than 25 mm was measured from the tip to the base of the lamina (Yourstone & Wallace 1990). Measurements were terminated at the onset of the dark chilling treatments.

The PI was determined to ensure that all dark chilling treatments commenced at the same stage of vegetative development in both genotypes and both N-treatments. Measurements were conducted each morning (2h after the start of the light period) on the same set of 20 plants (10 control and 10 for future dark chilling) from each genotype.

2.4.2 CO_2 assimilation

CO_2 assimilation was measured daily with a portable open circuit photosynthesis system (CIRAS-I, PP-systems, Hertz, UK) on the same set of 5 control and 5 dark chilled plants of both genotypes (Fig. 2.3).

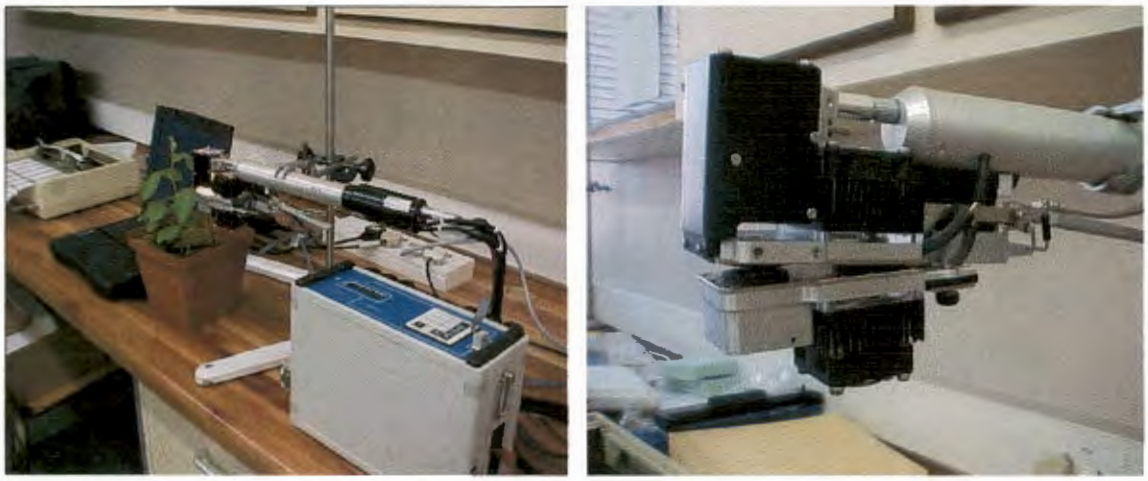


Figure 2.3. *CO_2 assimilation was measured in control and dark chilled plants with a portable photosynthesis system (left) and software-controlled broad leaf photosynthetic leaf chamber (right).*

Measurements started 3 h after the end of the dark period and were conducted at a leaf temperature of 23°C and a relative humidity close to ambient conditions. A 2.5 cm^2 section of the central leaflet of the youngest fully expanded trifoliate leaf was clamped in a software-controlled broad leaf photosynthetic leaf chamber (PLC). For the measurement of the relationship between CO_2 assimilation rate (A) and intercellular CO_2 concentration (c_i), a constant photosynthetic photon flux density (PPFD) of 1200 µmol m^{-2} s^{-1} was maintained in the PLC to ensure full activation of Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) during the measurements (Taylor & Terry 1984).

The operation of the CIRAS-I photosynthesis system is explained in Fig. 2.4.

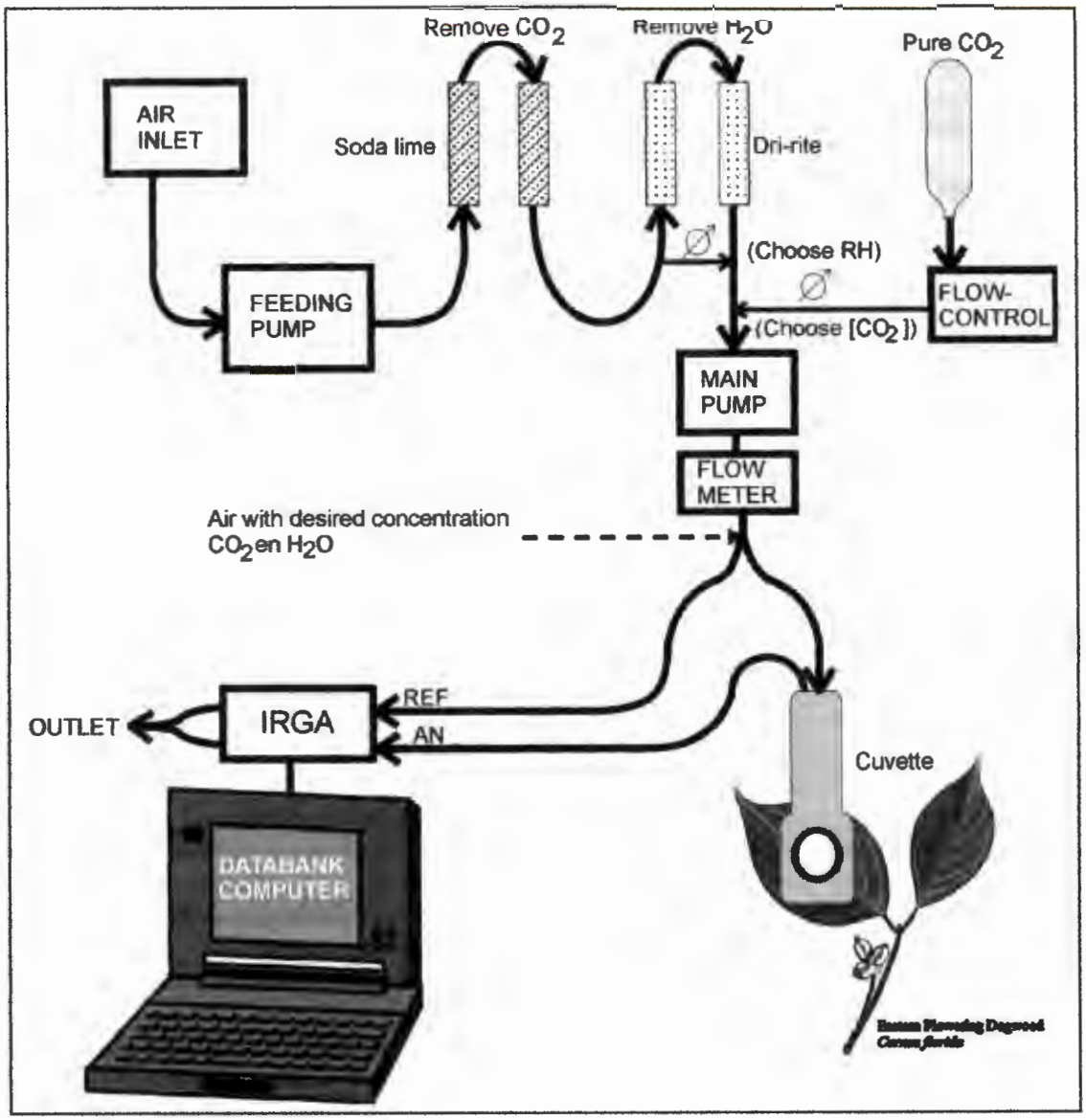


Figure 2.4. *The operation of the CIRAS-I photosynthesis system. The infrared gas analyser (IRGA) measures the difference in CO_2 concentration and water vapour of air directly imported into the IRGA (REF) and air imported through the photosynthetic leaf chamber (AN). This difference is then used to calculate CO_2 assimilation rate, stomatal conductance, intercellular CO_2 concentration and transpiration rate (From Krüger 2002).*

For the construction of A:c_i response curves, atmospheric CO_2 concentration (c_a) flowing over the leaf surface was increased with increments from 0 to 2000 µmol mol^{-1}. A typical A:c_i response curve and its main components is shown in Fig. 2.5.

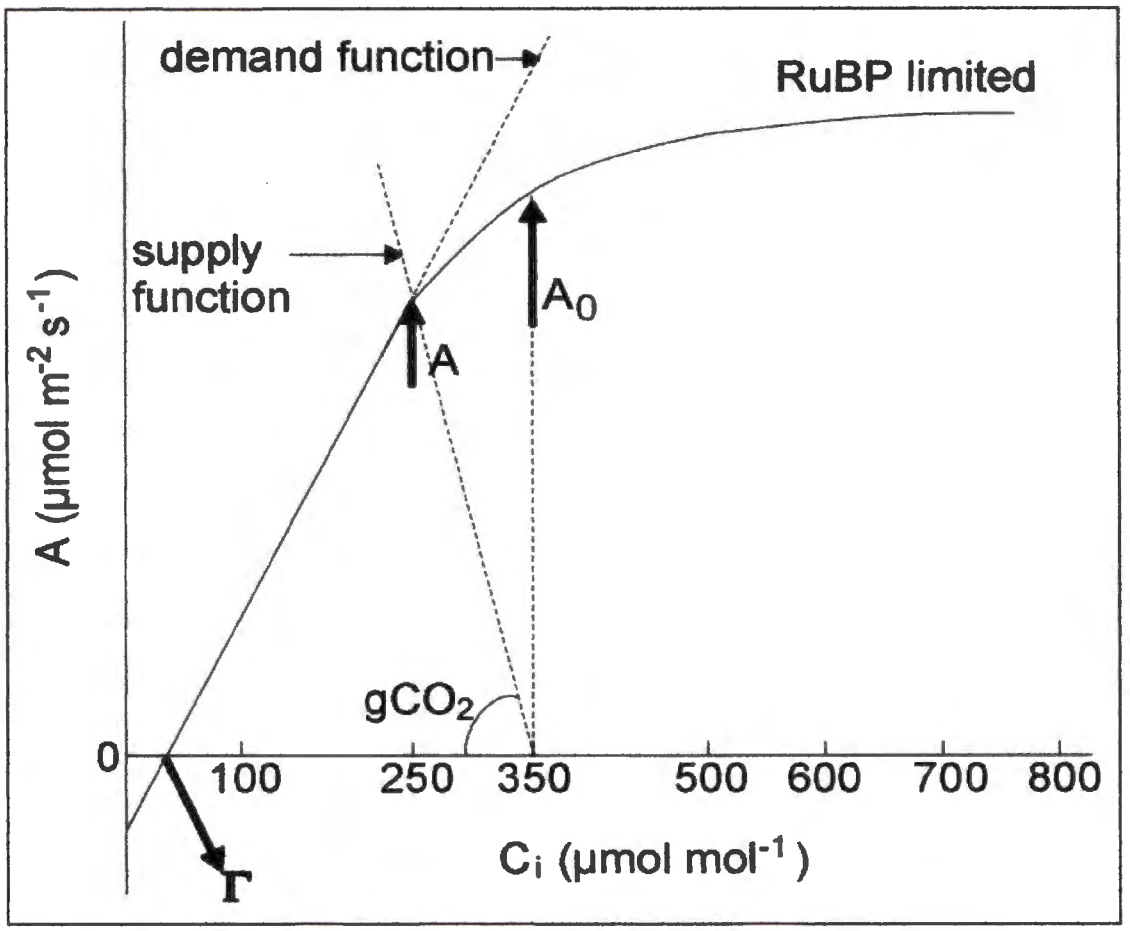


Figure 2.5. *A typical A:c_i response curve with its main component. (From Krüger 2002).*

A_0:	rate of photosynthesis when there is no stomatal resistance, in other words c_a=c_i (usually at c_i=350 µmol mol^{-1})
A:	rate of photosynthesis at c_a=350 µmol mol^{-1}
Γ:	CO_2 compensation concentration
Amax:	CO_2 saturated rate of photosynthesis

For each c_a, the ratio of A to c_i was automatically recorded. The initial slope ($\partial A/\partial c_i$) of the demand function of the A:c_i response (Fig. 2.5) at low c_i (CO_2 limiting) was computed by linear regression analysis. Relative stomatal limitation of photosynthesis (*l*) was calculated as the difference between the CO_2 assimilation rate when c_i = 350 µmol

mol^{-1} (A_0), and the actual rate at c_a = 350 µmol mol^{-1} (A), expressed as a percentage of A_0. All other calculations were done according to Farquhar & Sharkey (1982) and Chaves (1991).

2.5 Intrusive measurements

2.5.1 Ultrastructural studies

One hour before the end of the fourth night of dark chilling, the central leaflet of the youngest fully expanded trifoliate leaf of five control and five dark chilled plants was sampled for ultrastructural studies. Sampling occurred pre-dawn when starch levels in soybean leaves are at a minimum. The leaves were cut into 1 mm^2 pieces with a very sharp blade and placed in Todd's fixative (Todd 1986) for 24 h. The fixation schedule was conducted at room temperature. After 24 h, the leaf pieces were rinsed three times for 15 min in 0.05 M cacodylate buffer, followed by post-fixation with 1% aqueous osmium tetroxide (OsO_4) for 1 h. Buffers are used for dissolving fixatives, for the fixation of samples and for washing of samples between fixation steps. Osmium tetroxide is used as a secondary fixative to harden the tissue and as an electron dense stain. It stains tissue black and thus makes it more visible and easier to handle. The leaf pieces were then rinsed three times for 15 min in distilled water and stained for 30 min with 2% (w/v) uranyl acetate dissolved in distilled water. Tertiary fixatives like uranyl acetate, react fast with proteins and stabilise structures before extraction takes place.

All samples were dehydrated in an acetone series of 50%, 70% and 90% for 15 min and then twice for 15 min in 100% acetone. 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 three hours and then in 100% resin 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 the acetone was complete. The whole procedure was done in a fume cabinet, using a sample rotator. Each sample was then embedded in fresh 100% resin in a small flat mould and labeled (Fig. 2.6). Polymerisation took place at 65°C for 12 h.

Figure 2.6. *Embedded sample blocks for the study of the effects of dark chilling on leaf ultrastructure*

The embedded sample blocks were trimmed in the laboratory and ultra-thin cross sections (±0.5 µm thick) 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 (in 1% borax) at moderate heat, washed, dried and viewed under a light microscope. This was done to determine the quality of the fixation and the orientation of the sample. Ultra thin sections of ±100 nm were made and stretched with xylol vapour.

The sections were contrasted with 2% aqueous uranyl acetate for 5 min and lead citrate for 2 min (Reynolds 1963). To be of use in transmission electron microscopy (TEM) studies, a stain must have the ability to stop or strongly deflect the electrons of the electron beam so that they do not contribute to the final image. These stains are made up of heavy metal salts that are especially effective at deflecting electrons. One or more of

the sections were then placed on a G200 copper grid. Sections were observed and photographed with a Philips CM-10 transmission electron microscope operated at 80 kV.

2.5.2 Anatomical studies

One hour before the end of the fourth night of dark chilling, the central leaflet of the youngest fully expanded trifoliate leaf of five control and five dark chilled plants was sampled for anatomical studies. The leaves were cut in 4% formaldehyde (pH 7.4) with a very sharp blade to approximately 12 mm^2 sections. The leaf sections were then fixed overnight in formaldehyde after air trapped in the leaves was removed with a vacuum pump. The leaf sections were rinsed 3 times in 70% ethanol for 15 min and twice in absolute ethanol for 24 h. The leaf sections were then incubated twice in n-propanol for 24 h, and twice in n-butanol for 24 h. Pieces of wax were added to the n-butanol solution and which was then placed overnight in an oven (45°C). The next day excess n-butanol was allowed to evaporate. As soon as the n-butanol had evaporated, it was replaced twice with fresh wax. The leaf sections were embedded in the wax, which was then allowed to set. The sections were cut (8 µm thick) and stained with haematoxylin and safranine according to the method of Warmke & Lee (1976). The method was adjusted for staining wax sections. The sections were examined with a light microscope (Olympus BH2) connected to a computer by means of a video camera. Intercellular spaces were measured by FIPS (Flexible Image Processing System, CSIR, South Africa, version 2.15).

2.5.3 Analysis of inorganic element concentrations in leaf tissue

One hour before the end of the fourth night of dark chilling, and again 4 h after the start of the light period, the central leaflet of the youngest fully expanded trifoliate leaf of five control and five dark chilled plants was sampled. The leaves were dried between sheets of filter paper in a cool dry place.

When dried, the leaves were ground to a fine powder and mounted onto carbon double-sided conductive tape on a stub and examined under a scanning electron microscope (Philips XL 30) in conjunction with a software program (EDXAUTO version 1.8), which estimates the inorganic element concentrations in samples.

X-ray microanalysis gives information about the elemental composition of the specimen in terms of both quantity and distribution. The interaction of a high-energy electron with an atom may result in the ejection of an electron from an inner atomic shell. This leaves the atom in an ionized or excited state, with a vacancy in this shell. Di-excitation can occur by an electron from an outer shell filling the vacancy. The change in energy is determined by the electronic structure of the atom which is unique to the element. This "characteristic" energy can be released from the atom in two ways. One way is the emission of an X-ray photon with a characteristic energy specific to that transition and hence, to the element. The second way is releasing so called auger electrons. An X-ray spectrometer collects the characteristic X-rays. The spectrometer counts and sorts the X-rays, usually on the basis of energy (Energy Dispersive Spectrometry EDS). The resulting spectrum plots number of X-rays, on the vertical axis, versus energy, on the horizontal axis. Peaks on the spectrum correspond to the elements present in the sample.

2.6 Biochemical analysis

2.6.1 Sampling procedures

One hour before the end of each of the three consecutive nights of dark chilling, 3.14 cm^2 leaf discs were collected with a freeze clamp (cooled in liquid nitrogen) from the youngest fully expanded trifoliate leaves of six control and six dark chilled plants. Sampling occurred under dim green illumination. Leaf discs were again collected at 2-3 h intervals during the subsequent light period. During the day, sampling occurred at a light intensity of 1000 µmol m^{-2} s^{-1} and at 23°C. During daytime sampling, special care was taken not to shade the portion of leaf being sampled in order to prevent the deactivation of certain light-activated PCR cycle enzymes. All leaf discs were stored at –85°C for biochemical analysis. In the experiments where the effect of nitrate supplementation on the dark chilling response was investigated, sampling occurred only during the day following the third night of chilling.

2.6.2 Determination of initial and maximum ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity

Initial and maximum Rubisco activity was measured in leaf discs according to the method of Keys & Parry (1990). Initial activity is the activity of the enzyme under the growth conditions at the time of sampling. Maximum activity was obtained by activation of the extracted enzyme with bicarbonate after removal of all known tight-binding inhibitors of Rubisco. Each leaf disc was ground to a fine powder with liquid nitrogen in a pre-cooled mortar and rapidly extracted with 0.8 ml ice-cold extraction buffer containing 100 mM Bicine-NaOH (pH 8.0), 20 mM $MgCl_2$, 50 mM β-mercaptoethanol, 6 mM phenylmethylsulfonyl fluoride (PMSF) and 30 mg acid washed insoluble polyvinylpolypyrrolidone (PVPP). All pH adjustments were made with NaOH low in carbonate content. The crude extract was transferred to a pre-cooled micro centrifuge tube and centrifuged at 10 000 x g at 4°C for 1 min with an Eppendorf centrifuge (model 5804R) (Fig. 3.6 a & b). The clarified supernatant was immediately used for the measurement of initial Rubisco activity. The time between extraction and the start of the measurement was not more than 2 min. Initial Rubisco activity was measured in a scintillation vial in a total volume of 0.5 ml containing 100 mM Bicine-NaOH (pH 8.2), 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (0.5 μCi μmol^{-1}), 400 μM ribulose-1,5-bisphosphate (RuBP) and 25 μl leaf extract. After 1 min the reaction was terminated by addition of 200 μl 10 M formic acid.

Maximum Rubisco activity was measured after first incubating 100 μl leaf extract for 30 min in an equal volume of a solution containing 600 mM Na_2SO_4, 100 mM Bicine-NaOH (pH 8.0), 20 mM $MgCl_2$ and 50 mM β-mercaptoethanol. Incubation in the presence of Na_2SO_4 results in the removal of all known tight-binding inhibitors from Rubisco catalytic sites. After 30 min of incubation, 200 μl 60% (w/v) polyethylene glycol 4000 (PEG 4000) was added and the Rubisco protein precipitated for 30 min. The extract was then centrifuged at 10 000 x g at 4°C for 10 min and the supernatant (containing the unbound inhibitors) discarded. The pellet was washed with 0.5 ml of a solution containing equal volumes of extraction buffer and 60% PEG 4000 for 2 min and centrifuged at 10 000 x g at 4°C for 10 min and the supernatant discarded. The pellet was

finally resuspended in 100 µl extraction buffer and 25 µl used to measure maximum Rubisco activity. After a 3 min incubation period in reaction mixture containing 100 mM Bicine-NaOH (pH 8.2), 20 mM $MgCl_2$ and 10 mM $NaH^{14}CO_3$ to fully activate the enzyme, 400 µM RuBP was added and the reaction terminated after 1 min with 10 M formic acid. Acidified samples were evaporated to dryness in an oven. After addition of 0.4 ml distilled water and 3.5 ml scintillation cocktail (Packard Ultima Gold) to each scintillation vial, the ^{14}C incorporated into 3-phosphoglycerate was determined by liquid scintillation spectrometry using a liquid scintillation analyser (Beckmann LS 6000TA). The soluble protein content of the original supernatants (used for the measurement of initial activity) and resuspended pellets (used for the measurement of maximum activity) was determined with a spectrophotometer (Spectronic, Genesys 2) (Fig 2.7 c) according to the method of Bradford (1976).

2.6.3 Bradford Protein estimation

A 1 mg ml^{-1} stock solution of bovine serum albumin (BSA) was prepared, aliquoted in 1 ml quantities and frozen at -20°C. To correct for impurities in the BSA reagent, the actual protein content of this stock solution was quantified by measuring the absorbance at 280 nm in a quartz cuvette.

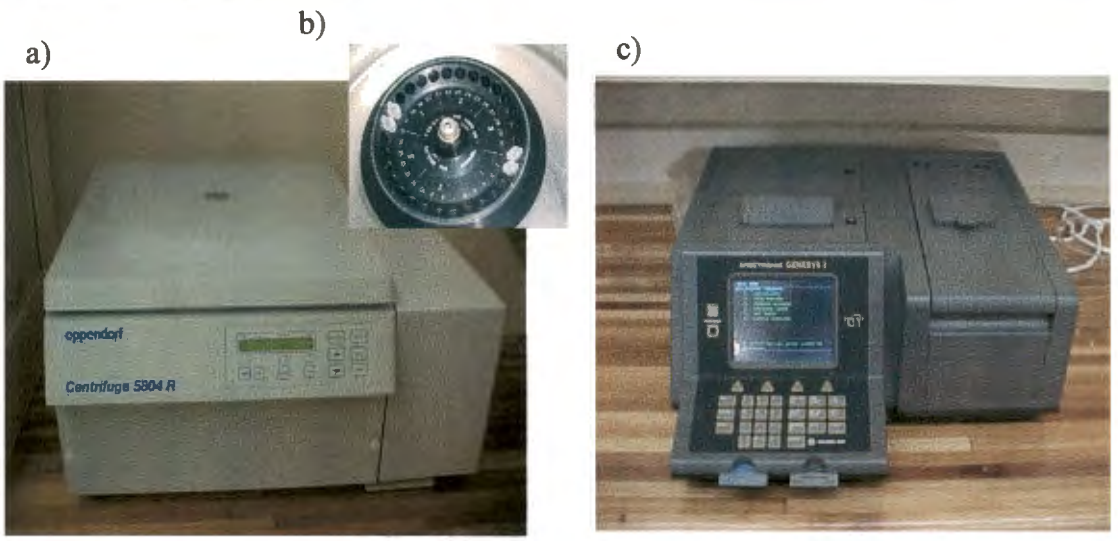


Figure 2.7. *Eppendorf centrifuge (model 5804R) with rotor (a & b) and Spectronic Genesys 2 spectrophotometer (c)*

Protein dye reagent (Bradford reagent) was prepared by dissolving 100 mg Coomassie Brilliant Blue G250 in 50 ml absolute ethanol and 100 ml 85% orto-phosphoric acid. Distilled water was then added to a final volume of 1 dm^3. The Bradford reagent was filtered and stored in the dark where it was stable for up to 4 months. The BSA stock solution was used to prepare a standard series of between 0 and 20 µg BSA in a total volume of 0.5 ml in micro centrifuge tubes. Bradford reagent (0.5 ml) was added to each tube, and the absorbance measured at 595 nm after 5 min. A standard curve was constructed and it was determined that a linear relationship ($R^2 > 0.95$) existed between absorbance and protein concentration between 2 - 15 µg. The concentration of BSA in this stock solution was 0.952 mg ml^{-1}.

2.6.4 Total chlorophyll content

Total chlorophyll content of leaf samples was determined using aliquots (50 µl) of the crude extracts (prior to centrifugation) according to the method of Wintermans & de Mots (1965). Fifty microliters of crude extract (prior to centrifugation) was added to 0.95 ml 96% (v/v) ethanol, thoroughly mixed, and then centrifuged at 10 000 x g for 5 min at 4°C. The absorbance of the chlorophyll containing supernatant was measured at 654 nm. The chlorophyll content was calculated with the following formula:

$$Chlorophyll\ content\ \left(\mu g\ ml^{-1}\right) = \frac{1000 \times A^{654}}{39.8}$$

2.6.5 Measurement of stromal fructose-1,6-bisphosphatase (FBPase) activity

Initial and total stromal FBPase activity was measured in leaf discs according to the method of Leegood (1993). Initial activity is the activity of the enzyme under the growth conditions at the time of sampling. Total activity was measured after incubation of the extracted enzyme at 25°C in the presence of dithiothreitol (DTT).

Each leaf disc was ground to a fine powder with liquid nitrogen in a pre-cooled mortar and rapidly extracted with 0.8 ml ice-cold extraction buffer containing 100 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% (v/v) Triton X-100, 6

mM PMSF, 2 mM benzamidine and 30 mg PVPP. The crude extract was transferred to a pre-cooled micro centrifuge tube and centrifuged at 10 000 x g at 4°C for 1 min. The supernatant was immediately used for the measurement of initial FBPase activity. The time between extraction and the start of the measurement was not more than 2 min.

Initial FBPase activity was measured in a total volume of 1 ml containing 100 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 0.05% (v/v) Triton X-100, 200 µM NAD, 3 units glucose-6-phosphate-dehydrogenase (G6PDH), 4 units phosphoglucose isomerase (PGI) and 50 µl supernatant. The reaction was started by the addition of 1 mM fructose 1,6-bisphosphate (FBP) and the rate of NAD reduction measured at 340 nm and 25°C.

Total FBPase activity was measured as described previously, but the reaction was only started after a 10 min incubation of 50 µl supernatant with 10 µl 0.5 M DTT containing 0.015% (v/v) Triton X-100. An incubation period of 10 min in the presence of DTT was found to be adequate for complete activation of stromal FBPase extracted from soybean leaves. After the 10 min incubation period, total FBPase activity was assayed in the presence of 100 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 0.05% (v/v) Triton X-100, 200 µM NAD, 3 units G6PDH and 4 units PGI. The reaction was started by the addition of 1 mM FBP and the rate of NAD reduction measured at 340 nm and 25°C. The soluble protein content of the supernatant was determined according to the method of Bradford (1976).

2.6.6 Measurement of NADP-dependent malate dehydrogenase (NADP-MDH) activity

Initial and total NADP-MDH activity was measured in leaf discs according to the method of Leegood (1993). The initial activity is the activity of the enzyme under the growth conditions at the time of sampling. Total activity was measured after incubation of the extracted enzyme at 25°C in the presence of DTT. The same extraction procedure used for FBPase was also used for NADP-MDH.

Initial NADP-MDH activity was measured in a total volume of 1 ml containing 100 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.05% (v/v) Triton X-100, 200 µM NADPH and 50 µl supernatant. The reaction was started by the addition of 500 µM neutralised (with KOH) oxaloacetic acid (OAA) and the rate of NADPH oxidation measured at 340 nm and 25°C.

Total NADP-MDH activity was measured after a 15 min incubation period at 25°C of 50 µl supernatant with 10 µl 0.5 M DTT containing 0.015% (v/v) Triton X-100. An incubation period of 15 min was found to be adequate for complete activation of NADP-MDH extracted from soybean leaves (Van Heerden *et al.* 2003). After the incubation period, total NADP-MDH activity was assayed in the presence of 100 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.05% (v/v) Triton X-100 and 200 µM NADPH. The reaction was started by the addition of 500 µM OAA.

2.6.7 Detection of proteins by Western Blot analysis

2.6.7.1 Extraction of proteins

Six leaf discs from each treatment were ground together to a fine powder with liquid nitrogen in a pre-cooled mortar and extracted with 3 ml ice-cold extraction buffer containing 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 3 mM DTT, 6 mM PMSF and 30 mg PVPP. An aliquot of the crude extract was transferred to a pre-cooled micro centrifuge tube and centrifuged at 10 000 x g at 4°C for 15 min. The soluble protein content of the supernatant was immediately determined according to the method of Bradford (1976).

The total soluble protein in the supernatant was diluted with ultra pure water to a concentration of either 4 µg (for the detection of Rubisco) or 10 µg (for the detection of FBPase and SBPase) in a final volume of 50 µl. Fifty µl SDS-PAGE sample buffer containing 125.5 mM Tris-HCl (pH 6.8), 10% (w/v) SDS, 0.287 mM β-mercapto-ethanol, 0.015 mM bromophenol blue and 20% (v/v) glycerol was added and the sample boiled for 5 min. Ten microliters of SDS-PAGE sample buffer was also added to 10 µl of western blotting molecular weight marker proteins and boiled for 5 min. The molecular weights of these marker proteins were 10, 20, 30, 45, 75 and 100 kDa. The boiled

samples and markers proteins were centrifuged at 10 000 x g and 4°C for 5 min and stored at –85°C.

2.6.7.2 Separation of proteins with SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of proteins and protein subunits. By this technique the proteins are denaturated with SDS, β-mercapto-ethanol and boiling.

Proteins were separated by SDS-PAGE with a mini-gel system (Mighty Small II, SE 250, Hoefer Scientific Instruments, San Francisco). The running gel solution contained 12% (w/v) acrylamide, 0.3% (w/v) bisacrylamide and 0.381 M Tris-HCl (pH 8.8) and was degassed prior to casting. The gel was casted between the glass plates after addition of 61 µl 10% (w/v) SDS, 61 µl 10% (w/v) ammonium persulphate and 10 µl TEMED. The stacking gel solution contained 4% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 0.118 M Tris-HCl (pH 6.8) and was degassed prior to casting. The gel was casted on top of the polymerised running gel after addition of 40 µl 10% (w/v) SDS, 21 µl 10% (w/v) ammonium persulphate and 20 µl TEMED. A plastic gel comb was immediately inserted into the stacking gel solution to form 10 sample wells in which the protein extractions and marker proteins were loaded. After the stacking gel has polymerised, the gel comb was carefully removed and the sample wells rinsed thoroughly with SDS running buffer consisting of 78 g glycine, 15.15 g Tris and 5 g SDS in 5 dm^3 distilled water. In sample wells 2 - 8, 10 µl extract (containing either 0.4 µg total soluble protein for Rubisco or 1 µg total soluble protein for FBPase and SBPase) was loaded with a micro syringe, while 10 µl of the marker proteins were loaded in sample wells 1 and 10. Proteins were separated for 75 min at 200 V and 4°C (Fig. 2.8).

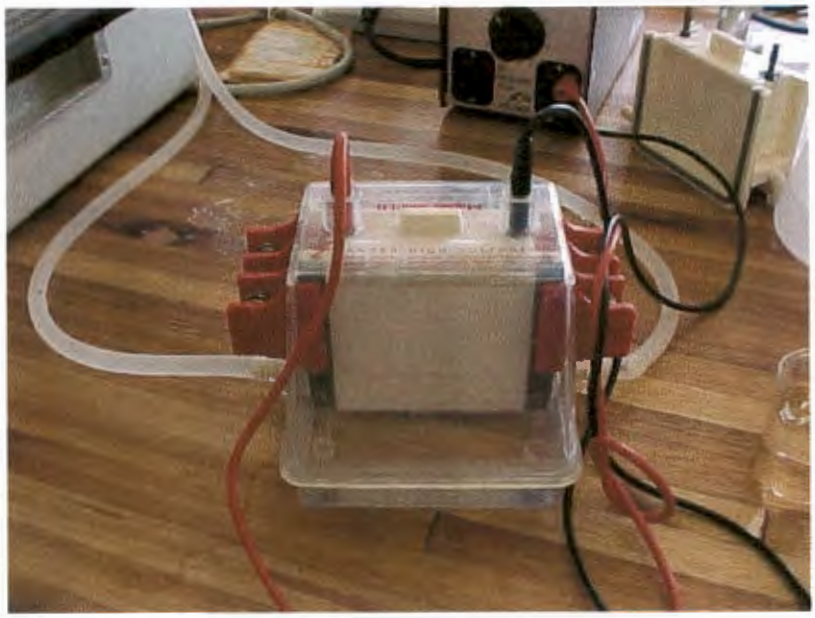


Figure 2.8. *Mini gel system used for the separation of proteins with SDS-PAGE.*

2.6.7.3 Protein transfer

After separation with SDS-PAGE, proteins were transferred from the running gel to a PVDF western blotting membrane (pore size of 0.45 μm). The high binding capacity, high mechanical strength and chemical resistance of the PVDF membrane makes it especially useful for protein transfer. Prior to protein transfer, the membrane was moistened with methanol for 3 s and then rinsed in ultra pure water twice for 1 min. The membrane, as well as two double layers of filter paper (Whatmann #1), was then soaked in transfer buffer containing 48 mM Tris, 39 mM glycine, 20% (v/v) methanol and 1.3 mM SDS for 30 min. The running gel was carefully removed from the glass plates and soaked for 1 min in transfer buffer. The membrane was placed on top of a double layer of filter paper on a semi-dry blotter (Trans-Blot SD, Bio-Rad Laboratories). The gel was then placed on top of the membrane and covered with a double layer of filter paper. Care was taken to remove any air bubbles between the different layers by using a small glass rod. Protein transfer from the gel to the membrane was conducted for 30 min at 15 V.

2.6.7.4. Binding of primary antibodies, blocking and labeling with secondary antibody

The PVDF membrane containing the transferred proteins was washed twice for 5 min in TBS [50 mM Tris-HCl (pH 7.5) and 150 mM NaCl]. The membrane was then blocked for

non-specific binding of antibodies by incubation (with gentle shaking) in 1% blocking solution [BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit), Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany] for 1 h. The membrane was then incubated for 1 h in 0.5% blocking solution containing the primary antibody specific to Rubisco, FBPase or SBPase at the recommended dilutions. The primary antibodies were all raised in rabbits and were received as generous gifts from three different sources. The source of these antibodies, recommended dilutions and amount of protein loaded onto the gel are outlined in table 2.1:

Table 2.1. *Technical information regarding the three different primary antibodies used*

Protein	Donor animal	Source of antibody	Amount of protein loaded on gel (µg)	Dilution used
Rubisco	Rabbit	Prof. Karl Kunert, FABI, University of Pretoria, Pretoria, South Africa	0.4	1 : 1000
FBPase	Rabbit	Dr. Tony Ashton, CSIRO Division of Plant Industry, Canberra, Australia	1.0	1 : 500
SBPase	Rabbit	Prof. Christine Raines, University of Essex, United Kingdom	1.0	1 : 1000

The membrane was washed three times in TBST [50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% (v/v) Tween 20] for 10 min, and twice with 0.5% blocking solution for 10 min. The membrane was then incubated for 30 min with 40 mU ml^{-1} horseradish peroxidase (POD)-labeled secondary antibody and 25 mU ml^{-1} antibody specific to the marker proteins, diluted in 0.5% blocking solution. The membrane was then washed four times with large volumes of TBST for 15 min each time to remove any unbound secondary antibody.

2.6.7.5 Chemiluminescent detection of labeled proteins

The whole procedure was conducted in a dark room. Excess buffer was drained off and the membrane placed in a tray with the protein-containing side facing upward. The pre-mixed detection reagent, luminescence substrate solution and starting solution (BM Chemiluminescence Western Blotting Kit) was added in a ratio of 100:1 and the membrane incubated for 60 s. Excess detection reagent was drained off and the membrane wrapped in Saran wrap. The membrane was inserted, protein-containing side facing upward, into a X-ray film cassette with a sheet of ECL Hyperfilm (High Performance Chemiluminescence Film, Amersham-Pharmacia Biotech, UK) on top off the membrane. The film was exposed for 10 s in the cassette. The film was then soaked in developer reagent (Ilford Phenisol, Ilford Imaging Limited, Mabberley Cheshire, England) for 5 min, in 3% acetic acid (stop reagent) for 1 min and in a standard fixer reagent for 2 min and then allowed to dry.

2.6.7.6 Optimising of Western blot procedure

The optimal amount of protein to be loaded on the gel for each of the primary antibodies (see Table 2.1) was determined. Amounts of protein, varying between 0.1 μg and 25 μg, were loaded on gels, separated by SDS-PAGE and then reacted with each of the primary antibodies. Generally it was found that below a certain protein amount loaded, specific to each primary antibody, bands were not clearly visible, while a too high amount of protein often resulted in non-specific binding of the primary antibody to other proteins. A good example of this is shown in Fig. 2.9 for the primary antibody specific to FBPase. When more than 2 μg of protein was loaded onto the gel, non-specific binding can be clearly seen. In Table 2.1 the optimal amounts of protein to be loaded on the gel for each of the primary antibodies are indicated. In the case of the FBPase primary antibody, two bands of slightly different molecular weights (44 kDa and 40 kDa) were also detected, even when only 1 μg of protein was loaded (Fig. 2.9).

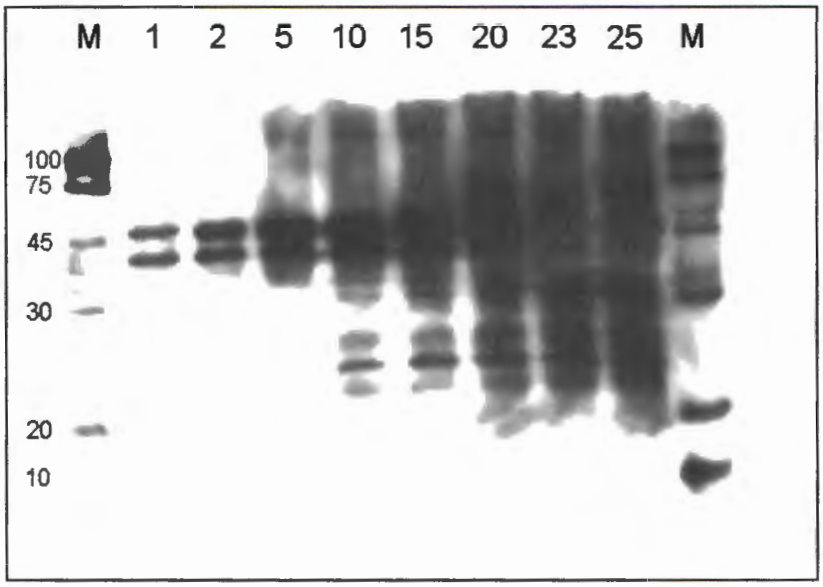


Figure 2.9. *Western Blot of FBPase with varying amounts of protein loaded onto the gel. The amounts (μg) of protein loaded in each sample well are indicated at the top of the figure. M = marker proteins used, with their respective molecular weights indicated.*

According to Raines *et al.* (1991), the FBPase holoenzyme is a homotetramer (4 subunits of identical molecular weight) in species investigated to date, although information for soybean is absent. If this is also true for soybean, there should theoretically be only one visible band when separated with SDS-PAGE. To test the possibility that one of the two bands was an artifact of non-specific binding, proteins were also extracted and separated under non-denaturing conditions (native-PAGE) to ensure that the holoenzyme remained intact. After cross-reaction with the FBPase primary antibody, only one band was revealed (Fig. 2.10), indicating that non-specific binding was not a likely cause of the two bands revealed under denaturing conditions. It appears that in the case of soybean, FBPase consists of four subunits of not precisely the same molecular weight, or the size of the subunits is altered slightly during the extraction procedure.

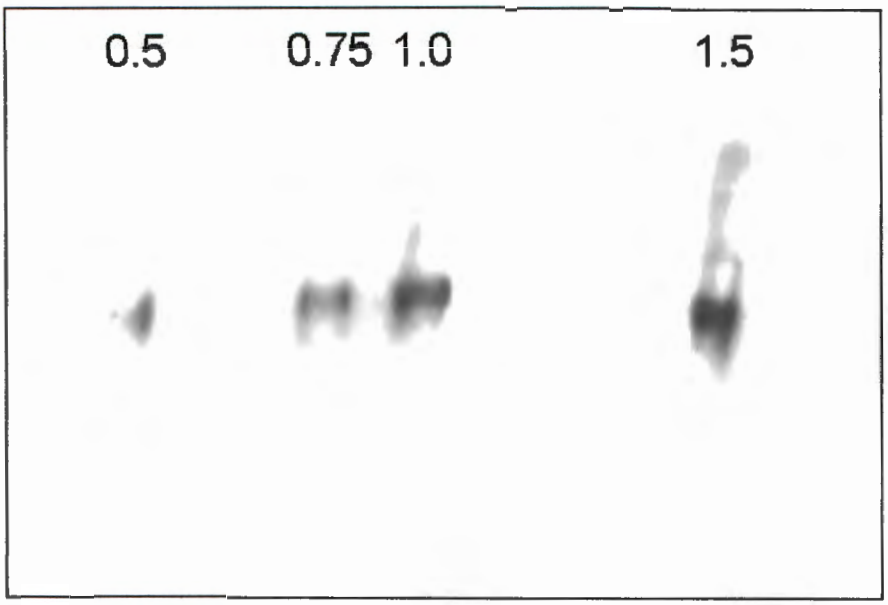


Figure 2.10. *Western blot of a native-PAGE gel, with varying amounts of protein loaded, and cross-reacted with FBPase primary antibody. The amounts (μg) of protein loaded in each sample well are indicated at the top of the figure.*

2.7 Data analysis

The software package, Statistica for Windows (version 6.0), was used for all statistical analysis. The Shapiro-Wilk W test was used in testing for normality (Shapiro *et al.* 1968). In data sets with parametric distributions, significant differences between treatment means were determined through analysis of variance (ANOVA) followed by post-hoc comparisons with the Tukey Honest Significant Difference (HSD) test. In data sets with non-parametric distributions, significant differences between treatment means were determined with the Kruskall-Wallis ANOVA (Kruskall 1952). Graphical presentation and curve fitting of data was performed with the software package SigmaPlot for Windows (2000 edition). Data analysis of Western blots was done using a Syngene Genius Bio Imaging System (Syngene, Beacon House, Nutfield Road, Cambridge CB4 1TF, UK). The software used to photograph the Western blots was GeneSnap (version 5.0) and the software used for estimating the protein content on the blots was GeneTools (version 3.00).

3. Results

3.1 The effect of dark chilling of roots and shoots on photosynthesis in N_2-fixing soybean plants

3.1.1 CO_2 assimilation

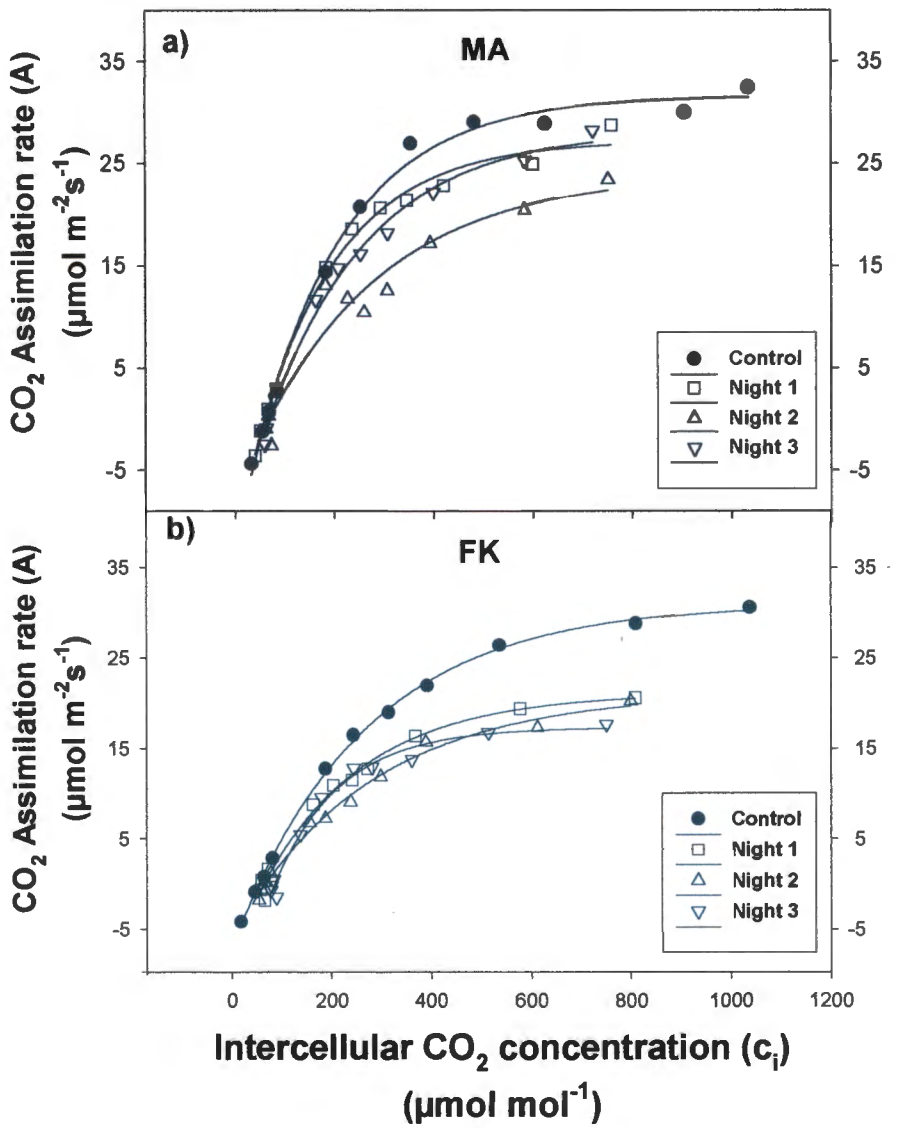


Figure 3.1. Relationship between CO_2 assimilation rate (A) and intercellular CO_2 concentration (c_i) in leaves from 'Maple Arrow' (a) and 'Fiskeby V' (b) following each of three consecutive nights of dark chilling. Measurements started 3 h after the end of each dark period and were conducted at a leaf temperature of 23°C and a light intensity of 1200 µmol m^{-2} s^{-1}. Data points represent the mean of 4 replicates.

Plants of 'Maple Arrow' (MA) and 'Fiskeby V' (FK) were dark chilled at 8°C for three consecutive nights. In this experiment, whole potted plants, including the roots, were chilled. Following each night of chilling, CO_2 assimilation measurements were conducted on these plants. The relationship between CO_2 assimilation rate (A) and intercellular CO_2 concentration (c_i) in response to dark chilling is shown in Fig. 3.1. Dark chilling induced changes in both the CO_2-limited and RuBP-limited regions of the A:c_i response curves for both MA (Fig. 3.1 a) and FK (Fig. 3.1 b). In order to compare the effects of dark chilling on the response in the two genotypes, the photosynthetic parameters deducible from the constructed A:c_i curves, were calculated and are summarised in table 3.1.

Table 3.1. The effect of dark chilling on key photosynthetic parameters calculated from the A:c_i response curves shown in Fig. 3.1. For each parameter the percentage change relative to the control value is shown. CE = carboxylation efficiency (the initial slope of the demand function); Γ = CO_2 compensation concentration; A_{max} = CO_2 saturated rate of photosynthesis; A = CO_2 assimilation rate at c_a = 350 µmol mol^{-1}; g_s = stomatal conductance at c_a = 350 µmol mol^{-1}; c_i = intercellular CO_2 concentration at c_a = 350 µmol mol^{-1}. Significant differences between the control and comparable treatment means are indicated by * and ** ($p < 0.05$ and $p < 0.01$ respectively) while ns = no significant difference.

'Maple Arrow'	**% Change**					
	CE	**Γ**	**A_{max}**	**A**	**g_s**	**c_i**
Control	0.1 ± 0.002	62.3 ± 3.7	32.5 ± 0.9	20.8 ± 1.5	515.9 ± 74.8	253.9 ± 9.7
Night 1	ns	ns	ns	ns	ns	ns
Night 2	-37**	ns	-28**	-43**	-64*	ns
Night 3	ns	ns	-13**	-29*	-60*	-17**
'Fiskeby V'						
	CE	**Γ**	**A_{max}**	**A**	**g_s**	**c_i**
Control	0.1 ± 0.003	56.4 ± 1.4	30.6 ± 1.2	16.5 ± 1.1	324.4± 31.7	245.6 ± 5.7
Night 1	ns	+18**	-36**	-41*	-62**	-20**
Night 2	-22*	+28**	-39**	-58**	-77**	-25**
Night 3	ns	+58**	-40**	-49**	-74**	-31**

In MA, a single night of dark chilling did not induce any significant change in the A:c_i response while large effects were already found in FK. In FK, a significant ($p < 0.01$) increase in the CO_2 compensation concentration (Γ) of 18% - 58% was observed during

the whole treatment period. In contrast, no significant increase was observed in MA. Large reductions in CO_2 saturated rates of photosynthesis (A_{max}) were observed, primarily in FK, where reductions of 36% - 40% persisted for the entire treatment period. Smaller reductions in A_{max} was observed in MA only after the second (28%) and third (13%) night of dark chilling. The decrease in A_{max} is commonly regarded as a symptom of reduced RuBP regeneration capacity by the stromal bisphosphatases (FBPase and SBPase) operating in the regeneration phase of the PCR cycle (Farquhar & Sharkey 1982). Distinct inhibition due to chilling (41% - 58%) of CO_2 assimilation rates (A) at c_a = 350 µmol mol^{-1} was observed in FK. In MA, no effect on A was observed after the first night. Whereas A was decreased by 49% after the third night in FK, MA only showed a decrease of 29%. Dark chilling of whole potted plants resulted in a significant and similar decrease in stomatal conductance (g_s) in both FK (after all three nights) and MA (only after the second and third night) of 60% - 74%. The decrease in A was accompanied by a decrease in intercellular CO_2 concentration (c_i) in MA only after the third night (17%) but throughout the treatment period in FK (20% - 31%). Analysis of the A:c_i response curves revealed that the decrease in the RuBP-limiting region of the response curve (indicated by A_{max}) was much more severe than the decrease in the CO_2-limiting region (indicated by the carboxylation efficiency, CE). This was especially evident in FK where A_{max} was decreased by 36% - 40% throughout the treatment period, while the decrease in CE that was observed only after the second night, did not exceed 22%. If we assume that limitations to CO_2 diffusion between the intercellular spaces and carboxylation sites are small compared to the limitation resulting from the carboxylation step itself, CE should be directly proportional to the Rubisco activity within the leaf (Von Caemmerer & Farquhar 1981). When comparing the response of A and A_{max} to dark chilling, an important difference between the two genotypes were observed. In MA significant acclimation to dark chilling was observed between the second and third night. For example, the decrease in both A and A_{max} after the third night (29% and 13% respectively) was considerably less than after the second night (43% and 28% respectively). In FK, only slight acclimation with respect to A was observed.

3.1.2 Stromal fructose-1,6-bisphosphatase (FBPase) activity

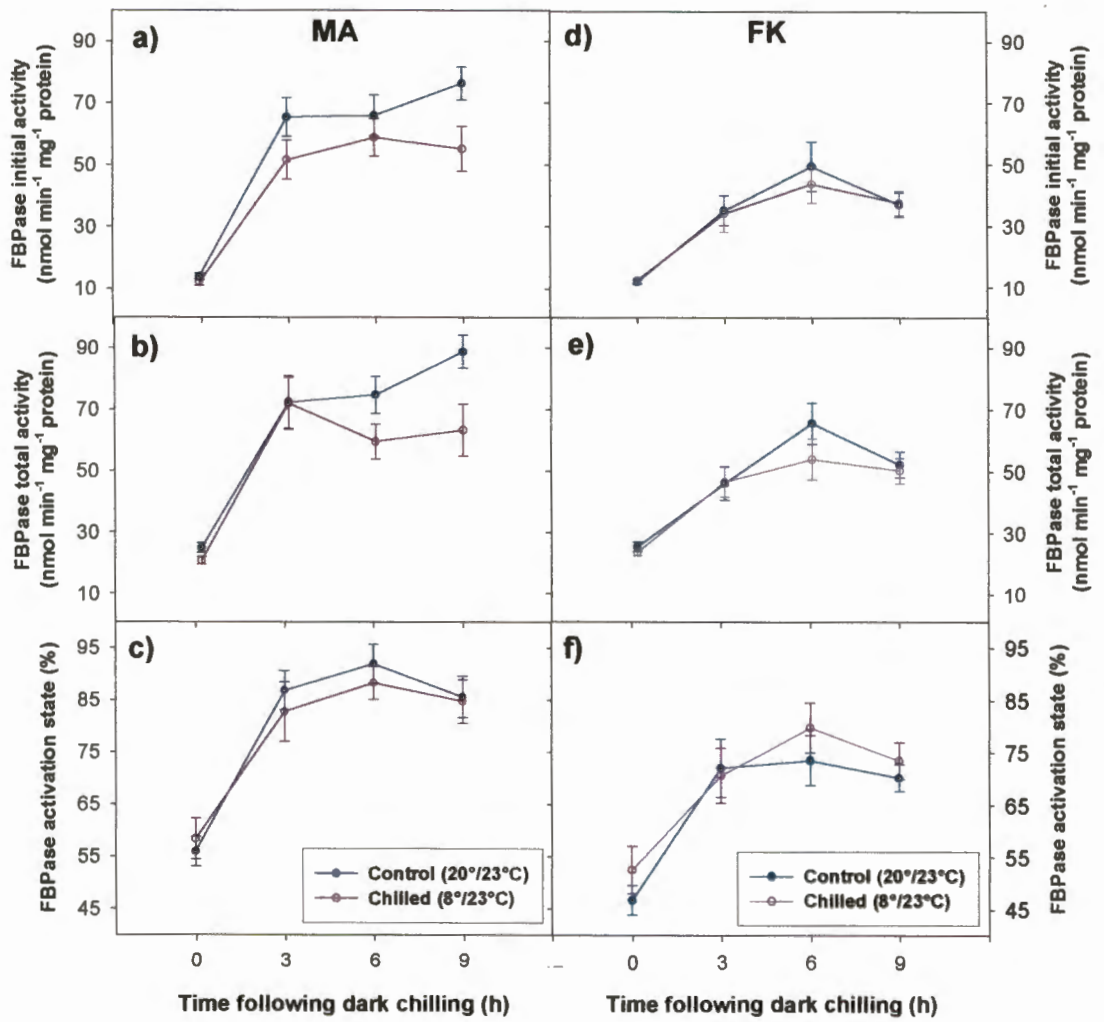


Figure 3.2. Initial (a & d) and total (b & e) activity and activation state (c & f) of stromal fructose-1,6-bisphosphatase in leaves of 'Maple Arrow' and 'Fiskeby V' at normal growth temperatures (control, solid circles) and in response to 3 nights of dark chilling (chilled, open circles). The activation state of FBPase represents the initial activity expressed as a percentage of the total activity. Leaf samples were collected in the dark (0 h) and at a light intensity of 1000 µmol m^{-2} s^{-1} and 23°C at regular intervals (3 h, 6 h and 9 h) following dark chilling. Data points represent the mean of 6 replicates ± SE.

Figure 3.2 illustrates the effect of dark chilling on stromal FBPase activity in the two genotypes. Because FBPase is an enzyme activated by light via the thioredoxin system, the intial activity in the dark (0 h) was found to be very low (± 10 nmol min^{-1} mg^{-1}

protein) in both genotypes and very similar for both the control and dark chilling treatment (Fig. 3.2 a & d). However, after 3 h of light exposure, full activation of FBPase was obtained and the initial activity was much higher (40 – 65 nmol min^{-1} mg^{-1} protein) than in the dark. Surprisingly the total activity of the enzyme also showed a similar pattern between dark and light conditions (Fig. 3.2 b & e), suggesting much lower steady state FBPase protein levels in the dark. Alternatively, the low total activity in the dark could indicate some unknown form of dark-dependent post-translational modification of the enzyme, rendering it insensitive to DTT activation *in vitro*. This type of modification, with respect to FBPase, however has not yet been reported in the literature. An interesting difference in FBPase activity between the control plants of the two genotypes was also observed. In the light, MA had a higher initial and total FBPase activity than Fiskeby V, suggesting higher steady state FBPase protein levels in MA. However, in FK also the activation state of FBPase was lower than in MA, suggesting a lower degree of light activation of available FBPase protein.

In MA, initial and total FBPase activity in the light was decreased by dark chilling. The inhibitory effect appeared to develop gradually during the light period. For example, initial FBPase activity was decreased by 23% ($p < 0.01$) and 28% ($p < 0.01$) following 6 h and 9 h of light exposure respectively. Over the same period, total activity was decreased by 20% ($p < 0.05$) and 29% ($p < 0.01$). Because both initial and total activity were decreased to a similar extent, no effect on FBPase activation state occurred (Fig. 3.2 c). Dark chilling did not induce a significant decrease in initial and total activity or activation state of FBPase in FK.

3.1.3 NADP-dependent malate dehydrogenase (MDH) activity

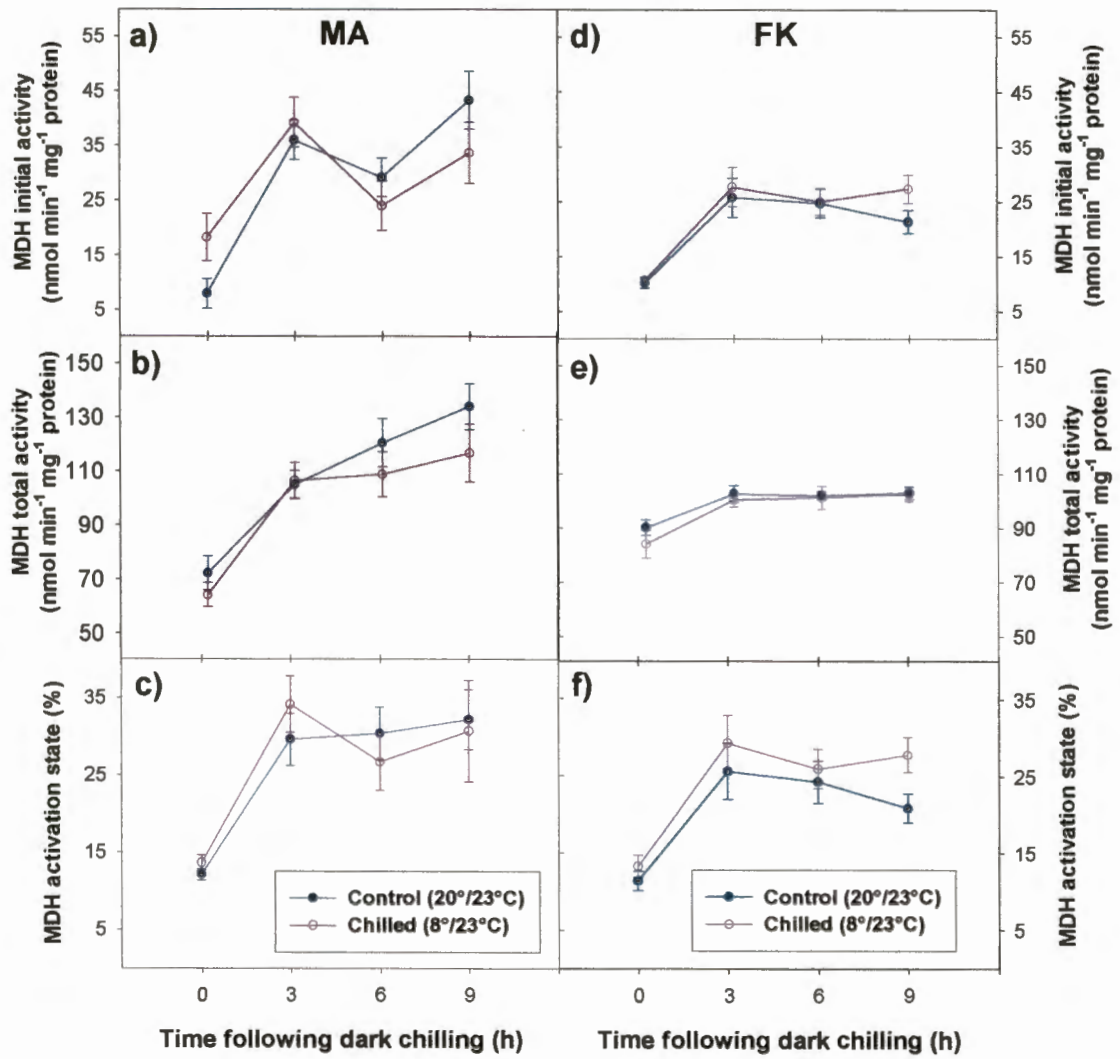


Figure 3.3. Initial (a & d) and total (b & e) activity and activation state (c & f) of NADP-dependent malate dehydrogenase in leaves of 'Maple Arrow' and 'Fiskeby V' at normal growth temperatures (control, solid circles) and in response to 3 nights of dark chilling (chilled, open circles). The activation state of MDH represents the initial activity expressed as a percentage of the total activity. Leaf samples were collected in the dark (0 h) and at a light intensity of 1000 μmol m^{-2} s^{-1} and 23°C at regular intervals (3 h, 6 h and 9 h) following dark chilling. The same leaf extracts used to determine FBPase activity was also used for MDH. Data points represent the mean of 6 replicates ± SE.

Because the activation state of MDH is regarded as a sensitive indicator of the $NADP^+$/NADPH ratio in the chloroplast stroma (Scheibe 1987; Harbinson *et al.* 1990),

the initial and total activity as well as the activation state of this enzyme was determined in the same leaf extracts used for the measurement of FBPase activity.

Figure 3.3 illustrates the effect of dark chilling on MDH activity in the two genotypes. Because MDH, similar to FBPase, is an enzyme activated by light, the initial activity in the dark (0 h) was found to be very low in both genotypes (Fig. 3.3 a & d). However, after 3 h of light exposure, full activation of MDH was obtained and the initial activity was much higher than in the dark. The only effect of dark chilling on MDH activity and activation state was observed in FK. Nine hours after the start of the light period, initial MDH activity (Fig. 3.3 d) in previously dark chilled plants was higher than in the control. In contrast, total MDH activity (Fig. 3.3. e) was unaffected. This suggests that the steady state protein level of MDH was not affected by dark chilling but that the activation state of the enzyme was elevated. The increase in activation state induced by dark chilling can be seen in Fig. 3.3 f. In contrast, MDH activation state in MA (Fig. 3.3 c) was not altered by dark chilling

3.1.4 Leaf nitrogen content

It is well established that symbiotic N_2 fixation is sensitive to environmental stress. (Purcell & King 1996). Inhibition of N_2 fixation by low root zone temperatures could result in nitrogen limitation within the leaves which in turn could reduce photosynthetic capacity, since enzymes involved in photosynthesis (especially rubisco) constitute a major nitrogen sink. In the present investigation I determined the effect of dark chilling on leaf nitrogen content in both genotypes by means of a scanning electron microscope in conjunction with a specialised software program to estimate the inorganic element concentrations in leaf samples.

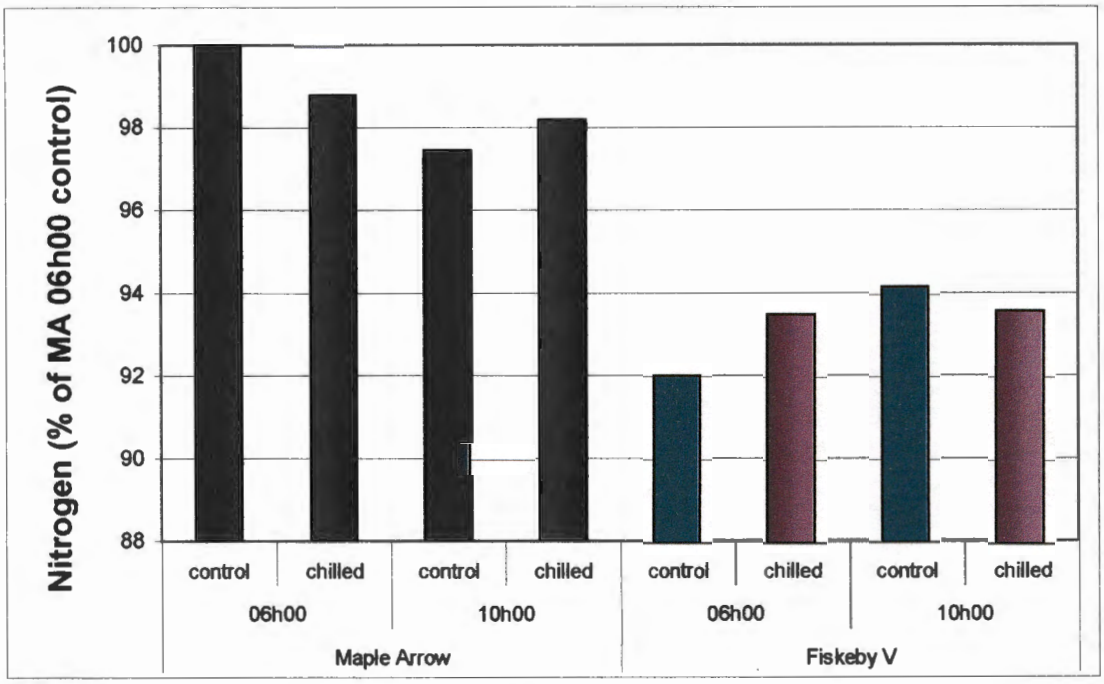


Figure 3.4. Leaf nitrogen content (expressed as a percentage of the control plants of MA sampled pre-dawn at 06h00) in N_2–fixing plants of 'Maple Arrow' and 'Fiskeby V' at normal growth temperatures (control) and after four consecutive nights of dark chilling (chilled). Leaf samples were collected in the dark (06h00) and 3 h after the start of the light period following dark chilling (10h00) at a light intensity of 1000 µmol m^{-1} s^{-1} and 23°C. Each value shown represents the nitrogen content of a collective sample from 6 leaves ground together and measured with a scanning electron microscope.

The leaf nitrogen content of control and dark chilled plants of MA and FK is shown in Fig. 3.4. Dark chilling did not cause a change in leaf nitrogen content. However, an interesting difference between control plants of the two genotypes was revealed. The leaf nitrogen content of MA was higher (by as much as 8%) than in FK. To evaluate the possible effect of this difference in leaf nitrogen content on photosynthetic capacity, the CO_2 assimilation rate at c_i = 350 µmol mol^{-1} (A^0), calculated from the A:c_i response curves (Fig. 3.1), maximum catalytic activity of Rubisco, total chlorophyll content and total soluble protein content were compared in the control plants of the two genotypes (Table 3.2).

Table 3.2. A comparison between CO_2 assimilation rate (at c_i = 350 µmol mol^{-1}), total chlorophyll and soluble protein content and maximum catalytic activity of Rubisco in leaves of control plants of 'Maple Arrow' and 'Fiskeby V'. All values presented are the mean of 4 – 6 replicates ± SE and are expressed on a leaf area basis.

	'Maple Arrow'	'Fiskeby V'
CO_2 assimilation rate (µmol m^{-2} s^{-1})	25 ± 0.5 (+16%)	21 ± 1.2
Total chlorophyll content (g m^{-2})	0.34 ± 0.006 (+12%)	0.30 ± 0.008
Total soluble protein content (g m^{-2})	4.58 ± 0.18 (+12%)	4.05 ± 0.21
Maximum Rubisco activity (µmol m^{-2} s^{-1})	12.1 ± 0.56 (+28%)	8.7 ± 0.49

MA had a 16% higher CO_2 assimilation rate, 12% higher chlorophyll content, 12% higher soluble protein content as well as a 28% higher maximum Rubisco activity than FK (Table 3.2). The 12% difference between the two genotypes with respect to chlorophyll and protein content compared well with the 8% difference in nitrogen content (Fig. 3.4). Since a large proportion of leaf nitrogen is invested in Rubisco, the large difference in maximum Rubisco activity between the two genotypes is not surprising. Taken together, these results indicate that MA has a higher photosynthetic capacity than FK under optimum growth conditions, which correlates well with the difference in leaf nitrogen content.

3.1.5 Leaf anatomy and ultrastructure

No anatomical differences were observed between the leaves of control plants of the two genotypes. Leaves of both genotypes are dorsiventral. Epidermal cells of both surfaces tend to be flat and tabular as seen in cross-section. All epidermal cell walls are thin, except on leaf margins where thick-walled cells are visible. It appears as if the abaxial epidermis has a larger number of stomata and that the cells are larger and more rounded than those of the adaxial epidermis.

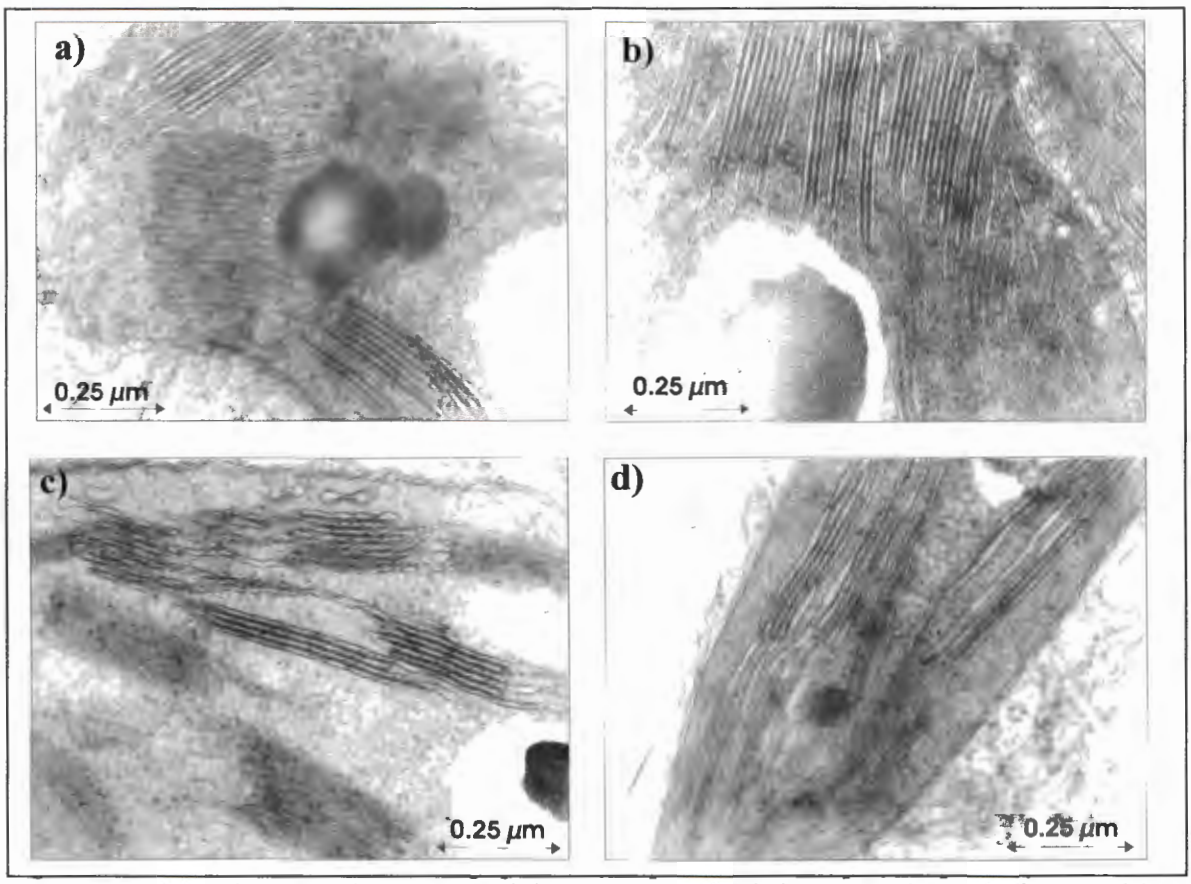
Immediately below the adaxial epidermis are two layers of columnar chlorenchyma cells, the palisade parenchyma. This tissue is largely responsible for photosynthesis, and its structure is remarkably well suited for it. The long, narrow cylindrical cells are rich in chloroplasts; these are pressed firmly against the plasmalemma by a large central vacuole. Seen in longitudinal leaf section nearly all the cells are enclosed by air spaces, so that almost the entire surface of each cell is exposed and carbon dioxide can circulate freely

around and between the cells, dissolving in water held by the cell walls. The chloroplasts are situated close to the cell wall, so that the carbon dioxide is immediately pulled out of the hyaloplasm and a steep diffusion gradient exists. Palisade cells are not separated from each other by too great a space, as the contact region between these cells and the upper epidermis forms a capillary system that functions to distribute water to the cells from the veins. All leaves measured had intercellular spaces of 45% to 50% of total leaf area in sections taken in longitudinal orientation through the palisade parenchyma.

Between the palisade parenchyma and the abaxial epidermis is the spongy mesophyll, a region of mesophyll in which the cells are very widely separated from each other with fewer chloroplasts. A consequence of the presence of spongy mesophyll is that the abaxial epidermis is separated from the palisade parenchyma, creating an extremely open aerenchyma and greatly enhancing the circulation of carbon dioxide. The spongy mesophyll allows the leaf to be flexible and the cells are elongated, with the presence of lobes that connect adjacent cells.

Soybean leaves have a reticulate venation. The veins have a sheath of tight-fitting thin-walled parenchyma cells with few chloroplasts. Sheath cells form bundle sheath extensions on both the adaxial and abaxial sides. Larger veins contain xylem and phloem, the latter with sieve tube members. A cambium is present in the main vein and may produce some secondary tissue. Tracheary elements of smaller veins have spiral wall thickenings.

When looking at the ultrastructural data, the following considerations have to be kept in mind. Material is subjected to conventional chemical fixation methods that can alter the morphology of cell components. Cells may also react differently compared to control plants to chemical fixatives when subjected to chilling temperatures.



'Maple Arrow' (a & b) and 'Fiskeby V' (c & d) leaves showing the thylakoid membranes in the control (a, c) and after exposure to four consecutive nights of dark chilling (b, d).

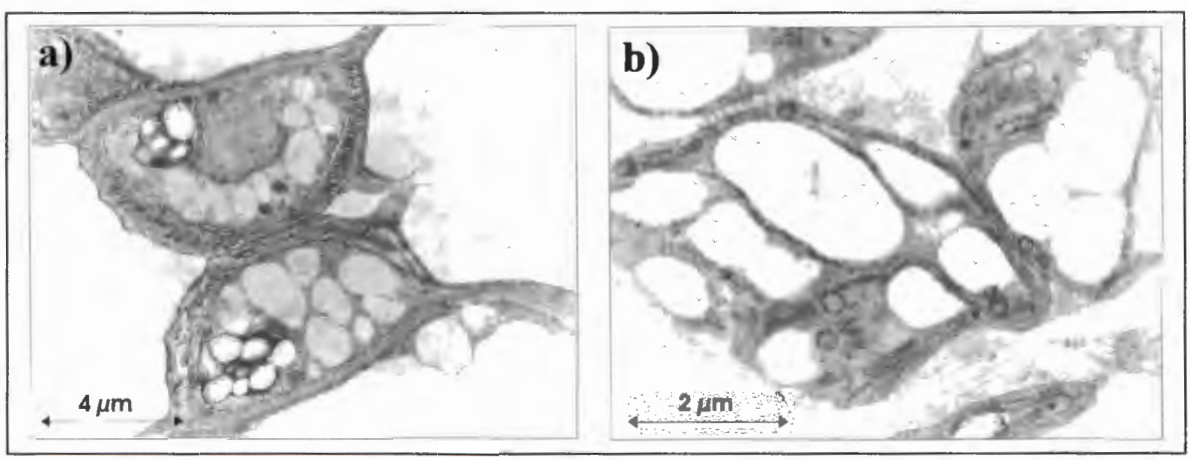


Figure 3.6. Transmission electron micrographs of 'Fiskeby V' leaf tissue after exposure to four nights of dark chilling. Transections are through (a) the stomates and (b) chloroplasts of palisade parenchyma cells.

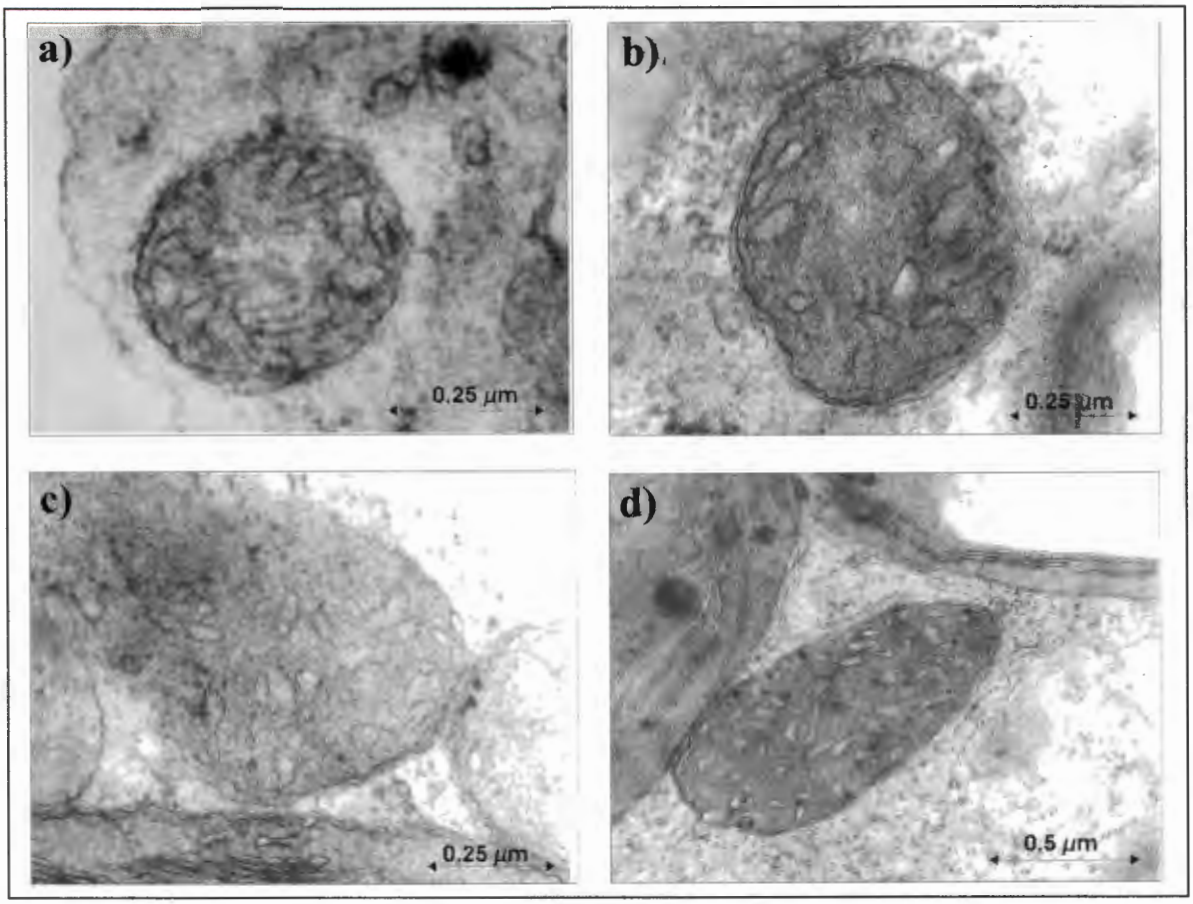


Figure 3.7. Transmission electron micrographs of the mitochondria within the palisade parenchyma cells of 'Maple Arrow' (a & b) and 'Fiskeby V' (c & d) leaves in the control (a, c) and after exposure to four consecutive nights of dark chilling (b, d).

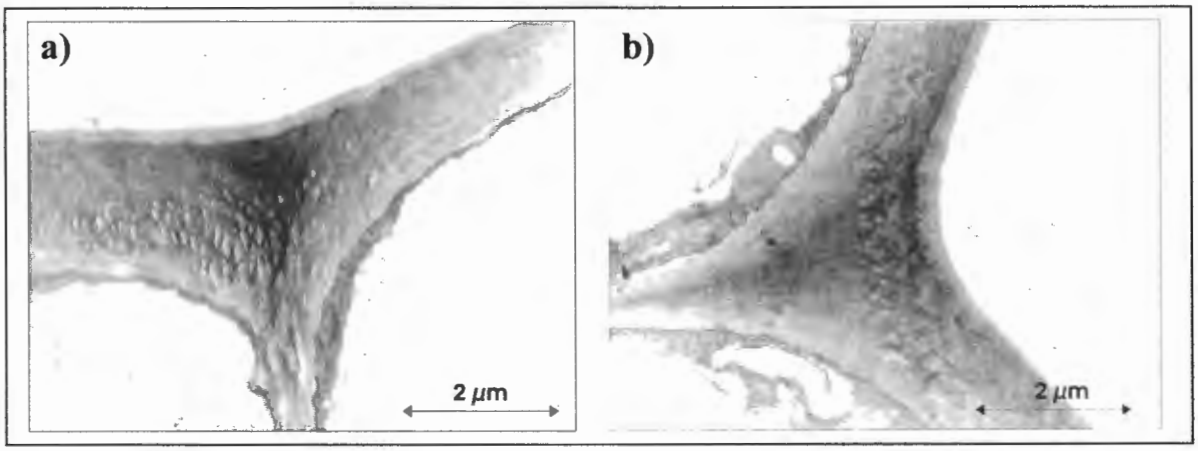


Figure 3.8. Transmission electron micrographs of the plasma membrane of palisade parenchyma cells in 'Fiskeby V' leaves before (a) and after (b) exposure to four nights of dark chilling.

Ultrastructurally, chloroplasts of palisade cells of dark chilled leaves exhibited few features that could be attributed to chilling injury (Fig. 3.5). Observed changes that could be attributed to chilling are widening of granal lamellae, vesiculation of lamellae and enlargement of vesicles (Fig. 3.5d) in FK, whereas no such changes were observed in MA. Large vesicles in stomatal cells of FK (Fig. 3.6a) and accumulation of lipid droplets in chloroplasts of palisade cells of FK (Fig. 3.6b) were observed. Starch depletion was not quantified and was not an obvious feature in any of the dark chilled leaves investigated ultrastructurally.

Mitochondrial structure was not drastically influenced by chilling, but possible enlargement of cristae was noted in some cells of MA (Fig. 3.7a & b) and in FK (Fig. 3.7c & d). The plasma membrane of chilled FK cells showed some signs of vesiculation (Fig. 3.8b), while no differences were observed in MA. No nuclear changes were observed in any of the two genotypes.

3.2 The effect of dark chilling of only the shoots on photosynthesis in N_2-fixing soybean plants grown with or without nitrate supplementation

In the series of experiments described below, only the above ground shoot portions of plants were dark chilled. The aim of these experiments was to evaluate the contribution of root chilling towards the inhibition of photosynthesis described in section 3.1. In addition, plants were grown with or without nitrate supplementation (+N and -N treatment respectively) prior and during exposure to dark chilling. The aim was to determine if supplementation of N_2-fixing soybean with nitrate had any beneficial effect on the photosynthetic capacity of the plant during and after exposure to chilling stress.

3.2.1 Vegetative growth

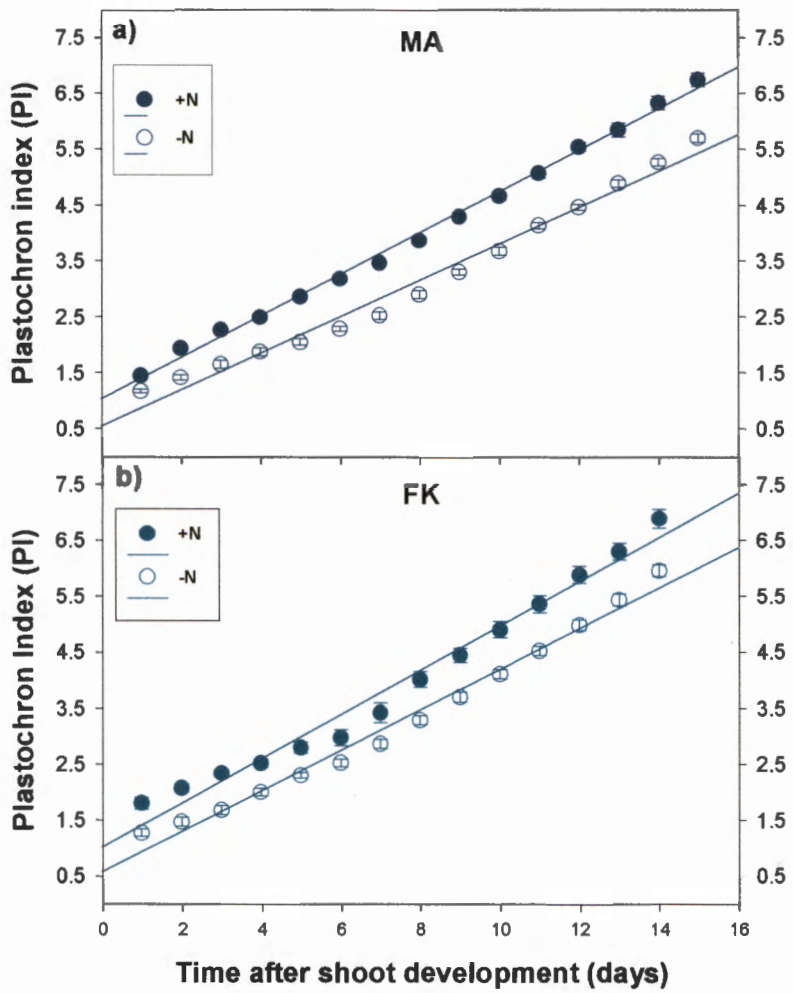


Figure 3.9. Change in Plastochron index of 'Maple Arrow' (a) and 'Fiskeby V' (b) plants with time that received either +N (solid circles) or -N nutrient solution (open circles). Plants were grown at 20°/23°C for a period of at least 12 days after the start of the two fertiliser treatments. Plants of each genotype and fertiliser treatment were dark chilled as soon as the plants reached a PI value of 6. Plants were dark chilled at 8°C for four consecutive nights. The PI was measured daily, 2 h after the start of each light period. Plotted values represent the mean of 10 replicates ± SE.

Because supplementation with nitrate may have resulted in differences in the rate of vegetative growth in the two genotypes, the plastochron index (PI) was employed as a sensitive index of vegetative growth. The main purpose was to ensure that dark chilling was induced in plants of comparable vegetative development. A linear increase in the PI

with time was observed in 'Maple Arrow' (MA) (Fig. 3.9 a) and 'Fiskeby V' (FK) (Fig. 3.9 b) plants fertilised with the –N and +N nutrient solutions. Plants that received the +N nutrient solution were characterised by a faster increase in the PI than plants receiving the –N nutrient solution. The difference in the increase in PI between the +N and –N treatments for both genotypes is summarised in table 3.3. For each treatment the slope and the R^2 value of the regression line is shown. From table 3.3 it can be seen that the +N treatment of MA resulted in a significant faster increase of 14% (steeper slope) in the PI than the –N treatment of MA ($p < 0.05$), while no significant differences were observed in different N treatments of FK. The –N treatment of FK had a highly significant faster increase (11.3%) than the same treatment plants of MA ($p < 0.01$). No significant differences were measured between the +N treatments of the two genotypes.

Table 3.3. Comparison of the effect of the –N and +N nutrient treatments on the increase in the plastochron index for MA and FK. The values presented represent the mean of 10 replicates ± SE.

Genotype	Treatment	Slope	R^2 value
MA	+N	0.3714 ± 0.008	0.996 ± 0.0019
	-N	0.3259 ± 0.004	0.982 ± 0.0012
FK	+N	0.3949 ± 0.008	0.978 ± 0.0022
	-N	0.3628 ± 0.004	0.985 ± 0.0016

3.2.2 CO_2 assimilation

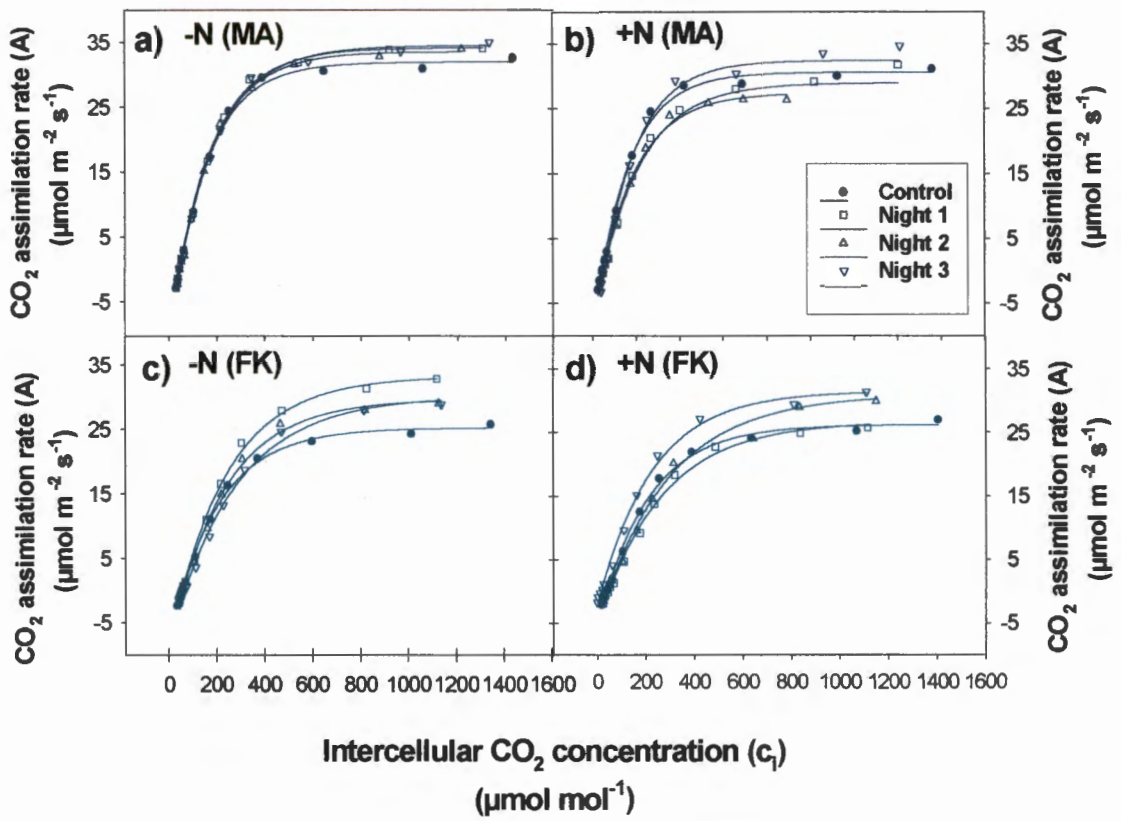


Figure 3.10. Relationship between CO_2 assimilation rate (A) and intercellular CO_2 concentration (c_i) in leaves of 'Maple Arrow' and 'Fiskeby V' receiving the -N (a, c) and +N nutrient solution (b, d) following each of three consecutive nights of dark chilling. Measurements started 3 h after the end of each dark period and were conducted at a leaf temperature of 23°C and a light intensity of 1200 µmol $m^{-2}s^{-1}$. Data points represent the mean of 4 replicates.

The relationship between CO_2 assimilation rate (A) and increasing intercellular CO_2 concentration (c_i) in response to dark chilling of only the shoots is shown in Fig. 3.10. Photosynthetic gas exchange measurements were conducted after each of three consecutive nights of dark chilling at 8°C on plants of MA and FK at a light intensity of 1200 µmol m^{-2} s^{-1} to ensure that Rubisco was fully activated during all measurements (Taylor & Terry, 1984).

Our comparative investigation of the A:c_i response curves as influenced by the interaction between dark chilling and N-nutrition revealed that the two genotypes were differentially

affected. No changes in CO_2 assimilation rate (A) and RuBP regeneration capacity (indicated by A_{max}) were observed in MA receiving the –N nutrient solution, while a decrease in A, but not in A_{max}, was observed in plants receiving the +N nutrient solution. In FK, dark chilling induced an increase in A_{max}, but not in A in plants receiving the –N nutrient solution, while both parameters increased in plants receiving the +N nutrient solution.

In order to compare the effects that dark chilling had on the response in the two genotypes and fertiliser treatments, the photosynthetic parameters deducible from the constructed A:c_i curves, were calculated and are summarised in table 3.4.

Table 3.4. The effect of dark chilling on key photosynthetic parameters calculated from the A:c_i response curves shown in Figure 3.10. For each parameter the percentage change relative to the control value is shown. CE = carboxylation efficiency (the initial slope of the demand function); A_{max} = CO_2 saturated rate of photosynthesis; A = CO_2 assimilation rate at c_a = 350 µmol mol^{-1}; g_s = stomatal conductance at c_a = 350 µmol mol^{-1}; c_i = intercellular CO_2 concentration at c_a = 350 µmol mol^{-1}. Significant differences between the control and comparable treatment means are indicated by * and ** ($p < 0.05$ and $p < 0.01$ respectively) while ns = no significant difference.

'Maple Arrow' (-N)	**% Change**				
	CE	**A_{max}**	**A**	**g_s**	**c_i**
Control	0.2 ± 0.01	33.8 ± 1.3	17.4 ± 0.6	179 ± 8.2	175.8 ± 5.7
Night 1	ns	ns	ns	ns	ns
Night 2	ns	ns	ns	-25**	-14*
Night 3	ns	ns	ns	ns	ns
'Maple Arrow' (+N)					
	CE	**A_{max}**	**A**	**g_s**	**c_i**
Control	0.2 ± 0.1	31.4 ± 1.2	17.8 ± 0.6	195.6 ± 7.6	185.8 ± 3.3
Night 1	ns	ns	-18*	ns	ns
Night 2	ns	ns	-25*	-26**	ns
Night 3	ns	ns	ns	ns	ns
'Fiskeby V' (-N)					
	CE	**A_{max}**	**A**	**g_s**	**c_i**
Control	0.1 ± 0.01	25.9 ± 0.8	11.1 ± 0.9	121.8 ± 8.6	176.8 ± 6.0
Night 1	ns	+27**	ns	ns	ns
Night 2	ns	ns	ns	ns	ns
Night 3	ns	ns	ns	ns	ns
'Fiskeby V' (+N)					
	CE	**A_{max}**	**A**	**g_s**	**c_i**
Control	0.1 ± 0.01	27 ± 0.9	12.6 ± 0.5	139.9 ± 7.4	177.9 ± 9.4
Night 1	ns	ns	+26**	+30*	ns
Night 2	ns	ns	+23*	+34**	ns
Night 3	ns	+16*	+23*	+28*	+37**

In MA and FK plants fertilised with the –N nutrient solution, dark chilling did not induce any significant decrease in the A:c_i response, while a larger decrease was found in MA plants fertilised with the +N nutrient solution and an increase in FK plants fertilised with the +N nutrient solution. Analysis of A:c_i curves (table 3.4) revealed that dark chilling had no significant effect on carboxylation efficiency (CE) for any of the treatments. In FK, a significant increase in CO_2 saturated rates of photosynthesis (A_{max}) of 27% and 16% was observed after the first night of chilling in the –N treatment and the third night

of dark chilling in the +N treatment, respectively. In MA, no significant effect of dark chilling on CE or A_{max} was observed for the two N treatments.

The plants used to determine the effect of dark chilling of the entire plant on photosynthesis (section 3.1), were fertilised with N-free nutrient solution. These plants can therefore be compared to the plants of the –N treatment to determine if dark chilling of the entire plant or only the shoots had a different effect on photosynthesis. When comparing the results shown in table 3.1 (entire plants chilled) with those in table 3.4 (-N treatment, shoot-chilled) it is clear that the effect on photosynthesis was very different for both genotypes. For example, dark chilling of the entire plant resulted in a decrease of A and A_{max} in both genotypes (albeit to a larger extent in FK), whereas chilling of only the shoots of these plants resulted in no inhibition. In addition, when entire plants were dark chilled, there was a clear distinction in the response of MA and FK. Under these conditions FK were decreased to a much larger extent than MA. However, in shoot-chilled plants, the response in MA and FK was much more similar and no clear genotypic difference existed between the two genotypes. Dark chilling of the entire plant also resulted in a much larger decrease in stomatal conductance (g_s) compared to shoot-chilled plants. Taken together, these results suggest that the presence of root chilling resulted in severe effects on photosynthesis, mediated most probably through the introduction of chill-induced drought stress in these plants. The large decrease in g_s can most probably be regarded as a symptom of the chill-induced drought stress.

3.2.3. Stromal fructose-1,6-bisphosphatase (FBPase) activity

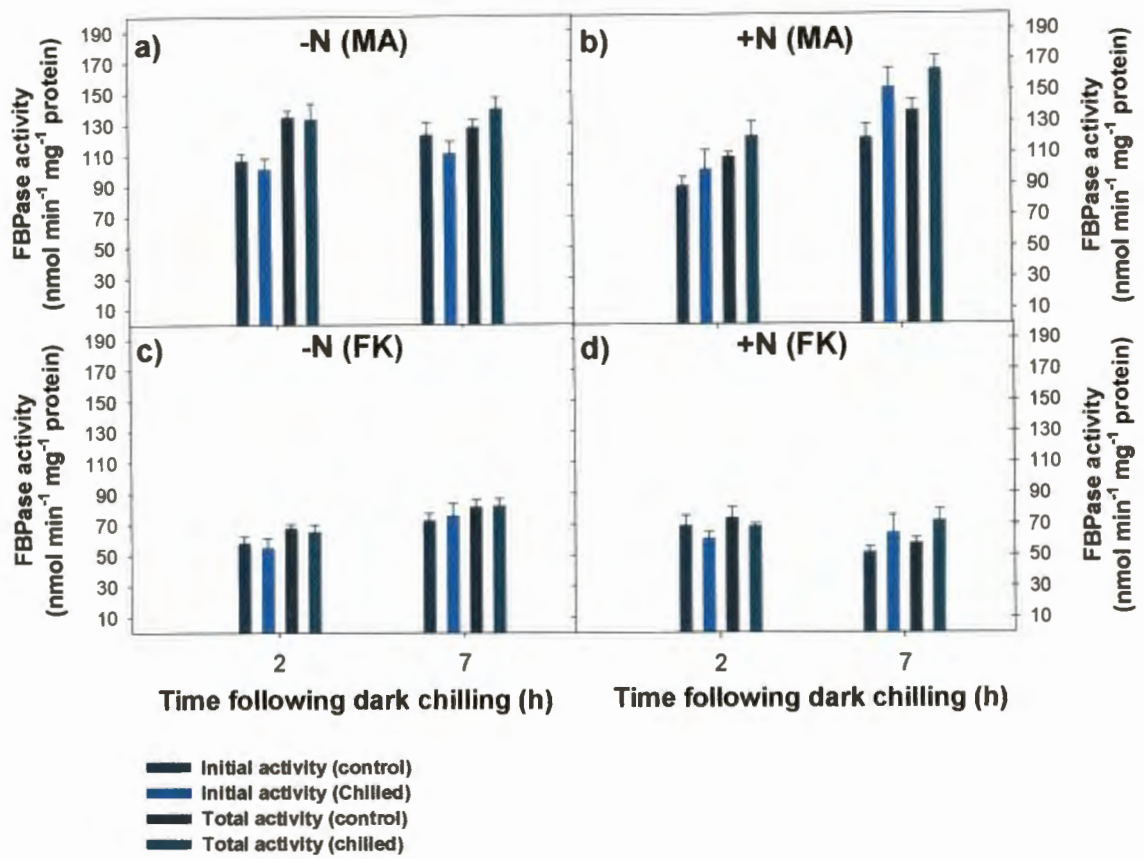


Figure 3.11. Initial and total FBPase activity in leaves of 'Maple Arrow' and 'Fiskeby V' receiving –N (a, c) and +N (b, d) nutrient solution at normal growth temperatures (control) and after 4 nights of dark chilling of only the shoots (chilled). Leaf samples were collected 2 and 7 h after the end of the dark period at a light intensity of 1000 µmol m^{-2} s^{-1} and 23°C. Each bar represents the mean of 6 replicates ± SE.

In the absence of root chilling, we determined that there was no effect on the initial and total activity of FBPase in FK treated with the –N and +N nutrient solution (Fig. 3.11 c & d). This finding is in accordance with the results obtained when the entire plant was dark chilled (Fig 3.4 d, e & f). In the absence of root chilling, no effect on FBPase activity in MA fertilised with the -N nutrient solution was observed (Fig. 3.11a). In the plants fertilised with the +N nutrient solution, a significant increase of 20% ($p < 0.05$) in total FBPase activity was seen 7 h after the end of the dark period in shoot-chilled plants (Fig. 3.11 b). Compared to the results shown in Fig 3.2, where dark chilling resulted in a

decrease in FBPase activity in MA, shoot-chilling did not result in any decrease in FBPase activity in the plants fertilised with the –N nutrient solution.

3.2.4 Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity

The effect of dark chilling on the activity of Rubisco in MA when roots were not subjected to chilling, is demonstrated in fig. 3.12.

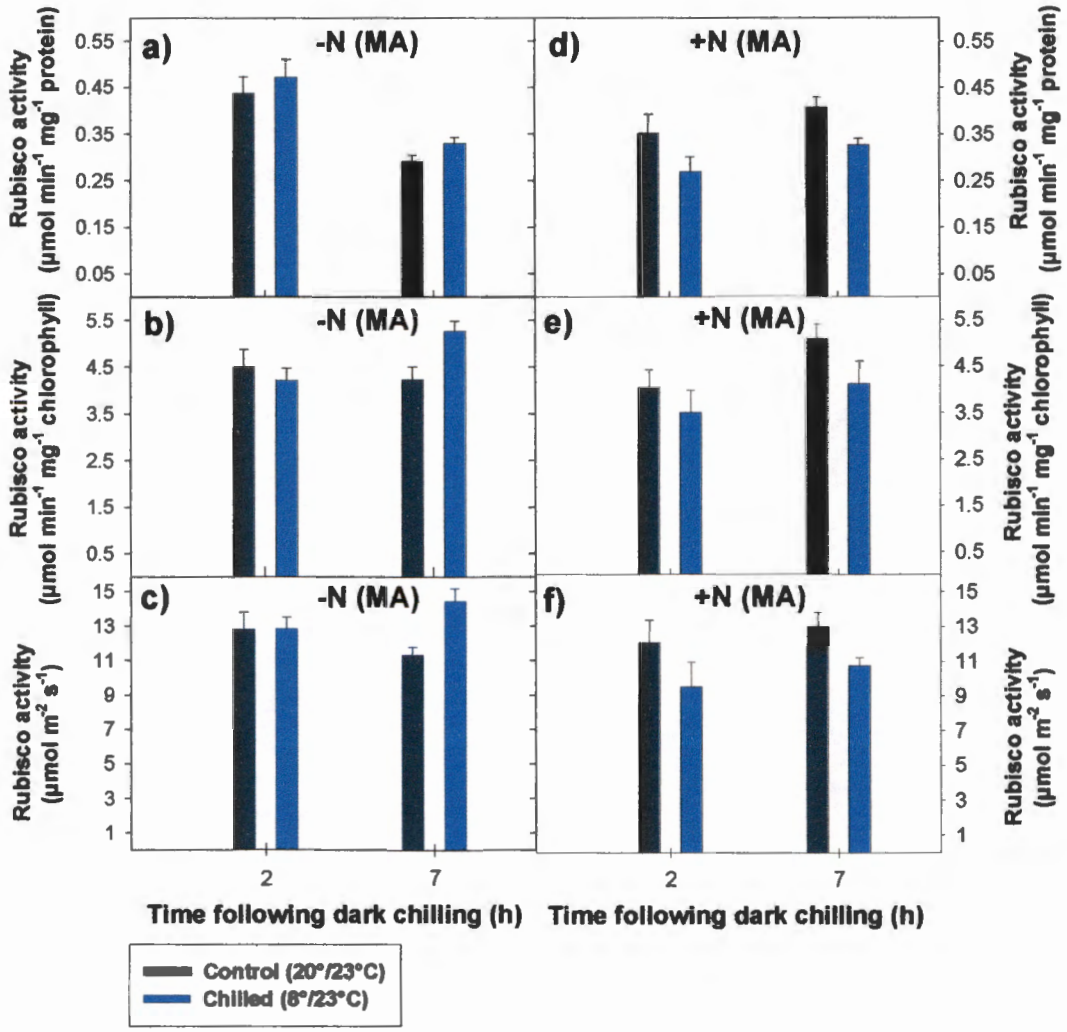


Figure 3.12. Rubisco activity, expressed on a total soluble protein (a, d), total chlorophyll (b, e) and leaf area basis (c, f) in 'Maple Arrow' plants receiving the –N and +N nutrient solutions at normal growth temperatures (control) and after 4 nights of dark chilling (chilled) of only the shoots. Leaf samples were collected 2 and 7 h after the end of the dark period at a light intensity of 1000 µmol m^{-2} s^{-1} and 23°C. Each bar represents the mean of 6 replicates ± SE.

Dark chilling resulted in a significant decrease of 20% ($p < 0.01$) and 17% ($p < 0.05$) in initial Rubisco activity in plants of MA treated with the +N nutrient solution when the activity was expressed on a soluble protein (Fig. 3.12d) and leaf area (Fig. 3.12f) basis respectively. This effect was observed 7 h after the end of the night period. In contrast, in plants of MA that received the –N nutrient solution, increases in Rubisco activity of 13% ($p < 0.05$), 25% ($p < 0.05$) and 27% ($p < 0.01$) were observed if activity is expressed on a soluble protein (Fig. 3.12 a), chlorophyll (Fig. 3.12 b) and leaf area basis (Fig. 3.12 c) respectively.

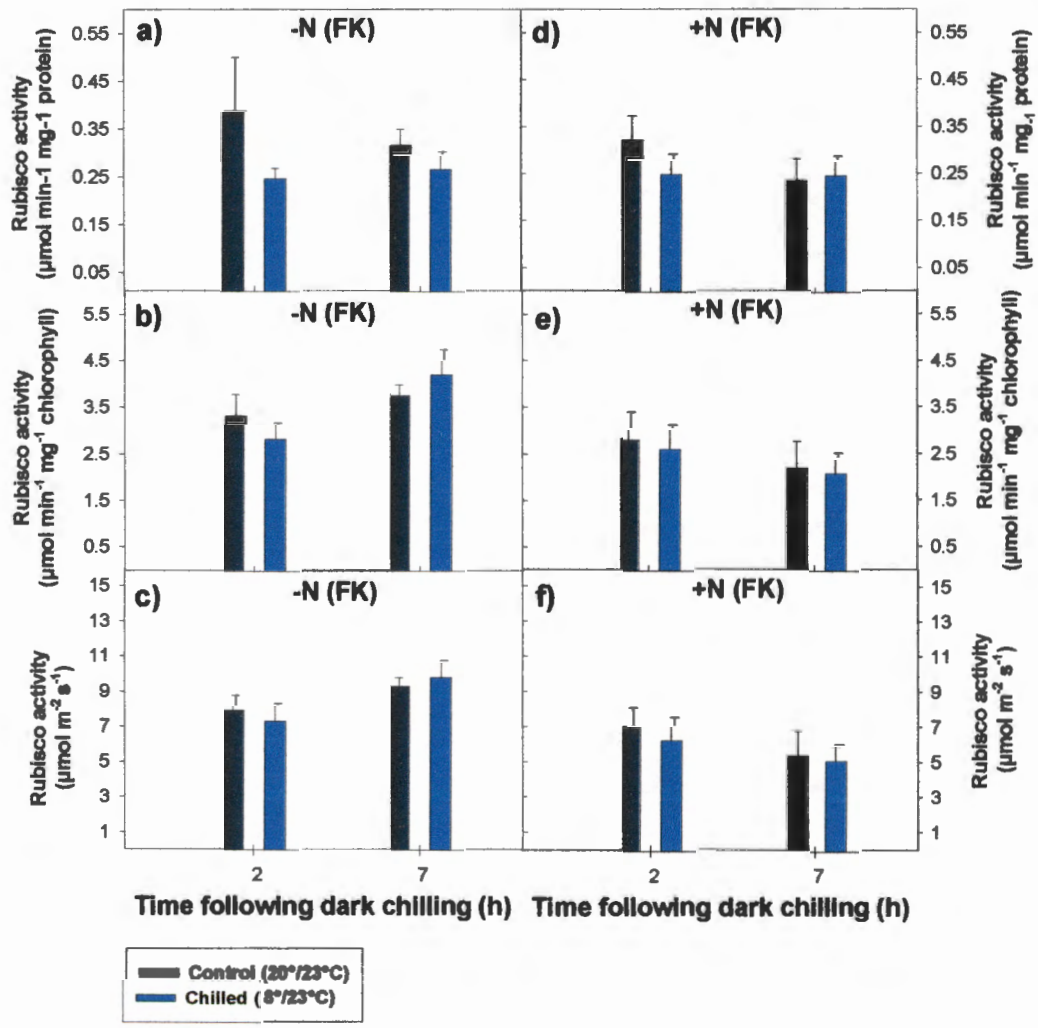


Figure 3.13. Rubisco activity, expressed on a total soluble protein (a, d), total chlorophyll (b, e) and leaf area basis (c, f) in 'Fiskeby V' plants receiving the –N and +N nutrient solutions at normal growth temperatures (control) and after 4 nights of dark chilling (chilled) of only the shoots. Leaf samples were collected 2 and 7 h after the end of the dark period at a light intensity of 1000 µmol m^{-2} s^{-1} and 23°C. Each bar represents the mean of 6 replicates ± SE.

In the case of FK, dark chilling did not result in any significant change in Rubisco activity when expressed on a total soluble protein, total chlorophyll or leaf area basis in plants fertilised with the -N or +N nutrient solution (Fig. 3.13 a - f).

3.2.5 Estimation of steady-state protein content of PCR-cycle enzymes

Western Blot analysis was used to estimate the steady state protein content of Rubisco, FBPase and SBPase following dark chilling of plants treated with the +N and -N nutrient solution.

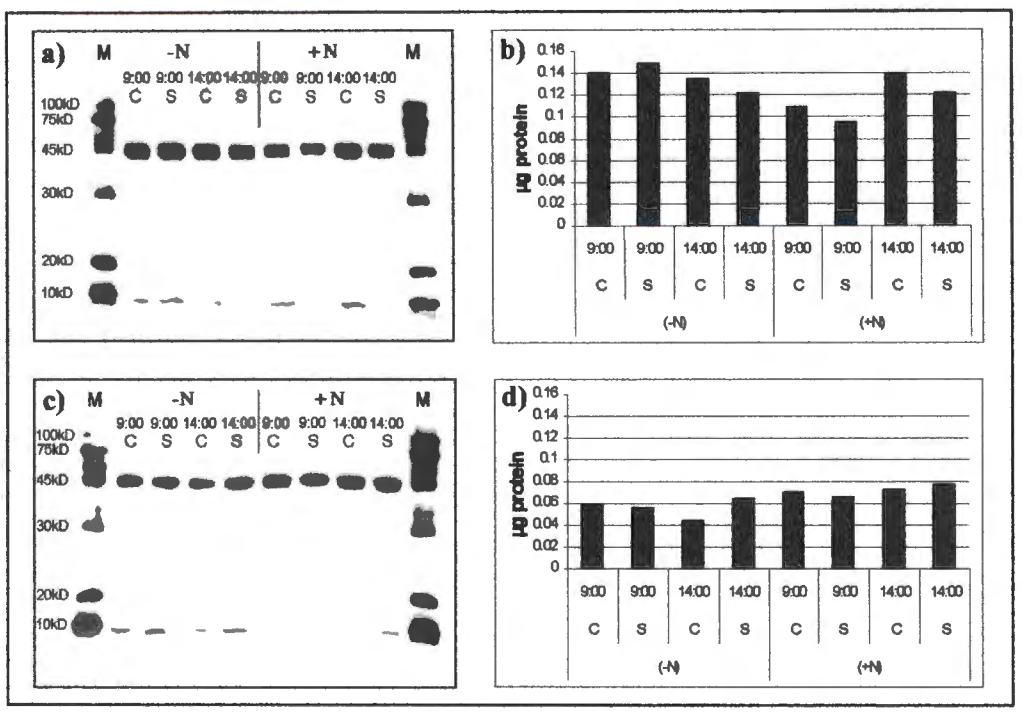


Figure 3.14. Western blots and estimated steady state protein content of Rubisco in leaves of 'Maple Arrow' (a & b) and 'Fiskeby V' (c & d) receiving the -N and +N nutrient solution. C = control plants; S = plants shoot-chilled for 4 consecutive nights; M = marker proteins. Proteins were extracted from collective leaf samples consisting of eight leaf discs collected in the light 2 h and 7 h after the end of the dark period.

For shoot-chilled plants of MA fertilised with the +N nutrient solution, a decrease of 12% in Rubisco protein content could be seen two and seven hours after the end of the dark period (Fig. 3.14a & b). This result supports the observation of decreased Rubisco activity in shoot-chilled plants of MA of the +N treatment (Fig. 3.12 d). On average, control plants of MA fertilised with the +N nutrient solution had a very similar Rubisco

protein content than those fertilised with the –N nutrient solution, while in control plants of FK, the +N treatment had a 27% higher Rubisco protein content than the –N treatment (fig. 3.14 c & d). In FK, dark chilling did not induce any changes in the protein content of Rubisco. An interesting observation was made when comparing Rubisco protein content in control plants of the two genotypes, namely that the Rubisco protein content was higher in MA than in FK. This supports the findings that MA had a higher Rubisco activity than FK and that MA has a higher photosynthetic capacity than FK under optimal growth conditions (Table 3.2).

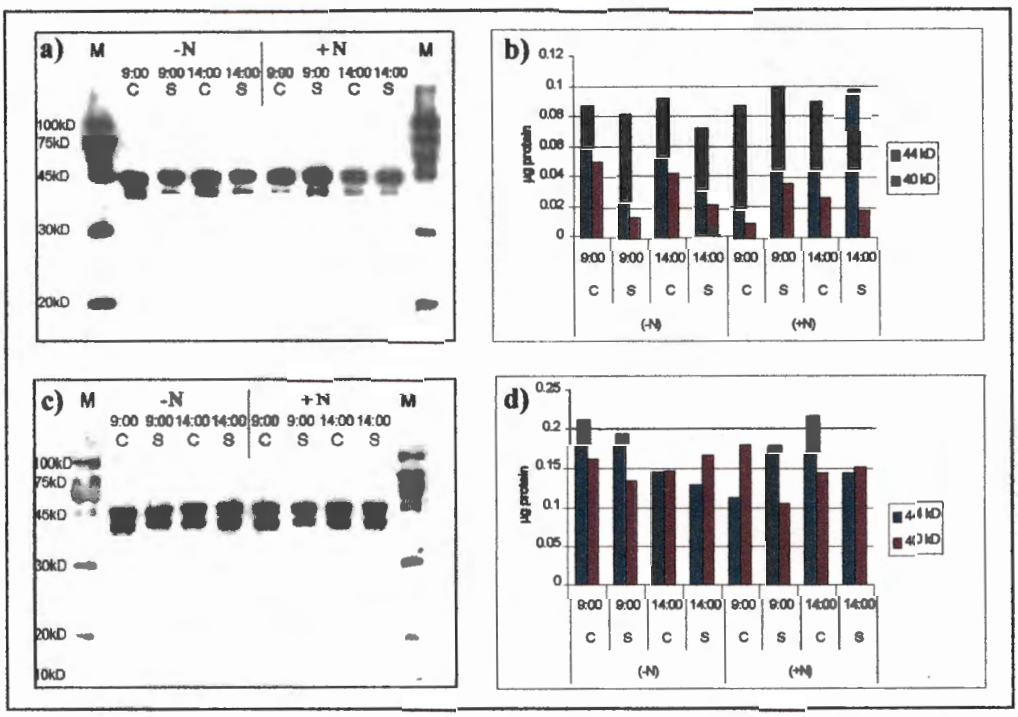


Figure 3.15. Western blots and estimated steady state protein content of stromal FBPase in leaves of 'Maple Arrow' (a & b) and 'Fiskeby V' (c & d) receiving the -N and +N nutrient solution. C = control plants; S = plants shoot-chilled for 4 consecutive nights; M = marker proteins. Proteins were extracted from collective leaf samples consisting of eight leaf discs collected in the light 2 h and 7 h after the end of the dark period.

In 'Maple Arrow' receiving the -N nutrient solution, FBPase protein content of the dark chilled plants was 31% and 30% lower than in the control following 2 h and 7 h of light exposure, respectively (Fig. 3.15 a & b).

Contrary to the above, dark chilled plants of the +N treatment had a higher (41%) FBPase protein content than the control plants sampled two hours after the end of the dark period

(Fig. 3.15 a & b). This finding corresponds to the higher FBPase activity (Fig. 3.11) that was observed in chilled plants of the +N treatment.

In FK receiving the -N nutrient solution, FBPase protein content in shoot-chilled plants was 12% lower than in the control following 2 h of light exposure. The +N treated plants showed a decrease of 18% in FBPase protein content in dark chilled plants following 7 h of light exposure (Fig. 3. 15 c & d).

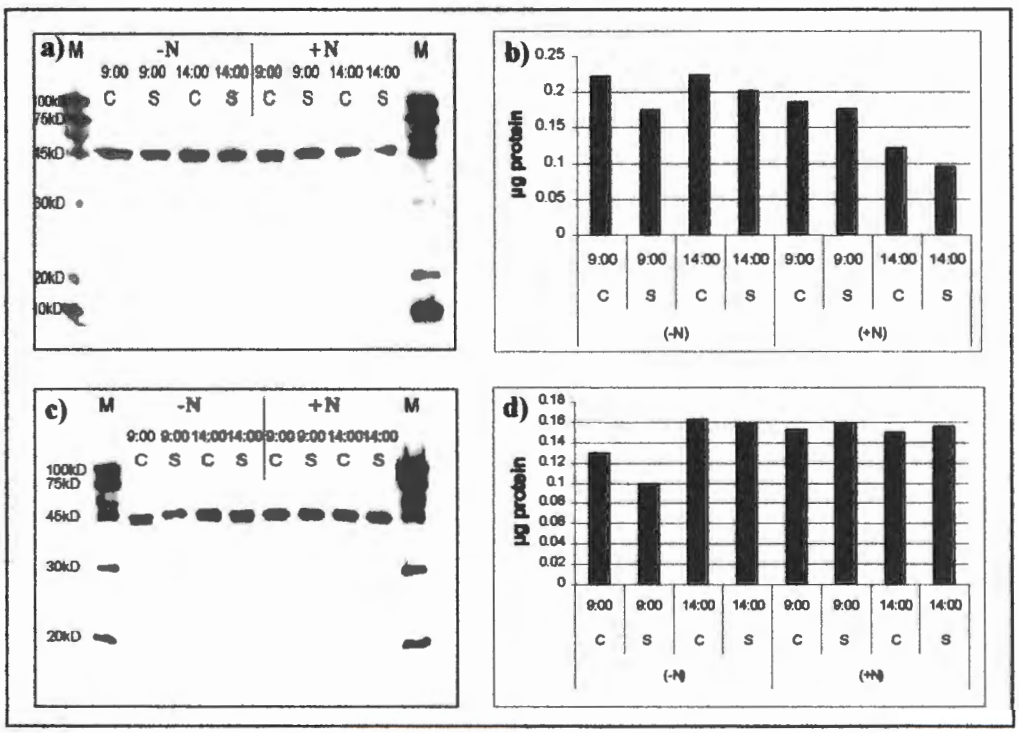


Figure 3.16. Western blots and estimated steady state protein content of SBPase in leaves of 'Maple Arrow' (a & b) and 'Fiskeby V' (c & d) receiving the -N and +N nutrient solution. C = control plants; S = plants shoot-chilled for 4 consecutive nights; M = marker proteins. Proteins were extracted from collective leaf samples consisting of eight leaf discs collected in the light 2 h and 7 h after the end of the dark period.

In MA receiving the -N nutrient solution, the SBPase protein content of the dark chilled plants was 21% and 9% lower than that of the control following 2 h and 7 h of light exposure respectively. In MA receiving the +N nutrient solution, the SBPase protein content of the dark chilled plants was 6% and 20% lower than the control following 2 h and 7 h of light exposure respectively. On average, SBPase protein content in MA was

30% lower in control plants of the +N treatment than in the –N treatment (Fig. 3.16 a & b).

In FK no effect of dark chilling or the different fertiliser treatments on SBPase protein content was observed (Fig. 3.16 c & d).

4. Discussion

4. Discussion

Photosynthetic control is the mechanism by which plants maintain a balance between energy conversion through electron transport and energy consumption by carbon assimilation (Foyer *et al.* 1990; Fisahn *et al.* 1995). If dark chilling disturbs this control, the rate of CO_2 assimilation will ultimately be inhibited during the subsequent day, even at optimal temperatures.

The results reported in this investigation demonstrate that dark chilling, followed by exposure to high light intensities, results in a significant decrease of photosynthesis in two soybean genotypes generally regarded as chilling tolerant. This observation is in accordance with findings by Van Heerden & Krüger (2000), Van Heerden *et al.* (2002) and Van Heerden *et al.* (2003). My results also contribute to the growing body of evidence that genotypic differences in the response of photosynthesis to dark chilling exist between chilling tolerant soybean genotypes. Evidence for the differential chilling sensitivity between the two genotypes is provided by the observation that photosynthesis was inhibited to a lesser extent in 'Maple Arrow' than in 'Fiskeby V'. Another significant finding was the large capacity for acclimation to chilling temperatures in 'Maple Arrow', whereas 'Fiskeby V' seems to lack this capacity. The difference in acclimation potential between the two genotypes grown at high light intensities was consistent with the difference in acclimation potential observed at much lower light intensities (Van Heerden *et al.* 2002). Available evidence therefore suggests that 'Maple Arrow' has the potential to tolerate chilling temperatures to a larger extent than 'Fiskeby V' under various growth conditions.

According to Van Heerden *et al.* (2002, 2003), both stomatal and mesophyll limitation contributed towards the inhibition of photosynthesis in these two genotypes upon exposure to relatively low light intensities following dark chilling. Gas exchange analysis conducted in this investigation revealed that mesophyll limitation (decrease in CO_2 assimilation rate due to the inhibition of the reactions of photosynthesis) was much more evident in 'Fiskeby V' than in 'Maple Arrow' when plants were exposed to high light intensities following dark chilling. This is in accordance with findings by Van Heerden

et al. (2003) who suggested a greater involvement of mesophyll limitation of photosynthesis in 'Fiskeby V' than in 'Maple Arrow' at low light intensities following dark chilling. The occurrence of stomatal limitation (decrease in CO_2 assimilation rate due to stomatal closure) of photosynthesis in the two genotypes is supported by the fact that the dark chilling-induced decrease in A was associated with a decrease in both g_s and c_i. Similar evidence for stomatal limitation was also reported by Perera *et al.* (1995), who observed that the reduction of A was associated with reductions in both g_s and c_i after long term exposure of rice to chilling temperatures. Studies by Van Heerden *et al.* (2003) indicated that the inhibition of CO_2 assimilation was characterised by a simultaneous decrease of g_s and c_i in 'Maple Arrow', whereas a similar decrease of g_s in 'Fiskeby V' occurred without any change in c_i. Based on the gas exchange analysis conducted in the current investigation, it appears that not only mesophyll limitation, but also stomatal limitation, was much more evident in 'Fiskeby V' when plants were exposed to high light intensities following dark chilling.

The dark chilling-induced decrease of photosynthesis that was observed is in accordance with numerous previous reports (Gourdon & Planchon 1982; Purcell *et al.* 1987; Caulfield & Bunce 1988; Holaday *et al.* 1992; Hurry *et al.* 1994; Van Heerden & Krüger 2000). However, in this investigation novel evidence is provided regarding the differential tolerance of closely related soybean genotypes to dark chilling, especially with respect to the physiological and biochemical response in the presence or absence of accompanying root chilling.

Dark chilling had diverse effects on many of the photosynthetic parameters measured in this investigation. For example, the effects of dark chilling on CO_2 assimilation, FBPase and Rubisco activity, as well as the steady-state protein levels of these enzymes, differed to a large extent between the two genotypes and was also influenced by the presence or absence of root chilling. Some of the changes (or absence thereof), especially on biochemical level, did not correlate with the observed loss of photosynthetic capacity in the two genotypes. This is somewhat surprising given the number of previous reports in the literature on rather simple relationships between photosynthetic capacity and chilling-

induced loss in the activities of photosynthetic enzymes such as Rubisco or FBPase (Brüggemann *et al.* 1992b; Brüggemann *et al.* 1994). The results presented in this investigation suggest that, at least in the case of chilling tolerant soybean genotypes, the relationship between biochemical changes and photosynthetic capacity are complex.

Consideration of the findings regarding mesophyll limitation suggests possible biochemical candidates responsible for the inhibition of photosynthesis. Stromal FBPase appeared to be most sensitive to dark chilling under the experimental conditions employed in the investigation. In contrast, gas exchange analysis indicated that dark chilling had much larger effects on CO_2 saturated rates of photosynthesis than Rubisco activity, since carboxylation efficiency was effected much less than CO_2-saturated rates of photosynthesis. Chilling-induced decreases in the activities of the thiol-modulated enzymes of the PCR cycle, particularly FBPase and SBPase, have been reported previously in tomato (Sassenrath *et al.* 1991) and in soybean (Van Heerden *et al.* 2003). Dark chilling decreased both initial and total FBPase activity in 'Maple Arrow', but not in 'Fiskeby V'. The decrease in FBPase activity in 'Maple Arrow', and lack thereof in 'Fiskeby V', is in accordance with previous findings by Van Heerden *et al.* (2003). The differential response of FBPase in the two genotypes therefore appears to be a true genotypic difference, even under conditions where the negative effects of dark chilling is intensified by exposure to high light intensities following the chilling event. If dark chilling-induced loss of FBPase activity did indeed limit CO_2 assimilation, it should have resulted in a greater loss of photosynthetic capacity in 'Maple Arrow' than in 'Fiskeby V', which was not the case. Decreased FBPase activity usually compromises the RuBP regeneration capacity within the leaf and will result in a decrease in the CO_2 saturated rate of photosynthesis (A_{max}) provided that photosynthesis is RuBP-limited under these conditions (Von Caemmerer & Farquhar 1981). Based on the much larger decrease of A_{max} in 'Fiskeby V', it was expected that FBPase activity would also have been decreased to a greater extent than in 'Maple Arrow'. However this was not the case. We provide novel evidence for the existence of an inverse relationship between loss of FBPase activity and loss of photosynthetic capacity in these two chilling tolerant soybean genotypes.

This observation leads to two important questions:

a) What factors, other than FBPase activity, might have resulted in the large decrease of A_{max} in 'Fiskeby V' following dark chilling?
b) What is the physiological significance of the observed decrease in FBPase activity under dark chilling of the whole plant in 'Maple Arrow', the genotype where A_{max} was affected the least?

Two hypotheses could explain the results observed for 'Fiskeby V'. Firstly, dark chilling may lead to a selective inhibition of end-product (sucrose) synthesis. This possibility is supported by Van Heerden *et al.* (2003), who reported a decrease in sucrose-phosphate-synthase (SPS) activity in 'Fiskeby V' but not in 'Maple Arrow'. Sucrose-phosphate-synthase is the principal enzyme responsible for sucrose synthesis in the cytosol. Inhibition of SPS activity by dark chilling will compromise sucrose synthesis that will result in an accumulation of phosphorylated metabolites in the cytosol (Leegood & Furbank 1986). Under these conditions, the level of Pi in the chloroplast becomes depleted to the point where photophosphorylation is Pi-limited (Herold 1980; Mächler *et al.* 1984; Leegood & Furbank 1986; Labate & Leegood 1988; Stitt & Grosse 1988). A decrease in phosphorylation potential (capacity for ATP synthesis) would cause a significant limitation on CO_2 assimilation (Huner *et al.* 1993), mainly because of an overall decrease in the activity of the PCR cycle, without necessarily affecting the potential catalytic activity of the individual enzymes (e.g. FBPase) *per se*. The decrease in the *in vivo* activity of the enzymes operating in the PCR cycle might be so small under these conditions that it would not be possible to detect the effect *in vitro*. This might explain the substantial decrease of A_{max} in the absence of any significant effects on FBPase activity in 'Fiskeby V'.

The second possibility is that PS II and PS I activity is inhibited by dark chilling to a larger extent than the PCR-cycle reactions. Under these conditions, NADPH consumption by the PCR cycle will exceed NADPH supply by the photochemical reactions, which will ultimately reduce the RuBP regeneration capacity in the leaf with a concomitant decrease of A_{max}. If NADPH is indeed in short supply, a decrease in the stromal redox state will

have been a clear symptom of this. However, the increase observed in the activation state of NADP-MDH in 'Fiskeby V' does not support this hypothesis. According to Scheibe (1987), the activation state of NADP-MDH is very sensitive to the stromal NADPH/$NADP^+$ ratio. The observed increase in the activation state of NADP-MDH in 'Fiskeby V' indicates that there was an actual accumulation of NADPH in the stroma, which suggests that the activity of the PCR cycle was inhibited more by dark chilling than the photochemical reactions. Taken together, the results presented in this investigation suggests that inhibition of end-product synthesis is the more likely hypothesis that could explain the large decrease of A_{max} in the absence of any direct effect on FBPase. The increase in stromal redox state in 'Fiskeby V', as indicated by the activation state of NADP-MDH, suggests a serious imbalance between the three main components of photosynthesis (photochemical reactions, PCR cycle and end-product synthesis) in this genotype. This imbalance could result in an even further decrease in photosynthetic capacity due to the formation of ROI and accompanying irreversible photooxidative damage, especially under the high light intensities used in this investigation.

It is important to consider the possible physiological significance of the decrease of FBPase activity in 'Maple Arrow', the genotype where A_{max} was affected the least. In this genotype, the activation state of NADP-MDH was not altered by dark chilling. This finding suggests that the stromal redox state remained optimal even though A_{max}, FBPase activity and possibly also PS II and PS I activity (Van Heerden *et al.* 2002) was inhibited by dark chilling. Similar to 'Fiskeby V', it appears that dark chilling inhibited photosynthesis in 'Maple Arrow' at a number of important sites. However, in this genotype it seemed as if dark chilling slowed the affected processes down to a very similar extent, thereby preventing the redox imbalance observed in 'Fiskeby V'. Tight metabolic control is necessary for a plant to adjust quickly to a changing environment. To achieve this, both fine and course regulation of various enzymes is necessary to adjust to changing metabolic requirements. The decrease in total FBPase activity that was observed in dark chilled plants of 'Maple Arrow' implies a decrease in the steady-state level of the enzyme, and not simply a decrease in activation state. In 'Maple Arrow', dark

chilling did not alter the activation state of FBPase, suggesting that the decrease in FBPase activity was most probably due to a decrease in transcript levels and associated protein synthesis. Control of enzyme activity on the level of transcription and translation in 'Maple Arrow' strongly suggests that the observed decrease in total FBPase activity is part of a concerted effort to down-regulate the activity of the PCR cycle, thereby preventing changes in stromal redox state following a dark chilling event. By preventing an increase in stromal redox state, the risk of ROI production and associated photooxidative damage was largely prevented. Taken together, the results presented in this study suggest that 'Maple Arrow' is capable of "sensing" dark chilling stress much better than 'Fiskeby V'. The advantage of this would be that 'Maple Arrow' could respond to changes in metabolism through various strategies (e.g. changes in FBPase activity), thereby preventing photooxidative damage to the photosynthetic machinery and limiting the negative effects that dark chilling had on CO_2-saturated rates of photosynthesis. 'Fiskeby V' on the other hand, lacks this tight metabolic control thereby exposing its photosynthetic machinery to photooxidative damage. Prevention of photooxidative damage may also explain, at least in part, the enhanced acclimation capacity in 'Maple Arrow' compared to 'Fiskeby V'.

Another important point to consider is the higher photosynthetic capacity (in control plants) of 'Maple Arrow' than 'Fiskeby V', associated with differences in leaf nitrogen, chlorophyll and soluble protein (especially Rubisco) content. The higher photosynthetic capacity in 'Maple Arrow' could be an advantage under conditions of stress, because it would allow this genotype the luxury of purposefully down-regulating its photosynthetic capacity without necessarily having any major impact on cellular carbon metabolism in general. The differences could also be as a result of different nitrogen binding status in the two genotypes. It may be that the local WB74 inoculant strain is not fully compatable with 'Fiskeby V' and that symbiotic nitrogen fixation in 'Fiskeby V' was affected by this after dark chilling treatments. This idea is supported by the increase in photosynthetic rates in 'Fiskeby V' after nitrogen application. Differences in photosynthetic capacity under optimal growth conditions were previously reported in other soybean genotypes (Ford & Shibles 1988). Because of less chlorophyll and therefore less light absorption,

'Fiskeby V' should in theory be less prone to photoinhibition during rewarming under high light intensities than 'Maple Arrow'. However, Xu *et al.* (1993) observed an opposite effect in soybean, where it was found that a chlorophyll-deficient soybean mutant was more susceptible to photoinhibition than the wildtype. One of the reasons proposed for this effect was that the chlorophyll-deficient mutant had a lower photosynthetic capacity. It is also known that soybean with lower chlorophyll content often have lower levels of carotenoids (Keck *et al.* 1970), which are known to protect against photoinhibition.

As mentioned above, only mild symptoms of CI were observed in 'Fiskeby V'. In 'Maple Arrow' only changes in mitochondrion structure were observed. This indicates that 'Maple Arrow' is more chilling tolerant than 'Fiskeby V'. The changes observed in ultrastructure as well as the measurements of photosynthetic capacity, indicated that 'Maple Arrow' was less affected by dark chilling than 'Fiskeby V'.

According to Kratsch & Wise (2000), the mitochondria appear to be resistant to chilling temperatures and only mitochondria in plants that are hypersensitive to chilling are visibly affected. Wise *et al.* (1983) proposed that ultrastructural CI develops progressively with time. The longer a plant is exposed to chilling conditions the more extensive and irreversible is the injury. In this study 'Maple Arrow' and 'Fiskeby V' were exposed to dark chilling for four consecutive nights and signs of disruption, which could be attributed to CI, were observed in the mitochondria of both genotypes. It has to be emphasised that no severe changes in the mitochondrial cristae were observed under the experimental conditions employed during this investigation.

Ultrastructural studies of chloroplasts in palisade cells of dark chilled leaves, exhibited few features that could be attributed to CI. Observed changes were widening of granal lamellae, vesiculation of lamellae and enlargement of vesicles in 'Fiskeby V', while no changes were observed in 'Maple Arrow'. According to Wise *et al.* (1983), chilling injury to chloroplasts is considerably delayed when chilling is performed in continuous darkness. They treated three species of differing chilling sensitivities to various

combinations of chilling, irradiance and water stress. The most CS species (*Gossypium hirsutum* L.) suffered damage within 24 h, with the response being total disruption of the chloroplast envelope and cell plasmolysis. A less CS species (*Phaseolus vulgaris* L.) showed a delayed response (within 144 h). The most CR species *(Brassica oleracea* L.) showed no signs of CI until exposed to prolonged chilling, light, and water stress simultaneously.

The plasma membrane of chilled 'Fiskeby V' plants shows signs of vesiculation, while no differences were observed in 'Maple Arrow'. According to Parkin *et al.* (1988) cellular membranes are the primary sites of damage during chilling stress. However, in this investigation both the roots and shoots of plants were exposed to dark chilling. Under these conditions it is unclear whether CI is a direct effect of temperature on the shoot or an indirect effect of drought stress induced by low root temperatures. It is well known that injury due to drought stress is closely related to CI (Wilson 1976). Soybean is classified as a drought sensitive species and much work has been done on the effects of drought stress on cell ultrastructure (Fellows & Boyer 1987).

Taken together, it was clear that no severe ultrastructural disruption was visible in these genotypes. This was as expected when considering that soybean is not an extremely CS species, compared to extremely CS species such as *Episcia reptans*, *Vigna radiata* and *Saintpaulia ionantha* (Kratsch & Wise (2000). Ultrastructurally and photosynthetically, 'Maple Arrow' showed more tolerance to dark chilling than 'Fiskeby V'.

According to Perera *et al.* (1995), leaf chilling tolerance increases in plants if only shoots are exposed to low temperature. The comparative investigation regarding the effects of dark chilling, in the presence or absence of root chilling, on the photosynthetic response in 'Maple Arrow' and 'Fiskeby V' confirmed that whole-plant dark chilling caused a greater decrease in photosynthetic capacity than shoot chilling alone. In fact, the plants subjected to shoot chilling did not show any major symptoms associated with chilling stress. This absence of response was rather unexpected but might be different if dark chilling is extended for longer periods of time. Because of the fact that dark chilling of only the shoots did not induce a decrease in photosynthetic capacity, the possible

ameliorating effect of nitrogen treatment on chilling tolerance could not be evaluated properly.

No conclusion regarding the relationship between dark chilling tolerance and different nitrogen treatments could be made. However, the decrease in the +N treated plants in both Rubisco activity and FBPase activity seven hours after the light period started, indicates a down regulation of photosynthesis. This effect is expected to be greater in the +N treated plants because of its greater leaf area. Because of the larger photosynthetic leaf area the plants reaches its starch capacity faster and it results in the down regulation of photosynthesis. This down regulation of photosynthesis in soybean was clearly demonstrated by unpublished results by our laboratory *(M.M. Viljoen, M.Sc. project, PU for CHE, 2002)*,

Although dark chilling of only the shoots did not induce a decrease in photosynthesis, evidence suggested that 'Maple Arrow' was more responsive to the change in temperature. Western Blot analysis revealed subtle changes in steady state levels of a number of PCR cycle enzymes in this genotype, while 'Fiskeby V' was much less responsive. It appeared as if 'Maple Arrow' was more efficient in adjusting carbon metabolism to changes in temperature than in the case of 'Fiskeby V', not only in the presence of root chilling, but also in its absence. During prolonged periods of sub-optimal temperature exposure, when decreased photosynthetic capacity is expected to occur, this might be to the advantage of 'Maple Arrow'.

In addition, the results from this comparative investigation provide novel evidence that the differences between 'Maple Arrow' and 'Fiskeby V', regarding the response of photosynthesis to dark chilling, is only revealed in the presence of root chilling. This finding suggests that 'Fiskeby V' is more sensitive to root chilling than 'Maple Arrow'. The possible underlying mechanism for this difference might be located at the level of BNF. If BNF is more sensitive to dark chilling in 'Fiskeby V' than in 'Maple Arrow', perturbations in nitrogen fixation, and possibly also nitrogen metabolism in the leaves, could explain some of the negative effects of dark chilling in this genotype. The response

observed can also be explained by the interaction of inoculant strain (WB 74) with 'Maple Arrow' and 'Fiskeby V'. It may be that the inoculant strain is not as compatible with 'Fiskeby V' than with 'Maple Arrow' and that dark chilling tolerance is affected by this difference. For future research it is recommended that the possible ameliorating effects of nitrate treatment on dark chilling stress should be investigated in the presence of root chilling. It would be very interesting to quantify the effects of nitrate treatment on the dark chilling tolerance of 'Fiskeby V', relative to 'Maple Arrow', under these conditions. The findings presented in this investigation also have important agricultural implications. The response of different genotypes to dark chilling under field conditions might differ quite considerably if low night temperatures coincide with the presence or absence of sub-optimal root zone temperatures (RZT). The possible involvement of low RZT in the dark chilling response of soybean will have to be considered carefully in field experiments aimed at selecting dark chilling tolerant genotypes.

5. Concluding remarks

5. Concluding remarks

This research project was aimed at increasing the current understanding about the physiological and biochemical basis for the limitation of photosynthesis by dark chilling in chilling tolerant soybean genotypes.

For the purpose of this investigation, two genetically related soybean genotypes of temperate origin, 'Maple Arrow' and 'Fiskeby V', both regarded as chilling tolerant (albeit differentially tolerant), were selected. It is known that the chilling tolerance of soybean genotypes from temperate origin, based on agronomic criteria, is heterogeneous. We hypothesised that a heterogeneous physiological and biochemical response to dark chilling must therefore also exist and that our diagnostic techniques would be sensitive enough to reveal genotypic differences, even between chilling tolerant genotypes.

In this investigation, novel evidence is provided regarding the differential tolerance of closely related soybean genotypes to dark chilling, especially with respect to the physiological and biochemical response in the presence or absence of accompanying root chilling. The difference in the sensitivity of photosynthesis upon dark chilling between 'Maple Arrow' and 'Fiskeby V' was one of the most striking observations. This finding contributes significantly toward the growing body of evidence that genotypic differences in the response of photosynthesis to dark chilling exist between chilling tolerant soybean genotypes. Evidence for the differential chilling sensitivity between the two genotypes is provided by the observation that photosynthesis was inhibited to a lesser extent in 'Maple Arrow', than in 'Fiskeby V'. Available evidence strongly suggest that 'Maple Arrow' has the potential to tolerate chilling temperatures to a larger extent than 'Fiskeby V' under various growth conditions. Based on the gas exchange analysis conducted in the current investigation, it appears that not only mesophyll limitation, but also stomatal limitation, was much more evident in 'Fiskeby V'.

The differential response of FBPase to dark chilling in the two genotypes appears to be a true genotypic difference. Novel evidence is provided for the existence of an inverse relationship between loss of FBPase activity and loss of photosynthetic capacity in the

two chilling tolerant soybean genotypes. The results presented in this investigation suggest that inhibition of sucrose synthesis is likely to explain the large decrease of A_{max} in the absence of any direct effect on FBPase, resulting in a serious imbalance between the three components of photosynthesis (photochemical reactions, PCR cycle and sucrose synthesis) in 'Fiskeby V'. In 'Maple Arrow' it seems as if dark chilling slowed the affected processes down to a very similar extent, thereby preventing the redox imbalance observed in 'Fiskeby V'. Taken together, the results presented in this study suggest that 'Maple Arrow' is capable of "sensing" dark chilling stress much better than 'Fiskeby V'. The advantage of this would be that 'Maple Arrow' could respond to changes in metabolism through various strategies, thereby preventing photooxidative damage to the photosynthetic machinery.

The changes in ultrastructure and photosynthesis indicated that 'Maple Arrow' was less affected by dark chilling than 'Fiskeby V'. No severe ultrastructural disruption was visible in these genotypes. This was expected when considering that soybean is not an extremely chilling sensitive species.

The comparative investigation regarding the effects of dark chilling, in the presence or absence of root chilling, on the photosynthetic response in 'Maple Arrow' and 'Fiskeby V' confirmed that whole-plant dark chilling caused a greater decrease in photosynthetic capacity than shoot chilling alone. No conclusion regarding the relationship between dark chilling tolerance and different nitrogen treatments could be made. Although dark chilling of only the shoots did not induce a decrease in photosynthesis, evidence suggested that 'Maple Arrow' was more responsive to the change in temperature. It appeared as if 'Maple Arrow' was more efficient in adjusting carbon metabolism to changes in temperature than 'Fiskby V', not only in the presence of root chilling, but also in its absence. During prolonged periods of sub-optimal temperature exposure, when decreased photosynthetic capacity is expected to occur, this might be to the advantage of 'Maple Arrow'.

In conclusion, the results strongly suggest a more dark chilling tolerant physiological and biochemical make-up in 'Maple Arrow' compared to 'Fiskeby V'. However, this effect was only visible during whole plant chilling. When roots remained at higher temperatures during chilling, this differential chilling sensitivity between the genotypes was cancelled, suggesting that 'Fiskeby V' is more sensitive to root chilling than 'Maple Arrow'. This may be further strengthened by the suggestion that the inoculant strain (WB 74) is not as compatible with 'Fiskeby V' than with 'Maple Arrow'. This is in accordance with results by Seddigh *et al.* (1988), indicating that 'Fiskeby V' are better adapted to chilled nights with respect of growth and yield. The possible involvement of low root zone temperatures in the dark chilling response of soybean will have to be taken into account in field experiments aimed at selecting dark chilling tolerant genotypes. For future research it is recommended that the possible ameliorating effects of nitrate treatment on dark chilling stress should be investigated in the presence of root chilling, as well as the effect of differences in inoculant strain preference for different genotypes.

6. Literature cited

6. LITERATURE CITED

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Appendix

Publications:

- I conducted the experimental work on FBPase activity presented in Figure 7 in the following publication:

  VAN HEERDEN, P.D.R. KRÜGER, G.H.J., LOVELAND, J.E., PARRY, M.A. & FOYER, C.H. (2003) Dark chilling imposes metabolic restrictions on photosynthesis in soybean. *Plant, Cell and Environment* (in press).

Conferences:

- First author of a poster presented at the annual congress of the South African Academy of Science and Art (2001, Potchefstroom).

  Title: "Die effek van lae nagtemperatuur en daaropvolgende blootstelling aan hoë ligintensiteit op fotosintesetempo en chloroplast fruktose-1,6-bisfosfatase aktiwiteit in *Glycine max* (L.) Merr."
- Co-author of a poster presented at the annual congress of the South African Association of Botanists (2002, Grahamstown)

  Title: "Bad hangovers in soybean leaves after a heavy night of chilling"
- Co-author of a poster presented at the annual congress of the South African Academy of Science and Art (2002, Stellenbosch).

  Title: "Lae nagtemperatuur versteur die regulering van sukrosefosfaat-sintase aktiwiteit in 'n kouesensitiewe sojaboonkultivar".
- First author of a poster presented at the annual congress of the South African Association of Botanists (2003, Pretoria).

  Title: "The impact of dark chilling on key biochemical reactions of photosynthesis and chloroplast ultrastructure in *Glycine max* (L.) Merr."


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