

**Limitation of photosynthetic carbon metabolism in South African
soybean genotypes in response to low night temperatures**

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List of abbreviations

A	CO ₂ assimilation rate at ambient CO ₂ levels
ABS/CS	Phenomenological energy flux (per cross section) for absorption
ABS/RC	The specific energy flux (per PSII reaction centre) for absorption
APS	Ammonium persulphate
ATP	Adenosine triphosphate
C _a	Atmospheric CO ₂ concentration
CCI	Chlorophyll content index
CFI	Chill factor index
Ci	Intercellular CO ₂ concentration
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N,N,N,N'-tetraacetic acid
ET ₀ /CS	Phenomenological energy flux (per cross section) for electron transport
ET ₀ /RC	Specific energy flux (per PSII reaction centre) for electron transport
F-6-P	Fructose-6-phosphate
FBP	Fructose-1,6-bisphosphate
cFBPase	Chloroplastic fructose-1,6-bisphosphatase
G-6-P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
G _s	Stomatal conductance
Hepes	(N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
HK	Hexokinase
J _{max}	Maximal CO ₂ assimilation rate at saturating CO ₂ concentration
NAD	β-Nicotinamide adenine dinucleotide
NADPH	β-Nicotinamide adenine dinucleotide phosphate
OEC	Oxygen-evolving complex
PGI	Phosphoglycoisomerase
Pi	Inorganic phosphate

PI	Plastochron index
PI _{ABS}	Performance index expressed on absorption basis
PMSF	Phenylmethylsulfonyl fluoride
PVPP	Polyvinylpolypyrrolidone
PSI	Photosystem I
PSII	Photosystem II
Φ_{E0}	Quantum yield of electron transport
Φ_{P0}	Maximum quantum yield of primary photochemistry
Ψ_0	Efficiency with which a trapped exciton, having triggered the reduction of Q_A to Q_A^- , can move an electron further than Q_A^- into the electron transport chain
Q_A	Primary quinone acceptor
RC	Photosystem II reaction centre
RC/CS	Photosystem II reaction centre per cross section
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
SBP	Sedoheptulose-1,7-bisphosphate
SBPase	Sedoheptulose-1,7-bisphosphatase
SC	Shoot chilling
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGR	Shoot growth rate
SNF	Symbiotic nitrogen fixation
SPS	Sucrose-phosphate synthase
TEMED	N,N,N',N'-Tetramethylethylenediamine
TR ₀ /CS	Phenomenological energy flux (per cross section) for excitation energy trapping
TR ₀ /RC	Specific energy flux (per reaction centre) for excitation energy trapping
Tris	Tris(hydroxymethyl)-aminomethan
UDPG	Uridine 5'-diphosphoglucose
WPC	Whole plant chilling

Preface

Over the past 30 years the production of soybean in South Africa has increased about ten-fold, mainly due to the increase in demand for usage of soybean oil cake in the animal-feeds industry. However, soybean is sensitive to low night temperatures (dark chilling), thus limiting its yield and making it difficult to successfully cultivate over a broad geographic range within South Africa. Regions of high-altitude, where soybean is predominantly cultivated, often experience episodes of dark chilling, thus creating a scenario which seriously compromise efforts aimed at increasing yield per unit area. Apart from low night air temperatures, sub-optimal root zone temperatures could further aggravate the unsuitable performance of soybean in some of these regions. Successful breeding programmes aimed at developing more chilling tolerant soybean genotypes which deliver higher yields, would greatly enhance existing production in South Africa. However, the chilling tolerance of current South Africa soybean genotypes is poorly characterised. Although international breeding programmes have successfully developed genotypes which are more chilling tolerant, these genotypes do not adapt well to South African conditions. Thus local breeding programmes will offer the best solution in developing chilling tolerant soybean genotypes which are sustainable for use under South African conditions. Out of a practical perspective, identification of chilling tolerant candidates within the South African soybean gene pool was the main driver for the research reported in this thesis. This would be the first step towards increasing the chilling tolerance of South African soybean genotypes, since these candidate genotypes could be used in local breeding programmes aimed at developing more chilling tolerant genotypes delivering higher yields in areas of South Africa where dark chilling is a limiting factor.

The research embodied in this thesis involved the screening of thirty South African soybean genotypes for dark chilling tolerance, and in addition, provide novel insights into the physiological and biochemical factors underpinning the differences in chilling tolerance between genotypes of contrasting chilling tolerance. Moreover, this

research provides a resource of information that could be utilised in further endeavours aimed at developing more chilling tolerant soybean genotypes for commercial use.

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Abstract

Limitation of photosynthetic carbon metabolism in South African soybean genotypes in response to low night temperature.

Soybean (*Glycine max* (L.) Merr.) is a key source of protein for humans and animals, but is unfortunately chilling sensitive. A single episode of low night temperature (dark chilling) is sufficient to inhibit pod formation in soybean. Genotypes respond differently to dark chilling, with some exhibiting a degree of tolerance, while others are extremely sensitive. Since many soybean-producing regions in South Africa are located at high altitudes with very low daily minimum temperatures, production potential is often limited. Identification of genotypes best suited for cultivation in areas that experience dark chilling episodes could provide parental material for inclusion in breeding programs aimed at developing more dark chilling tolerant genotypes, ensuring higher yields per unit area.

The main objectives of this study were to evaluate a large number of South African soybean genotypes for chilling tolerance and to benchmark their response against those of two foreign genotypes of well-known chilling tolerance. Moreover, a detailed physiological and biochemical characterisation of the chilling response of two of these local genotypes of contrasting chilling tolerance was undertaken with emphasis on the contributing role of low soil temperatures towards the inhibition of photosynthesis.

Thirty South African soybean genotypes of unknown chilling tolerance and two foreign genotypes of well known, but contrasting chilling tolerance, were grown under controlled growth conditions in growth chambers. Chlorophyll *a* fluorescence measurements were employed as a rapid screening tool for dark chilling tolerance. Plants were dark chilled (6°C) for seven consecutive nights, but kept at normal day temperatures (26°C). Chlorophyll *a* fluorescence (O-J-I-P) transients were recorded before the end of each night of dark chilling and analysed by the so-called JIP-test to translate stress-induced alterations in the transients to changes in biophysical parameters quantifying the stepwise energy flow through photosystem II (PSII). The performance index (PI_{ABS}), a

multi-parametric expression that combines the three main functional steps taking place in PSII, was used as a measure of dark chilling tolerance. It was successfully demonstrated how the O-J-I-P chlorophyll *a* fluorescence transients could be utilised in screening for dark chilling tolerance in large numbers of soybean genotypes. Elaboration of the PI_{ABS} resulted in the formulation of a novel parameter, the so-called chill factor index (CFI), which revealed large differences in dark chilling response among the genotypes. The CFI of the two foreign reference genotypes correlated with their known difference in chilling tolerance. The CFI was used to rank the thirty genotypes according to dark chilling tolerance, and two local genotypes were selected for further detailed studies, namely Highveld Top, representing a chilling tolerant genotype, and PAN809, representing a highly chilling sensitive genotype.

Further investigations focused on the effects of low soil temperatures, in combination with low air temperatures (whole plant chilling, WPC), compared to the situation where only low air temperatures, but normal soil temperatures, were experienced (shoot chilling, SC). This was an important consideration because besides the inhibition of photosynthesis by low air temperatures, low soil temperatures could also inhibit symbiotic nitrogen fixation, which may indirectly aggravate effects on photosynthesis.

Besides chlorophyll *a* fluorescence, these detailed studies also involved monitoring of vegetative development with the plastochron index, CO₂ gas exchange analysis, determination of chlorophyll and ureide content in leaves, and assaying the activities and contents of key photosynthetic enzymes.

Although a clear distinction could be made between the overall effects induced by the WPC and SC treatments, there were also similarities, especially regarding initial effects. This included the reduction of stomatal conductance, inhibition of PSII function and inhibition of CO₂ saturated rates of photosynthesis ($J_{\max}$), which were similar in both WPC and SC treatments of PAN809. These initial effects could therefore be ascribed mainly to chilling stress experience by shoots and leaves. However, reduced soil

temperatures aggravated the effects of dark chilling, especially in PAN809. When above and below-ground metabolism was taken into consideration, it was clear that continued exposure to dark chilling set into motion a sequence of events in PAN809 leading to severe inhibition of photosynthesis.

The inhibition of PSII function in WPC-treated plants of PAN809 was further aggravated when low soil temperatures inhibited symbiotic nitrogen fixation, causing reduced ureide production and export from the nodules to the shoot, leading to a chlorotic phenotype. Similar symptoms were absent in Highveld Top. When leaf ureide content dropped below a certain threshold level in the WPC-treated plants of PAN809, additional constraints on photosynthesis developed. As N-limiting conditions developed in these leaves, chloroplastic fructose-1,6-bisphosphatase (cFBPase) was targeted specifically, leading to reduced cFBPase protein content and specific activity. As a result, RuBP regeneration capacity was severely compromised as indicated by decreased RuBP content and further suppression of photosynthetic rates that was observed exclusively in WPC-treated plants of PAN809.

In conclusion, the study demonstrated that the genetic pool of South African soybean genotypes exhibits a large degree of variation in dark chilling tolerance. The genotypes Highveld Top and PAN809 were investigated further, focusing on the effect of sub-optimal soil temperatures on above and below-ground metabolism. These treatments facilitated unravelling of the sequence of events leading to the development of a chlorotic shoot phenotype in PAN809 and additional constraints being imposed on photosynthesis of which selective targeting of cFBPase was a key factor. It was clearly demonstrated that, in a chilling sensitive genotype such as PAN809, the response of photosynthesis to dark chilling depended on both shoot and root-derived factors. The large difference in the response of the two genotypes to the WPC-treatment made it possible to identify the key-sites of dark chilling inhibition.

KEY WORDS: Calvin-cycle, chilling tolerance, chlorophyll *a* fluorescence, dark chilling, low soil temperature, photosynthesis, PSII function, soybean.

Opsomming

Beperking van fotosintetiese koolstofmetabolisme in Suid-Afrikaanse sojaboon genotipes in reaksie op lae nagtemperatuur

Sojaboon (*Glycine max* (L.) Merr.) is 'n belangrike bron van proteïene vir beide mens en dier, maar is ongelukkig ook koue sensitief. Blootstelling aan selfs net een nag van lae nagtemperatuur (donkerverkoeling) is voldoende om peulvorming te rem. Genotipes reageer verskillend op donkerverkoeling - sommige vertoon 'n mate van bestandheid, terwyl ander uiters sensitief is. Aangesien sojaboon produserende gebiede in Suid-Afrika in hoogliggende gebiede geleë is, met lae minimum daaglikse temperatuur, word produksie-potensiaal gereeld beperk. Genotipes wat die beste geskik is vir kultivering in hierdie areas kan geskikte ouermateriaal verskaf vir telingsprogramme wat daarop gemik is om meer koue bestande genotipes te ontwikkel en sodoende 'n groter opbrengs per eenheidsarea verseker.

Die hoof doelwitte van hierdie studie was om 'n groot aantal Suid-Afrikaanse sojaboon genotipes vir bestandheid teen donkerverkoeling te evalueer, en om hulle reaksie te vergelyk met dié van twee buitelandse genotipes waarvan die koue-bestandheid welbekend is. Verder is 'n diepgaande fisiologiese- en biochemiese studie uitgevoer op die reaksie van twee plaaslike genotipes teenoor donkerverkoeling, met die klem op die bydrae wat lae grondtemperatuur lewer op die remming van fotosintese.

Dertig Suid-Afrikaanse sojaboon genotipes met onbekende koue-bestandheid, asook twee buitelandse genotipes met bekende maar kontrasterende koue-bestandheid, is onder gekontroleerde toestande in groeikamers gekweek. Chlorofil-a fluoressensie metings is gebruik as 'n vinnige graderingsmetode vir bestandheid teen donkerverkoeling. Plante is vir sewe agtereenvolgende nagte donkerverkoel (6°C), maar by normale dagtemperatuur (26°C) gehou. Chlorofil-a fluoressensie (O-J-I-P) krommes is aan die einde van elke donkerverkoelingsperiode gemeet en met die sogenaamde JIP-toets geanaliseer om stres-geïnduseerde veranderings in die krommes te herlei na

veranderings in biofisiese parameters wat die stapsgewyse energievloei deur fotosisteen II (PSII) kwantifiseer. Die vitaliteitsindeks (PI_{ABS}), 'n multi-parametriese uitdrukking wat die drie hoof funksionele stappe in PSII saamvat, is as maatstaf vir donker koue-bestandheid gebruik. Dit is suksesvol gedemonstreer dat die O-J-I-P chlorofil-a fluoressensie krommes aangewend kan word as 'n graderingsmetode vir donker koue-bestandheid by 'n groot aantal sojaboon genotipes. Verdere verwerking van die PI_{ABS} het tot die formulering van 'n nuwe parameter, die sogenaamde koue faktor indeks (CFI) gelei, wat groot verskille in die reaksie van genotipes op lae nagtemperatuur laat blyk het. Die CFI van die twee buitelandse genotipes het ooreengestem met hulle reeds bekende verskil in koue-bestandheid. Die CFI is gebruik om die dertig plaaslike genotipes volgens koue-bestandheid te rangskik, en twee van hierdie genotipes is gekies vir verdere diepgaande studies, naamlik Highveld Top wat koue-bestand is, en PAN809 wat uiters koue-sensitief is.

Verdere studies het op die invloed van lae grondtemperatuur in kombinasie met lae lugtemperatuur (heelplant verkoeling, WPC) gefokus, in vergelyking met die situasie wanneer slegs lae lugtemperatuur (normale grondtemperatuur) teenwoordig is (bogrondse verkoeling, SC). Hierdie was 'n belangrike aspek omdat fotosintese nie net deur lae lugtemperatuur gerem word nie, maar lae grondtemperatuur ook simbiotiese stikstoffiksering rem en dus indirek die effek op fotosintese kan vererger.

Behalwe chlorofil-a fluoressensie, het verdere studies die monitering van vegetatiewe groei m.b.v. die plastochronindeks, fotosintetiese CO_2 gaswisselingsmetings, bepaling van chlorofil en ureïed inhoud van blare, asook die bepaling van aktiwiteit en inhoud van sleutel fotosintetiese ensieme, ingesluit.

Alhoewel duidelike onderskeid getref kon word tussen die algehele effekte van WPC en SC behandelings, was daar ook ooreenkomste teenwoordig, veral rakende die aanvanklike effekte. Dit het die afname in stomageleiding, asook remming van PSII funksie en CO_2 versadigde tempo's van fotosintese (J_{max}) ingesluit, wat soortgelyk was in beide WPC en SC behandelings van PAN809. Hierdie aanvanklike effekte kon dus

hoofsaaklik toegeskryf word aan koue stremming wat deur die blare en stingels ervaar is. Lae grondtemperatuur het egter die effek van donkerverkoeling vererger, vernameelik in PAN809. Wanneer bogrondse sowel as ondergrondse metabolisme in ag geneem is, was dit duidelik dat aaneenlopende blootstelling aan donkerverkoeling 'n reeks gebeure in werking gestel het in PAN809, wat daartoe gelei het dat fotosintese intensief gerem word.

Die remming van PSII funksie in WPC behandelde plante van PAN809 is verder vererger wanneer lae grondtemperatuur simbiotiese stikstoffiksering gerem het, en sodoende gelei het tot verlaagde ureïed produksie en die vervoer daarvan vanaf wortelknoppies na bogrondse dele, om 'n chlorotiese fenotipe tot gevolg te hê. Soortegelyke simptome was afwesig by Highveld Top. Wanneer die ureïed inhoud van die blaar onder 'n sekere drempelwaarde geval het in WPC behandelde plante van PAN809, het verdere beperkings op fotosintese te voorskyn gekom. Sodra N-beperkende toestande in die blare ontwikkel het, is chloroplastiese fruktose-1,6-bisfosfatase (cFBPase) geteiken, wat tot verlaagde cFBPase proteïeninhoud en spesifieke aktiwiteit gelei het. Gevolglik is RuBP regenerering aansienlik benadeel, soos aangedui word deur verlaagde RuBP vlakke en beperkte fotosintese tempo's wat spesifiek waargeneem is in die WPC behandelde plante van PAN809.

Die studie het dus suksesvol aangetoon dat die genetiese poel van Suid-Afrikaanse sojaboon genotipes 'n groot mate van variasie toon ten opsigte van donker koue-bestandheid. Die genotipes Highveld Top en PAN809 is verder ondersoek, met spesifieke fokus op die effek van sub-optimale grondtemperatuur op bogrondse en ondergrondse metabolisme. Hierdie behandelings het dit moontlik gemaak om die reeks gebeure, wat lei tot die ontwikkeling van 'n chlorotiese fenotipe in PAN809, asook verdere beperkings wat op fotosintese geplaas word, te ontrafel. Die spesifieke teikening van cFBPase speel 'n sleutelrol in dié verband. Daar kon duidelik aangetoon word dat, in 'n koue sensitiewe genotipe soos PAN809, die reaksie van fotosintese op donkerverkoeling afhanklik is van beide bogrondse- en ondergrondse faktore. Die groot verskil in reaksie van die twee genotipes op die WPC-behandeling het dit moontlik gemaak om die sleutelgebiede van donkerverkoeling remming te identifiseer.

SLEUTELTERME: Calvinsiklus, chlorofil-a fluoressensie, donkerverkoeling, fotosintese, koue-bestandheid, lae grondtemperatuur, PSII-funksie, sojaboon.

Chapter 1

Literature Review

1.1 Soybean: Agricultural Importance

Native to eastern Asia, and produced worldwide, soybean (*Glycine max* L. Merrill) is a short season leguminous crop that grows well in warm climates with intermediate to heavy rainfall. With seeds containing approximately 20% oil and 43% protein (Deosthali et al, 2005), it is the most important source of edible vegetable oil and protein, supplying the world with food for both humans and animals (Wilcox, 1987). For centuries soybeans and derived products have been used as a staple food source in Eastern Asia (Young et al, 1984). An increase in world population, as well as a deficit of protein from the diets of many developing countries, provides an ideal opportunity to enhance the utilisation of soybean protein for both human and animal consumption. This would seem an especially good opportunity, since soybeans have an annual global production of 88 million metric tones (Hume et al, 1985).

A growing market for soybean products in South Africa is evident from the increase in soybean production (Hinze et al, 2004) – from 137000 tonnes in 2003/2004 to 220000 tonnes in 2004/2005 (Animal Feed Manufacturers Association Chairman's Report, 2004/2005). Already soybean-based protein products are available in various forms in South Africa. Most of the locally processed soybeans are used as full fat soybean meal by manufacturers of animal feed (Kemp, 2002).

The protein in soybean seeds contains most of the required essential amino acids for a balanced diet (Duxbury et al, 1990). Apart from nutritional value, soybean seeds also contain potentially anti-cancerous compounds, eg. protease inhibitors and phytic acids, and may reduce cholesterol levels (Sijtsema et al, 2002). Since it is also a concentrated source of isoflavones, it is hypothesized that soybeans could play an important role in reducing the risk of contracting various chronic diseases (Messina, 1999).

To keep up with the growing demand for soybean, both locally and in the rest of Africa, it is vital to collect as much knowledge as possible about the different mechanisms of chilling tolerance in soybean, with the aim to increase chilling tolerance (Ceccarelli and Grando, 1996). Like many other warm-climate crop species (e.g. cucumber, tomato and maize), soybean is sensitive to sub-optimal growth temperatures. Many soybean-producing regions in South Africa are located at high altitudes where dark chilling limits soybean production potential (Smith, 1994). In the following section the particular problems associated with soybean cultivation under conditions of low night temperature (dark chilling) are reviewed.

1.2 Sensitivity to and effects of low night temperatures (dark chilling)

The optimal production of agricultural crops, as well as their geographical distribution, is often limited by a range of environmental factors (Allen and Ort, 2001). In the case of soybean, the average annual rainfall in a specific area should be more than 700 mm, with 450 mm of this occurring during the growing season (Smith, 1994). Suitable temperatures for optimum soybean growth range between 20°C and 30°C, while temperatures below 15°C are known to have negative effects on growth, development and ultimate seed yield (Gass et al, 1996). In cases where soybean experience only short term chilling, the changes in metabolism seem to be reversible upon rewarming (Musser et al, 1984). The effect of dark chilling on soybean phenology in particular is dependant upon the specific stage of development, and also varies greatly between genotypes (Seddigh et al, 1989). Frost temperatures of below 0°C cause extensive tissue damage and is fatal for the plant, especially when the plant is acclimated to much higher temperatures (Hume and Jackson, 1981). In soybean there seems to be no difference in frost tolerance among genotypes (Hume and Jackson, 1981). Since soybeans are already susceptible to drought, a limited supply of water could further aggravate the effect of other factors, which in turn would contribute to further inhibition.

Effects of chilling stress on photosynthesis are identified mostly through changes in the chloroplast, the centre of photosynthetic activity. On a structural level, dark chilling disrupts grana arrangement, although photo-activation does seem to lead to

partial reconstruction (Gartska et al, 2005). Furthermore, thylakoids swell up and become distorted, the number of starch granules decreases, and a peripheral reticulum, originating from the inner membrane of the chloroplast, becomes visible (Kratsch and Wise, 2000).

Acclimation to chilling temperatures is known to occur, with some resistant species or genotypes within a species being able to adjust their photosynthetic apparatus in order to maintain near optimal function (Hjelm and Ögren, 2003; Van Heerden et al, 2004b). The ability to acclimate to chilling temperatures is greatly influenced by the stage of plant development at the given time, since certain growth phases are more sensitive than others (Hällgren and Öquist, 1990). Adjustment to the combined exposure to high light intensity and chilling stress is also of great importance in order to minimize photo-inhibition, while the time of exposure to chilling stress is also a determining factor (Huner et al, 1993). Acclimation entails many biochemical changes in the plant, including accumulation of certain molecules and the appearance of new ones, as well as alterations to membrane composition (Alberdi and Corcuera, 1991). In cases of long-term exposure to dark chilling, photosystem II (PSII) is down-regulated by reductions in the photochemical efficiency in order to match the decreased energy requirements for CO₂ fixation (Huner et al, 1993). These are but some of the levels on which plants can be characterized as chilling sensitive or chilling tolerant. On a molecular level, genes involved in both stress tolerance and responses have been identified (Yamaguchi-Shinozaki and Shinozaki, 2006), and more particularly in soybean, where possibilities to enhance tolerance through bio-technology have been explored more recently (Funatsuki et al, 2003).

An important phenomenon that has not received adequate attention is the effect of chilling on plants where the soil does not show a drastic decline in temperature. Many studies have focused only on cases where the shoots and leaves, as well as the roots were exposed to episodes of sub-optimal temperatures, although some investigations into normal root but suboptimal shoot temperatures have been reported (Castle et al., 2006). In cases where roots are exposed to chilling in the presence of light, it sometimes leads to chill-induced drought stress with subsequent leaf wilting. This is due to reduced root

hydraulic conductance restricting water uptake and supply to the transpiring leaves (Allen and Ort 2001, Bloom et al. 2004, Melkonian et al. 2004b). The same, however, is not true when only shoots and leaves of plants are exposed to chilling. When only the shoots of plants experience chilling, the lower demand for nutrients causes reduced uptake via the roots (Svenning et al., 1996), also leading to an accumulation of N in the roots (Castle et al., 2006). However, low soil temperatures could directly affect uptake and transport of required nutrients (Lee et al, 2004), thus compromising growth (Hatch and Macduff, 1991) and even photosynthesis. Interestingly, a degree of chilling tolerance seems to be induced when only the shoots experience low temperatures (Fennell et al, 1990). Exposure of shoots of soybean plants to chilling temperatures during the period of floral induction, depending on the period of exposure, inhibits production of floral primordia, and leads to malformation of pods at later stages (Musser et al, 1986).

In mature and developing leaves, stress caused by exposure to chilling results mostly in damage to the chloroplast, inhibiting photosynthesis and causing premature senescence (Nie and Baker, 1991). In order to predict net primary production of a species such as soybean, it is necessary to assess the different limitations that are imposed on photosynthesis during growth, as a result of altered environmental conditions such as dark chilling (Kosugi et al., 2003).

1.3 Chilling effects on photosynthesis

1.3.1 The photosystems:

1.3.1.1 Photosystem I (PSI):

The PSI complex contained in plants consist of 19 protein subunits, roughly 175 chlorophyll molecules, 2 phylloquinones and 3 Fe₄S₄ clusters (Ben-Sham et al, 2003), with the main task of the photosystem being to generate negative redox potential and to utilize light for electron transport from plastocyanin on the luminal side of the thylakoid membrane to ferredoxin on the stromal side of the thylakoid membrane (Jensen et al, 2007).

Photodamage of PSI in particularly C₃ plants is known to occur when chilling conditions are combined with light stress, causing preferential damage to PSI (Scheller and Haldrup, 2005). Both oxygen and electron transport are required for this inhibition and it involves the oxidative destruction of FeS clusters found in PSI (Sonoike, 1996). Repair of PSI damage as a result of photo-inhibition involves the degradation of the core complex, but very little is known about the processes and enzymes involved in the breakdown and re-use of components to form new PSI complexes (Jensen et al, 2007). Damage to PSI, e.g. as a result of chilling stress etc, is considered a notable factor in the limitation of growth and yield in crops that grow in temperate regions. Thus a better understanding of PSI characteristics would no doubt promote attempts to improve, among other things, the yield of important crops.

1.3.1.2 Photosystem II (PSII):

The electrochemical energy required for the oxidation of water is converted from light energy by photosystem II (PSII), which is a large pigment-protein complex embedded in the lipid layer of the thylakoid membrane. PSII consists of more than 25 subunits, of which nine interact to reduce plastoquinone and evolve oxygen (Barber et al, 1997), with the main subunits being D₁, D₂, the cytochrome b559 dimer, CP47 and CP43 (Hankamer et al, 1997). The oxygen evolving complex (OEC) is made up of the three remaining polypeptides, along with a manganese cluster. Light is harvested by other subunits which act as antenna, cooperating with the LHCII complex, containing a large amount of the grand total of pigments. These pigments include chlorophyll a and b, as well as carotenoids, and serve to collect light and transfer the energy to the reaction centres (RC's) of PSII (Ventrella et al, 2007).

As is the case with PSI, photo-inhibition reduces the activity of PSII, however photosynthetic organisms do possess the ability to overcome the toxic effect of light by means of the repair of damaged PSII units (Aro et al, 1993). Thus the balance between damage and repair of PSII determines the extent to which photo-inhibition affects plants.

Although several schemes for the mechanism of photodamage have been noted in the past, the most recent findings suggest a two-step process: firstly the light dependent destruction of the Mn cluster of the OEC occurs, and secondly the PSII RC is inactivated by light that is absorbed by chlorophyll pigments (Ohnishi et al, 2005). The repair of PSII is inhibited by reactive oxygen species, thus further enhancing the extent of photo-inhibition. Repair of PSII is mostly suppressed by decreased synthesis of the D1 protein (Nishiyama et al, 2001). It is assumed that the redox signals that would normally activate the relevant translation process are interrupted by reactive oxygen species under oxidative conditions (Murata et al, 2007).

Changing environmental conditions enhance the photodamage to PSII, with its activity sometimes declining more rapidly as a result of these changes than of other physiological parameters (Aro et al, 1993). It has, however, been proven that it is in fact the process of PSII repair which is more sensitive to unfavourable environmental conditions, rather than photodamage of PSII itself (Nishiyama et al, 2001). Chilling stress in particular is known to promote the photo-inhibition of PSII under high irradiance (Bertamini et al, 2005; 2006; Öquist et al, 1993; Van Heerden et al, 2003c). It would seem that photodamage of PSII is not really affected by chilling temperatures, and Murata et al (2007) found that the process of PSII repair was more susceptible to this kind of stress. As already noted, it would seem that the synthesis of D1 proteins is the process of PSII repair mostly suppressed by chilling temperatures (Allakhverdiev and Murata, 2004).

The temperature at which PSII starts to undergo denaturation is highly influenced by the fluidity of the thylakoid membranes (Raison et al, 1982). It is known that, at chilling temperatures, membrane lipids of species sensitive to chilling temperatures start to undergo lateral phase separation, with minor components starting to freeze (Raison and Orr, 1986). However, studies have shown that this can be regarded as a 'primitive' condition, with plants showing more suitable adaptations for colder climates (Terzaghi et al, 1989). More recent findings show that at low temperatures, the lipid and fatty acid composition of membranes tend to undergo changes, enabling the cell to maintain the

chloroplast function, even if only at a minimum (Routaboul et al, 2000). Thus chilling-induced resistance would seem to be accelerated by the unsaturation of the PSII thylakoid membranes (Zhu et al, 2007).

1.3.2. Photosynthetic CO₂ gas exchange:

Utilisation of CO₂ for carbon assimilation and photosynthesis begins with uptake from the atmosphere, contributing to canopy photosynthesis of the plant. The dependence of photosynthesis on CO₂ concentrations ultimately affects plant growth and productivity (Kirschbaum, 1994). Limitations such as decreased leaf area, closing stomata and decreased efficiency to assimilate carbon all result in reduced canopy photosynthesis (Frederick et al., 1989). In the case of stomatal closure, the mechanism acts as protection, reducing the loss of water through transpiration (Cochard et al., 2002; Souza et al., 2004). However, this mechanism simultaneously also limits the amount of CO₂ available for carbon assimilation. It is now widely accepted that stomatal closure account to a large part for the inhibition of photosynthesis during drought stress (Flexas et al., 1999). Sufficient water promotes gas exchange and results in significant increases in net photosynthesis rate, stomatal conductance and apparent carboxylation efficiency (Niu et al., 2004).

Photosynthesis is one of the first processes affected by chilling (Flexas et al., 1999) causing a less responsive behaviour to CO₂ (Kirschbaum, 1994), even at elevated levels, although not to the same extent as under ambient conditions (Melkonian et al., 2004a). Short-term exposure to chilling night or day temperatures causes reductions in CO₂ assimilation and stomatal conductance, with no effects on internal CO₂ (Melkonian et al., 2004a; Singh et al., 2005; Kościelniak and Biesaga-Kościelniak, 2006). Reduced activity of Rubisco, as well as reduced capacity for electron transport, are some of the main reasons for the inhibition of assimilation rate at low temperatures (Farquhar and Sharkey, 1982).

Levels of CO₂ assimilation in leaves that developed during short term chilling would seem to return to normal only several weeks after exposure, and with only partial

acclimation of stomatal conductance (Melkonian et al., 2004a). Recovery of photosynthesis after chilling episodes seems to be more rapid at elevated levels of CO₂ (Melkonian et al., 2004a). However, in some cases when plants are grown to maturity under chilling conditions, there seems to be no effects on photosynthetic parameters (Singh et al., 2005). In plants known to be sensitive to chilling, i.e. tropical and sub-tropical species, irreversible inhibition of CO₂ assimilation is known to occur, accompanied by a decrease in RuBP regeneration capacity (Zhou et al., 2004a; b), with disruption of stomatal control of CO₂ supply (Allen and Ort, 2001). Even in cases where chilling sensitive species showed no significant inhibition of CO₂ assimilation in the first couple of hours of rewarming after a chilling period, reductions were ultimately visible by midday, due to increased stomatal limitation and inhibition of Rubisco (Allen et al. 2000). In soybean in particular, the same is true with regard to the effect of chilling night temperatures on carbon metabolism. Both CO₂ assimilation rate and stomatal conductance show decreases upon exposure to dark chilling, even in genotypes from a more temperate origin (Van Heerden et al., 2004b).

The enzymes of the Calvin-cycle are all involved in photosynthetic gas exchange to a greater or a lesser degree. Thus any alterations in these enzymes could be reflected in the gas exchange capacity a plant exhibits. The following section focuses on the relevant enzymes of the Calvin-cycle which had been investigated in the present study.

1.3.3. The Calvin cycle

Situated in the stroma of the chloroplast, the photosynthetic carbon reduction cycle (Calvin cycle) is the primary pathway for CO₂ fixation. The products of the light reactions of photosynthesis, namely ATP and NADPH, are used by the Calvin cycle to fix atmospheric CO₂ into compounds for carbohydrate synthesis, i.e. starch and sucrose. Eleven different enzymes are involved in the cycle, catalyzing thirteen different reactions (Fig. 1.1). Control of carbon assimilation is shared between several of these enzymes, including ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), sedoheptulose-1,7-bisphosphatase (SBPase), fructose-1,6-bisphosphatase (FBPase) and aldolase

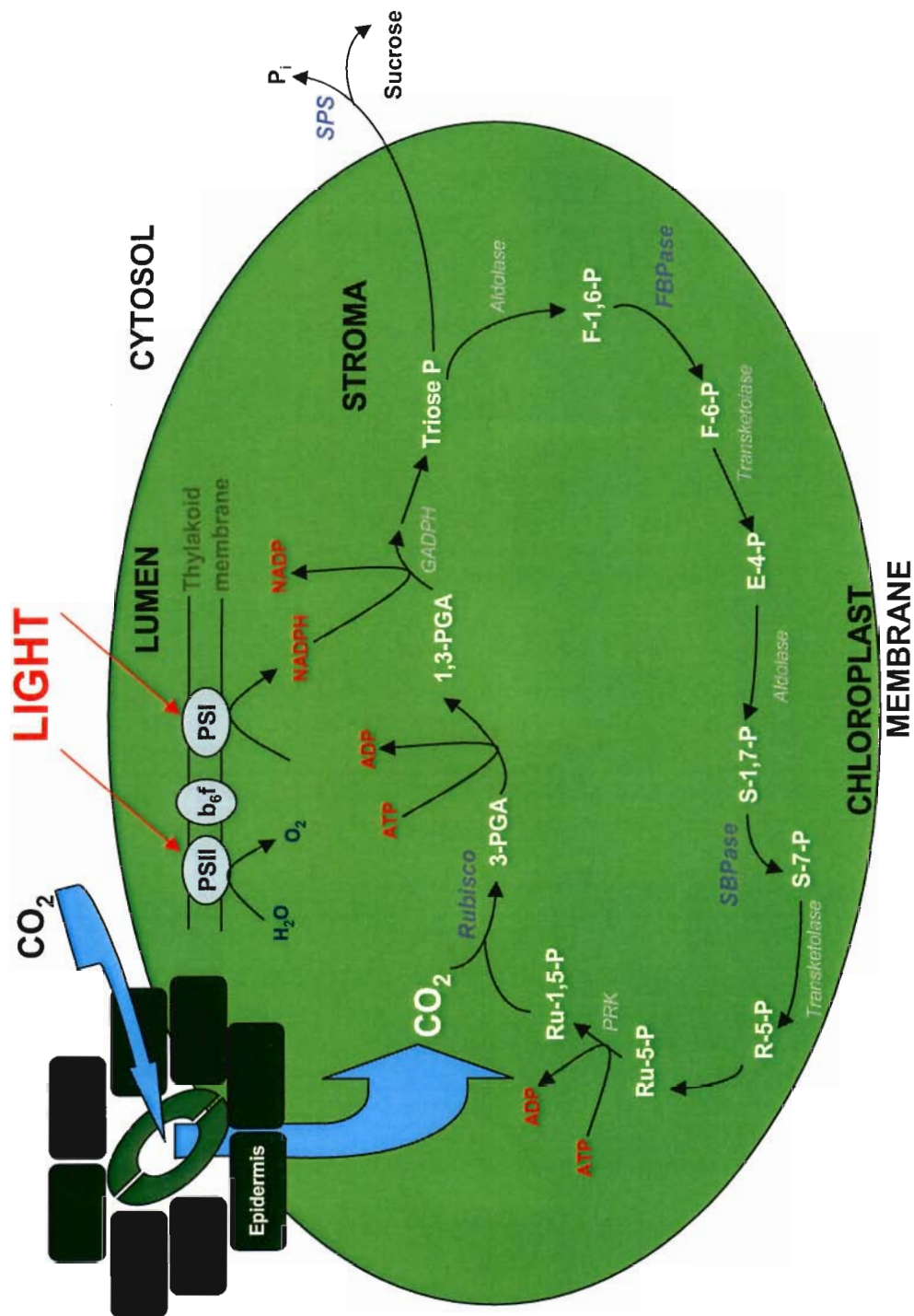
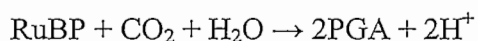


Figure 1.1 The Calvin cycle, showing all the relevant enzymes investigated in the study (dark blue), as well as the remaining enzymes of the cycle (light blue).

(Harrison et al, 1998; Ölçer et al., 2001), and the contribution of each of these enzymes differs according to environmental conditions (Raines et al., 2000).

1.3.3.1 Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco):

The most abundant protein on earth, Rubisco (EC 4.1.1.39) (Kung, 1976; Ellis, 1979) catalyzes the first reaction of the Calvin cycle, namely the conversion of ribulose-1,5-bisphosphate (RuBP) to phosphoglycerate (PGA):



thus assimilating CO₂ to sugar-form (Weisbach et al, 1956; Jensen and Bahr, 1977). A large number of reviews regarding its function and properties have been published (e.g. Portis, 1992; Spreitzer and Salvucci, 2002; Andersson, 2008).

It can act as a bifunctional enzyme, catalysing incorporation of both O₂ and CO₂, i.e. carboxylase and oxygenase activities. The ratio of these two reactions determines the efficiency with which CO₂ is assimilated (Jensen and Bahr, 1977), since oxygenation acts as competitive inhibitor of carboxylation, thus providing glycolate for the photorespiratory pathway (Ogren, 1984). Together with the capacity for RuBP regeneration, Rubisco is considered one of the main rate-limiting steps in photosynthetic carbon assimilation (Hudson et al, 1992; Savitch et al., 2000), especially under saturated light conditions in the field, although Stitt and Schulze (1994) found that plants are able to adjust the balance between Rubisco and photosynthetic apparatus at different light intensities, thus avoiding limitation of photosynthesis by Rubisco alone. Antisense studies have surprisingly shown that reducing Rubisco activity with up to 50%, had no significant effect on photosynthesis, even under a wide range of growth conditions (Raines, 2003). However, it is very clear that Rubisco's biochemical characteristics play a role in it being one of the main factors limiting the uptake of atmospheric carbon. Similar to SPS, Rubisco is also characterised by circadian regulation, since the transcriptional activity of genes encoding for Rubisco activase is known to be controlled by an endogenous rhythm (Martino-Catt and Ort, 1992). Levels of Rubisco also largely

determine partitioning of nitrogen for other photosynthetic components, since it is by far the largest sink for nitrogen among these components (Makino et al, 1994).

Consisting of eight large and eight small subunits, Rubisco is converted to an active form by light (Leegood, 1993). The subunits are assembled by means of coordinated biochemical processes taking place in different cellular compartments. The expression of the gene *rbcS* in the nuclear genome is modulated by light, and produces a precursor polypeptide of the small subunit, which is translated by cytosolic ribosomes. The gene *rbcL*, situated in the chloroplast genome, codes for the mature form of the large subunit, which is translated on chloroplast ribosomes. The enzyme is finally assembled from its constituent subunits in the chloroplast, assisted by special modulating proteins called chaperonins (Roy, 1989; Buchanan and Wolosiuk, 1998).

The amounts of active or inactive forms of Rubisco are determined by pH, CO₂ and Mg²⁺ (Lorimer et al., 1976), but the activity of the enzyme is independent of substrate levels or inhibitors (Portis, 1992). Rubisco activity is, however, decreased by deactivation via post-translational regulation, involving a lysine residue on the active site being decarbamylated (Andrews and Lorimer, 1987).

Chilling has species-dependent effects on the activity and levels of Rubisco. In general, both content and activity of the enzyme is reduced by chilling stress in species sensitive to chilling (Bertamini et al., 2005; Brüggeman et al., 1992; Zhou et al, 2004a), with breakdown of the enzyme that can already start at a temperature of 15°C (Kingston-Smith and Foyer, 2000) as well as disruption of its circadian regulation (Martino-Catt and Ort, 1992). In bean, simultaneous exposure of plants to light and chilling temperatures of 10°C decreases activation of Rubisco within minutes, with a further decrease evident as exposure is extended (Holaday et al, 1992). At low temperatures Rubisco kinetics is linked to, and also largely determines photosynthetic performance (Brüggeman et al, 1992; Yamori et al, 2006). A decrease in Rubisco content observed during prolonged exposure to chilling is also considered one of the reasons for photosynthetic limitations, even after a period of recovery (Pietrini et al., 1999). In more chilling resistant species,

changes in the optimum temperature of carboxylation can lead to a degree of acclimation of photosynthesis during chilling (Yamori et al, 2005). As with many other enzymes, cold-hardening in chilling tolerant species, such as winter rye, could also lead to a considerable increase in the activity of Rubisco and its relevant metabolites, thus maintaining normal photosynthetic capacities (Hurry et al., 1994). Although Brüggeman (1992a) found that long-term chilling had no effect on Rubisco activity in soybean, these studies were conducted under low irradiance, minimising photo-inhibition. Van Heerden et al (2003a) carried out studies of several nights of dark chilling, with each treatment period being followed by normal daylight irradiance, and found that Rubisco activity was subsequently lowered, in association with decreased CO₂ assimilation.

1.3.3.2 Fructose-1,6-bisphosphatase (FBPase):

The enzyme FBPase (EC 3.1.3.11) serves as one of the branch points between the regenerative phase of the Calvin cycle and biosynthesis of starch and catalyses the irreversible breakdown of fructose-1,6-bisphosphate to fructose-6-phosphate and Pi (Kossmann et al., 1994; Tamoi et al., 2006), which is a regeneration step in the Calvin cycle for recovering the CO₂ receptor, RuBP (Sahrawy et al., 2004). There are two isoforms of FBPase in a cell, namely a cytosolic form, which is involved in sucrose synthesis, and a chloroplastic form (cFBPase), which is involved in the regulation of the CO₂ assimilation pathway (Chueca et al., 2002). Cytosolic FBPase is regulated by fructose-2,6-bisphosphate (Fru2,6bisP), a signalling metabolite, of which the concentration is modulated by either inhibiting or activating metabolites (Stitt et al, 1984; Neuhaus et al., 1990). Redox potential regulates cFBPase via the ferredoxin/thioredoxin system, which in turn modulates the activity of the enzyme during dark-light transitions (Buchanan, 1991; Ocón et al., 2001).

Both cytosolic and cFBPase are homotetramers, about 350 amino acids in size, with a molecular mass of approximately 40kDa for each subunit (Chiadmi et al., 1999). Synthesis of cFBPase is regulated by light and occurs at the transcriptional level (Sahrawy et al., 1990). Partial activation by high levels of Mg⁺² also occurs, even in the absence of a reductant (Ruelland and Migonac-Maslow, 1999). In its oxidized form,

cFBPase has a very low activity, but after reduction its specific activity increases markedly (Ruelland and Migonac-Maslow, 1999). The presence of Ca^{2+} and FBP, in combination with the factors of pH, Mg^{2+} and thioredoxins are all involved in light-activation of cFBPase, thus making it a slow process compared to the rate of catalysis (Chueca et al., 2002). As a result, the activity of cFBPase can be divided into two steps, namely activation and catalysis (Schürmann and Wolosiuk, 1978).

Under conditions where plants experience colder day/night temperature regimes of 18/15°C, the activity of cytosolic FBPase increases slightly, although this effect is not carried through to photosynthesis (Makino et al., 1994). Activation of chloroplastic bisphosphatases by thioredoxins are disrupted by chilling, causing inhibition of reductive activation of cFBPase upon illumination (Sassenrath et al., 1990; Hutchison et al., 2000), although in some cases low activation is observed (Holaday et al., 1992), with only partial restriction of normal capacity when exposed to low light intensities (Fisahn et al., 1995). In soybean in particular, the activity and activation state of cFBPase during the light period is inhibited following nights of dark chilling (Van Heerden et al., 2003a), possibly being the result of inhibition of hysteresis regulation of the enzyme, where the active form is locked by its substrate after it has been activated by thioredoxin *f* (Van Heerden et al., 2003a). In this case, concomitant decreases in photosynthetic CO_2 assimilation were also observed. Antisense studies have shown that a decrease of up to 34% in cFBPase activity is required to mirror an affect in photosynthesis in plants grown under low light intensities (Raines, 2003).

1.3.3.3. Sedoheptulose-1,7-bisphosphatase (SBPase):

The second of the two chloroplastic bisphosphatases, SBPase (EC 3.1.3.37), catalyses the irreversible dephosphorylation of sedoheptulose-1,7-bisphosphate to sedoheptulose-7-phosphate, and similar to cFBPase plays a determining role in allocating carbon to synthesis of sugars and starch (Geiger and Servaites, 1994; Raines et al., 1999; Ruelland et al., 1999). Of these two bisphosphatases, SBPase appears to be the most important factor in RuBP regeneration in the Calvin cycle (Tamoi et al., 2006). It is a homodimeric polypeptide, consisting of two identical subunits with a molecular weight of

35 to 38 kDa (Cadet and Meunier, 1988). The enzyme is not active in darkness, but shows increased activity within 15 minutes of exposure to light (Laing et al., 1981). As is the case with cFBPase, light activation of SBPase is achieved by reduced thioredoxin (Nishizawa and Buchanan, 1981), leading to a 12-fold increase in activity (Breazeale et al., 1978). Both the pH of the stroma as well as levels of Mg^{2+} also regulate SBPase activity, since both factors are affected by changes in light conditions (Nishizawa and Buchanan, 1981). Further control is also enforced by the levels of the catalyzed reaction products, namely inorganic phosphate and sedoheptulose-7-phosphate (Schimkat et al., 1990).

Light conditions, sugar levels and development control gene expression of SBPase, with light exposure increasing SBPase transcript levels 20-fold in previously dark-grown seedlings of some species (Willingham et al., 1994). Metabolic feedback also affects gene expression by reducing levels of Calvin cycle mRNA, including SBPase (Jones et al., 1996).

Being one of the key regulatory enzymes in the Calvin cycle, changes in SBPase activity are also closely linked to the rate of photosynthesis, with reduced SBPase activity resulting in limitation of CO_2 assimilation rates (Harrison et al., 1998) and reduction in the capacity to regenerate RuBP (Harrison et al., 2001). Under saturated light conditions, a reduction of just 20 to 35% in SBPase activity is sufficient to significantly limit photosynthetic capacity. Carbohydrate levels, in particular starch, are reduced as a result of decreased SBPase activity, although these changes also depend on leaf development (Ölçer et al., 2001; Lefebvre et al., 2003).

The effect of chilling on SBPase is similar to that of its bisphosphatase counterpart cFBPase, with activity of the enzyme being reduced upon illumination at temperatures as low as 4°C due to disruption of thioredoxin mediated activation (Sassenrath et al., 1990; Hutchison et al., 2000), although this has only been found in certain species.

1.3.3.4 Other Calvin cycle enzymes

Some of the other Calvin cycle enzymes of note are glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (EC.1.2.1.13), phosphoribulokinase (PRK) (EC.2.7.1.19), aldolase (EC 4.1.2.13) and transketolase (TK) (EC.2.2.1.1).

GAPDH consists of two subunits, GapA and GapB, of 36 kDa and 41 kDa, respectively (Cerff, 1982). The subunits are very homologous with one another at the nucleotide and amino acid levels (Shih et al, 1986). Prokaryotic features are evident in the amino acid sequences of the enzyme subunits, making it likely that GAPDH evolved from other lineages (Shih et al, 1986). Binding of the NAD co-enzyme to GAPDH triggers a process of sequential structural changes, and a model of how these changes enable the enzyme to perform its functions was proposed by Skarzynski and Wonacott (1988).

The enzyme PRK is light regulated via the reversible reduction by thioredoxin. It plays an important role in the reductive pentose phosphate pathway by catalysing the reaction which provides intermediates for CO₂ fixation (Michels et al, 2005). This entails the transfer of a γ -phosphoryl group from ATP to ribulose 5-phosphate to produce ribulose 1,5-bisphosphate, which serves as the acceptor molecule for CO₂. The PRK enzymes can be divided into two classes, Class I and II, with Class II being found in terrestrial plants (Martin and Schnarrenberger, 1997).

TK functions in the nonoxidative branch of the pentose phosphate pathway and is responsible for catalysing the reversible transfer of two-carbon glycol aldehyde units from ketose phosphates to aldolase phosphates (Schenk et al, 1998; Veitch et al, 2004). Co-factors for TK include thiamine diphosphate and bivalent cations Ca²⁺ and Mg²⁺ (Sevostyanova et al, 2006). Sugars such as xylulose 5-phosphate, sedoheptulose 7-phosphate, fructose 6-phosphate and erythrose 4-phosphate serve as donor substrates for TK (Sevostyanova et al, 2006).

Aldolase possesses a 38 kDa subunit which combines to form tetramers which represent the active enzyme complex (Raines et al, 1991). Together with triosephosphate isomerase (EC 5.3.1.1) it continually catalyses two of the reactions in the photosynthetic carbon reduction cycle, namely the rapid and reversible ketose-aldolase isomerization of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (G-3-P), followed by reversible aldol condensation of these two compounds to form fructose-1,6-bisphosphate (FBP) (Ma et al, 2007). The enzyme has both a cytosolic and chloroplastic isoform, which exhibit similar, although not identical catalytic and structural characteristics (Schnarrenberger and Kruger, 1986).

GADPH and PRK are known to be negligible when it comes to control of the flux through the Calvin cycle (Raines, 2003). In the case of TK, although reduced activity of the enzyme has an influence on photosynthetic capacity and sucrose metabolism, a notable change in total biomass is only evident after activity is reduced below 60% of normal values (Raines, 2003). Since aldolase catalyses a reversible reaction, it is assumed not to have regulatory control over the flux through the Calvin cycle, and is thus not of great importance in the control of growth or metabolism. For these reasons, this study did not focus on the activities of Calvin cycle enzymes other than Rubisco, cFBPase and SBPase.

1.3.4 Ribulose-1,5-bisphosphate (RuBP)

Ribulose-1,5-bisphosphate (RuBP) plays the role of acceptor in Rubisco's catalysed fixation of CO₂ to 3-PGA. RuBP originates during the regenerative phase of the Calvin cycle by conversion of triose phosphates through a series of reactions. Most of the triose phosphate molecules produced in the Calvin cycle are locked into the cycle for regeneration of RuBP (Raines, 2003).

Farquhar et al (1980) proposed a model stating that photosynthetic rate is equal to the minimum rate of RuBP regeneration, as well as RuBP carboxylation, and the effect of temperature changes on these two partial reactions mirrors any changes in the photosynthesis rate. Changes in the optimum temperature for photosynthesis are believed

to be the result of changes in the balance between the regeneration and carboxylation of RuBP in response to temperature shifts (Farquhar and von Caemmerer, 1982). Furthermore, regeneration of RuBP is also dependent on electron transport (von Caemmerer, 2000; Harrison et al, 2001), a factor also heavily dependent on temperature changes, as well as by limitation of the capacity of certain Calvin cycle enzymes (Hurry et al, 1998). Thus, the regeneration of RuBP is a complex process involving several temperature-dependent factors.

Considering factors affecting levels of RuBP, even a small change in the activity of SBPase reduces RuBP regeneration capacity, thus limiting CO₂ assimilation (Harrison et al, 2001), while it would seem that reduced growth temperatures decrease the rate of RuBP regeneration (Labate et al, 1990; Yamori et al, 2005).

1.3.5 Carbohydrate metabolism

1.3.5.1 Sucrose-phosphate synthase (SPS)

Sucrose-phosphate-synthase (EC 2.4.1.14) is a soluble enzyme located in the cytoplasm, and being a key enzyme in the pathway of sucrose synthesis (Stitt et al, 1988), it catalyzes the reaction uridine diphosphoglucose (UDPGlc) + fructose-6-phosphate = sucrose-6-phosphate + UDP (Huber and Israel, 1982). SPS activity is characterized by a circadian rhythm in species like tomato (Jones and Ort, 1997; Winter and Huber, 2000), while an ultradian rhythm occurs in species such as soybean (Kerr et al, 1985). Together with invertase and sucrose synthase, SPS modulates carbon partitioning since it supplies carbohydrates and regulates source-sink ratios by amplification of appropriate signals (Roitsch, 1999). The steady-state levels of the SPS enzyme protein is modulated by three different mechanisms, namely allosteric regulation, i.e. activation by glucose-6-phosphate (G6P) and inhibition by inorganic phosphate (Pi) (Doehlert and Huber, 1983; Huber et al, 1990; Krause and Stitt, 1992); reversible covalent modification induced by the presence of light (Stitt et al, 1988) or osmotic stress (Toroser and Huber, 1997); and regulation on transcriptional level during the transition of plant organs from sink to source (Klein et al, 1993). Activation of SPS by light occurs in as little as 15 minutes, with inactivation by darkness taking up to 30 minutes (Huber et al, 1989). The SPS activation state is thus

linked to photosynthesis rate (Battistelli et al, 1991), since the rate possibly alters the levels of the metabolites involved in the control of the enzyme (Isopp et al, 2000). It has been shown in tomato that SPS is a critical point of control of photosynthesis, since overexpression results in increased sucrose/starch ratios and photosynthetic capacity under high levels of CO₂ and saturating light (Galtier et al, 1995). Furthermore, mild water stress reduces SPS activity in *Phaseolus vulgaris* as a result of photosynthetic inhibition, since restricted CO₂ supply causes stomatal closure, thus inhibiting photosynthesis (Vessey et al, 1991) as well as an increase in cytosolic Pi (Castrillo, 1992). However, overexpression does not necessarily lead to increased SPS activity because of posttranslational regulation of the enzyme (Winter and Huber, 2000). During dark phases, in the absence of photosynthesis, SPS is converted into a low activity form by phosphorylation at the Ser-158 residue (Huber, 2007). On a protein level, SPS is regulated by a number of signals, including plant development, the environment, and nutritional needs (Huber, 2007).

Regulation of SPS differs between species, allowing for classification into three categories: predominantly being activated by light (Class I and II) (Huber and Huber, 1992) or not (Class III). Soybean belongs to the latter group. Interestingly, however, there does seem to be some differences in regulation of SPS between soybean genotypes of early and late maturity groups, with early maturity groups showing significant light activation of SPS (Huber and Huber, 1996). Thus a possible difference in the effect of dark chilling on photosynthesis in soybean could be explained by this occurrence, since these genotype-specific differences are not known to be present in other species.

In C₃-plants, it is known that chilling causes inhibition of SPS activity, leading to decreased levels of sucrose (Labate and Leegood, 1990). Since SPS is under circadian regulation, mistiming of the endogenous rhythm as a result of chilling stress could be an important factor in the sensitivity of certain plant species toward chilling (Jones et al, 1998). Since the circadian control of SPS is linked to the activity of protein phosphatases, it is most likely that chilling delays the de-phosphorylation of SPS by inhibiting the expression of the gene which encodes for SPS phosphatase (Jones et al, 1998). However,

prolonged exposure to chilling temperatures in chilling resistant species could lead to an increase in SPS synthesis (Guy et al, 1992) and activity (Hurry et al, 1995), accompanied by increased sucrose levels (Guy et al, 1992). Studies with *Arabidopsis* have shown that over-expression of SPS under CO₂ enriched conditions causes an increase in sucrose levels in leaves (Signora et al, 1998). At low temperatures this could reduce the inhibition of photosynthesis (Singh et al, 2005) and lead to the development of freezing tolerance (Strand et al, 2003). In some cases, plant species found in extreme cold conditions maintain high levels of SPS activity, leading to the abnormal accumulation of sucrose, as a means to enhance chilling tolerance and plant survival (Zúñiga-Feest et al, 2005).

1.3.5.2 Carbohydrates

In higher plants, the fixation of CO₂ via the Calvin cycle results in the formation of metabolites which can either be used for starch synthesis in the stroma, or exported to the cytosol for sucrose synthesis. Maintaining a correct balance between levels of sucrose and starch is of high importance, and is mostly determined by the current needs of the plant as well as environmental conditions (Sahrawy et al., 2004), with sucrose formation indirectly controlling the rate of starch formation (Huber and Israel, 1982). Larger amounts of sucrose and/or starch are accumulated during shorter days, compared to long days (Britz, 1990). The ratio of sucrose:starch is linked to several enzymes, with decreases in sFBPase activity causing the ratio to reach a near maximum value (Kossmann et al., 1994; Sahrawy et al., 2004), while the same is true when an increase in SPS activity occurs (Signora et al., 1998).

Low growth temperatures lead to reduced biomass, i.e. carbohydrate accumulation in maize, although acclimation at higher temperatures partially restores sucrose levels to normal in some genotypes, coupled with increased photosynthesis (Pietrini et al., 1999). In chilling resistant species, maintaining optimal photosynthesis at lower temperatures would seem to be promoted by inducing higher levels of carbohydrate synthesis, although this may lead to feedback inhibition of photosynthesis at a certain point (Labate and Leegood, 1990). In cases where high levels of carbohydrates are successfully maintained, it usually points to disturbed balances between photosynthesis

and consumption of carbohydrates, but could possibly also serve as an adaptive mechanism in helping to stabilize proteins and membranes (Venema et al., 1999).

1.3.5.2.1 Sucrose:

Sucrose is synthesised in the cytosol, catalysed by sucrose-phosphate synthase (SPS) and sucrose synthase (SS) (Noël and Pontis, 2000), and is the major form of sugar transported via the phloem (Walker and Ho, 1977; Winter and Huber, 2000). It is now known that even gene expression is regulated directly or indirectly by levels of sucrose (Winter and Huber, 2000; Tabaei-Aghdaei et al, 2003; Thibaud et al, 2004). Since sucrose synthesis utilises triose phosphates originating from the chloroplast, less of these intermediates are available for starch synthesis. The combination of two triose phosphate molecules, coupled with the release of inorganic phosphate (Pi), leads to formation of one molecule F6P, which can subsequently be converted to either G6P or G1P. Accumulation of sucrose leads to a decrease in the synthesis thereof and suppresses photosynthesis by reducing Pi cycling and depletion of ATP levels (Labate et al., 1990). Regulation of cytosolic FBPase determines the levels of FBP, which in turn regulates the conversion of triose phosphates to hexose phosphates (Krause and Stitt, 1992). Sucrose is irreversibly converted to glucose and fructose by invertase, while SS catalyses its reversible breakdown.

Chilling temperatures lead to increased levels of sucrose and fructans in some species (Guy et al., 1992; Miao et al., 2006). Accumulation of sugars usually leads to inhibition of photosynthesis and less active enzymes in the Calvin cycle, although in cases where decreased activity of FBPase occurs as a result of possible environmental stress, it serves to promote photosynthetic activity (Sahrawy et al., 2004). In some chilling resistant species, it is believed that accumulation of sugars under extreme environmental conditions such as cold serves as a means of protection and attempts to hardiness (Tognetti et al., 1990; Signora et al, 1998; Strand et al, 2003), and this accumulation is known to be in parallel with increased activity of SPS (Guy et al., 1992; Zúñiga-Feest et al., 2005). Rapid mobilization for metabolic requirement is the most probable reason why sucrose in particular would be ideal as storage carbohydrate under

changing environmental conditions (Guy et al., 1992). In the case of drought stress, increases in sucrose levels are also evident, with accompanied higher activities of SPS (Zrenner and Stitt, 1991).

1.3.5.2.2 Starch:

Starch is the major medium of carbohydrate storage in nearly all plants, as well as the second most abundant carbohydrate in the biosphere, and is formed in chloroplasts via the process of photosynthesis. Light predominantly determines the levels of starch in leaves, accumulating during the daytime when respiration and translocation is exceeded by the photosynthetic rate (Labate and Leegood, 1990; Blennow et al., 2002). Starch molecules are formed by donation of glucose units from adenosine diphosphoglucose, and the reaction is regulated by ADPG pyrophosphorylase (ADPGase) (Preis, 1982). Activation of ADPGase is achieved by 3-phosphoglycerate while it is inhibited by Pi. The ratio of these two regulators determines the state of starch synthesis. More recent findings indicate that trehalose 6-phosphate also plays an important role in the regulation of starch metabolism, since an increase in its levels leads to a marked stimulation in starch synthesis in a very short period of time (Kolbe et al, 2005). Degradation of starch to glucose is catalysed by α -amylase, β -amylase and starch phosphorylase (Dennis and Blakeley, 2000), among others, and while several other enzymes may also play a role in this process, many of the mechanisms and enzymes involved in this process remain to be clarified (Smith et al, 2005).

In soybeans in particular the accumulation of starch correlates with the length of the photoperiod (Chatterton and Silviu, 1979), with Pi limitation during prolonged photoperiods leading to an increase in the levels of foliar starch (Rao et al., 1993). Limitations in supply of nitrogen to the plant also have a stimulatory effect on levels of starch, in association with decreased photosynthetic rates (Robinson, 1996).

Although long-term moderate chilling temperatures result in increased starch accumulation in chloroplasts due to reduced export (Schaffer et al., 1991; Venema et al., 1999), chilling temperatures of 6°C impair starch formation (Brüggeman et al., 1992).

One major reported symptom of this occurrence is premature chlorosis (Schaffer et al., 1991). Drought stress is also characterised by decreased starch levels (Vessey et al., 1989; Zrenner and Stitt, 1991).

1.3.6 Nitrogen fixation

Legumes, including soybean, contain specialised bacteria in their roots which utilises N from the air, while nitrate is taken up directly from the soil by the roots (Harper, 1987). The root hairs of soybean are infiltrated by the N-fixing bacteria *Bradyrhizobium japonicum*, where it then multiplies. Nodules are formed on the roots as a result of this infection, and the bacteria housed in the nodules fixes the N it absorbs from the air around the roots to form ammonia (NH_3). Since this is a mutually beneficial partnership for both the plant and the bacteria, the process is called symbiotic nitrogen fixation (SNF) (Adjei et al, 2002). Transport of fixed N from the roots to other parts of the plants occurs in the form of ureides via the xylem sap (McClure and Israel, 1979).

The catabolism of ureides such as allantoin and allantoate accounts for the greatest part of N utilised by the soybean plant (Winkler et al, 1987). Firstly, the enzyme allantoinase converts allantoin into allantoate. In soybean, allantoate can then follow one of two possible directions, being catabolized by either allantoate amidinohydrolase or allantoate amidohydrolase (Van der Drift et al, 1970; Vogels and Van der Drift, 1976). Further processing of ureide catabolites entails incorporation into crucial proteins such as enzymes in the form of amino acids.

Drought is known to be the cause of ureide accumulation in petioles and leaves of soybean (Vadez et al, 2000). This is most likely the result of decreased catabolism of ureides in the leaves, apart from the fact that nodule activity also declines under these conditions (Vadez et al, 2000). The accumulation of ureides and certain amino acids in leaves is most probably a main cause of decreased nodule activity, acting as a feedback mechanism (Serraj and Sinclair, 1996). Legros and Smith (1994) found that low soil temperatures result in decreased levels of N within soybean. Also, it appears that SNF is more sensitive to low soil temperature than growth. It would seem that the key point of

SNF inhibition by chilling stress in soybean root nodules is nitrogenase activity (van Heerden et al., 2008). In the chilling sensitive soybean genotype, PAN809, the loss of SNF was found to be the result of inhibition of nodule respiration, in conjunction with inefficient regulation of the oxygen diffusion barrier, thereby leading to irreversible inactivation of nitrogenase by high O₂ concentrations (Van Heerden et al., 2008). In the more tolerant genotype, Highveld Top, the ability of nodule respiration to acclimate to chilling temperatures, and more effective control of the oxygen diffusion barrier, protected nitrogenase from similar inactivation (Van Heerden et al., 2008).

1.4.1 Objectives of the study:

The main objectives of this investigation were:

- i. the evaluation of thirty commercial South African soybean genotypes to assess their response to dark chilling under controlled environmental conditions in growth chambers for identification of genotypes exhibiting contrasting degrees of chilling tolerance;
- ii. to benchmark the chilling tolerance of the South African soybean genotypes against those of two foreign soybean genotypes of well-known chilling tolerance;
- iii. to conduct a detailed physiological and biochemical characterisation of the chilling response of selected South African soybean genotypes exhibiting contrasting chilling tolerance with emphasis on the contributing role of low soil temperatures in the inhibition of photosynthesis by dark chilling.

1.4.2 Research hypothesis:

It is hypothesised that within the South African soybean gene pool, a sufficient degree of variation in dark chilling tolerance would exist, enabling selection of genotypes which can subsequently be used for unravelling the mechanism of inhibition of photosynthesis by dark chilling. It is further hypothesised that, through the inhibition of symbiotic nitrogen fixation by low soil temperatures, the negative effects of dark chilling on photosynthetic metabolism would be aggravated and that the selected genotypes

would respond differently depending on their chilling tolerance, thereby further facilitating identification of key metabolic sites of inhibition.

Chapter 2

Materials and Methods

2.1 Selection of plant material

Two soybean (*Glycine max* (L.) Merrill) genotypes PAN809 and Highveld Top were used in all the dark chilling experiments. These two genotypes were selected from a collection of thirty commercial soybean genotypes, which are currently cultivated by farmers in various parts of South Africa (De Beer and Pretorius, 2002). The selection process was based on screening of the thirty genotypes for dark chilling tolerance by means of chlorophyll *a* fluorescence measurements and subsequent application of a novel parameter, the so-called chill factor index (CFI) (Section 2.4.3). The CFI was used for ranking the thirty genotypes according to chilling tolerance (Table 3.1). PAN809 obtained the lowest CFI value of all the genotypes, while Highveld Top ranked third highest. Both these genotypes were selected for further investigations, respectively considered as chilling sensitive and chilling tolerant. Although Highveld Top did not have the highest CFI, it was selected because of its popularity as a commercial genotype due to several desirable agronomic traits.

2.2 Growth conditions

For all experiments conducted, seeds of the genotypes were sown in 1.5 dm³ plastic pots containing a mixture of commercial potting soil, sand and vermiculite (ratio of 4:2:1 respectively). The total water soluble nitrogen content of the potting soil mixture was 4.9 mg l⁻¹. In all cases the seeds were inoculated with *Bradyrhizobium japonicum* (bacterial strain WB 74) at the time of sowing to ensure root nodule development and symbiotic nitrogen fixation (SNF). Seedlings were grown in a Conviron PGW 36 growth chamber (Controlled Environments Ltd., Winnipeg, MB, Canada, R3H 0R9) under rigorously controlled growth conditions: 15h/9h and 26°C/20°C light/dark cycle with a light intensity of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the level of the plant canopy. Four seeds were sown in each pot and emerging seedlings were reduced to two per pot ten days after germination. The CO₂ concentration in the growth chamber was maintained at 350 $\mu\text{mol mol}^{-1}$ throughout the diurnal cycle. Potted plants were rotated daily to compensate for any

variations in light intensity, especially along the sides of the growth chamber. Plants were given distilled water daily and fertilised two times a week with a full strength nitrogen-free Hoagland nutrient solution (Hoagland and Arnon, 1950) which contained 2 mM MgSO_4 , 1 mM KH_2PO_4 , 90 μM Fe-EDTA, 5 mM CaCl_2 , 5 mM KCl, as well as the recommended amount of trace elements.

2.3 Dark chilling treatments

For the initial screening of the 30 soybean genotypes, eight plants of each genotype were transferred to a refrigerated chamber controlled at 6°C for one entire dark period. The remaining plants were kept under normal conditions in the growth chamber at 20°C and represented the control treatment. Only the shoots and leaves of plants were dark chilled, and the roots were kept at 20°C by inserting the pots into custom designed pot-incubators (Analytical Scientific Instruments, PO Box 5832, Weltevreden Park, 1715, South Africa) that circulated warm air around the pots. This strategy minimised the possible occurrence of chill-induced drought stress and associated leaf wilting, a potential artifact that could interfere with initial interpretations of dark chilling tolerance in the large number of genotypes used for screening purposes. At the end of the dark period the chilled plants were returned to the growth chamber containing the control plants for the subsequent light period at 26°C. This temperature regime was repeated for seven consecutive light/dark cycles on the same set of plants.

For all further experiments with PAN809 (chilling sensitive) and Highveld Top (chilling tolerant) a group of plants of each genotype was transferred to a refrigerated chamber controlled at 6°C for one entire dark period (9h). The remaining plants were kept under normal conditions in the growth chamber and represented the control treatment. Two different chilling regimes were employed to compare the effects of whole plant chilling (WPC) with that of only shoot chilling (SC). Low soil temperatures often result in chill-induced drought stress due to reduced root hydraulic conductance restricting the uptake of water (Melkonian et al. 2004b), but these symptoms do not occur when only the shoots and leaves of plants are chilled. Although many studies have investigated the effects of chilling stress on photosynthesis, metabolism and water

relations of plants, most of these studies employed whole-plant chilling, which could have resulted in secondary effects such as chill-induced drought stress. Furthermore, in nodulating legumes, inhibition of SNF and possibly also irreversible effects such as premature root nodule senescence might be induced by low root temperatures. Since we are not aware of any detailed studies that have focused on a comparison of the effects of WPC and SC on warm-climate legumes such as soybean, we aimed to provide novel information by employing such an experimental design in this study. For the SC treatment, the shoots and leaves of plants of each genotype were exposed to 6°C, while the roots were kept at 20°C as described above. Maintenance of normal root temperatures in the SC treatment prevented any direct chill-induced effects on the root systems and nodules. For the WPC treatment, both the shoots and roots of plants of each genotype were exposed to 6°C. At the end of the dark period, the chilled plants were returned to the growth chamber or glasshouse containing the control plants for the subsequent light period at normal daytime temperatures. This temperature regime was repeated for twelve consecutive light/dark cycles on the same set of plants.

2.4 Non-intrusive measurements

2.4.1 The plastochron index

The vegetative development of the plants prior to induction of dark chilling was quantified by repeated measurements of the plastochron index (Erickson and Michelini 1957). The time interval between the initiations of consecutive leaves on a plant is termed the “plastochron”. The plastochron index (PI) is considered a very sensitive indicator of plant development and has been used extensively for various physiological investigations, especially in legumes grown under controlled environmental conditions (Snyder and Bunce 1983, Yourstone and Wallace 1990, Jamadagni et al. 1994). Among its applications, the PI enables precise quantification of shoot growth rates (Snyder and Bunce 1983). Three weeks after seedling emergence, the PI of selected plants of each genotype was measured. These measurements were repeated on the same set of plants on alternate days until the onset of the dark chilling treatment. All trifoliate leaves with central leaflets exceeding a reference length of 25 mm (L_{ref}) were counted. The length of the youngest central leaflet longer than or equal to 25 mm, as well as the length of the

central leaflet (shorter than 25 mm) on the next trifoliate leaf was measured. The PI of each plant was calculated using the following formula:

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

where n = number of trifoliate leaves with central leaflets longer than the reference length of 25 mm (L_{ref}), L_n = length of the central leaflet on trifoliate leaf L_n (which by definition is longer than or equal to L_{ref}) and L_{n+1} = length of the central leaflet on trifoliate leaf L_{n+1} (which by definition is shorter than L_{ref}). The PI was used to ensure that the plants of both genotypes were at a comparable stage of vegetative development at the onset of dark chilling stress. Dark chilling stress was initiated in each genotype when a PI value of five was obtained (central leaflet of fifth trifoliate leaf equal or longer than 25 mm). Shoot growth rates (SGR = increase in plastochron index units per day) during the period of chilling were calculated as: $SGR = (PI_{last\ night\ of\ chilling} - PI_{1st\ night\ of\ chilling})/total\ number\ of\ nights\ of\ chilling$.

2.4.2 Chlorophyll *a* fluorescence measurements

One hour before the end of the dark period, on the day prior to commencement of the dark chilling treatment, chlorophyll *a* fluorescence measurements were conducted on the central leaflet of the youngest fully expanded trifoliate leaf of selected plants of each genotype (control plants and plants to be chilled during the next dark period). Chlorophyll *a* fluorescence transients (O-J-I-P transients) were recorded in the growth chamber at 20°C under dim green light with a Plant Efficiency Analyser (PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 30 4NE, UK), featuring a data acquisition rate of 10 μ s for the first 2 ms and every 1 ms thereafter, and 12 bit resolution (Strasser et al., 1995). O-J-I-P transients were induced by a red light (peak at 650 nm) of 3200 μ mol $m^{-2} s^{-1}$ (sufficient excitation intensity to ensure closure of all PSII reaction centers to obtain a true fluorescence intensity of F_M) provided by the PEA through an array of six light-emitting diodes. All measurements were conducted on fully dark-adapted attached leaves. Every third day, for the following twelve days, fluorescence measurements were conducted on the same leaves one hour before the end of the dark

period, in the growth chamber at 20°C and in the refrigerated chamber at 6°C (for the control and dark chilled plants, respectively). In the screening experiments of the thirty commercial genotypes, the dark chilling period was limited to seven nights, with measurements conducted after every night of treatment.

2.4.2.1 The O-J-I-P fluorescence transient

The fluorescence transient, also called the Kautsky transient (Kautsky et al., 1960), shows a fast rise completed in less than one second, and subsequent slower decline towards a steady state (Strasser and Tsimilli-Michael, 2001). The polyphasic fluorescence rise during the first second of illumination includes phases labelled O, J, I and P (Figure 2.1). The transient changes its shape in accordance to environmental conditions, and provides information about the structure, conformation and function of the photosynthetic apparatus (Strasser et al., 1996).

The O-J part of the transient is considered as the photochemical phase, i.e. the phase reflecting single turnover events (Q_A reduction by PSII activity), while the J-P phase reflects multiple turnover events, i.e. Q_A^- accumulation as the net result of Q_A reduction by PSII and Q_A^- reoxidation by the electron transport chain (Strasser et al. 1995).

This distinction is supported by the finding that the *in vivo* exhibited O-J phase coincides with the monophasic (O-J-I-P) fluorescence rise exhibited in the presence of an inhibitor of Q_A^- reoxidation (e.g. DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea), when both are normalised from 0 to 1 (Strasser and Strasser, 1995). Optimisation of the time at point J, i.e. the lowest possible slope or if existing, the time at slope zero, determined a fixed time of 2 ms to be used for the J step. If 1 or 3 ms is used, the value would show a very small change. The JIP-test uses and depends on the digital calculation of the slope at the origin of the fluorescence transient from time zero to time j (e.g. 2 ms). The maximal rate of primary photochemistry is proportional to this slope, dF/dt_0 , or if normalised, dV/dt_0 . If this variable transient is normalised to the J-step, it becomes V_t/V_J , which represents the single turnover domain for time 0 to time J (e.g. 2 ms) with or without DCMU.

Consequently, the maximal fractional velocity of Q_A reduction is represented by the normalised original slope $dV/dt_0/V_J$, which equals $dQ_A/Q_{A,total}/dt_0$ (Strasser et al., 1995).

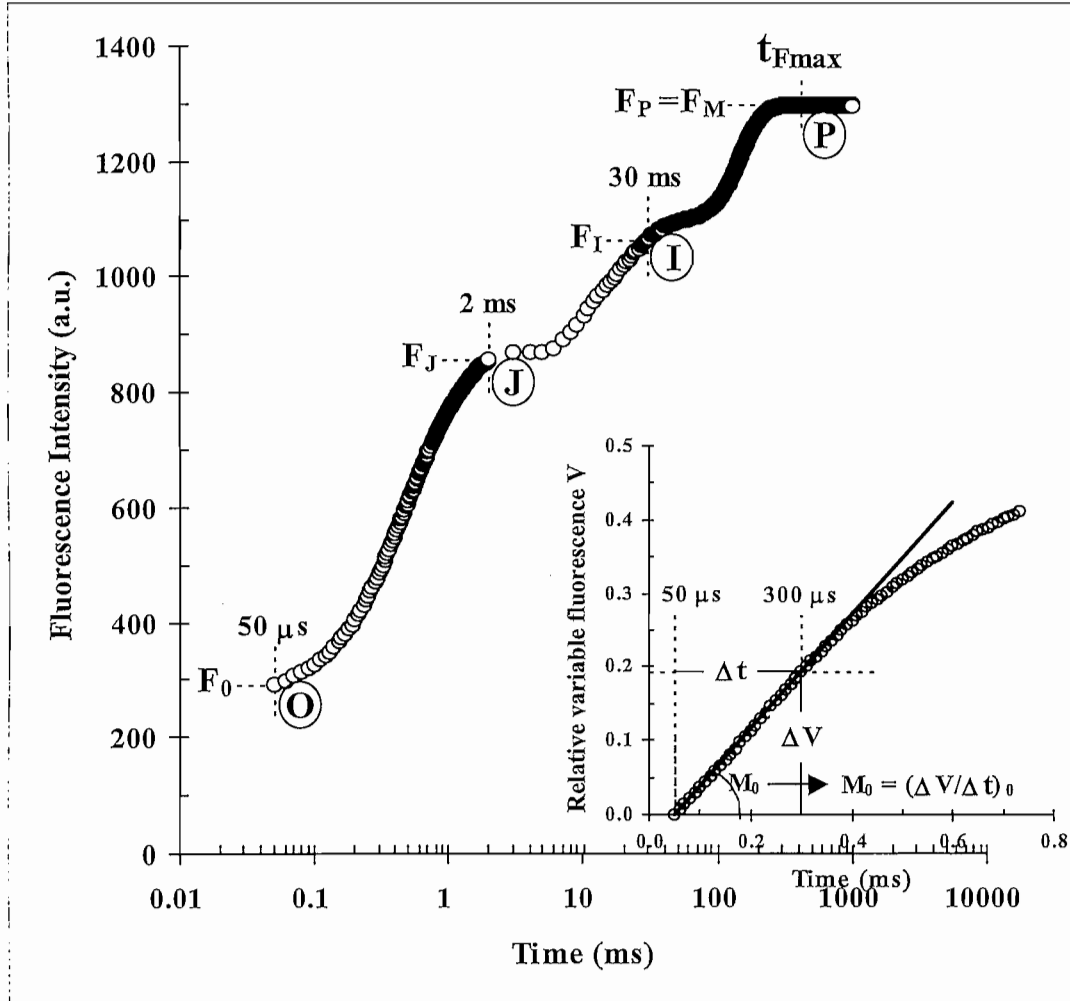


Figure 2.1 The transient shows a Chl a polyphasic fluorescence rise O-J-I-P, plotted on a logarithmic time scale from 50 μ s to 1 s (Strasser and Tsimilli-Michael, 2001).

2.4.2.2 Analysis of O-J-I-P fluorescence transients with the JIP-test

The JIP-test (Strasser and Strasser, 1995) was employed to analyse each O-J-I-P transient. The following data from the original measurements were used: maximal fluorescence intensity (F_M); fluorescence intensity at 50 μ s (considered as F_0); fluorescence intensity at 300 μ s ($F_{300\mu s}$) required for calculation of the initial slope (M_0)

of the relative variable fluorescence (V) kinetics; and the fluorescence intensity at 2 ms (the J step) denoted as F_J .

The JIP-test represents translation of the original recorded data to biophysical parameters that quantify the step-wise energy flow through PSII. The parameters which all refer to time zero (onset of fluorescence induction) are: (i) the specific energy fluxes (per reaction centre) for absorption (ABS/RC), trapping (TR_0 /RC), dissipation at the level of the antenna chlorophylls (DI_0 /RC) and electron transport (ET_0 /RC); (ii) the flux ratios or yields, i.e. the maximum quantum yield of primary photochemistry ($\phi_{P_0} = TR_0/ABS = F_V/F_M$), the efficiency ($\psi_0 = ET_0/TR_0$) with which a trapped exciton can move an electron into the electron transport chain further than Q_A^- , the quantum yield of electron transport ($\phi_{E_0} = ET_0/ABS = \phi_{P_0} \cdot \psi_0$); (iii) the phenomenological energy fluxes (per excited cross section, CS) for absorption (ABS/CS), trapping (TR_0 /CS), dissipation (DI_0 /CS) and electron transport (ET_0 /CS). The fraction of active PSII reaction centres per excited cross section (RC/CS) is also calculated. The formulae showing how each of the above-mentioned biophysical parameters can be calculated from the original fluorescence measurements is shown in Appendix I and has also been described previously in detail (Krüger et al. 1997; Force et al. 2003; Van Heerden et al. 2003b). After every night of dark chilling, the average value of each of these parameters was calculated for control and dark chilled plants of each genotype.

The initial stage of photosynthetic activity of a RC complex is regulated by three functional steps namely absorption of light energy (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport (ET). Strasser et al. (2000) introduced a multi-parametric expression of these three independent steps contributing to photosynthesis, the so-called performance index (PI_{ABS}):

$$PI_{ABS} = \frac{\gamma}{1-\gamma} \cdot \frac{\phi_{P_0}}{1-\phi_{P_0}} \cdot \frac{\psi_0}{1-\psi_0}$$

For full definitions and derivations of the performance index, see Appendix II. The PI_{ABS} was used in the calculation of a CFI for each of the 30 genotypes (see section 2.4.3).

2.4.3 The Chill Factor Index (CFI)

A novel parameter, the so-called chill factor index (CFI), was introduced, which allocated degrees of importance to the measured chilling-induced reduction in the PI_{ABS} during the seven consecutive nights of exposure to dark chilling in the initial screening experiments:

$$CFI = \log A + 2 \log B + 4 \log C$$

A is the average relative PI_{ABS} during the first treatment (period A) of e.g. two nights of chilling, B is the average relative PI_{ABS} during the second treatment (period B) of e.g. the third, fourth and fifth night of chilling, and C is the average relative PI_{ABS} of the third treatment (period C) e.g. during the sixth and seventh night of chilling. The relative PI_{ABS} for each night of dark chilling was calculated as $PI_{ABS(\text{dark chilled})} / PI_{ABS(\text{control})}$. The CFI is premised on the assumption that chilling tolerant genotypes, capable of tolerating dark chilling stress for prolonged periods, would be able to maintain higher PI_{ABS} values following long-term dark chilling treatment than chilling sensitive genotypes. By definition, the CFI regards a reduction in the PI_{ABS} in any given genotype during period B or C more significant (by a factor of two and four respectively) than a reduction in the PI_{ABS} during period A. As a consequence, chilling sensitive genotypes that exhibit the largest reduction in the PI_{ABS} during the latter stages of dark chilling treatment will also have the lowest (most negative) CFI values.

The CFI is intended as a practical tool for the classification of chilling tolerance. The different requirements regarding the CFI are considered in the equation, where the total period of dark chilling treatment is divided into three shorter periods (A, B and C). If the particular stress treatment under investigation is dark chilling, the CFI of period A (first two nights of dark chilling) may be of greater importance in regions where chilling night temperatures occur only for short spells, than the CFI after seven nights of dark chilling. The same is true for the next period of time, i.e. AB (the first five nights of dark chilling). Since the change in CFI over the period of treatment is not necessarily linear, it

may be important for the applicator of the index to get a clear indication of the response at the different periods of stress.

2.4.4 Chlorophyll content

The chlorophyll content of leaf discs (3.14 cm²) collected from soybean leaves of different stages of development (young to senescent) were measured with a hand-held chlorophyll Content Meter (CCM-200, Opti-Sciences, Inc., 164 Westford Road #4, Tyngsboro, MA 01879, USA), which recorded a chlorophyll content index (CCI) for each leaf disc. The total extractable chlorophyll content of each leaf disc was subsequently determined according to the method of Wintermans and De Mots (1965). The actual chlorophyll content of each leaf disc was then plotted against its corresponding CCI value. This calibration curve revealed a highly linear relationship ($r^2 > 0.96$) between actual chlorophyll content and a wide range of recorded CCI values (Fig 2.2), thus justifying the use of the measured index values. In the subsequent dark chilling experiments, the CCI values of the fourth trifoliate leaf (also used for photosynthesis and fluorescence measurements) of control and dark chilled plants of both genotypes were measured on every third day during the twelve-night dark chilling period.

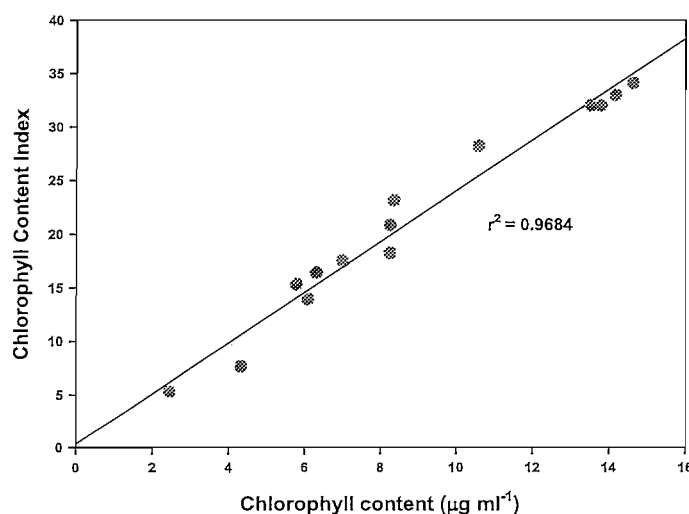


Figure 2.2 Calibration curve relating chlorophyll content index (CCI) values of soybean leaf discs, as measured with a portable chlorophyll meter, with actual extractable chlorophyll content of the same samples.

2.4.5 CO₂ assimilation

CO₂ assimilation was measured using a portable photosynthesis system (CIRAS-1, PP Systems, Hertz, UK) before the onset of the chilling stress treatment and again after every third night of exposure to dark chilling. Measurements were conducted on four plants each of the control, SC and WPC treatments in the two genotypes PAN809 and Highveld Top. A 2.5 cm² section of the central leaflet of the fourth trifoliate leaf was clamped in a photosynthetic leaf chamber (PLC) with light and temperature control. Humidity in the PLC was maintained close to ambient conditions. Measurements were started 3 hours after the end of the dark period and conducted at a leaf temperature of 26°C, an irradiance of 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In order to determine the relationship between CO₂ assimilation rate (A) and intercellular CO₂ concentration (C_i), C_a was increased with increments from 0 to 2000 $\mu\text{mol mol}^{-1}$. For each C_a the ratio of A to C_i was automatically recorded. The initial slope ($\delta A/\delta C_i$) of the A:C_i response curve at low C_i (CO₂ limiting) was determined by linear regression. Relative stomatal limitation ($\mathcal{L}$) was calculated as the difference between the CO₂ assimilation rate when C_i = 350 $\mu\text{mol mol}^{-1}$ (A₀), and the actual rate at C_a = 350 $\mu\text{mol mol}^{-1}$ (A), expressed as a percentage of A₀. All other calculations were done according to Farquhar and Sharkey (1982), Chaves (1991) and Makino et al. (1994).

2.5 Collection of leaf samples

On the day before the onset of dark chilling, and on every third day thereafter during the 12-night dark chilling treatment, leaf discs were collected for various biochemical procedures. Leaf discs (3.14 cm²) were collected with a freeze clamp, cooled in liquid nitrogen, from the fourth trifoliate leaf of control and dark chilled plants. Sampling occurred at 11:00 in the growth chamber, under normal growth conditions of 26°C and irradiance of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the level of the pots and a CO₂ concentration of 350 $\mu\text{mol mol}^{-1}$.

2.6 Biochemical analysis of dark chilled plants

2.6.1 Photosynthetic enzymes

2.6.1.1 Sucrose-phosphate synthase (SPS, EC 2.4.1.14) activity

Leaf discs were ground with liquid nitrogen and extracted with 800 μ l extraction buffer containing 50 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol 0.1% (v/v) Triton X-100, 6 mM PMSF and 2 mM benzamidine (Stitt et al. 1988). A small pellet ($\pm$ 30 mg) of acid-washed insoluble polyvinylpolypyrrolidone (PVPP) was also added to the mortar during extraction. After extraction the crude extract was centrifuged for 2 min at 10000 x g and 4°C. The supernatant was desalted by centrifugation according to the method of Helmerhorst and Stokes (1980). Polystyrene columns, packed with a 2 ml bed volume of Sephadex G-25 superfine gel, and equilibrated with 6 ml buffer containing 50 mM Hepes-KOH (pH 7.4), 4 mM $MgCl_2$ and 1 mM EDTA, were used to desalt 400 μ l of supernatant by centrifugation at 700 x g and 4°C for 2 min.

Desalted supernatant was used immediately to assay SPS activity according to the method of Guy et al. (1992), using limiting substrates containing inorganic phosphate (Pi) (V_{lim} assay) or saturating substrates without Pi (V_{max} assay). Reaction mixtures for the V_{lim} assay contained 35 μ l desalted supernatant, 6 mM fructose-6-phosphate (F-6-P), 24 mM glucose-6-phosphate (G-6-P), 10 mM uridine 5'-diphosphoglucose (UDPG), 15 mM K_2HPO_4 , 50 mM Hepes-KOH (pH 7.4), 4 mM $MgCl_2$ and 1 mM EDTA, in a final volume of 70 μ l. K_2HPO_4 was excluded from the V_{max} assay, with the concentrations of F-6-P and G-6-P increased to 20 mM and 80 mM, respectively. Desalted supernatant in a reaction mixture containing 10 mM UDPG, but in the absence of F-6-P and G-6-P was included as a control for each sample. The reaction was performed at 25°C for 15 minutes and terminated by addition of 70 μ l 30% (w/v) KOH. Samples were then incubated at 100°C for 10 minutes and briefly cooled on ice, after which 1 ml of resorcinol reagent was added (1% Resorcinol solution in ethanol, added to 30% HCl in a 1:3 ratio) (Huber and Israel, 1982). After incubation for 10 minutes at 80°C, the formation of sucrose-

phosphate was determined at 520 nm with a spectrophotometer (Camspec, Camspec Ltd., Sawston, Cambridge, UK).

2.6.1.2 Chloroplast fructose-1,6-bisphosphatase (FBPase, E.C. 3.1.3.11) activity

The initial and total activity of chloroplast FBPase was determined according to the method of Leegood (1993). Initial activity is the activity of the enzyme under growth conditions at the time of sampling. Total activity was measured after incubation of the enzyme at 25°C in the presence of dithiothreitol (DTT), which fully activates FBPase *in vitro*.

Leaf discs were ground with liquid nitrogen and extracted with 1ml extraction buffer containing 100 mM Hepes-KOH (pH 8.0), 10 mM MgCl₂·6H₂O, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 0.1 PMSF and 2 mM benzamidine. A small pellet (± 30 mg) of acid-washed insoluble polyvinylpyrrolidone (PVPP) was also added to the mortar during extraction. The crude extract was centrifuged at 10000 x g and 4°C for 1 min, and the supernatant immediately used to determine initial FBPase activity. Initial activity was measured in a total volume of 1 ml, which contained 50 µl supernatant, 40 mM NAD, 0.5 M DTT, 3 units glucose-6-phosphate-dehydrogenase (G6PDH), 4 units phosphoglucose isomerase (PGI) and 900 µl assay buffer. The assay buffer consisted of 100 mM Hepes-KOH (pH 8.0), 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.05% Triton X-100. The reaction was started by addition of 100 mM FBP and the rate of NADH formation was measured spectrophotometrically at 340 nm.

Total activity was measured after a 15 minute incubation of 50 µl supernatant at 25°C with 10 µl 0.5 mM DTT. After incubation, 900 µl assay buffer (as described above), 40 mM NAD, 3 units G6PDH and 4 units PGI was added to the supernatant. The reaction was conducted as described above.

2.6.1.3 Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39) activity

The method of Keys and Parry (1990) was used to determine initial and total Rubisco activity. The initial activity is the activity of the enzyme under growth conditions at the time of sampling. Total activity is determined by activation of the extracted enzyme with bicarbonate prior to the reaction.

Leaf discs were ground with liquid nitrogen and extracted with 1 ml extraction buffer containing 100 mM Bicine-NaOH (pH 8.0), 20 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 50 mM mercaptoethanol and 0.1 M PSMF. A small pellet (± 30 mg) of acid-washed insoluble polyvinylpolypyrrolidone (PVPP) was also added to the mortar during extraction. The extract was centrifuged at $10000 \times g$ at 4°C for 1 min and the supernatant was immediately used to measure initial Rubisco activity. Initial Rubisco activity was measured in a total volume of 500 μl containing 100 mM Bicine-NaOH (pH 8.2), 20 mM MgCl_2 , 10 mM $\text{NaH}^{14}\text{CO}_3$ ($0.5 \mu\text{Ci } \mu\text{mol}^{-1}$), 400 μM ribulose-1,5-bisphosphate (RuBP) and 25 μl leaf extract. The reaction was terminated after 1 minute with 200 μl 10 M formic acid. Total Rubisco activity was determined after 25 μl of extract had been incubated in 415 μl extraction buffer (without mercaptoethanol) for 3 minutes in the absence of RuBP to fully activate the enzyme. The reaction was then started by addition of RuBP and terminated after 1 minute with 200 μl of 10 M formic acid.

The acidified samples were dried in an oven at 60°C , whereafter 0.5 ml H_2O and 3.5 ml Packard Ultima Gold scintillation cocktail (PerkinElmer Life And Analytical Sciences, Inc., Waltham, Massachusetts, USA) was added to each vial. A liquid scintillation analyzer (Beckman LS 6000TA) was used to determine the incorporation of ^{14}C into 3-phosphoglycerate.

2.6.1.4 Sedoheptulose-1, 7-bisphosphatase (SBPase, E.C. 3.1.3.37) activity

SBPase activity was assayed by phosphate released, according to the method of Harrison et al. (1998). Leaf discs were ground with liquid nitrogen and extracted with 1 ml extraction buffer, containing 50 mM Hepes-KOH (pH 8.2), 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1

mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100, 10 mM DTT, 0.5 mM PMSF and 2 mM benzamidine. The extract was centrifuged at 14000 x g and 4°C for 1 min, after which 400 µl supernatant was desalted through polystyrene columns packed with Sephadex G-25 superfine gel as described previously.

A volume of 20 µl of desalted extract was added to 66 µl assay buffer containing 50 mM Tris-HCl (pH 8.2), 15 mM MgCl₂, 1.5 mM EDTA and 10 mM DTT. The reaction was started by addition of 2 mM SBP (kindly provided by Prof Christine Raines, Department of Biological Sciences, University of Essex, UK) and conducted at room temperature for 5 min. The reaction was stopped by addition of 50 µl 1 M perchloric acid followed by centrifugation at 14000 x g and 4°C for 10 min. For each reaction, a control was included in the same assay buffer already containing 50 µl of 1 M perchloric acid. Samples, controls and phosphate standards (0.125 to 4 nmol PO₄³⁻) were incubated for 25 minutes at room temperature with Biomol Green reagent (BIOMOL International, L.P., 5120 Butler Pike Plymouth Meeting, Pennsylvania, USA) and the absorbance measured at 620 nm using a microplate reader (Power Wave Select, Bio-Tek Instruments, Inc. Highland Park, USA). For all enzymes described above, the soluble protein content of supernatants used in the assays was determined according to the method of Bradford (1976).

2.6.2 Quantification of RuBP content

Leaf discs were ground in liquid nitrogen and 400 µl ice-cold 0.46 M trifluoroacetic acid was added and extracted while thawing. The extract was centrifuged at 10000 x g and 4°C for 5 minutes. Supernatant (100 µl) was transferred to a scintillation vial and dried down *in vacuo* over NaOH pellets and anhydrous CaCl₂. The RuBP content of the residue in the scintillation vials was determined according to the method of Latzko and Gibbs (1972). Reaction mixture (400 µl) containing 100 mM Bicine-NaOH (pH 8.2), 20 mM MgCl₂, 10 mM NaH¹⁴CO₃ (0.5 µCi µmol⁻¹) and 50 µg purified wheat rubisco was added. Prior to use the purified rubisco was activated according to the method of Gutteridge et al. (1982) at 37°C for 40 min in the presence of 100 mM Bicine-

NaOH (pH 8.2), 20 mM MgCl₂ and 10 mM NaHCO₃. The reaction was terminated after 40 minutes by addition of 100 µl 10 M formic acid. Standards containing known amounts of RuBP (10 nmol) were also included. Acidified samples were dried in an oven at 60°C and incorporation of ¹⁴C into 3-phosphoglycerate determined by liquid scintillation spectrometry after addition of 3.5 ml Ultima Gold scintillation cocktail (PerkinElmer Life And Analytical Sciences, Inc., Waltham, Massachusetts, USA).

2.6.3 Leaf ureide content

Leaf discs were dried at 60°C in an oven prior to ureide (allantoin and allantoic acid) extraction. Ureides were extracted from leaf discs with 1 ml 0.2 M NaOH whereafter the crude extracts were boiled for 20 min. After brief cooling on ice, the samples were centrifuged at 10 000 x g for 10 min. The ureide concentrations of the clarified supernatants were measured spectrophotometrically as the phenylhydrazone derivative of glyoxylate (Young and Conway 1942). The procedure of the analysis is detailed elsewhere (Peoples et al. 1989).

2.6.4 Determination of soluble sugars and starch content

Soluble sugars and starch were extracted from leaf discs in hot ethanol (80% v/v) to denature any degrading enzymes. Samples were ground with liquid nitrogen, after which a volume of 1 ml hot ethanol (± 75°C) was added to the fine powder in a microfuge tube. After 15 seconds the tube was closed, mixed vigorously using a vortex mixer and then incubated at 80°C for 20 minutes. The samples were then cooled on ice and centrifuged at 10000 x g for 10 min. An aliquot (50 µl) was removed for the estimation of chlorophyll content according to the method of Wintermans and De Mots (1965) and the remaining supernatant was stored at -20°C for enzyme-based determination of soluble sugars.

For enzymatic digestion of starch to glucose, 184 µl of 50 mM Na-acetate (pH 4.8) and 16 µl starch degrading enzymes, containing 10 U amyloglycosidase and 10 U α-amylase, was added to 200 µl of resuspended pellet. Digests were mixed gently and placed in an incubator at 37°C for 48 h, and mixed again halfway through the incubation

period. Digests were centrifuged for 5 minutes before use and the supernatant diluted 20 fold with water.

The soluble sugars glucose, fructose and sucrose were measured enzymatically as described by Jones et al. (1977). The assay is based on a change of absorbance at a wavelength of 340 nm when NADP is converted to NADPH, coupled to the conversion of glucose 6-phosphate to 6-phospho-gluconate. The sequential addition of hexokinase, PGI and invertase enables glucose, fructose and sucrose to be determined in sequence. The assay mixture is prepared containing everything apart from hexokinase, phosphoglucisomerase (PGI) and invertase. When hexokinase (HK) is added, glucose and fructose are phosphorylated to glucose 6-phosphate and fructose 6-phosphate, respectively. Glucose 6-phosphate is then converted into gluconate by G6PDH which measures glucose. PGI converts fructose 6-phosphate to glucose 6-phosphate, which is taken through the same reaction, measuring fructose. Invertase cleaves sucrose to glucose and fructose, which are then taken through the reactions and sucrose is quantified.

For the sucrose assay, 2.5 μ l of supernatant was added to 142.5 μ l reaction mixture containing 100 mM Imidazole-HCl (pH 6.9) and 10 mM $MgCl_2$, as well as 1.1 mM ATP, 0.5 mM NAD, 1.4 U G6PDH, 0.75 U HK and 0.35 U PGI. Absorbance was determined at 340 nm in a micro plate reader (Power Wave Select, Bio-Tek Instruments, Inc. Highland Park, USA) until a stable value was obtained. To start the reaction, 250 U Invertase was added and the total increase in absorbance determined.

For the glucose and fructose assay, 25 μ l of supernatant was added to 123 μ l reagent mixture containing 100 mM Imidazole-HCl buffer (pH 6.9) and 10 mM $MgCl_2$, as well as 1.1 mM ATP, 0.5 mM NAD and 1.4 U Glucose-6-phosphate dehydrogenase (G6PDH). Absorbance was determined at 340 nm until a stable value was obtained and all G-6-P had been converted to 6-PG. The next reaction was started by addition of 0.75 U HK and absorbance was determined at 340 nm until a stable value was obtained. Glucose was converted to G-6-P by HK and G-6-P was converted to 6-PG by G6PDH, determining the levels of glucose. Fructose was also converted to F-6-P. The next

reaction was started by addition of 0.35 U of PGI and absorbance was determined at 340 nm until a stable value was obtained. F-6-P was converted to G-6-P by PGI and G-6-P to 6-PG by G6PDH, determining the levels of fructose.

For the starch assay, 25 μ l of the 20 fold diluted starch digest was added to a reagent mixture containing 100 mM Imidazole-HCl buffer (pH 6.9) and 10 mM $MgCl_2$, as well as 1.1 mM ATP, 0.5 mM NAD and 1.4 U Glucose-6-phosphate dehydrogenase (G6PDH). Absorbance was determined at 340 nm until a stable value was obtained. The next reaction was started by addition of 0.75 U HK to convert all glucose produced during starch digestion to G-6-P, and G-6-P to 6-PG by G6PDH. The corresponding increase in absorbance at 340 nm due to NAD reduction was measured until an end-point value was obtained. Sugar and starch content was expressed as hexose equivalents on a chlorophyll basis.

2.7. Detection of proteins by Western Blot Analysis

2.7.1 Extraction of proteins

Leaf discs were ground with liquid nitrogen and extracted with 1.5 ml ice-cold extraction buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 3 mM DTT, 30 mg insoluble PVPP and 15 μ l protease inhibitor cocktail (for plant cell extracts, containing AEBSF, Pepstatin A, Leupeptin, bestatin and E-64 in DMSO (Sigma-Aldrich Chemie GmbH, 89552 Steinheim, Germany)). The crude extracts were centrifuged at 10000 x g at 4°C for 15 min. The soluble protein content of the supernatants was determined according to the method of Bradford (1976).

A volume of 250 μ l 4x SDS-PAGE sample buffer containing 250 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 0.287 mM mercaptoethanol, 0.015 mM bromophenol blue and 40% (v/v) glycerol was added to 750 μ l supernatant and then boiled for 5 min. Samples were stored at -20°C until further analysis was performed.

2.7.2 Protein separation and detection with antibodies

Proteins and protein subunits were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a mini-gel system (Mini-PROTEAN 3 Electrophoresis Module, Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California, USA). The running gel solution contained a 2.5 ml 40% acrylamide/bisacrylamide mixture (Bio-Rad Laboratories) and 2.5 ml 1.5 M Tris-HCl (pH 8.8). The gel was cast between the glass plates after addition of 0.1 ml 10% (w/v) SDS, 0.1 ml 10% (w/v) ammonium persulphate and 10 μ l TEMED. The stacking gel solution contained 0.2 ml 40% acrylamide/bisacrylamide mixture and 0.5 ml 0.5 M Tris-HCl (pH 6.8). The gel was casted on top of the polymerised running gel after addition of 20 μ l 10% (w/v) SDS, 25 μ l 10% (w/v) ammonium persulphate and 5 μ l 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. Sample wells were rinsed thoroughly with SDS running buffer consisting of 192 mM glycine, 25 mM Tris and 0.5% SDS. An amount of between 10 and 20 μ g total soluble protein (depending on the protein under investigation) was loaded in the sample wells, together with 10 μ l of a broad range prestained SDS-PAGE protein standard (Bio-Rad Laboratories). Proteins were separated for 120 min at 75 V and 4°C.

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

For immunoblotting, the Hybond C-extra nitrocellulose membrane containing the transferred proteins was incubated in blocking solution containing 25 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1 mM EDTA and 1% (w/v) skimmed milk powder for about 3 hours. The membrane was probed overnight with primary anti-rabbit antibody according to recommended dilution ratios for a particular antibody, being 1:250 for Rubisco LSU and SSU, 1:250 for FBPase, and 1:1000 for SBPase. The membrane was thereafter washed 3 times (10 minutes each) with blocking solution, and probed with anti-rabbit IgG secondary Horse Radish Peroxidase linked antibody for 2 hours (1:1000 dilution). The membrane was again washed 3 times (10 minutes each) with blocking solution, after which it was rinsed 3 times with ultrapure water.

For ECL blotting the same procedure as described above was used, with the only difference being the composition of the blocking solution. The nitrocellulose membrane was incubated in a 5 % TBS-T blocking solution containing 1 M Tris-HCl (pH 7.6), 100 mM NaCl and 0.1% Tween 20.

For immunoblotting, the nitrocellulose membrane was incubated in a detection reagent containing 50 mM Tris-HCl (pH 7.6), chloronaphtol and 30% H₂O₂, for 15 minutes. The development of the membrane was stopped by replacing the detection reagent with ultrapure H₂O.

For chemiluminescent detection, the whole procedure was conducted in a dark room. Excess buffer was drained off and the membrane was placed in a tray with the protein-containing side facing upward. The ECL Western blotting detection reagent (Amersham) was added 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 30 seconds in the cassette. The film was then soaked in developer reagent (Ilford Phenisol, Ilford Imaging Limited,

Mabberley Cheshire, England) for 5 min and in a standard fixer reagent for 2 min and then allowed to dry.

2.8 Data analysis

Statistical analysis was conducted with the software package Statistica for Windows version 6 (StatSoft, Inc. 2300 East 14th Street, Tulsa OK 74104, USA). Normal distribution of data was determined using the Shapiro-Wilk W test (Shapiro et al. 1968). In data sets with parametric distribution, significant differences between treatment means were determined using Student's t-test. In data sets with non-parametric distribution, significant differences between treatment means were determined with the Mann-Whitney U-test (Mann and Whitney 1947).

Chapter 3

Evaluation of dark chilling tolerance in thirty commercial South African soybean genotypes*

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3.1 Introduction:

Soybean genotypes respond differently to dark chilling, with some exhibiting a degree of tolerance, while others are extremely sensitive (Lawn and Hume, 1985; Seddigh et al., 1989). Finding an efficient and rapid method for selection of dark chilling tolerance among large numbers of genotypes allows identification of those genotypes best suited for cultivation in areas that experience dark chilling episodes. Another important advantage would be the identification of parental material that could be included in breeding programs aimed at developing more dark chilling tolerant genotypes, ensuring higher yields per unit area. This is especially important for agricultural practices in South Africa, where soybean is frequently grown in regions of high altitude that often experience low minimum night temperatures, which limits optimal cultivation (Smith 1994).

Most selection procedures are based on subjective visual scoring or yield measurement in the target environment, but more specific selection techniques for use under controlled conditions are also required to facilitate more rapid progress in the development of genotypes with higher chilling tolerance (Wery et al., 1994; Bertin et al., 1996). Since photosynthesis forms such an integral part of plant metabolism, and is known to be extremely sensitive to dark chilling stress (Caulfield and Bunce, 1988; Allen and Ort, 2001), *in vivo* chlorophyll *a* fluorescence measurements has been used frequently in the past as a convenient and non-intrusive method to determine the tolerance of different species and genotypes to chilling (Neuner and Larcher, 1990; Brüggeman, 1992b; Adams and Perkins, 1993; Wery et al., 1994; Sthapit et al., 1995; Bertin et al., 1996; Fracheboud et al., 1999; Van Heerden et al., 2003c).

Chlorophyll *a* fluorescence studies indicate that a primary target for chilling stress is located at the reaction center of photosystem II (PSII) (Agati et al., 1996). Illuminating a dark-adapted leaf results in characteristic changes in the intensity of chlorophyll *a* fluorescence, known as the Kautsky effect (Kautsky and Hirsch, 1931). It is postulated that the rising phase of the transient reflects the primary reactions of photosynthesis (Krause and Weis, 1991). When the Kautsky transient is plotted on a logarithmic time scale, a polyphasic rise exhibiting the steps J and I (Strasser and Govindjee, 1992) or I₁ and I₂ (Schreiber and Neubauer, 1987) between the initial O (F₀) and maximum P level (F_P), appears. Strasser and Strasser (1995) have developed the JIP-test, which is used to translate the original fluorescence measurements of these O-J-I-P transients into several phenomenological and biophysical expressions that quantify PSII-function (Strasser et al., 2000; Strasser and Tsimilli-Michael, 2001; Strasser et al., 2004). The JIP-test is highly suited for *in vivo* investigations of the behaviour of the photosynthetic apparatus, since the shape of the O-J-I-P fluorescence transient is sensitive to stress caused by changes in many environmental conditions (Krüger et al., 1997; Tsimilli-Michael et al., 1998; Tsimilli-Michael et al., 1999).

Strasser et al. (2000) introduced a multi-parametric expression, the so-called performance index (PI_{ABS}), which takes the three main functional steps of photosynthetic activity by a PSII-reaction center complex, namely light energy absorption, trapping of excitation energy, and conversion of excitation energy to electron transport, into consideration. Studies on soybean have indicated a positive relationship between the effect of dark chilling on the PI_{ABS} and various other photosynthetic parameters, as well as known differences in dark chilling tolerance of genotypes of temperate and tropical origin (Van Heerden et al., 2004a; 2004b). For example, Van Heerden et al., (2004a; 2004b) demonstrated that the effect of dark chilling on the PI_{ABS} in the chilling tolerant (Hume and Jackson, 1981; Lawn and Hume, 1985) genotype, Maple Arrow, was much less than in a chilling sensitive (Lawn and Hume, 1985) tropical genotype, Java 29. This evidence suggests that the PI_{ABS} might be a suitable parameter for evaluating a large number of genotypes for dark chilling tolerance.

In this chapter it is demonstrated how analysis of recorded O-J-I-P fluorescence transients was employed for the evaluation of dark chilling tolerance in thirty South African soybean genotypes under controlled growth conditions. It is illustrated how dark

chilling affects the shape of these transients and the PI_{ABS} in these genotypes. In addition, the PI_{ABS} was used to define a novel parameter, the chill factor index (CFI) (refer to section 2.4.3), capable of revealing large differences in dark chilling response between the genotypes. The underlying hypothesis was that the JIP-test, and more specifically the PI_{ABS} and CFI, would be sensitive enough to evaluate the response in the genotypes and to ultimately rank them according to dark chilling tolerance.

3.2 Results:

The typical O-J-I-P fluorescence transients recorded in fully expanded leaves of untreated and dark chilled plants of two South African soybean genotypes, Sonop and PAN809, are shown in Figure 3.1. The respective transients illustrate the average response in the two genotypes during a treatment period of seven consecutive nights of dark chilling. All the O-J-I-P transients were normalised between O and P in order to reveal changes in the shape of the transients between these two steps more clearly. Upon excitation of dark-adapted leaves of untreated plants with a saturated light pulse, there was a rapid initial rise in fluorescence intensity from O to the first intermediate step J (ca. 2 ms). During this O to J phase, mainly single turnover events with respect to Q_A reduction occurred (Strasser and Govindjee, 1992). This phase was followed by a further rise to the second intermediate step I within ca. 30 ms and to the final peak P in ca. 200 ms where multiple turnover redox reactions occurred (Strasser and Govindjee, 1992). In both genotypes the response of untreated plants was very similar. The O-J-I-P fluorescence transient reflects the filling of the electron acceptor side of PSII (Q_A , Q_B and PQ pool) with electrons from the donor side of PSII (Papageorgiou, 1975; Lavorel and Etienne, 1977; Strasser and Govindjee, 1992). The relationship of these events to the O-J-I-P fluorescence transient was suggested by Strasser et al., (1995) to be the following: O, minimal Chl a fluorescence yield (highest yield of photochemistry); O to J, reduction of Q_A to Q_A^- (photochemical phase, light intensity dependent); J to I to P, reduction of the PQ pool (non-photochemical phase). Since the O-J-I-P fluorescence transient reflects the kinetics and heterogeneity involved in filling the PQ pool with electrons, it can be used as a sensitive tool to investigate the photosynthetic apparatus *in vivo* (Strasser et al., 1995). From the data shown in Figure 3.1, two important observations regarding the effect of dark chilling on the O-J-I-P fluorescence transients can be made: a) the shape of the O-J-I-P fluorescence transients recorded in leaves of dark chilled plants of both genotypes differed from those recorded in untreated plants, and b) a conspicuous difference existed

between the two genotypes with regard to the extent to which the shape of the transients was altered by dark chilling, especially at the J-peak (2 ms). Dark chilling resulted in a much larger increase in the fluorescence intensity at 2 ms (J-peak) in PAN809 than in Sonop.

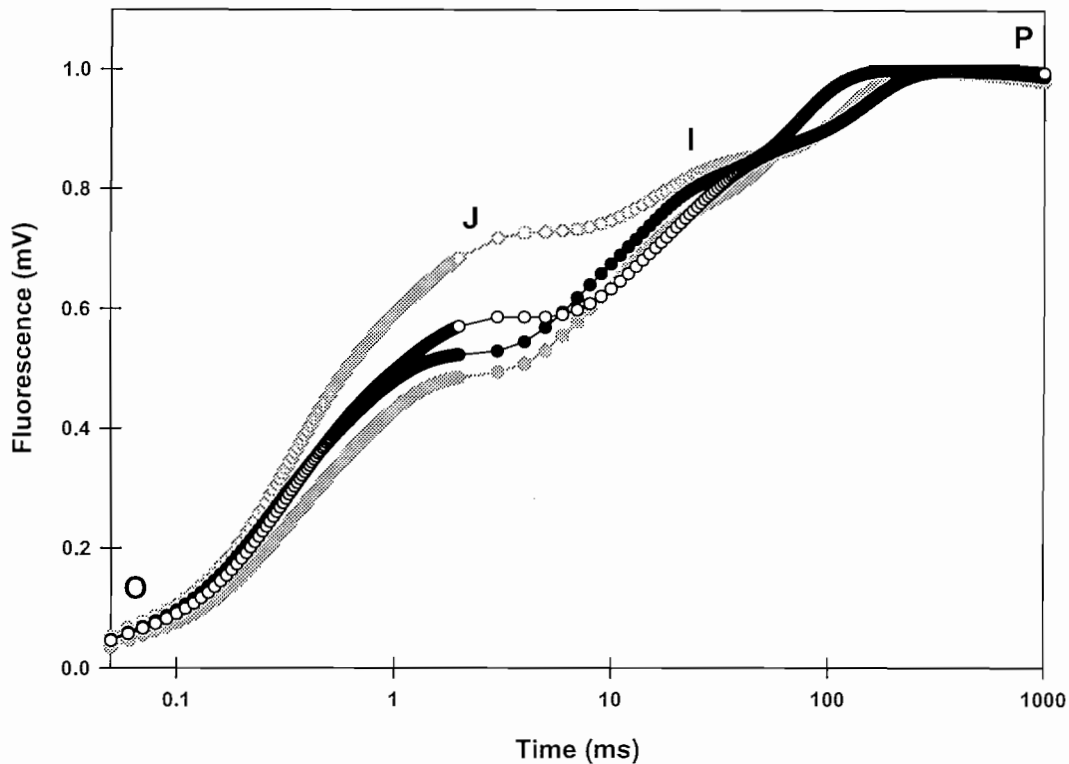


Figure 3.1 Typical O-J-I-P chlorophyll *a* fluorescence transients in untreated (closed circles) and dark chilled (open circles) plants of two South African genotypes, Sonop (black transients) and PAN809 (grey transients), showing the average response in the two genotypes during a treatment period of seven consecutive nights of dark chilling.

Using the JIP-test, a number of useful parameters quantifying energy flow through PSII can be derived from the O-J-I-P fluorescence transient (Appendix I) (Force et al., 2003; Strasser et al., 2004). One of these parameters is the so-called performance index (PI_{ABS}), of which the relationship with the O-J-I-P fluorescence transient has been described in detail (Strasser et al., 2004). Although dark chilling decreased the PI_{ABS} in all the genotypes studied, three contrasting response types were identified among the genotypes. The results shown in Fig. 3.2 illustrate the effect of seven consecutive nights of dark chilling on the PI_{ABS} in Sonop, HV1 and PAN809, representative genotypes of each of these three response types, respectively. From the results shown in Fig. 3.2 it is clear that the response of the three genotypes to dark chilling differed in two major ways.

Firstly, the genotypes differed to the extent to which the values of the PI_{ABS} were decreased by dark chilling. In the case of Sonop, the average decrease of the PI_{ABS} during the seven nights of dark chilling was only 13% (Fig. 3.2a), compared to 26% (Fig. 3.2b) and 65% (Fig. 3.2c) in HV1 and PAN809, respectively. Secondly, the genotypes also differed with respect to the pattern by which the PI_{ABS} was decreased by dark chilling. In fully expanded leaves of control plants of all genotypes the PI_{ABS} values showed a clear increase during the progression of the experimental period. This is a common phenomenon in plants since photosynthetic activity increases during leaf development and gradually reaches a maximum in the mature, fully expanded leaf (Gepstein, 1988). In the case of dark chilled plants of Sonop (Fig. 3.2a), and other genotypes belonging to the same group (least chilling sensitive), this phenomenon was still evident because the PI_{ABS} values, although consistently lower than the control, showed a gradual increase (27% in the case of Sonop) during the progression of the dark chilling treatment. In the other two genotypes, however, dark chilling completely stalled the progressive increase in the PI_{ABS} (Fig. 3.2b and c). Previous studies obtained similar data in Maple Arrow and Java 29, two genotypes of well-known, but contrasting chilling tolerance (Van Heerden et al., 2004a). Although dark chilling decreased the PI_{ABS} in these two genotypes, only in Java 29 (the chilling sensitive genotype) did dark chilling stall any further increase in the PI_{ABS} during the treatment period.

In Fig. 3.3 the effect of dark chilling on the three main partial responses involved in PSII function, together with the PI_{ABS} and the quantum yield of primary photochemistry (F_v/F_m) is shown for the three representative genotypes Sonop, HV1 and PAN809. By presenting the data in this fashion, insight is gained into the relative contribution of each of the partial responses towards the overall inhibition of PSII activity by dark chilling. From the data it is clear that the quantum yield of primary photochemistry (F_v/F_m) was affected least by dark chilling, revealing very few differences between the genotypes. This confirms previous findings by Van Heerden et al., (2004a) who demonstrated that F_v/F_m (ϕ_{Po}) is not a sensitive parameter for the assessment of dark chilling stress in soybean. Furthermore, it is evident that dark chilling-induced decrease in PI_{ABS} in the chilling sensitive representative PAN809, is mostly the result of a loss in electron transport capacity, as indicated by the large decrease in $\psi_o/(1 - \psi_o)$.

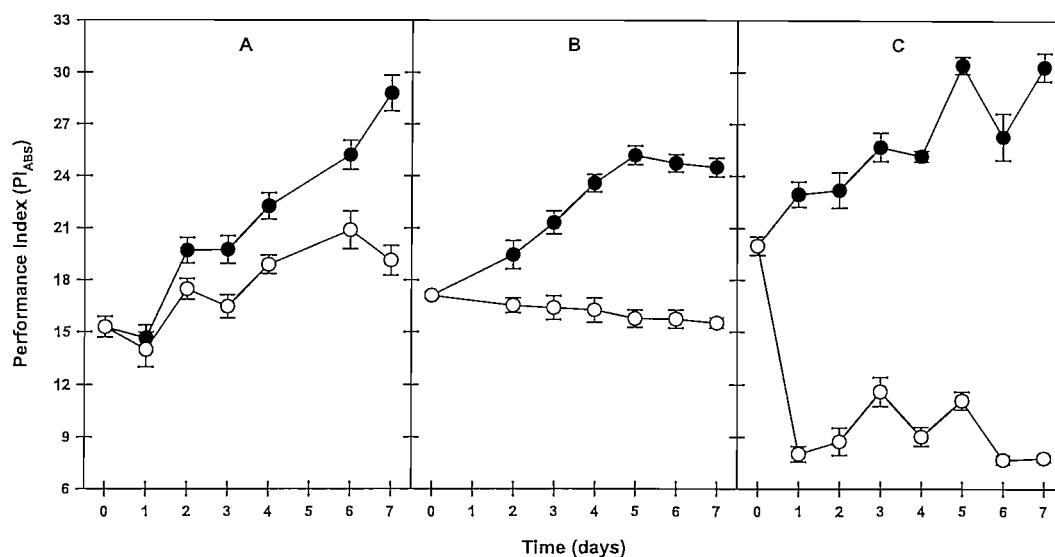


Figure 3.2 The effect of seven consecutive nights of dark chilling on the performance index (PI_{ABS}) in Sonop (A), HV1 (B) and PAN809 (C), representative genotypes of each of the three response types that were typically observed among the thirty South African soybean genotypes. Closed and open circles represent control and dark chilled plants, respectively. Each data point represents the average of eight plants $\pm$ standard error.

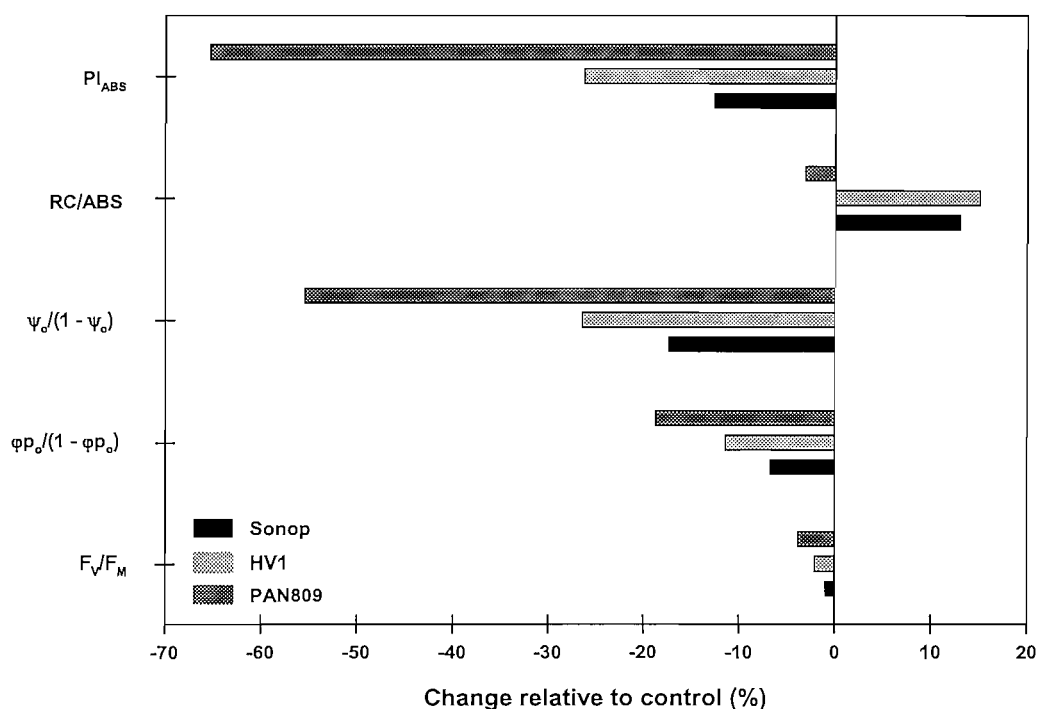


Figure 3.3 The average effect of seven consecutive nights of dark chilling on the PI_{ABS} and its partial responses, namely the density of working photosystems (reaction centre per chlorophyll, RC/ABS), the efficiency of primary photochemistry (trapping) ($\phi_{p_o}/(1 - \phi_{p_o})$) and the efficiency of the conversion of excitation energy to electron transport ($\psi_o/(1 - \psi_o)$), as well as the maximum quantum yield of primary photochemistry (F_v/F_m) in the three representative South African genotypes, Sonop, HV1 and PAN809. The horizontal bars indicate the response in dark chilled plants relative to that of control plants. Negative percentage values indicate a chilling-induced decrease in the PI_{ABS} and its partial responses. Each bar represents the average of eight plants.

Since large differences in the response of the thirty genotypes to dark chilling could successfully be established by using the PI_{ABS} , it was further employed in the calculation of a novel parameter, the chill factor index (CFI). By definition the CFI regards a reduction in the PI_{ABS} in any given genotype during the latter stages of dark chilling treatment progressively more significant (important) than a reduction in the PI_{ABS} during the first and second night of chilling. As a consequence, chilling sensitive genotypes, which exhibit the largest reductions in the PI_{ABS} during the latter stages of dark chilling treatment, will also have the lowest CFI values. The CFI for the thirty South African genotypes, as well as the two reference genotypes of known chilling tolerance, Maple Arrow (chilling tolerant) and Java 29 (chilling sensitive) are shown in Table 3.1. The known difference in dark chilling tolerance between the two reference genotypes was confirmed by their respective CFI values. The chilling tolerant genotype Maple Arrow had a CFI of -0.56, compared to -1.95 of the chilling sensitive genotype Java 29. The South African genotypes were subsequently ranked from number one to thirty according to their CFI value. The majority of the South African soybean genotypes had intermediate CFI values between Maple Arrow and Java 29. A group of five genotypes had similar or slightly higher CFI values than Maple Arrow. On the other hand, eight genotypes had CFI values lower than Java 29.

The relationship between the CFI of each genotype and the average reduction in the relative PI_{ABS} [$\log(PI_{ABS(stress)}/PI_{ABS(control)})$] after each of the seven nights of dark chilling is shown in Fig 3.4. A positive relationship ($r^2 = 0.94$) between the CFI and the decrease in relative PI_{ABS} could be established. Genotypes having the highest CFI (least negative) also exhibited the smallest reduction in relative PI_{ABS} during the entire dark chilling period. On the other hand, genotypes having the lowest CFI (most negative) also exhibited the largest reduction in the relative PI_{ABS} . Based on this relationship, a distinct group of five genotypes (LS677, LS555, Highveld Top, Wenner, Sonop) were identified that exhibited dark chilling responses comparable to that of Maple Arrow, the chilling tolerant reference genotype. On the other hand, eight genotypes (AG5601, AG6101, PAN854, Prima2000, Tallboy, Egret, Stork, PAN809) were identified that exhibited even poorer dark chilling responses than Java 29, the chilling sensitive reference genotype.

Table 3.1 Calculated chill factor index (CFI) values for thirty South African soybean genotypes and the two foreign genotypes (indicated in bold), Maple Arrow (chilling tolerant) and Java 29 (chilling sensitive), following an exposure period of seven nights to dark chilling. The three genotypes with the same CFI value (Spitfire, SCS1 and CRN5550) were ranked according to the average decrease in relative PI_{ABS} during the entire dark chilling period (refer to Fig. 3.4).

Genotype number	Genotype	Chill Factor Index
1	LS677	-0.40
2	LS555	-0.45
3	Highveld Top	-0.52
4	Wenner	-0.54
Chilling tolerant	Maple Arrow	-0.56
5	Sonop	-0.69
6	A5409	-0.80
7	Maruti	-0.87
8	Marula	-0.98
9	Knap	-1.06
10	LS666	-1.08
11	PAN564	-1.11
12	Amstel	-1.25
13	HV1	-1.28
14	PAN660	-1.42
15	AG5602	-1.49
16	LS599	-1.68
17	Potties	-1.70
18	Spitfire	-1.71
19	SCS1	-1.71
20	CRN5550	-1.71
21	Dumela	-1.75
22	SNK500	-1.78
Chilling sensitive	Java 29	-1.95
23	AG5601	-2.07
24	AG6101	-2.36
25	PAN854	-2.41
26	Prima2000	-2.46
27	Tallboy	-2.62
28	Egret	-2.98
29	Stork	-3.07
30	PAN809	-3.50

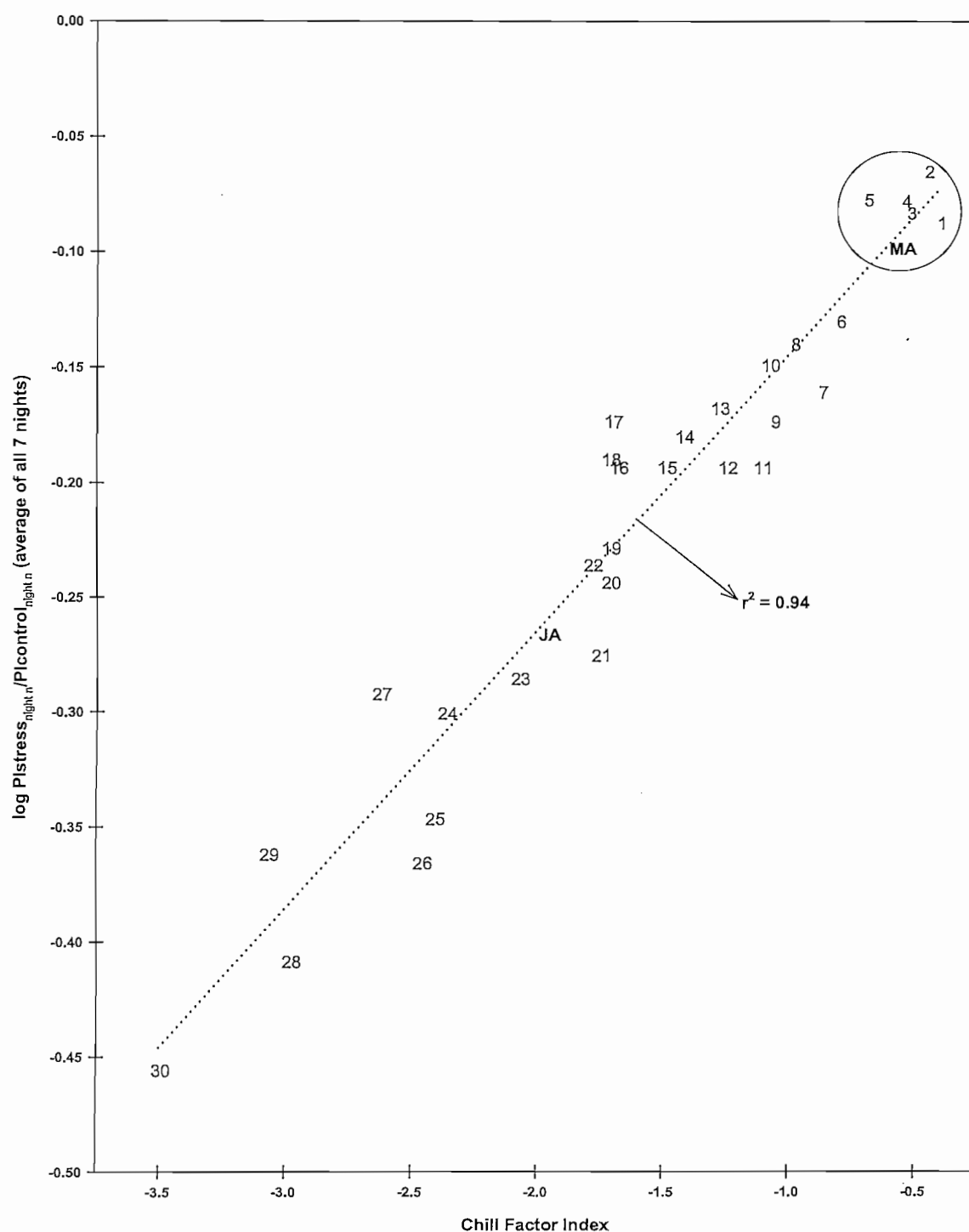


Figure 3.4 The relationship between chill factor index and average reduction in relative PI_{ABS} ($\log \text{PI}_{\text{stress}} / \log \text{PI}_{\text{control}}$) during seven consecutive nights of dark chilling in thirty South African soybean genotypes. The positions of the genotypes are indicated by numbers ranging from one to thirty according to the data in Table 3.1. The position of the two reference genotypes, Maple Arrow (MA) and Java 29 (JA), are also indicated in bold. The circle indicates a group of five genotypes with dark chilling response comparable to that of Maple Arrow.

3.3 Discussion:

Studies have shown that in the Kautsky transient, an increase in fluorescence intensity at 2 ms is usually interpreted as evidence of accumulation of the fraction of reduced Q_A^- pool (Strasser et al., 1995; Lazár and Ilík, 1997), possibly due to a decrease in electron transport beyond Q_A^- (Haldimann and Strasser, 1999). In the case of soybean, both short-term (Van Heerden and Krüger, 2000) and longer-term (Van Heerden et al., 2003c) experiments have indicated that dark chilling decreases the efficiency of electron transport beyond Q_A^- . Thus in the case of Sonop, PSII electron transport was inhibited to a much lesser extent by dark chilling than in PAN809 (Fig. 3.1), suggesting that the O-J-I-P fluorescence transient is sensitive enough to reveal these differences in dark chilling response.

In the context of the O-J-I-P fluorescence transient, the PI_{ABS} is a function of the fluorescence extrema F_o (O) and F_M (P), the intermediate step J (note again the large effect of dark chilling at this point) and the slope at the origin of the fluorescence rise, whereas the expression F_v/F_M is a function of only the fluorescence extrema F_o (O) and F_M (P), independent of which trajectory the fluorescence intensity reaches its maximal value by. Clearly, under already mild (absence of low root temperatures) dark chilling stress conditions, the shape of the fluorescence transient changes, particularly at the J-peak, long before the ratio of the extrema F_o and F_M changes. The O-J-I-P fluorescence transient and quantification of stress-induced changes in its shape (kinetics) with the PI_{ABS} , thus provide us with a rapid and non-intrusive diagnostic tool for early detection of dark chilling stress that can be employed to evaluate large numbers of soybean genotypes for dark chilling tolerance.

Considering the effect of dark chilling on PI_{ABS} , three different responses among the genotypes could be identified, namely least chilling sensitive (Fig. 3.2a), moderately chilling sensitive (Fig. 3.2b) and highly chilling sensitive (Fig. 3.2c). All the genotypes evaluated for dark chilling tolerance in this study responded similarly to one of these three representative genotypes, with the majority of genotypes belonging to the moderately and highly chilling sensitive groups that are incapable of withstanding long-term dark chilling stress.

The dark chilling-induced loss of electron transport capacity, as indicated by the large decrease in $\psi_o/(1 - \psi_o)$ (Fig. 3.3), is the reason for the large increase in fluorescence intensity at the J-step of the O-J-I-P fluorescence transient (Fig. 3.1) in chilling sensitive genotypes such as PAN809. Taken together, it would appear that the effect of dark chilling on electron transport beyond Q_A^- is one of the main factors that determine the magnitude of reduction in the PI_{ABS} in different soybean genotypes, since it is one of the partial responses of the photosynthetic apparatus that define the multi-parametric PI_{ABS} expression. The extent of the increase in fluorescence intensity at the J-step, i.e. increase in the size of the Q_A^- pool, also appears to be a good indicator of the degree of mesophyll limitation of photosynthesis following dark chilling stress. It has been previously demonstrated that stomatal limitation is largely responsible for the inhibition of photosynthesis in the chilling tolerant reference genotype Maple Arrow (small increase in fluorescence intensity at the J-step), whereas mesophyll limitation is the main cause of the inhibition of photosynthesis in the chilling sensitive reference genotype Java 29 (large increase in fluorescence intensity at the J-step). The severe inhibition of photosynthetic capacity in Java 29 was characterized by a large decrease in the PI_{ABS} , loss of RuBP regeneration capacity, and inhibition of stromal fructose-1,6-bisphosphatase and sucrose-phosphate synthase activity (Van Heerden et al., 2004b).

Although a positive relationship between the newly formulated CFI and the decrease in relative PI_{ABS} was established in the study, an advantage of using the CFI is that calculation of the CFI can be adapted to suit a specific case best, by arbitrarily allocating different weights to the chilling response of the plants over consecutive time periods represented in the formula. Due to the particular nature of the chilling response in soybean, the study regarded a reduction in the PI_{ABS} in any given genotype during period B (third, fourth and fifth night of chilling) or C (sixth and seventh night of chilling) more significant (by a factor of two and four respectively) than a reduction in the PI_{ABS} during period A (first two nights of dark chilling). In screening trials evaluating chilling tolerance in other plant species, however, the reduction in the PI_{ABS} during period A, for example, may be of greater importance, especially in regions where chilling night temperatures occur seldom or only for a few nights. The CFI, therefore, allows the particular investigator the freedom of altering the duration and significance factors of the different periods (A, B, C etc.) accordingly.

In conclusion, it could convincingly be demonstrated that large genotypic variation in the dark chilling tolerance of thirty South African soybean genotypes exists. Through the measurement of O-J-I-P fluorescence transients, and analysis of these transients by the JIP-test, the effect of dark chilling on the PI_{ABS} , a very sensitive indicator of dark chilling stress in soybean, could be determined. The effect of dark chilling on the PI_{ABS} during the various phases of the treatment period was used to introduce a novel parameter, the CFI, which made it possible to evaluate the chilling sensitivity of each genotype during the entire period of dark chilling stress. The CFI of the two reference genotypes, Maple Arrow and Java 29, correlated with their known difference in chilling tolerance. The CFI was used to rank the thirty genotypes according to dark chilling tolerance and identified five genotypes with comparable dark chilling response to Maple Arrow under experimental conditions that excluded low root temperatures. These five genotypes have indeed been confirmed to be very popular in agricultural practices on the South African highveld (personal communication, Mr A de Lange, ARC, Potchefstroom) where cool night temperatures are often experienced during the soybean growth season. The dark chilling tolerance of these five genotypes should, however, be evaluated under field conditions in order to establish their potential value as parental material in breeding programs aimed at increasing the dark chilling tolerance of South African soybean genotypes. Finally, it can be proposed that the PI_{ABS} and CFI could be used as sensitive indicators of dark chilling stress in soybean. These indexes could possibly also be of great value as screening tools for chilling tolerance in other chilling sensitive crop species in the future, as well as for studies focusing on different environmental factors, e.g. drought stress and heat, etc. Indeed, the concept of the CFI developed in this chapter was recently used to define a so-called “drought factor index” for the purpose of screening barley genotypes for drought tolerance (Oukarroum et al., 2007).

Chapter 4

Characterisation of the role of low soil temperature in the inhibition of growth and PSII function during dark chilling in two South African soybean genotypes of contrasting chilling tolerance*

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4.1 Introduction

The degree of chilling stress that plants experience depends on various environmental conditions, resulting in different physiological and biochemical responses. For example, if roots are exposed to low temperatures, it often results in chill-induced drought stress with subsequent leaf wilting due to reduced root hydraulic conductance restricting water uptake (Allen and Ort 2001, Bloom et al. 2004, Melkonian et al. 2004b). However, these symptoms do not occur when only the shoots and leaves of plants are chilled (shoot chilling, SC). Many studies have investigated the effects of chilling stress on photosynthetic metabolism and water relations of plants (e.g. Anderson et al. 1995, Aroca et al. 2003, Aroca et al. 2005, Bertamini et al. 2005). In most of these studies, however, both the shoots and roots of plants were chilled (whole-plant chilling, WPC), which could have resulted in secondary effects such as chill-induced drought stress. In addition, in most studies plants were exposed to both sub-optimal night and day temperatures. For example, although Musser et al. (1984) investigated the effects of SC in soybean, plants were exposed to chilling temperatures on a continual (24h) basis for seven days. Very few studies have considered the scenario where plants experience low night but normal daytime temperatures, which is a common phenomenon in many soybean-producing areas, including South Africa. Although Bertamini et al. (2005, 2006) recently investigated these effects in grapevine, potted plants were exposed to WPC. The possible presence of secondary effects on roots during chilling stress experiments has been acknowledged previously. In an attempt to minimize these effects, Shen et al. (2000) incubated pots in a water bath at 20°C to increase root temperatures in cucumber

plants following exposure to chilling temperatures of 3°C. Alternatively, previously chilled plants were first acclimated under low light conditions before exposure to full irradiance (e.g. Bertamini et al. 2006). The association between the roots of soybean and *Bradyrhizobium japonicum* bacteria leads to formation of specific organs, called root nodules, where symbiotic nitrogen fixation (SNF) takes place. The main products of SNF in soybean root nodules, namely ureides (allantoin and allantoic acid), are exported to the rest of the plant where they are incorporated into amino acids and proteins. In nodulating legumes, however, inhibition of SNF (Duke et al. 1979), and possibly also irreversible effects such as premature root nodule senescence, might be induced by low root temperatures, even after rewarming. No detailed studies that have focused on a comparison of the effects of WPC and SC on warm-climate legumes such as soybean exist in the scientific literature.

In Chapter 3 O-J-I-P chlorophyll *a* fluorescence transients, and subsequent analysis of these transients by the JIP-test, were employed in the ranking of thirty South African soybean genotypes according to dark chilling tolerance. Based on this ranking procedure, two genotypes, representing contrasting degrees of chilling tolerance, namely Highveld Top (chilling tolerant) and PAN809 (chilling sensitive), were selected for further detailed investigations. Refer to Chapter 3 (table 3.1) for the ranking positions of these two genotypes.

In this chapter, the effects of dark chilling on vegetative development, PSII function, and leaf ureide content in these two genotypes were investigated. Moreover this investigation also encompassed a comparison of WPC and SC effects, thereby providing information on the modulation of chilling response by these treatments.

4.2 Results

4.2.1 Dark chilling reduces shoot growth rates (SGR's) and chlorophyll content

Both the WPC and SC treatments inhibited shoot growth in the chilling sensitive genotype PAN809, while there was no visual effect in Highveld Top (Fig. 4.1). In Fig. 4.2 the increase in plastochron index over time for each treatment during the 12-night

period of chilling stress is shown. The slope of each linear regression represents the SGR (increase in plastochron index units per day) for a particular treatment and is given in the top left-hand side of each figure. In PAN809, SGR's were reduced by 13% (SC, $p<0.01$) and 21% (WPC, $p<0.01$), while no significant reduction was observed in Highveld Top (Fig. 4.2). The WPC treatment also led to severe chlorosis in PAN809 (Fig. 4.1). After twelve nights of chilling treatment, the chlorophyll content of the fourth trifoliolate leaves of PAN809 was 23% (SC, $p<0.01$) and 57% (WPC, $p<0.01$) lower than in the control, while in Highveld Top, the chlorophyll content was only decreased by 15% ($p<0.01$) in plants of the WPC treatment (Fig. 4.7A). In control plants, Highveld Top had a higher chlorophyll content than PAN809 at this stage of leaf development, which corresponded with differences in light and CO₂ saturated rates of photosynthesis in the same leaves (see Chapter 5).

4.2.2 Changes in O-J-I-P fluorescence rise kinetics during dark chilling

Excitation of dark-adapted leaves with a saturated light pulse induced typical O-J-I-P fluorescence transients in the control (Fig. 4.3A, D), WPC (Fig. 4.3B, E) and SC (Fig. 4.3C, F) treatments. The original O-J-I-P transients (average of 24 measurements) recorded at regular intervals over the 12-night treatment period are shown. Upon excitation, there was an initial rapid rise in fluorescence intensity from O (F_0) to the first intermediate step J at 2 ms. A further rise then followed to the second intermediate step I at 30 ms, finally reaching P (F_M) at ca. 200 ms. In terms of actual fluorescence intensities and kinetics, genotypic differences in the chilling response of Highveld Top and PAN809 are clearly visible. Dark chilling induced a large decrease in maximal fluorescence intensities (F_M) in PAN809 but not in Highveld Top. This decrease occurred in a chilling duration-dependent fashion, especially in the WPC treatment (Fig. 4.3e). In the case of Highveld Top, both the chilling treatments caused a slight and very similar decrease in maximal fluorescence intensities. It is also clear that the WPC treatment (Fig. 4.3E) caused larger changes in fluorescence values and kinetics than the SC treatment (Fig. 4.3F) in PAN809.



Figure 4.1 Shoot development in Highveld Top (chilling tolerant) and PAN809 (chilling sensitive) plants exposed for 12 consecutive nights to either whole plant chilling, shoot chilling or kept under control conditions.

Highveld Top

PAN809

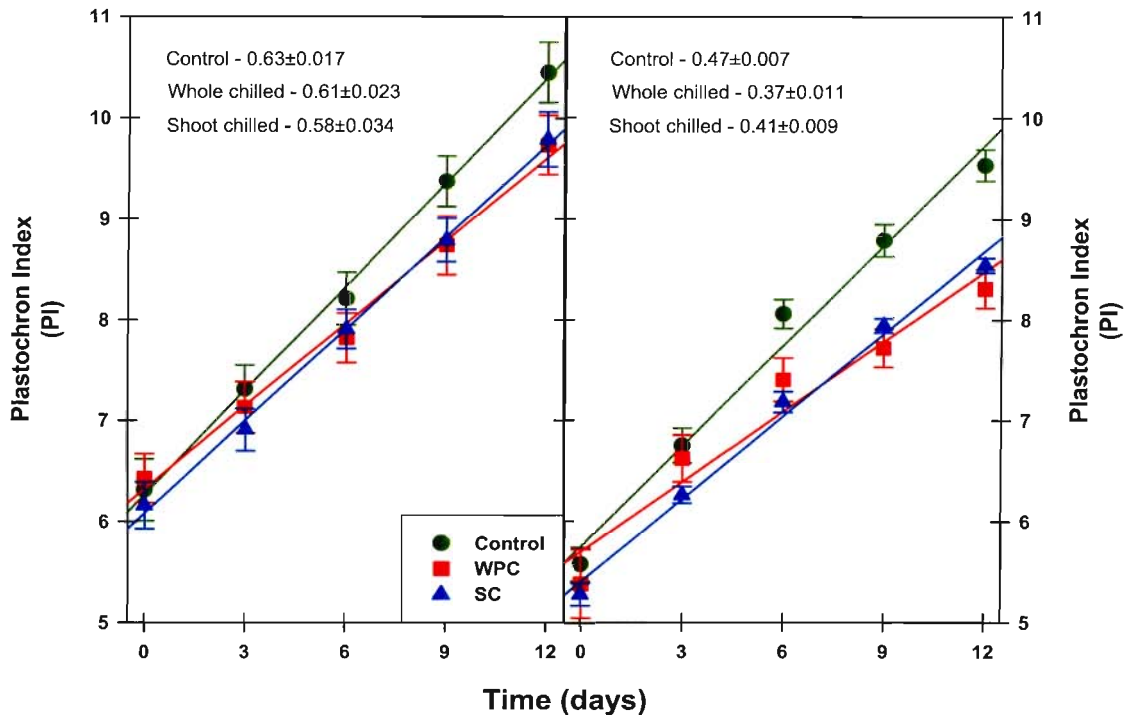


Figure 4.2 Shoot development, as quantified by the plastochron index (PI) in Highveld Top (chilling tolerant) and PAN809 (chilling sensitive) plants exposed to 12 consecutive nights of either whole plant chilling, shoot chilling or kept under control conditions. The actual shoot growth rates (increase in PI units day⁻¹) during the treatment period are shown. All values indicate the mean of 4 replicates $\pm$ SE.

The treatment-dependent changes in fluorescence rise kinetics were investigated further by the construction of so-called difference in variable fluorescence (ΔV) curves (Fig. 4.4). For the construction of ΔV curves the original transients shown in Fig. 4.3 were first normalised between the two fluorescence extremes (O and P), whereafter the normalised values of treated plants at each time point (nights 3, 6, 9 and 12) were subtracted from the normalised values of the control plants at the same time points. The black horizontal zero baseline represents the values obtained at each time point after subtraction of the normalised values of control plants from itself. These curves revealed the presence of two clearly defined peaks, namely a ΔK -peak (at ca. 300 μ s) and a ΔJ peak (at ca. 2 ms), dependent on genotype and type of chilling treatment.

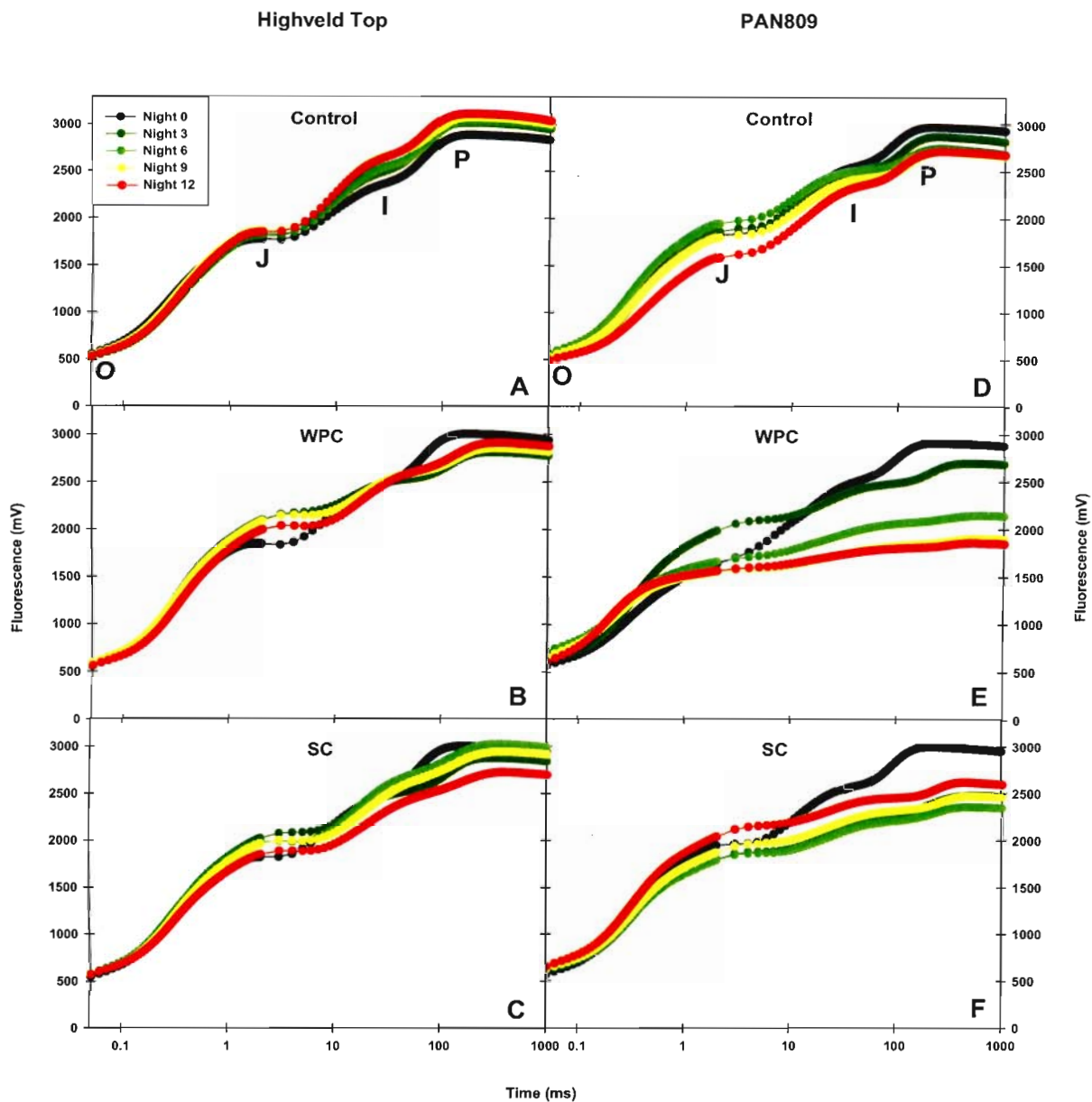


Figure 4.3 Effects of dark chilling on O-J-I-P fluorescence transients recorded in fully expanded leaves of control (A, D), WPC (B, E) and SC (C, F) plants of Highveld Top and PAN809. Transients represent the average of 24 measurements (obtained from 6 plants per treatment) and were recorded at regular intervals (0, 3, 6, 9 and 12 nights) during the treatment period. The position of the main steps (O, J, I and P) is indicated in the top figures.

The ΔJ peak was induced by the WPC and SC treatments in both genotypes, but the size of the peaks in both dark chilling treatments of PAN809 (Fig. 4.4C, D) was much larger than in Highveld Top (Fig. 4.4A, B), with a progressive increase over the treatment period. The appearance of the ΔK -peak was much more specific. A clearly defined ΔK -peak was only induced by the WPC treatment in PAN809 (Fig. 4.4D), showing progressive development over time and being most pronounced after nine and twelve nights of dark chilling when the same leaves also had 32% and 57% lower chlorophyll contents (also see Fig. 4.7A) than the control (negative percentage values shown at the ΔK -peaks in Fig. 4.4D).

4.2.3 Dark chilling inhibits PSII electron transport

The effects of dark chilling on one of the JIP-test parameters, the PI_{ABS} , as well as the F_V/F_M ratio, are shown in Fig. 4.5. In this figure the F_V/F_M ratio is included to emphasise its insensitivity to dark chilling. Firstly, dark chilling, irrespective of the type of treatment, caused a large reduction in the PI_{ABS} values but not in the F_V/F_M ratio. Secondly, a clear genotypic difference in the effect of dark chilling on the PI_{ABS} values was observed, with PAN809 (Fig. 4.5B) being much more affected than Highveld Top (Fig. 4.5A), irrespective of the type of chilling treatment. Thirdly, in PAN809, but not in Highveld Top, the WPC treatment resulted in larger reductions in PI_{ABS} values than the SC treatment. Over the twelve nights of chilling the PI_{ABS} values in Highveld Top decreased on average by 31% (WPC) and 27% (SC) compared to values at the start of the experiment (night 0), while in PAN809 the decreases were much more pronounced, namely 83% (WPC) and 55% (SC). In control plants of Highveld Top the PI_{ABS} values increased progressively during the experimental period, while in control plants of PAN809, values only showed an increase between nine and twelve nights. Dark chilling prevented similar developmental changes in PI_{ABS} values in both genotypes.

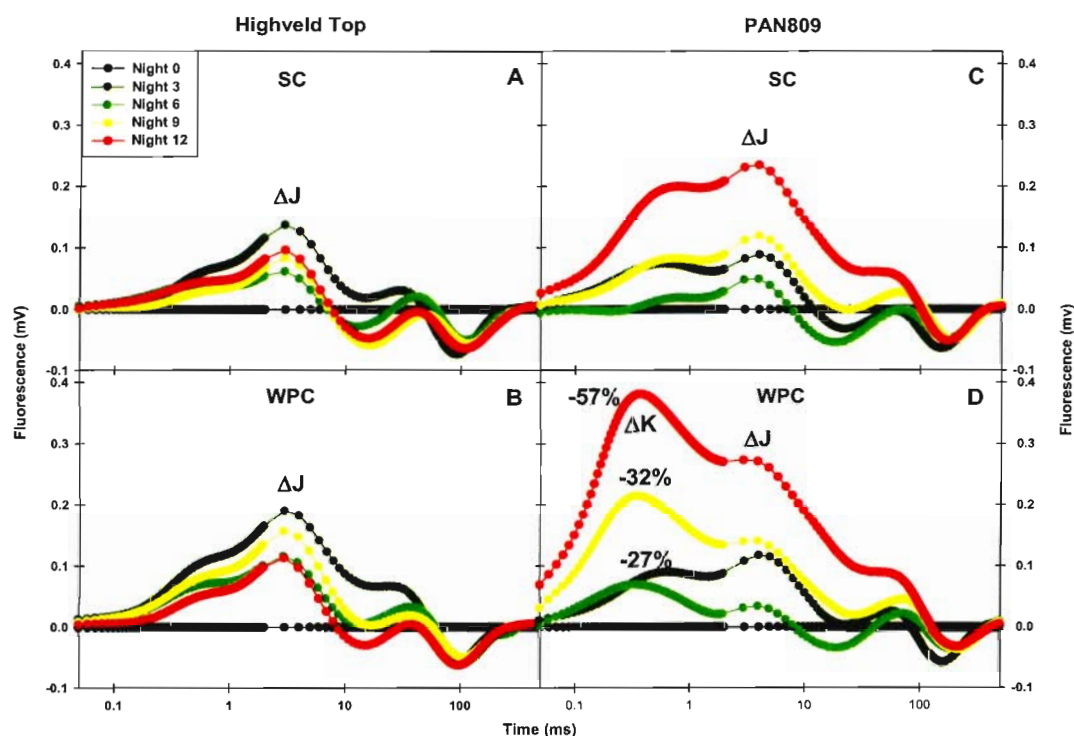


Figure 4.4 Effects of dark chilling on the kinetics of O-J-I-P fluorescence transients recorded in fully expanded leaves of SC (A, C) and WPC (B, D) plants of Highveld Top and PAN809. Difference in variable fluorescence (ΔV) curves was constructed by subtraction of the normalised (between O and P) values of the O-J-I-P transients recorded in the SC and WPC plants (Fig. 4.2) from those of the control plants at each respective time point. The black zero line represents subtraction of the normalised values of the control transients from itself. Note in particular the increases in fluorescence intensity at 300 μ s (ΔK peak) and 2 ms (ΔJ peak). The percentage decrease in chlorophyll content in leaves of WPC plants of PAN809 with development of the ΔK peak is indicated as inserts. All curves represent the mean response obtained from six plants per treatment.

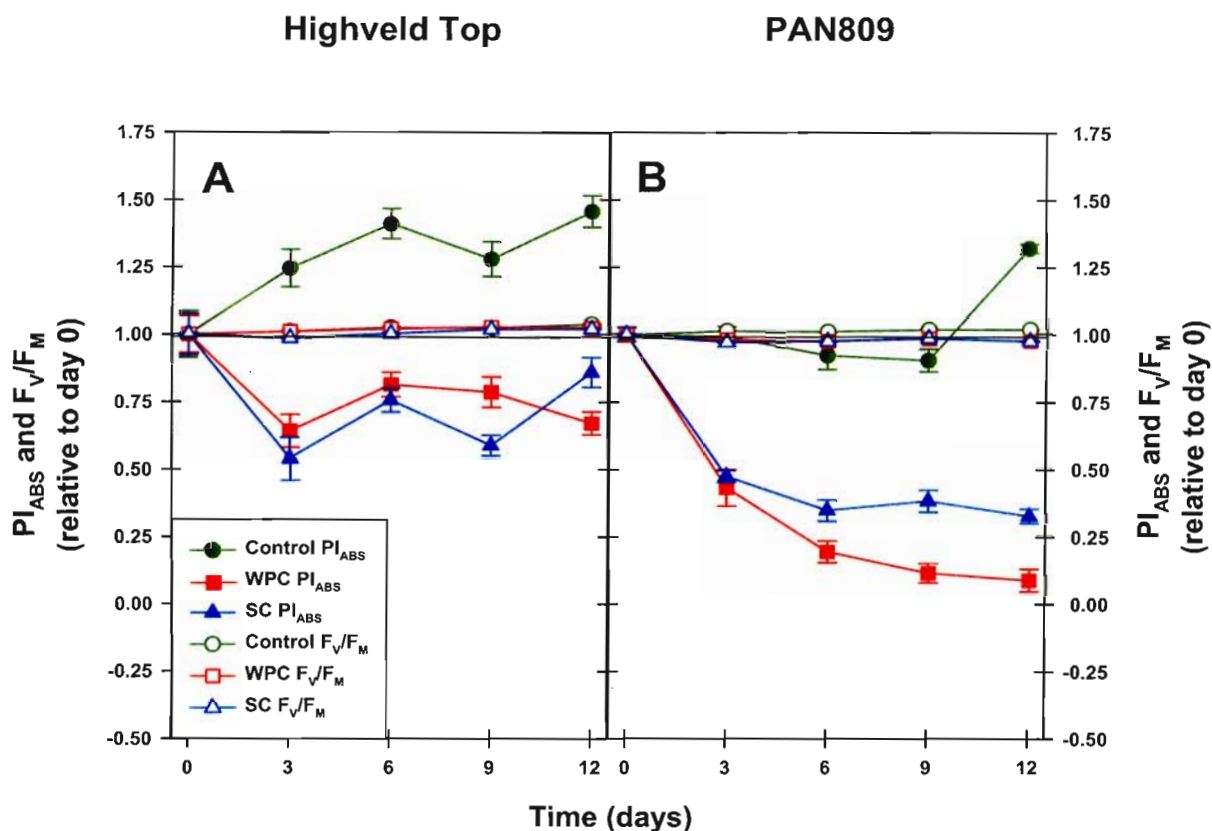


Figure 4.5 The effects of dark chilling on the performance index (PI_{ABS}) and maximum quantum yield of primary photochemistry (F_V/F_M) in Highveld Top (A) and PAN809 (B). Actual values were normalised relative to values measured before the onset of the chilling treatment (night 0) to reveal differences in the response of the PI_{ABS} and F_V/F_M ratio more clearly. Open and closed symbols depict normalised F_V/F_M and PI_{ABS} values, respectively, while circles, triangles and squares depict control, SC and WPC treatments, respectively. Each data point represents the mean of six replicates $\pm$ SE.

The response of PS II function in the two genotypes during dark chilling stress was investigated further by detailed analysis of the recorded O-J-I-P fluorescence transients with the JIP-test. The effects of the WPC and SC treatments on the maximum quantum yield of primary photochemistry ($F_V/F_M = \phi_{Po}$) and the specific (per RC) and phenomenological (per excited CS) energy fluxes for light absorption, excitation energy trapping and electron transport are presented in the form of radar plots (Fig. 4.6A and B). For comparative purposes the effects of chilling stress on the PI_{ABS} are also incorporated. All the parameters calculated in the control plants were normalised to the numeric value

of one. The radar plots therefore show the fractional increase or decrease in each of the parameters calculated in the plants exposed to the WPC and SC treatments. The large effects of both the WPC and SC treatments on PI_{ABS} in the case of PAN809 (Fig. 4.6B), and the much smaller effect on PI_{ABS} in Highveld Top (Fig. 4.6A), can clearly be seen.

Based on the degree of deviation from the green reference line (control plants) in the case of PAN809, it is clear that severe and more intermediate effects on PS II function were induced by the WPC and SC treatments, respectively. In contrast, the effects of chilling stress on most parameters in Highveld Top were much less pronounced. In PAN809 the WPC treatment led to deactivation of PS II reaction centres (RC/CS) on the one hand, and a large increase in the apparent antenna size per remaining active RC (ABS/RC) on the other hand. The increase in ABS/RC compensated for the large decrease in RC/CS because excitation energy trapping per active RC (TR_o/RC) was also increased, while electron transport per active RC (ET_o/RC) decreased to a lesser extent. The large compensation on the part of ABS/RC concomitantly led to an increase in the phenomenological energy flux for absorption (ABS/CS) and maintenance of excitation energy trapping flux (TR_o/CS). This compensatory response was, however, not sufficient to prevent a chilling stress-induced decrease in the phenomenological energy flux for electron transport (ET_o/CS). The overall insensitivity of the F_v/F_m ratio towards dark chilling, compared to the large effect on the PI_{ABS} , is also clearly visible.

4.2.4 Time-dependent changes in chlorophyll and ureide content resulting from dark chilling

The changes in chlorophyll and ureide content of fully expanded leaves over the entire dark chilling treatment are presented in Fig. 4.7A and B, respectively. Significant reductions in chlorophyll content commenced after only three nights of dark chilling in both WPC and SC treatments of PAN809, as well as the WPC treatment of Highveld Top (Fig. 4.7A). A slight, but not significant ($P>0.05$) increase in chlorophyll content, relative to control plants, occurred in SC treated plants of Highveld Top.

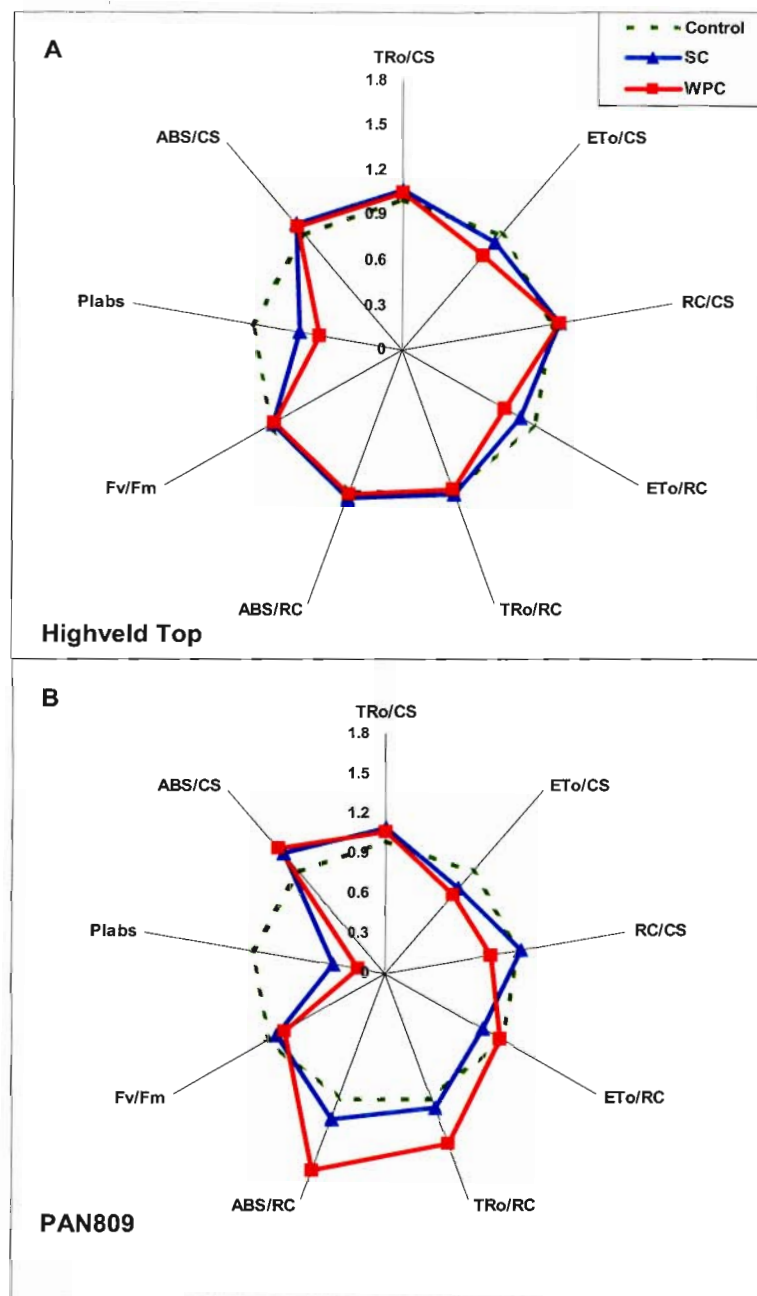


Figure 4.6 Radar plots showing the effect of dark chilling on the specific (per RC) and phenomenological (per CS) fluxes for light absorption (ABS), excitation energy trapping (TR_o) and electron transport (ET_o) in Highveld Top (A) and PAN809 (B). The effects of chilling on the fraction of active reaction centres per excited cross section of leaf (RC/CS), F_v/F_m, and PI_{ABS} are also incorporated into the plots. Values for all the parameters measured in plants exposed to WPC (red squares) and SC (blue triangles) treatments were normalised against values measured in the respective control plants (dashed green reference line).

Further exposure to dark chilling lead to an increasing reduction in chlorophyll content in the WPC treated plants of PAN809, while chlorophyll content in the other treatments decreased to a much lesser extent. The very large reduction in chlorophyll content that occurred between 9 and 12 nights of chilling in the WPC treatment of PAN809 was preceded (between 6 and 9 nights) by a large decrease in leaf ureide content in the same leaves (Fig. 4.7B). From Fig. 4.7B it is clear that leaf ureide content was reduced to the largest extent in the WPC treatment of PAN809.

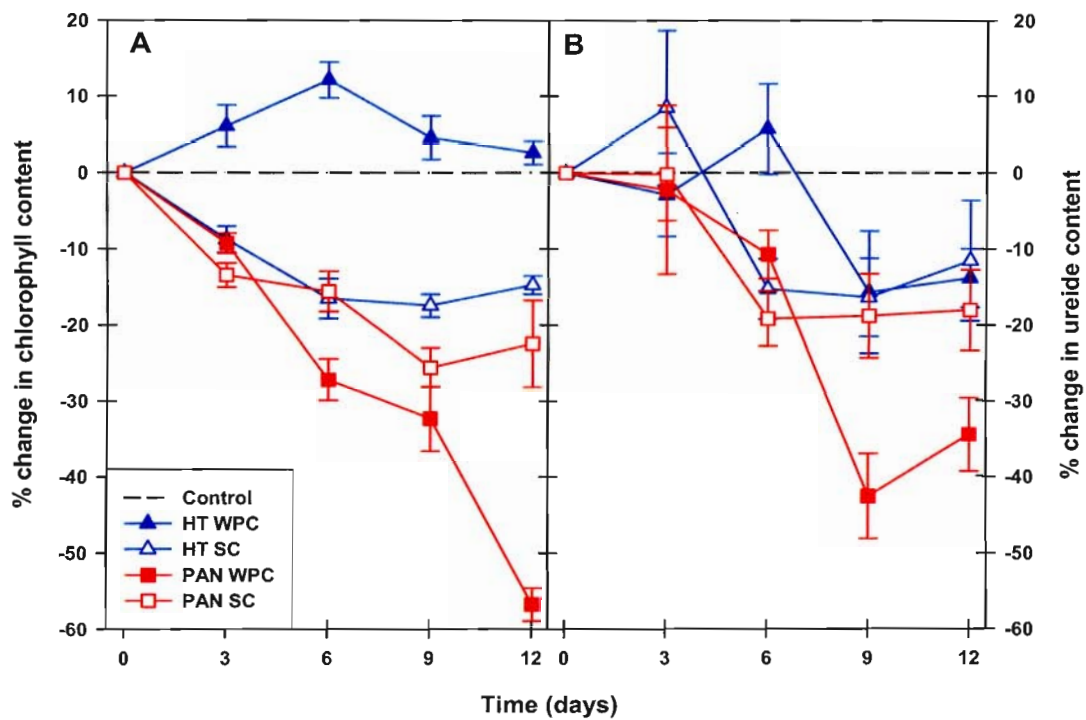


Figure 4.7 Progressive effects of dark chilling during a twelve-night treatment period on chlorophyll content (A) and leaf ureide content (B). To facilitate comparison between these parameters, and present time-dependent changes more clearly, values are expressed as percentages relative to control values at each respective time point. Each value represents the mean of six replicates $\pm$ SE.

4.3 Discussion

Chilling-associated decreases in PSII function occur in many plants due to a multitude of possible perturbations including oxidative damage to thylakoids (Tambussi et al. 2004), inhibition of electron transport by downstream regulation to match a lower ATP and NADPH demand during reductive carbon metabolism (Zhou et al. 2004b) and conformational changes/dissociation of PSII proteins (Higuchi et al. 2003, Parvanova et al. 2004). Bertamini et al. (2006) recently demonstrated pronounced dark chilling-induced degradation of the D1 reaction centre protein in grapevine plants.

Recording of O-J-I-P fluorescence transients during the period of WPC and SC treatment, followed by analysis with the JIP-test, allowed the quantification of biophysical parameters that gave insight into the changes occurring in PSII function as a result of these treatments. This demonstrated the clear genotypic differences in chilling response between the two genotypes as well as differential effects induced by the WPC and SC treatments. Moreover, these fluorescence measurements were coupled with the quantification of SGR's, chlorophyll and ureide content.

In both genotypes, but especially in plants of PAN809 exposed to WPC, distinct changes in the kinetics of the O-J-I-P transients emerged. The ΔJ -peak that emerged in both genotypes under both chilling treatments can be interpreted as evidence of accumulation of the Q_A^- pool (Strasser et al. 1995, Govindachary et al. 2004), most probably because of decreased electron transport downstream of Q_A^- (Haldimann and Strasser 1999). The exclusive appearance of a ΔK -peak in the WPC treatment of PAN809, on the other hand, points towards uncoupling of the oxygen-evolving complex (OEC) (Srivastava et al. 1995). It is well known that the donor side of PSII (i.e. the OEC) is sensitive to changes in temperature (Terashima et al. 1989, Shen et al. 1990, Enami et al. 1994), but the current study revealed for the first time that in specifically whole chilled soybean plants, this disruption is not triggered directly by chilling of leaves *per se*, but rather indirectly by chilling effects on the roots. Thus novel evidence is provided, demonstrating that in the chilling sensitive genotype, low soil temperatures disrupt the OEC, in addition to a more general (in terms of genotype and treatment) chilling-induced

restriction on electron transport downstream of Q_A^- that depends more on chilling of the leaves *per se*.

Exposure to specific environmental stressors over a prolonged period often leads to the development of visible symptoms during later stages. Out of a breeding/biotechnology perspective, however, early detection of stress is critical to identify the signals that trigger or block corresponding molecular processes. The PI_{ABS} values in chilled leaves were already decreased to a large extent after only 3 nights of chilling, compared to the effects on chlorophyll content and leaf ureide content that developed progressively during the duration of the treatment period. The aggravation of chilling stress effects by the WPC treatment was reflected by larger reductions in the PI_{ABS} values in these plants. Changes in the kinetics of O-J-I-P transients, and quantification of these effects by the PI_{ABS} , thus appears to be a sensitive tool for differentiating between WPC and SC effects in soybean genotypes of differing dark chilling tolerance.

Although decreases in electron transport was observed in both the WPC and SC treatments of PAN809, RC deactivation only occurred in the WPC treatment, indicating that repair processes (e.g. D1 protein turnover) were disrupted by this treatment, similar to recent observations in grapevine (Bertamini et al. 2006).

Differences in chilling stress sensitivity between the two genotypes, as reflected by O-J-I-P fluorescence rise kinetics, correlated with the growth response of the genotypes. Out of an agricultural perspective this is important, because reduced vegetative growth will ultimately manifest itself in the form of reduced flowering, pod formation and yield. Moreover, aggravation of chilling stress effects by the WPC treatment, as indicated by the ΔK -peak, was also reflected by the severe chlorosis that distinguished the WPC from the SC treatment in PAN809.

On a mechanistic level, the appearance of a ΔK -peak (Fig. 4.4), coupled to the decrease in SGR's (Fig. 4.2), chlorophyll content (Fig. 4.7A), ureide content (Fig. 4.7B)

and soluble protein content (see Chapter 5), provides information about how the WPC treatment aggravated chilling stress in PAN809. Interestingly, the appearance of a K-peak in the O-J-I-P transient has been observed previously when insufficient supply of nitrogen from roots to shoots occurs (Strasser et al. 2004). It is therefore hypothesised that the WPC treatment led to the progressive development of N-limitation, most probably because of a disruption of bacterial nitrogenase (EC 1.18.6.1) activity and nodule metabolic potential in chilled root systems of PAN809. Symbiotic nitrogen fixation in soybean root nodules is inhibited by low soil temperatures (Walsh and Layzell 1986), and in addition, decreased distribution of nitrogen from the roots to the rest of the plant has been reported in other legumes at low temperatures (Castle et al. 2006). Inhibition of nitrogenase activity, coupled to decreased distribution of nitrogenous compounds (mostly ureides in soybean and other warm-climate legumes) to the foliage, will lead to chlorosis and reduced growth rates. The inhibition of SNF, with concomitant development of N-limitation, as indicated by the large reduction in leaf ureide content (Fig. 4.7B), might be an important underlying factor contributing towards the chilling sensitivity of PAN809. Very recent results obtained with the acetylene reduction assay have shown much larger chilling-induced reductions in nitrogenase activity and nodule ureide content in PAN809 than in Highveld Top during a short period of WPC treatment (Van Heerden et al. 2008).

In conclusion, this chapter provides novel evidence linking changes in O-J-I-P fluorescence rise kinetics to changes in nitrogen metabolism in nodulated soybean during chilling stress, which ultimately manifests itself in the form of symptoms (e.g. chlorosis and reduced growth rates) on the whole-plant level. The sensitivity of the O-J-I-P fluorescence transient as a tool to visualise these effects, makes it suitable for use in field-applied research as well as in more in-depth studies combining different biochemical and biophysical approaches.

Chapter 5

Characterising the inhibition of photosynthesis in soybean by comparing responses to low air and soil temperatures, with specific focus on key photosynthetic enzymes

5.1 Introduction

As previously discussed, many studies have investigated the effects of chilling stress on plants, but in the majority of these, however, entire plants were chilled which could have resulted in secondary effects. Another important consideration when investigating the effects of low soil temperatures in legumes, such as soybean, is that a cluster of nitrogen-fixing root nodules are situated just below the soil surface at the base of the primary root where the risk of exposure to low temperatures is high. This could lead to additional constraints, because besides the inhibition of photosynthesis by low air temperatures, low soil temperatures can also inhibit symbiotic nitrogen fixation (Guy et al., 1997), which may indirectly aggravate effects on photosynthesis. In nitrogen-fixing legumes this makes it difficult to dissect the relative contribution of low soil and air temperatures towards the overall inhibition of photosynthesis in the case of experiments where both the roots and shoots of plants were chilled.

In Chapter 4, the effect of chilling temperatures on two soybeans genotypes of previously established contrasting chilling tolerance (Chapter 3) was investigated, exposing experimental plants to low night temperatures of 6°C (9 hours) and normal day temperatures of 26°C (15 hours) for up to 12 consecutive diurnal cycles. Furthermore, this part of the study also compared the response of plants where both the roots and shoots were chilled with plants where only the shoots were chilled. It was concluded that in the presence of low soil-temperatures, nitrogen fixation in root nodules of the chilling sensitive genotype is disrupted, leading to a decrease in leaf ureide content so that fully expanded leaves gradually become chlorotic. However, the mechanistic basis underpinning the more severe inhibition of photosynthesis that was observed in whole-chilled plants on the one hand and the large genotypic difference between the two

genotypes on the other hand, was not explored beyond the effects of these treatments on photosystem II (PSII) function.

The effects of dark chilling on photosynthesis are complex and do not only involve direct effects on PSII function. In fact, some effects on PSII could be a down-regulatory response to downstream perturbations since chilling temperatures are known to often inhibit the photosynthetic carbon reduction (Calvin) cycle reactions proportionately more than the energy-transducing light reactions (Sassenrath et al., 1990). Eleven different enzymes comprise the Calvin-cycle, and control of carbon assimilation is shared between several of these, including ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), sedoheptulose-1,7-bisphosphatase (SBPase), chloroplastic fructose-1,6-bisphosphatase (cFBPase) and aldolase (Harrison et al., 1998; Ölçer et al., 2001). The relative contribution of each of these enzymes towards the flux through the Calvin-cycle differs according to environmental conditions (Raines et al., 2000). Furthermore, since the Calvin cycle provides precursors for the formation of carbohydrates, it also influences the activity of enzymes such as sucrose-phosphate synthase (SPS), which is known to be sensitive towards unfavorable shifts in temperature (Labate and Leegood, 1990). Dark chilling, however, may also influence SPS activity directly in species such as tomato, for example through disruption of the enzyme's circadian rhythm (Jones et al., 1998). Generally, chilling stress also causes inhibition of CO₂ assimilation through reduced stomatal conductance (Melkonian et al., 2004b; Singh et al., 2005; Kościelniak and Biesaga-Kościelniak, 2006) indicating a complex interplay between both diffusive and metabolic factors in the inhibition of photosynthesis.

This chapter discusses further experiments conducted on the two soybean genotypes of well-defined but contrasting chilling tolerance, for an in-depth empirical appraisal of the mechanisms involved in the differential inhibition of photosynthesis, firstly between the two genotypes and secondly where low soil temperatures were present or absent during nighttime exposure to low air temperatures. Novel results showed that low soil temperatures aggravate the inhibition of photosynthesis in the chilling sensitive

soybean genotype through highly selective intensification of effects on specific target enzymes.

5.2 Results

5.2.1 Dark chilling alters CO₂ response kinetics in a genotype dependent fashion

Construction of A:C_i response curves, which show the relationship between CO₂ assimilation rate (A) and intercellular CO₂ concentration (C_i), revealed differential responses in the two genotypes towards dark chilling. In Highveld Top (Fig 5.1A, C, E), the changes in CO₂ response kinetics induced by both types of chilling, i.e. whole plant chilling (WPC) and shoot chilling (SC), over time, were much smaller than in PAN 809 (Fig 5.1B, D, F). In PAN809, the effects of dark chilling on these kinetics were particularly pronounced at high C_i values (refer to section 5.2.3 for more details).

5.2.2 Dark chilling reduced stomatal conductance (G_s) and ambient CO₂ assimilation rates (A) irrespective of genotype and type of treatment

In general dark chilling reduced G_s and A in both genotypes and under both conditions (WPC and SC) of chilling stress. In Highveld Top only WPC treated plants showed a significant ($P < 0.05$) negative response after three nights of chilling, with an inhibition of 73% in A compared to control plants (Fig. 5.2A). However, with further nights of exposure to chilling, both WPC and SC treated plants reacted very similarly with inhibition of between 43% and 54% ($P < 0.05$) in A attained after twelve nights. In PAN809 on the other hand, A was significantly inhibited by 57% ($P < 0.01$) in both WPC and SC treated plants after only three nights of chilling (Fig. 5.2B). With continued nightly exposure to chilling temperatures, WPC treated plants of PAN809 experienced very large inhibitions of up to 75% ($P < 0.01$) in A, while the inhibition in SC treated plants was less (Fig. 5.2B). The response of G_s mimicked the pattern of inhibition of A by dark chilling in Highveld Top (Fig. 5.2C). In Highveld Top, after twelve nights of chilling, reductions in G_s of 53% ($p < 0.01$) and 56% ($p < 0.05$) were evident in WPC and SC treated plants, respectively. However, in PAN809, where A was inhibited to a different extent in WPC and SC treated plants, values for G_s only reflected this clearly

after 12 nights of chilling where it was reduced by 63% ($P < 0.05$) and 32% ($P < 0.05$) in WPC and SC treated plants, respectively (Fig. 5.2D).

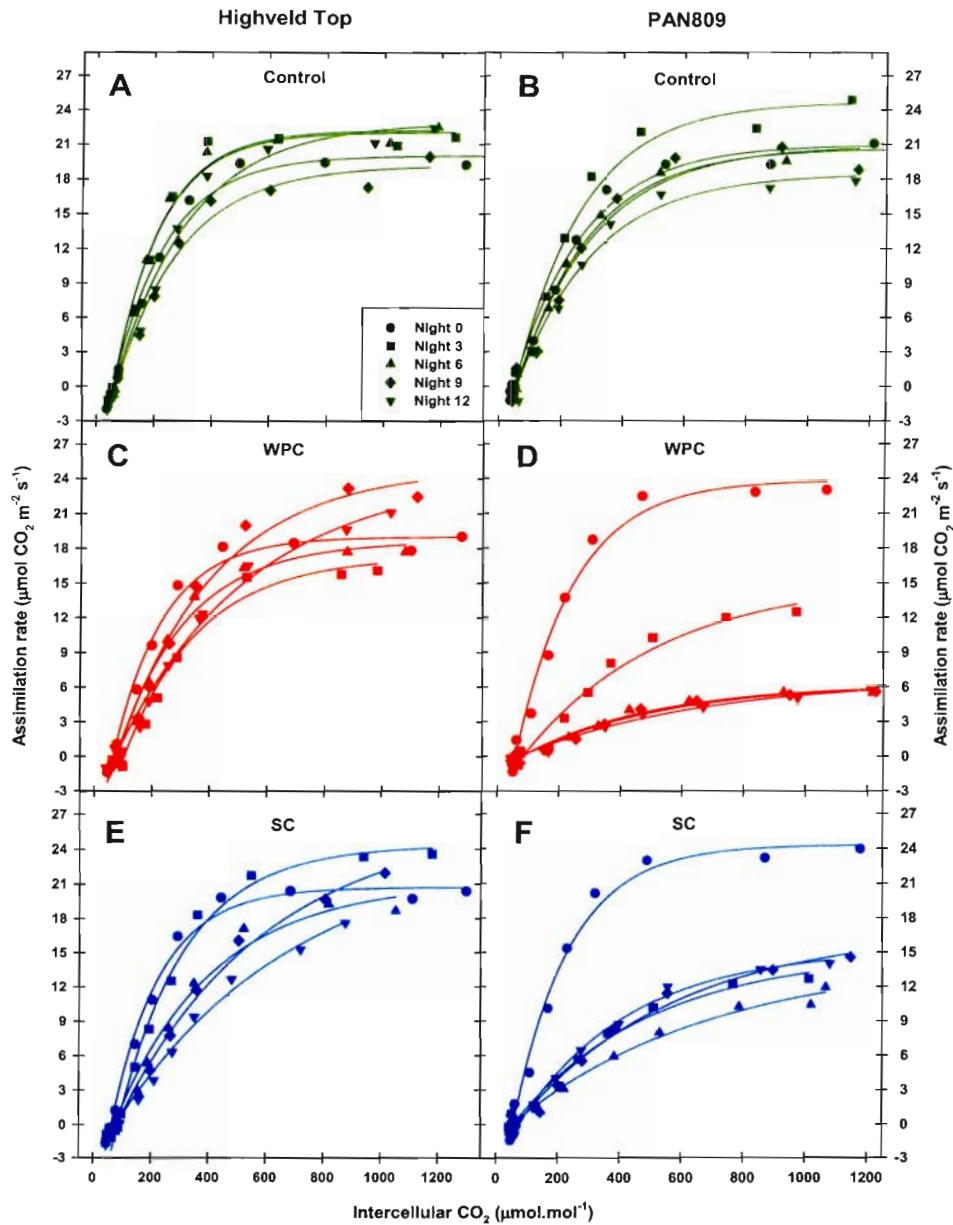


Figure 5.1 Relationship between CO_2 assimilation rate and intercellular CO_2 concentration in control, whole chilled (WPC) and shoot chilled (SC) plants of Highveld Top (left) and PAN809 (right) over a period of 12 consecutive nights of treatment, measured at $1200 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Each data point represents the mean of four replicates.

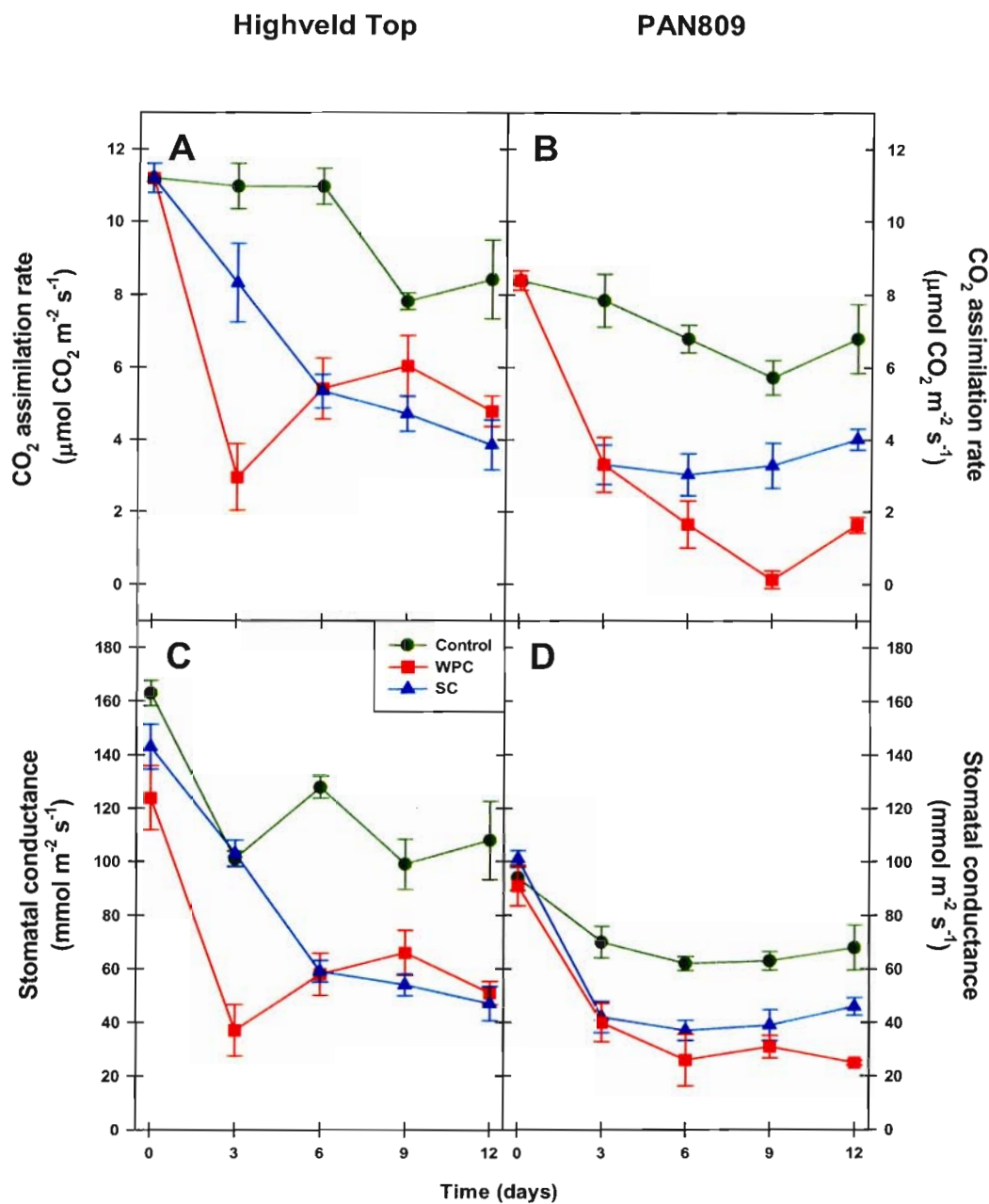


Figure 5.2 Effects of dark chilling on CO₂ assimilation rate and stomatal conductance in Highveld Top (A, C) and PAN809 (B, D) over a period of 12 consecutive nights of treatment, measured at 1200 μmol m⁻² s⁻¹ and ambient CO₂. Each data point (Control ●, WPC-treatment ■ and SC-treatment ▲) represents the mean of four replicates ± SE.

The relationship between A and G_s as a whole revealed a similar response in the two genotypes towards dark chilling over the period of twelve nights, with a decrease in A being accompanied by a decrease in G_s (Fig. 5.3A & B), but without consistent differentiation of response between WPC and SC treated plants. For example, the more severe inhibition of A in WPC treated plants of PAN809 were not consistently associated with significantly lower G_s values when compared to SC treated plants. The relationship between A and intercellular CO_2 concentration (C_i) revealed a noteworthy shift in WPC treated plants of PAN809 where the decrease in A was accompanied by a large increase in C_i compared to control values (Fig. 5.3D). A similar shift in C_i was not observed in Highveld Top (Fig. 5.3C).

5.2.3 Dark chilling inhibited CO_2 -saturated rates of photosynthesis (J_{max}) in a genotype and treatment dependent fashion

The $A:C_i$ response curves for PAN809 revealed pronounced effects of dark chilling on CO_2 -saturated rates of photosynthesis (J_{max}) at high C_i values (Fig. 5.1D, F). After three nights of chilling, J_{max} was inhibited by 46% ($P < 0.01$) in both the WPC and SC treated plants of PAN809 (Fig. 5.4B), correlating with the similarity in treatment responses observed for the other gas exchange parameters at this particular time point (Fig. 5.2). Upon continuation of chilling treatment, a clear treatment difference in the inhibition of J_{max} developed in PAN809. In the WPC treatment the inhibition of J_{max} increased to 73% ($P < 0.01$) after six nights of dark chilling and was maintained at this low level for the remainder of the treatment period. In SC plants of PAN809 the inhibition of J_{max} was less, being only 41%, 33% and 21% after six, nine and twelve nights of dark chilling, respectively. In the case of Highveld Top, no significant ($P > 0.05$) change in J_{max} as a result of dark chilling could be measured in either WPC or SC treated plants (Fig. 5.4A). Because J_{max} is regarded as an indicator of ribulose-1,5-bisphosphate (RuBP) regeneration capacity by the Calvin cycle, the effects of the chilling treatments on RuBP content were determined. In WPC treated plants of PAN809, RuBP content was reduced by between 48% and 53% ($P < 0.01$) on a leaf area basis after nine and twelve nights of chilling compared to the control. In the SC treated plants, RuBP content did not differ significantly from the control (Fig. 5.4D). In Highveld Top, RuBP

content was not reduced by either of the chilling stress treatments (Fig 5.4C), which is consistent with the absence of any effects of these treatments on $J_{\max}$ (Fig. 5.4A).

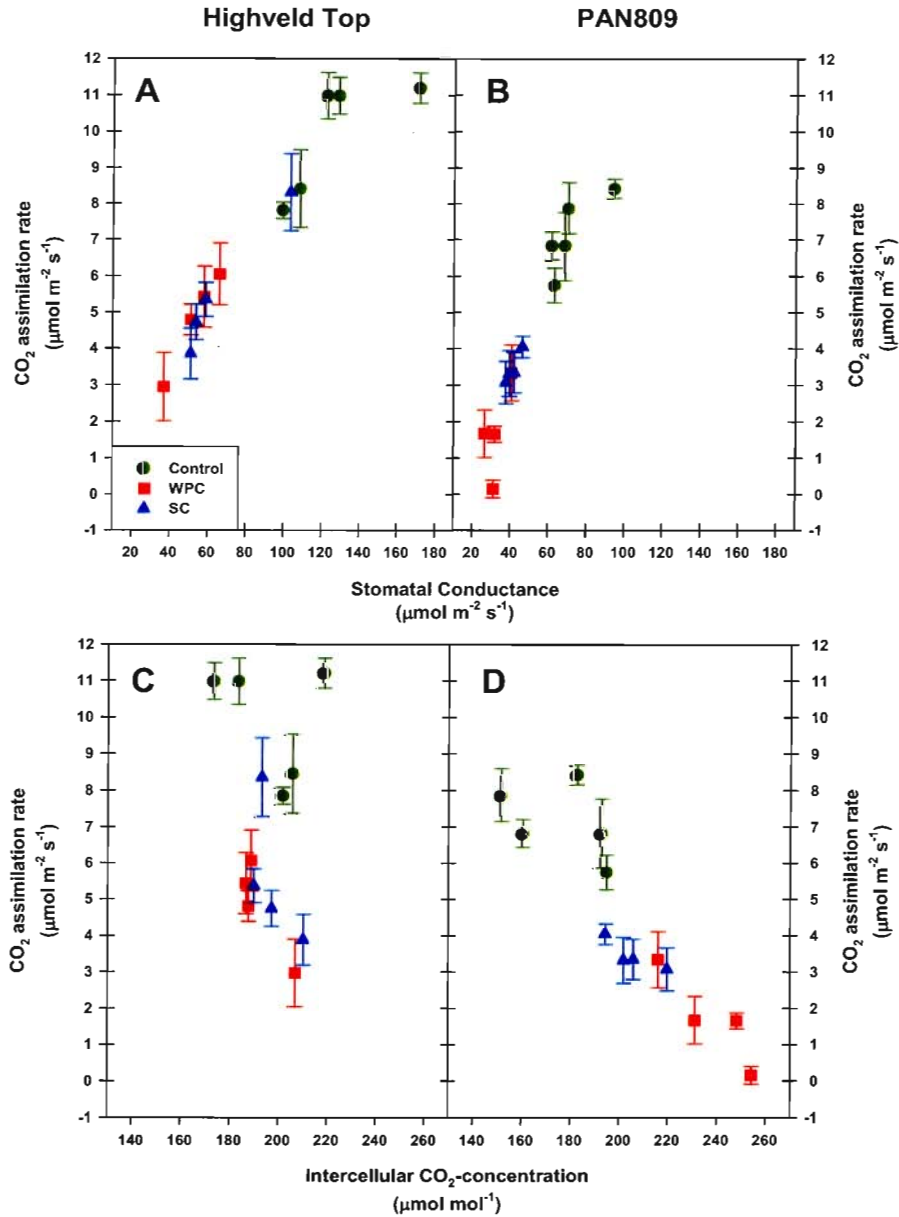


Figure 5.3 The effects of dark chilling on the relationship between CO₂ assimilation rate and stomatal conductance or intercellular CO₂ in Highveld Top (A, C) and PAN809 (B, D) over a period of 12 consecutive nights of treatment, measured at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ and ambient CO₂. Each data point (Control ●, WPC-treatment ■ and SC-treatment ▲) represents the mean of four replicates $\pm$ SE.

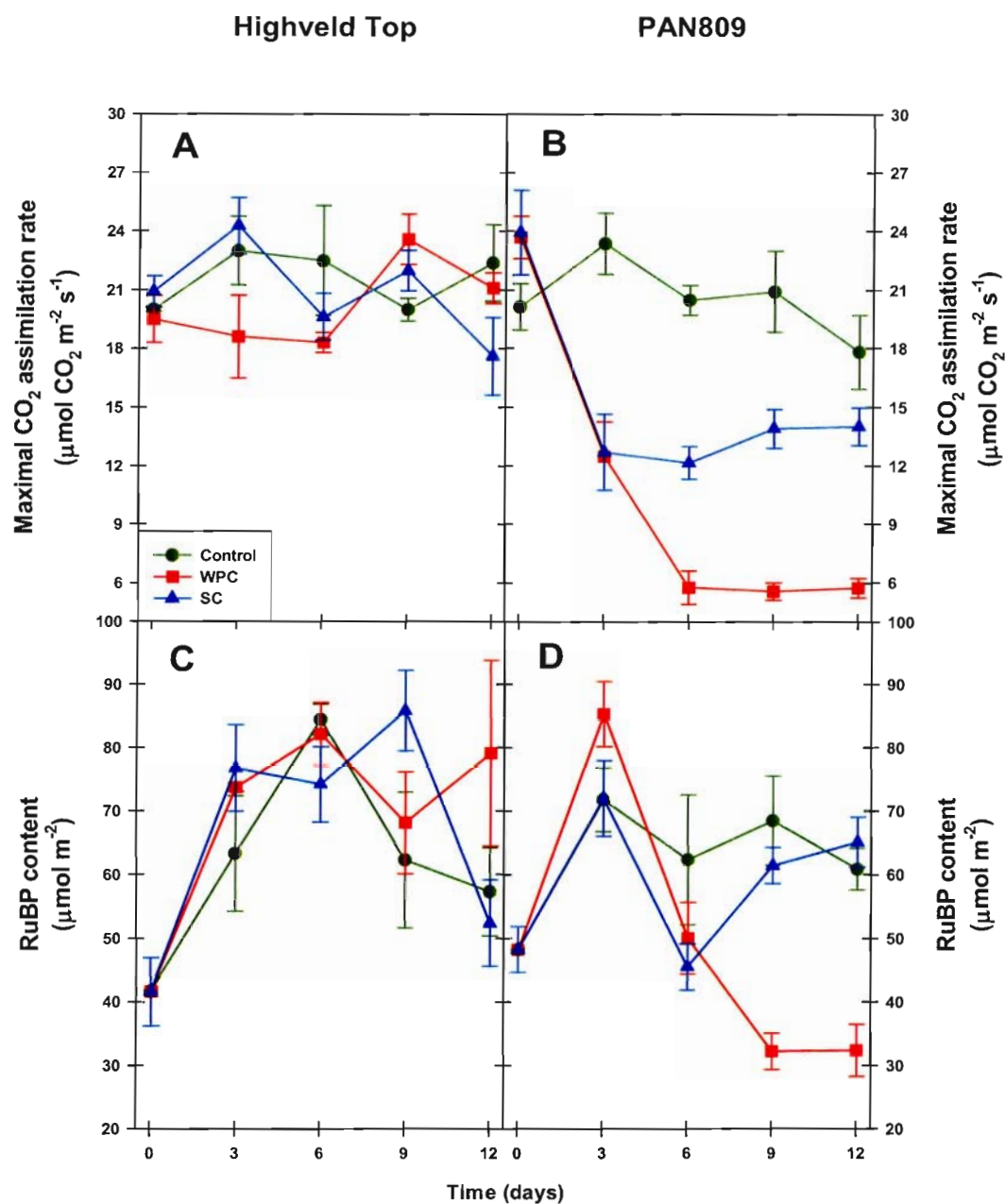


Figure 5.4 Effects of dark chilling on the CO₂ saturated rate of photosynthesis ($J_{\max}$), measured at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, as well as RuBP content of leaves in Highveld Top (A, C) and PAN809 (B, D) over a period of 12 consecutive nights of treatment. Each data point (Control ●, WPC-treatment ■ and SC-treatment ▲) represents the mean of four replicates $\pm$ SE.

5.2.4 Chloroplast fructose-1,6-bisphosphatase (cFBPase), but not sedoheptulose-1,7-bisphosphatase (SBPase) or ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), was selectively targeted by dark chilling in a treatment dependent fashion

Initial and total cFBPase activity of Highveld Top remained unchanged by dark chilling in both WPC and SC treated plants until the twelfth night of treatment, where significant ($P < 0.01$) inhibition of 34% and 29% occurred in initial cFBPase activity and 23% and 31% in total cFBPase activity of WPC and SC treated plants, respectively (Fig. 5.5A & C). PAN809, on the other hand, was more sensitive and experienced earlier and larger inhibition of both initial and total cFBPase activity (Fig. 5.5B & D). After six nights of chilling, a significant inhibition of initial and total cFBPase activity was already observed in both the WPC and SC treated plants. Upon further chilling, a clear treatment effect developed with both initial and total cFBPase activity being inhibited more in the WPC treated plants. It is noteworthy that the inhibition in cFBPase activity of between 48% - 55% during this part of the treatment period in WPC treated plants of PAN809 corresponded closely with the reduction in RuBP content of between 48% and 53% in the same leaves (Fig. 5.4D, nights nine and twelve). cFBPase activity was also expressed on a leaf area basis (results not shown) and showed the same genotype and treatment differences as when expressed on a soluble protein basis (Fig. 5.5).

The decrease in total cFBPase activity in response to chilling stress pointed towards a possible decrease in the steady state content of this enzyme. This was investigated further through immunoblotting, which revealed a clear decrease in cFBPase protein abundance, but only in the WPC treated plants of PAN809. This effect became visible after 9 nights of chilling and was also evident after twelve nights (Fig. 5.5E). The largest effect on both initial and total cFBPase activity in WPC plants of PAN809 developed beyond 6 nights of chilling (Fig. 5.5B & D), suggesting strongly that the intensification of inhibition was linked to a chilling-induced decrease in cFBPase protein content. Even though moderate inhibition of total cFBPase activity was also observed in Highveld Top after twelve nights of chilling, no effect on cFBPase protein content was detected.

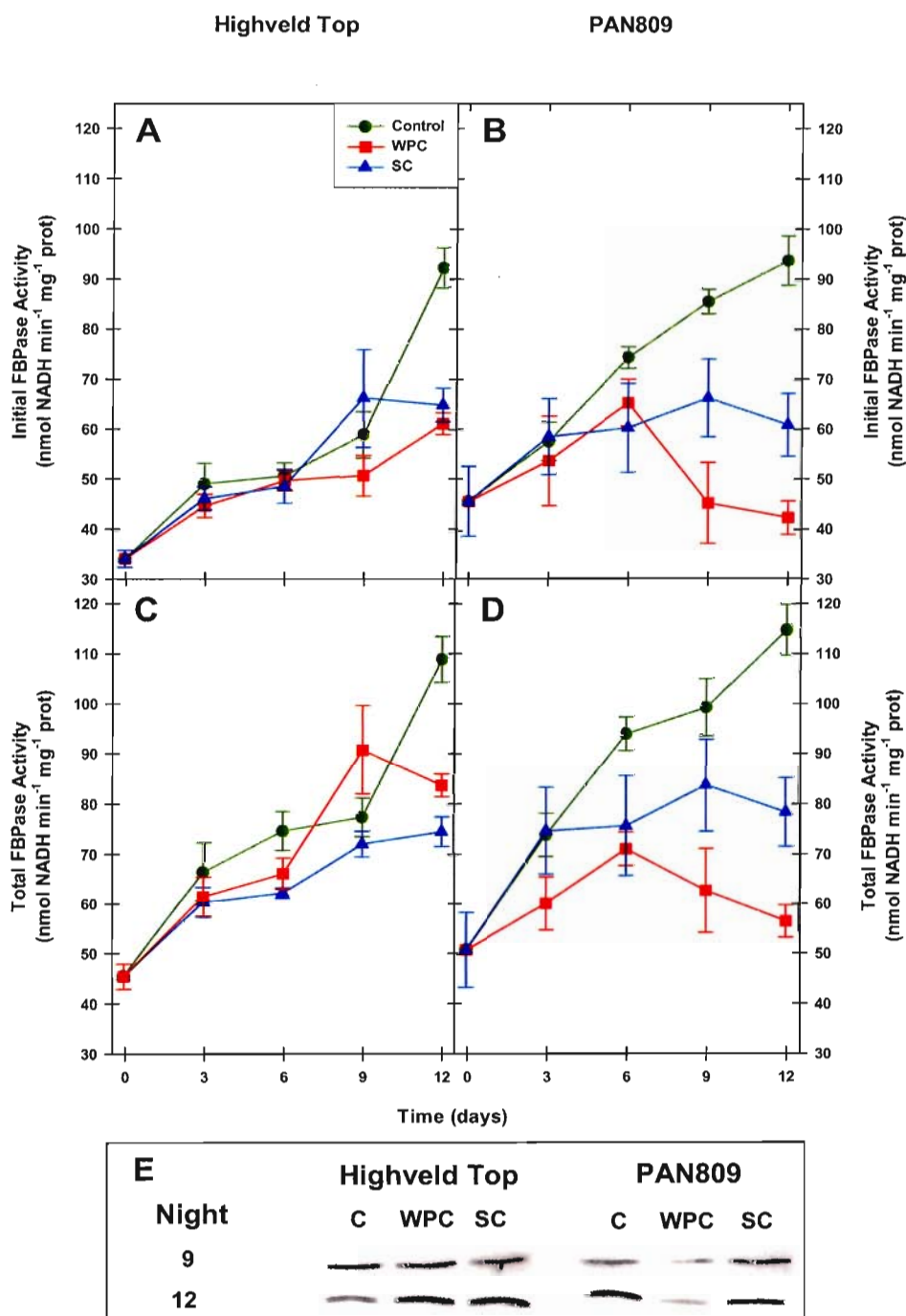


Figure 5.5 Effects of dark chilling on initial and total FBPase activity in leaves of Highveld Top (A, C) and PAN809 (B, D) as well as on FBPase protein content (E), sampled at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ and ambient CO_2 . Each data point (Control ●, WPC-treatment ■ and SC-treatment ▲) represents the mean of five replicates $\pm$ SE.

Since the two chloroplastic bisphosphatases, cFBPase and SBPase, operate in tandem in the Calvin cycle and share similar regulatory properties, the effects on SBPase activity was also assessed. Surprisingly, it was found that the effects of chilling on SBPase activity were much less clear than for cFBPase. For example, whereas cFBPase activity was inhibited in Highveld Top after 12 nights of chilling, SBPase activity was not inhibited over the entire period of chilling treatment (Fig. 5.6A). Although there was a tendency of lower SBPase activity in WPC and SC treated plants of PAN809 (Fig. 5.6B), these differences were mostly not significant ($P > 0.05$). In addition, contrary to cFBPase activity, no differentiation in response between WPC and SC treated plants could be discerned. Confirming this lack in response further, no decline in the steady state protein content of SBPase was detected either (Fig. 5.6C).

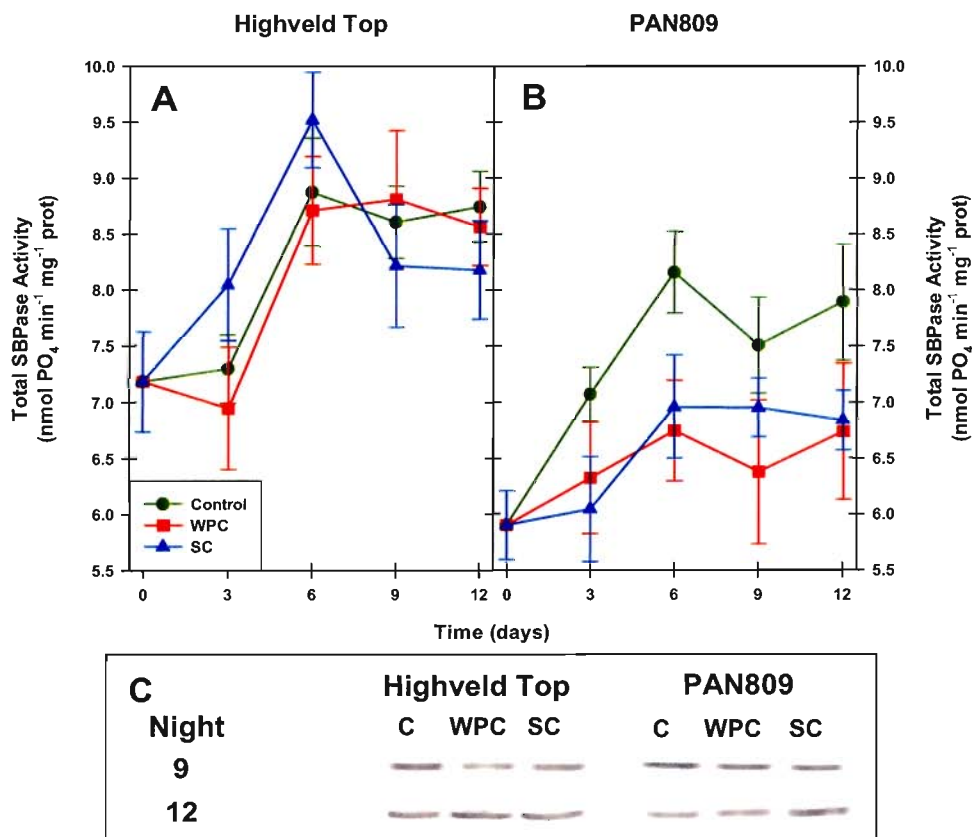


Figure 5.6 Effects of dark chilling on total SBPase activity in leaves of Highveld Top (A) and PAN809 (B) as well as on SBPase protein content (C), sampled at 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and ambient CO_2 . Each data point (Control ●, WPC-treatment ■ and SC-treatment ▲) represents the mean of five replicates $\pm$ SE.

Neither initial nor total Rubisco activity was inhibited by WPC and SC treatment in the case of Highveld Top (Fig. 5.7A & C). Dark chilling also had no negative effect on initial Rubisco activity in PAN809 (Fig. 5.7B). In PAN809, the only significant change in total Rubisco activity was evident after twelve nights of treatment with inhibitions of 43% ($p < 0.01$) and 37% ($p < 0.01$) in WPC and SC treated plants, respectively (Fig. 5.7D). Besides the fact that no clear distinction in response between WPC and SC treated plants could be made, the difference in activity between these treatments and the control was not really because of a chilling-induced decrease in Rubisco activity in chilled plants *per se*, but rather because of a large increase in activity in control plants between nine and twelve nights. This contrasts with cFBPase where actual decreases in specific activity were observed in WPC treated plants (Fig. 5.5B & D).

5.2.5 Dark chilling inhibited sucrose-phosphate-synthase (SPS) activity but without clear correlation with treatment-dependent effects on $J_{\max}$

Published results indicate that SPS activity is closely related to RuBP regeneration capacity ($J_{\max}$) (Galtier et al., 1995; Strand et al., 2000). Because chilling stress inhibited $J_{\max}$ in PAN809, the effects on $V_{\lim}$ and $V_{\max}$ SPS activity were established. In contrast to the absence of inhibition of $J_{\max}$, and the general insensitivity or delayed inhibition of the other measured enzymes in Highveld Top, $V_{\lim}$ SPS activity was inhibited ($P < 0.01$) by 36% and 30% after nine and twelve nights of WPC treatment, respectively (Fig. 5.8A). PAN809 was more severely affected with significant reductions visible after six nights of dark chilling in both $V_{\lim}$ and $V_{\max}$ SPS activity of WPC and SC treated plants (Fig. 5.8B & D). Although $V_{\lim}$ SPS activity of SC treated plants of PAN809 was inhibited by 33% ($P < 0.05$) after six nights of chilling, this inhibition was not statistically maintained for the remainder of the experimental period, in contrast to the sustained inhibition of $J_{\max}$ (Fig. 5.4B). After nine and twelve nights of chilling the $V_{\lim}$ SPS activity of WPC treated plants of PAN809 was inhibited to a larger extent than in SC treated plants. However, this treatment difference occurred later than for $J_{\max}$, where the difference between WPC and SC treated plants was already fully established after six nights of chilling (Fig. 5.4B).

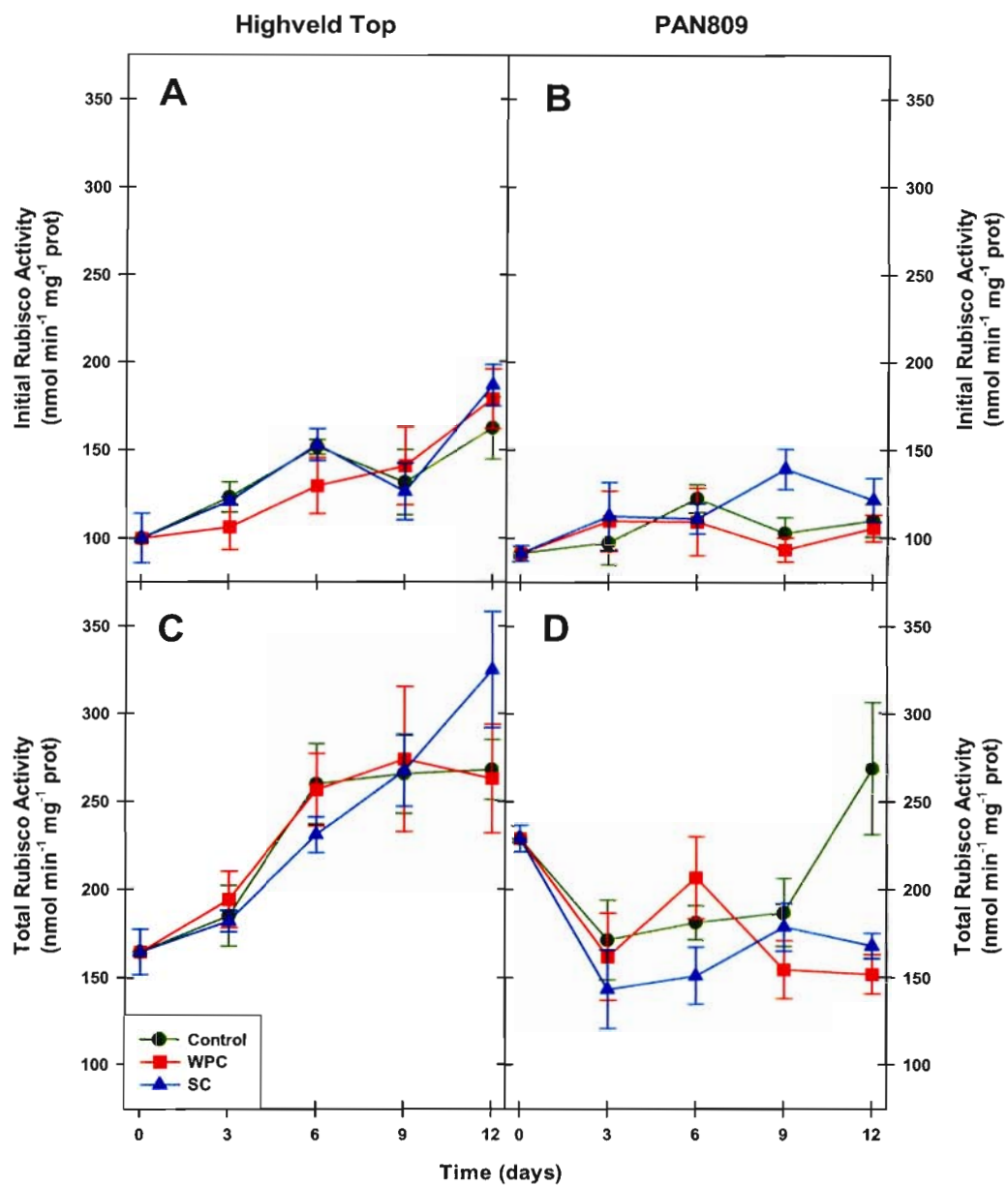


Figure 5.7 Effects of dark chilling on initial and total Rubisco activity in leaves of Highveld Top (A, C) and PAN809 (B, D) over a period of 12 consecutive nights of treatment, sampled at 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and ambient CO_2 . Each data point (Control ●, WPC-treatment ■ and SC-treatment ▲) represents the mean of five replicates $\pm$ SE.

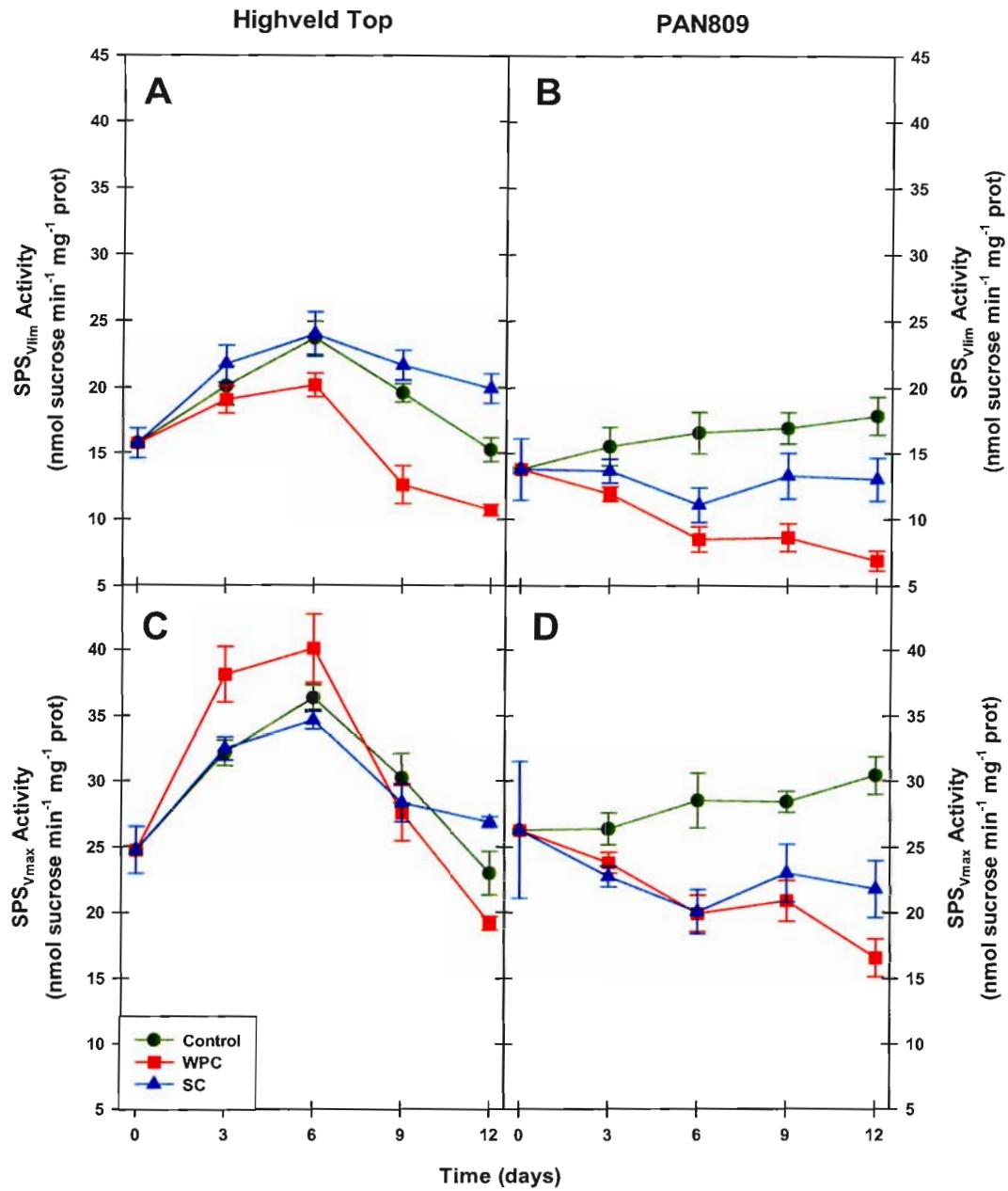


Figure 5.8 Effects of dark chilling on V_{lim} and V_{max} SPS activity in leaves of Highveld Top (A, C) and PAN809 (B, D) over a period of 12 consecutive nights of treatment, sampled at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ and ambient CO_2 . Each data point (Control ●, WPC-treatment ■ and SC-treatment ▲) represents the mean of five replicates $\pm$ SE.

Although $V_{\max}$ SPS activity of WPC and SC treated plants of PAN809 was clearly lower than the control, a clear treatment difference developed very slowly and was only significant after twelve nights of chilling (Fig. 5.8D). This corresponded poorly with the large difference in $J_{\max}$ between these two groups of plants that was already fully established after the sixth night of chilling. In Highveld Top no significant inhibition in $V_{\max}$ SPS activity occurred in WPC or SC treated plants (Fig. 5.8C). Taken together, the above results showed that dark chilling inhibited $V_{\lim}$ SPS activity in both genotypes and $V_{\max}$ SPS activity in PAN809 only, but that these effects were either inconsistent or delayed relative to the clear treatment and genotype differences in the response of $J_{\max}$ to dark chilling.

5.3 Discussion

This part of the investigation was aimed at gaining a better insight into the mechanistic basis underpinning the large differences in the inhibition of photosynthesis in two soybean genotypes of contrasting chilling tolerance that was reported in Chapter 4. Because chilling of entire (roots and shoots) N_2 -fixing soybean plants makes it difficult to dissect the relative contribution of low soil and air temperatures towards the inhibition of photosynthesis, experiments were designed so that chilling responses in WPC and SC treated plants could be compared. The discussion below focuses on novel results obtained in these experiments showing that low soil temperatures aggravate the inhibition of photosynthesis in the chilling sensitive soybean genotype PAN809 through effects on specific target enzymes.

In most cases throughout the experimental period, the inhibition of CO_2 assimilation rate (A) induced by dark chilling was accompanied by a large decrease in stomatal conductance (G_s), with this decrease being very similar in both chilling treatments and genotypes. Increased stomatal limitation of photosynthesis would therefore be a good candidate to explain at least in part the inhibition of A during dark chilling, especially since it has been shown to be involved in the inhibition of photosynthesis in other species following chilling (Allen et al., 2000). However, the

effects of dark chilling on G_s cannot satisfactorily explain the differences in chilling-sensitivity of photosynthesis between the two genotypes or treatments.

A contrasting picture emerged when the relationship between A and C_i was determined. In Highveld Top, C_i remained unchanged during exposure to both SC and WPC treatments, while a notable increase in C_i occurred in PAN809, especially in WPC-treated plants. An increase in C_i during chilling stress is usually regarded as indirect evidence of mesophyll limitation of photosynthesis, where the normal function of the chloroplast is impaired (Markhart, 1986; Kubien et al., 2003). Under these conditions the inhibition of photosynthesis should not be easily reversible during exposure to high CO_2 concentrations, in contrast to the situation when stomatal limitation dominates. This was confirmed through measurement of J_{max} , which was reduced to a large extent in chilled plants of PAN809 but not in Highveld Top. These measurements, coupled to determination of RuBP content, provided further support in favour of mesophyll constraints being the main factor involved in the dark chilling-induced inhibition of photosynthesis in PAN809, especially in the WPC treatment.

Two major role-players that are involved in RuBP regeneration are the chloroplastic bisphosphatases cFBPase and SBPase (Kossmann et al., 1994; Ashton, 1998; Raines, 2006). The regulatory role of both these enzymes in photosynthesis has been studied extensively in the past (e.g. Kossmann et al., 1994; Lefebvre et al., 2005). Moreover, these chloroplastic bisphosphatases appear to be sensitive to chilling in the light (Sassenrath et al., 1990). In contrast to chilling in the light, very little information exists on the effects of dark chilling on these enzymes, in particular SBPase. Previous studies (Van Heerden et al., 2003a; 2004b) have shown that cFBPase activity in WPC-treated soybean plants is decreased by dark chilling involving short-term treatments of between 1 – 3 nights, but these studies did not compare root versus shoot chilling responses and also did not consider effects on SBPase. Until now, similar effects of chilling on these two chloroplastic bisphosphatases have been assumed. The current investigation provides new insights into the effects of dark chilling on these two enzymes. Firstly, in the chilling tolerant genotype Highveld Top, cFBPase activity was

only moderately inhibited after extended (12 nights) exposure to dark chilling and without any effects on $J_{\max}$ or RuBP content, while in PAN809 the decrease in activity occurred already at a much earlier stage. Secondly, in Highveld Top, both the WPC and SC treatments elicited a similar response, while in PAN809, a clear treatment effect was seen with activities reduced severely in WPC-treated plants. Thirdly, SBPase did not share the same degree of chilling sensitivity as cFBPase and no clear treatment effect could be discerned.

Since cFBPase and SBPase operate in tandem in the Calvin cycle and share similar regulatory properties (Nishizawa and Buchanan, 1981; Kelly, 2001), the finding that they responded differently to dark chilling is very intriguing. The effects of dark chilling on photosynthetic electron transport in Highveld Top and PAN809 have been characterised in Chapter 4, and based on those findings, it is possible that disruption of light activation of certain Calvin-cycle enzymes could have occurred during the light periods following dark chilling. Since both cFBPase and SBPase are activated through the Fd/thioredoxin system, decreased electron transport could have resulted in lower chloroplastic bisphosphatase activity. Since RuBP regeneration is also dependent on the supply of reducing equivalents by electron transport this could have contributed further towards this inhibition. However, if reduced light activation and reductant supply were the main causes for the large inhibition of cFBPase activity, it is reasonable to assume that it would have affected SBPase activity similarly. The current investigation provides strong evidence that dark chilling specifically targets cFBPase and that the degree of inhibition, which depends on the presence or absence of low root temperatures, influences the magnitude of reduction in $J_{\max}$ and RuBP regeneration capacity. This is supported further by the western blot analysis, which showed that the content of cFBPase, but not SBPase, was reduced in PAN809, and only in WPC-treated plants.

Aggravation of chilling effects on cFBPase in the WPC treatment suggests that this is not caused by chilling effects on the shoots *per se*, but rather that chilling of the roots must be somehow involved. Although chilling of entire soybean plants does result in mild chilling-induced perturbations in leaf water status (Van Heerden et al., 2003a),

these effects are transient with any signs of leaf wilting disappearing soon (within 2 h) after return to normal daytime temperatures. It is unlikely that such mild and transient effects on leaf water status would have been involved, especially since the biochemical reactions of photosynthesis are known to be rather tolerant to decreases in leaf water status (Medrano et al., 2002). After 6 nights of chilling, no changes in cFBPase protein content were visible on western blots (results not shown). The differential effects of the WPC and SC treatments on total cFBPase activity started only after 6 nights of chilling (Fig. 5.5D) and were associated with lower cFBPase protein contents (Fig. 5.5E) that developed somewhere between 6 and 9 nights of chilling. Consideration of the onset of these responses is important, because it was shown that leaf ureide content severely decreased in WPC-treated plants of PAN809 between 6 and 9 nights of chilling, predisposing these plants to N-limitation, which ultimately caused the characteristic chlorotic shoot phenotype (Chapter 4 Section 4.2.4). Atkins et al. (1988) demonstrated the role of low ureide content in the progressive development of chlorosis in another ureide-exporting legume, cowpea. The large decrease in leaf ureide content was absent from SC-treated plants, suggesting that direct chilling effects on root nodules and inhibition of symbiotic nitrogen fixation (SNF) was causing the response in WPC-treated plants. Indeed, very recent evidence has shown that SNF is highly sensitive to dark chilling in PAN809, mainly because of the inability of nodule respiration to acclimate to chilling and the lack of a counter-response against elevated internal O₂ concentrations by the variable oxygen diffusion barrier within the nodules (Van Heerden et al., 2008). In Highveld Top, nodule respiration undergoes acclimation to dark chilling and, most importantly, electron dense occlusions form within the intercellular spaces in the nodule cortex and infected zone, restricting flux of O₂ through the nodule, thereby protecting nitrogenase from O₂-induced deactivation (Van Heerden et al., 2008).

The decrease in leaf ureide content therefore appears to trigger the onset of leaf senescence in WPC-treated plants of PAN809 (Chapter 4 Section 4.2.4) and the results presented here strongly suggest that cFBPase is an important target during this process, even before the chlorotic phenotype becomes visible (between 9 and 12 nights of chilling, Chapter 4 Section 4.2.4). Crafts-Brandner et al. (1996) found that in bean, limited

availability of N results in premature senescence of especially the older leaves. Evidence for the specific effects that lower cFBPase activity exerted on leaf carbohydrate metabolism was seen in the ratio of soluble sugars to starch. In transgenic potato plants with reduced cFBPase levels, Kossman et al. (1994) found that the activity and content of none of the other key photosynthetic enzymes were affected by plant transformation and that reduced cFBPase activity was responsible for the observed increase in the ratio of soluble sugars to starch. In WPC-treated plants of PAN809, a 12% increase in the ratio of soluble sugars to starch was observed after 12 nights of dark chilling (results not shown), highlighting the significance of the selective inhibition of cFBPase activity on carbohydrate metabolism.

The question remains why cFBPase, but not SBPase, is selectively targeted in WPC-treated plants? Since the reduction in cFBPase protein content seems to be related to the inhibition of SNF, rather than to low temperature effects on the leaves *per se*, this is an important question because any environmental factor that leads to lower leaf ureide contents could perhaps trigger similar effects. This would certainly be an interesting hypothesis to test, since recent evidence suggest that ureides cannot only be seen as a transportable N-metabolite, but that it also functions as a regulator of SNF (Ladrera et al., 2007) and as a cellular scavenger of reactive oxygen species (Brychkova et al., 2008). Exogenous application of ureides to senescing *Arabidopsis* leaf discs led to diminished H₂O₂ accumulation and delayed progression of chlorophyll degradation (Brychkova et al., 2008). Consistent with this is the observation that severe chlorosis in WPC-treated plants of PAN809 only developed after leaf ureide content dropped to 60% of that of control plants (Chapter 4 Section 4.2.4). Based on the results presented here it is hypothesised that the reduction in leaf ureide levels in WPC-treated plants of PAN809 below a certain threshold predisposes the leaf to senescence and protein degradation. Kingston-Smith et al. (2005) have shown that foliar nitrogen limitation in white clover does indeed alter protease isoform composition in the leaves. Protein degradation in chloroplasts during senescence is carried out by, among others, Clp and FtsH proteases (Lindahl et al., 1996; Adam et al., 2006; Adam, 2007) and is closely coupled with adaptation to changing environmental conditions (Sakamoto, 2006). Although these and

some other chloroplast proteases have been characterised to some extent, information on their physiological functions, specific substrates and substrate recognition are still very poorly understood (Adam, 2007). Although much remains to be resolved, the important role of a proteinase inhibitor in enhancing dark chilling tolerance in tobacco was recently demonstrated (Prins et al., 2008). Constitutive expression of the cysteine proteinase inhibitor, oryzacystatin I in transformed tobacco (Van der Vyver et al., 2003) results in enhanced chilling tolerance because it protects Rubisco from dark chilling-induced degradation by cysteine proteinases in transformed lines (Prins et al., 2008). These effects appeared to have been highly specific to Rubisco, suggesting that different chloroplast proteins might have specific proteases involved in their degradation. Perhaps, in soybean, cFBPase and SBPase are degraded by different proteases, which show different activation responses towards decreased ureide levels.

Decreased CO₂ assimilation rates in rice as a result of chilling temperatures are more closely related to reduced RuBP regeneration capacity than changes in activity of Rubisco (Hirotsu et al., 2005). The results reported here support this also for soybean because inhibition of Rubisco activity in PAN809 occurred much later than for cFBPase, and in addition no distinction between WPC and SC treatments could be made. This suggests that Rubisco is unlikely to be a key aspect in the chain of events occurring in dark chilled plants of PAN809, and more specifically where low soil temperatures are involved.

Published results indicate that SPS activity is closely related to RuBP regeneration capacity (Galtier et al., 1995; Strand et al., 2000) and that it is also sensitive to dark chilling (Jones et al., 1998). Dark chilling appears to interfere with the circadian regulation of SPS activity, at least in the case of tomato (Jones et al., 1998). However, although the results obtained in the current investigation showed that SPS activity appears to be very sensitive to dark chilling in the soybean genotypes chosen for this study, its relationship with RuBP regeneration capacity is less clear. Although SPS V_{lim} activity in WPC-treated plants of both Highveld Top and PAN809 was inhibited, a reduction in RuBP content and J_{max} only occurred in the latter genotype. In contrast to

SPS V_{lim} activity, which was inhibited in both genotypes, only PAN809 experienced an inhibition in SPS V_{max} activity. Important, however, is that a clear treatment difference between WPC and SC-treated plants was very slow to develop (only after 12 nights of chilling), in contrast to cFBPase activity (already between 6 and 9 nights of chilling). Therefore, although decreased SPS activity could have certainly contributed towards the more severe inhibition of photosynthesis in PAN809, the enzyme that reflected the differential treatment effects of dark chilling on J_{max} and RuBP regeneration capacity in the current investigation the best was cFBPase.

Based on the results presented here, it could be assumed that the response of photosynthesis to dark chilling in chilling sensitive soybean genotypes like PAN809 is determined by both shoot and root-derived factors. Upon exposure of only the shoots to low air temperatures (SC treatment), reduced PSII function (Chapters 3 and 4), stomatal conductance, cFBPase and SPS activity all contributed towards the moderate inhibition of photosynthesis, but without reducing RuBP content or inducing a chlorotic shoot phenotype. However, under conditions where root nodules are also exposed to low temperatures (WPC treatment), SNF is rapidly inhibited in PAN809 (Van Heerden et al., 2008), which gradually leads to reduced leaf ureide content and the development of a characteristic chlorotic shoot phenotype. When leaf ureide content drops below a certain threshold, additional constraints on photosynthesis, over and above those occurring in the SC treatment, are imposed. Under these N-limiting conditions, cFBPase is specifically targeted leading to reduced cFBPase protein content. This lowers cFBPase activity below the level required to sustain RuBP content with severe consequences for photosynthesis. In Highveld Top, SNF is much more tolerant against chilling (Van Heerden et al., 2008) preventing the chlorotic shoot phenotype and the additional constraints on photosynthesis. It is concluded that the sensitivity of SNF towards dark chilling plays a major role in the inhibition of photosynthesis under conditions where low soil temperatures are experienced in combination with low air temperatures.

Chapter 6

General Discussion and Recommendations

The main aims of this study were, firstly, to evaluate a large number of South African soybean genotypes for chilling tolerance in order to determine which genotypes showed contrasting responses towards dark chilling; secondly, to benchmark the chilling tolerance of the South African soybean genotypes against those of two foreign soybean genotypes, Maple Arrow and Java 29, of well-known chilling tolerance; and thirdly, to conduct a detailed physiological and biochemical characterisation of the chilling response of two of these local genotypes of contrasting chilling tolerance with emphasis on the contributing role of low soil temperatures towards the inhibition of photosynthesis.

It was hypothesised that, within the South African soybean gene pool, a sufficient degree of variation in dark chilling tolerance would exist, enabling selection of genotypes which could subsequently be used for unravelling the mechanism of inhibition of photosynthesis by dark chilling. It was further hypothesised that, through the inhibition of symbiotic nitrogen fixation by low soil temperatures, the negative effects of dark chilling on photosynthetic metabolism would be aggravated and that the selected genotypes would respond differently depending on their chilling tolerance, thereby further facilitating identification of key metabolic sites of inhibition.

By employing the JIP-test, the existence of large genotypic variation in the dark chilling tolerance of thirty South African soybean genotypes could convincingly be demonstrated. Previous studies have shown that the performance index (PI_{ABS}), a multi-parametric expression derived from the JIP-test, is a highly sensitive indicator of dark chilling stress in soybean (Van Heerden et al., 2004a; 2004b), and the analysis of measured O-J-I-P fluorescence transients in this study confirmed this. Based on the effects exerted on the PI_{ABS} , a large degree of variation in response to dark chilling was demonstrated between the thirty soybean genotypes investigated in this study. The effects of dark chilling on the PI_{ABS} during the various phases of the treatment period were used to formulate a novel parameter, the chill factor index (CFI), which made it possible to

rank the genotypes according to chilling tolerance. The CFI of the two foreign soybean genotypes, Maple Arrow and Java 29, which served as reference genotypes, correlated with their known difference in chilling tolerance. Five of the local genotypes, including Highveld Top, exhibited CFI values comparable to that of the chilling tolerant reference genotype, Maple Arrow. It was established that these genotypes have previously proven to be very popular in agricultural practices on the South African Highveld where the growth season is characterised by low night temperatures (personal communication, Mr A de Lange, ARC, Potchefstroom). On the other hand, the genotype PAN809 obtained the lowest ranking of all the genotypes evaluated and exhibited even poorer dark chilling tolerance than Java 29, the chilling sensitive reference genotype.

Based on the successful ranking of the thirty soybean genotypes, two genotypes were selected for further detailed studies, namely Highveld Top, representing a chilling tolerant genotype, and PAN809, representing a highly chilling sensitive genotype. Furthermore, the effect of low soil temperatures, in combination with low air temperatures (whole plant chilling, WPC) were compared with the situation where only low air temperatures, but normal soil temperatures, were experienced (shoot chilling, SC).

Although a very clear distinction could be made between the overall effects induced by the WPC and SC treatments over the treatment period of twelve nights, there were also similarities, especially regarding initial effects experienced during the first three nights of dark chilling. Examples of this include the reduction of G_s , inhibition of PSII function (as indicated by PI_{ABS}) and the inhibition of J_{max} which were similar in both WPC and SC treatments of PAN809 after three nights of chilling. These similarities could therefore be ascribed more exclusively to chilling stress experience by shoots and leaves. However, it was evident that reduced soil temperatures aggravated the effects of dark chilling, especially in PAN809. When the metabolism occurring above and below-ground was taken into consideration, it was clear that a sequence of events was set into motion by dark chilling in PAN809. This study provided novel evidence showing that the response of photosynthesis to dark chilling in chilling sensitive soybean genotypes like

PAN809 was determined by both shoot and root-derived factors, and that the changes which occurred in carbon and nitrogen metabolism during chilling stress were ultimately manifested in the form of symptoms (e.g. reduced growth rate and chlorosis) on the whole-plant level.

The proposed sequence of events causing these severe symptoms in PAN809 is depicted in Fig. 6.1. In both SC and WPC-treated plants, photosynthesis in leaves was initially impaired in a similar way, with the rate of CO₂ assimilation (A) being inhibited due to reduced stomatal conductance (G_s) (step 1 in Fig. 6.1). At this early stage of dark chilling, impairment of PSII function also occurred in both SC and WPC-treated plants (step 2 in Fig. 6.1), being characterised mainly by an accumulation of the Q_A⁻-pool because of decreased electron transport downstream of Q_A⁻ (Haldimann and Strasser 1999). The main site of PSII inhibition at this stage was revealed by the characteristic appearance of a J-peak in O-J-I-P transient, which was visible in plants of both the WPC and SC treatments and in both genotypes (Fig. 4.4). However, in response to further dark chilling exposure, a progressive deterioration of PSII function (appearance of K-peak in O-J-I-P transient and more pronounced reduction in PI_{ABS}) could be seen in WPC-treated plants of PAN809 (intensification of effects indicated by the thicker white arrow in Fig 6.1 at step 2), with uncoupling of the oxygen-evolving complex (OEC) taking place. Although it is known that the donor side of PSII (i.e. the OEC) is sensitive to changes in temperature (Terashima et al. 1989, Shen et al. 1990, Enami et al. 1994), the results reported in this thesis provided novel evidence that in WPC-treated plants of PAN809, this disruption was not caused by chilling of leaves *per se*, but rather indirectly through chilling effects on the roots/root nodules.

The WPC treatment caused the progressive development of N-limitation (low ureide content) in leaves of PAN809 plants because of disruption of bacterial nitrogenase activity (step 3 in Fig. 6.1). Recent findings have shown that nitrogenase activity in PAN809 is extremely sensitive to WPC-treatment due to the inability of nodule respiration to acclimate to low temperature and the inability of the variable diffusion barrier to respond to elevated O₂ concentrations within the nodules (van Heerden et al.,

2008). The more effective operation of these mechanisms in Highveld Top protects nitrogenase from deactivation, and constitutes a main difference in the dark chilling response of these two genotypes. The O-J-I-P kinetics revealed this genotypic difference through the appearance of a characteristic K-peak, which only developed in the WPC-treated plants of PAN809. The development of the K-peak coincided with the decrease in leaf ureide content, which supports the use chlorophyll *a* fluorescence transients and analysis by the JIP-test as a non-intrusive diagnostic tool for detecting changes in below-ground metabolism of soybean in response to dark chilling. It was this gradual reduction in leaf ureide content (N-limitation) that led to the development of the chlorotic phenotype and more reduced growth rates in WPC-treated plants of PAN808.

When leaf ureide content dropped below a certain threshold level in the WPC-treated plants of PAN809, additional constraints on photosynthesis developed. As N-limiting conditions developed in these leaves, cFBPase was targeted specifically (step 4 in Fig. 6.1), leading to reduced cFBPase protein content and much larger reductions in its specific activity. As a result of this, RuBP regeneration capacity was severely compromised as indicated by the decrease in RuBP content that was observed exclusively in WPC-treated plants of PAN809 (Fig. 5.4D) and the much larger decrease in $J_{\max}$ (Fig. 5.4B). The reasons for the specific targeting of cFBPase remains elusive, but could possibly be ascribed to senescence-related effects. When leaf ureide content in WPC-treated plants of PAN809 reached a certain threshold minimum, it presumably predisposed these leaves to senescence-related protein degradation. The noteworthy observation that cFBPase, but not SBPase, was targeted might indicate that these two enzymes, which share very similar regulatory properties, are degraded by different proteolytic systems, which show different activation responses towards decreased ureide levels. This would be an interesting hypothesis to test through precise assessment of changes in the protease profiles in leaves differing in ureide content.

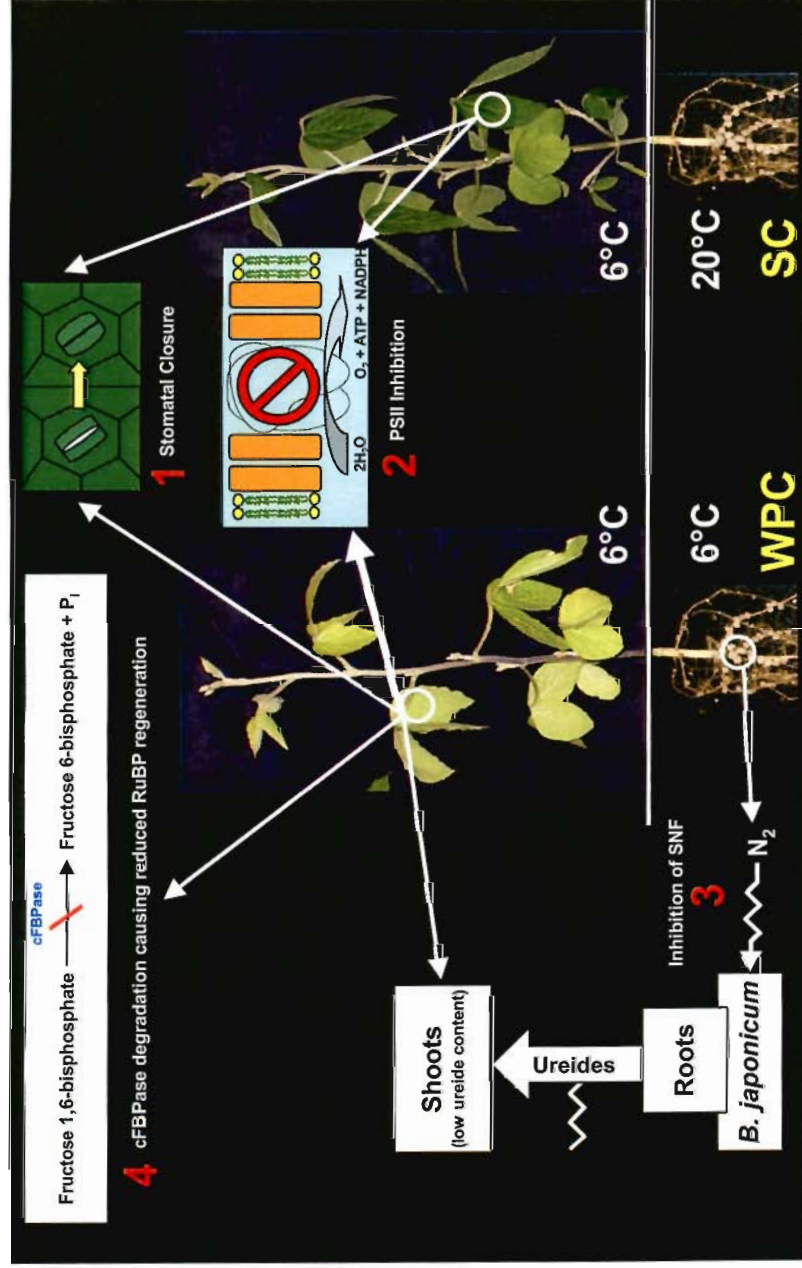


Figure 6.1 A schematic model illustrating the main target sites where whole plant chilling (WPC, left) and shoot chilling (SC, right) affects above and below-ground metabolism in the chilling sensitive soybean genotype PAN809. In both WPC and SC-treated plants, chilling stress effects on the leaves lead to stomatal closure (1) and inhibition of PSII function (2), initially causing similar inhibition of photosynthesis. However, with continued exposure to dark chilling, inhibition of PSII function in WPC-treated plants is further aggravated (indicated by thicker white arrow) when low soil temperatures inhibit symbiotic N-fixation (3), causing reduced ureide production and export from the nodules to the shoot, leading to a chlorotic phenotype. Inhibition of these two key processes is indicated by the resistance symbols. When leaf ureide content drops below a certain threshold, cFBPase is specifically targeted (4), considerably aggravating the inhibition of photosynthesis through reduced RuBP regeneration capacity. This model illustrates the interplay that exists between shoot and root-derived factors involved in the inhibition of photosynthesis under these conditions.

In conclusion, this study demonstrated that the genetic pool of South African soybean genotypes exhibits a large degree of variation in dark chilling tolerance. Screening of this genetic diversity was conducted successfully by rapid and non-intrusive chlorophyll *a* fluorescence measurements. The genotypes Highveld Top and PAN809 were identified as exhibiting a great contrast in chilling tolerance, and investigated further in detail, focusing on the effect of sub-optimal soil temperatures on above and below-ground metabolism. These treatments facilitated unravelling of the sequence of events leading to the development of a chlorotic shoot phenotype in PAN809 and additional constraints being imposed on photosynthesis, of which the selective targeting of cFBPase was a key factor. It was clearly demonstrated that in a chilling sensitive genotype such as PAN809, the response of photosynthesis to dark chilling depended on both shoot and root-derived factors. The large difference in the response of the two genotypes to the WPC-treatment made it possible to identify the key-sites of dark chilling inhibition.

Recommendations for possible practical application and future research, based on the results of this study, include the following:

- i. Since the thirty soybeans genotypes used in this study were successfully ranked according to chilling tolerance, more reliable recommendations could be made regarding the best choice of genotypes for cultivation in those parts of South Africa prone to dark chilling.
- ii. Analysis of O-J-I-P chlorophyll *a* fluorescence transients shows potential for use in legumes as a sensitive and rapid diagnostic tool to detect above and below-ground perturbations induced by chilling stress and perhaps also by other environmental stressors.
- iii. The concept underlying the chill factor index (CFI) could possibly be extended to other crops and other stress screening programmes. This was recently successfully done in a study that focused on drought tolerance screening in barley genotypes, where the CFI was adapted to define a so-called “drought factor index” (Oukarroum et al., 2007).

- iv. Since cFBPase appears to be a main target of dark chilling stress in soybean under conditions where SNF is inhibited, genetic modification, e.g. through over-expression of cFBPase or expression of protease inhibitors directed against the putative proteases involved in cFBPase degradation, could be considered as possible strategies to enhance the resilience of photosynthetic carbon metabolism under these conditions.

Chapter 7

References

- Adam, Z., 2007. Protein Stability and Degradation In Plastids. Trends in Current Genetics, 19: 315-338.
- Adam, Z., Rudella, A., van Wijk, K.J., 2006. Recent advances in the study of Clp, FtsH and other proteases located in chloroplasts. Current Opinions in Plant Biology 9: 234-240.
- Adams, G.T., Perkins, T.D., 1993. Assessing cold tolerance in Picea using chlorophyll fluorescence. Environmental and Experimental Botany, 33: 377-382.
- Adjei, M.B., Quesenberry, K.H., Chambliss, C.G., 2002. Nitrogen Fixation and Inoculation of Forage Legumes. SS-AGR-56, Agronomy Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, pp. 1-5.
- Agati, G., Mazzinghi, P., Di Paola, M.L., Fusi, F., Cecchi, G., 1996. The F685/F730 chlorophyll fluorescence ratio as indicator of chilling stress in plants. Journal of Plant Physiology, 148: 384-390.
- Alberdi, M., Corcuera, L.J., 1991. Cold acclimation in plants. Phytochemistry, 30(10): 3177-3184.
- Allakverdiev, S.I., Murata, N., 2004. Environmental stress inhibits the synthesis de novo proteins involved in the photodamage-repair cycle of photosystem II in Synechocystis sp. PCC 6803. Biochimica et Biophysica Acta, 1657: 23-32.
- Allen, D.J., Ratner, K., Giller, Y.E., Gussakovsky, E.E., Shahak, Y., Ort, D.R., 2000. An overnight chill induces a delayed inhibition of photosynthesis at midday in mango (*Mangifera indica* L.). Journal of Experimental Botany, 51(352): 1893-1902.
- Allen, D.J., Ort, D.R., 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends in Plant Science, 6: 36-42.
- Anderson, M.D., Prasad, T.K., Stewart, C.R., 1995. Changes in isosyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. Plant Physiology, 109:1247-1257.

- Andersson, I., 2008. Catalysis and regulation in Rubisco. *Journal of Experimental Botany*, 59: 1555-1568.
- Andrews, T.J., Lorimer, G.H., 1987. Rubisco: Structure, mechanism and prospects for improvement. In: *The Biochemistry of Plants*. Hatch, M.D., Boardman, N.K. (eds) New York Academic Press. Vol. 10 pp. 132-219.
- Aro, E.M., Virgin, I., Andersson, B., 1993. Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochimica et Biophysica Acta*, 1143: 113-134.
- Aroca, R., Irigoyen, J.J., Sánchez-Díaz, M., 2003. Drought enhances maize chilling tolerance. II. Photosynthetic traits and protective mechanisms against oxidative stress. *Physiologia Plantarum*, 117: 540-549.
- Aroca, R., Amodeo, G., Fernández-Illescas, S., Herman, E.M., Chaumont, F., Chrispeels, M.J., 2005. The role of aquaporins and membrane damage in chilling and hydrogen peroxide induced changes in the hydraulic conductance of maize roots. *Plant Physiology*, 137: 341-353.
- Ashton, A.R., 1998. Sedoheptulose-1,7-bisphosphate activity of chloroplast fructose-1,6-bisphosphate: identification of enzymes hydrolyzing fructose-1,6-bisphosphate and sedoheptulose-1,7-bisphosphate in stromal extracts from chloroplasts of spinach (*Spinacia oleracea*). *Australian Journal of Plant Physiology*. 25: 531-537.
- Atkins, C.A., Sanford, P.J., Storer, P.J., Pate, J.S., 1988. Inhibition of nodule functioning in cowpea by a xanthine oxidoreductase inhibitor, allopurinol. *Plant Physiology* 88: 1229-1234.
- Barber, J., Nield, E.P., Morris, D., Zheleva, B., Hankamer, B., 1997. The structure, function and dynamics of photosystem II. *Physiologia Plantarum*, 100: 817-827.
- Battistelli, A., Adcock, M.D., Leegood, R., 1991. The relationship between the activation state of sucrose phosphate synthase and the rate of CO₂ assimilation in spinach leaves. *Planta*, 183: 620-622.
- Ben-Sham, A., Frolov, F., Nelson, N., 2003. Crystal structures of plant photosystem I. *Nature*, 426: 630-635.

- Bertamini, M., Muthuchelian, K., Rubinigg, M., Zorer, R., Nedunchezian, N., 2005. Low-night temperature (LNT) induced changes of photosynthesis in grapevine (*Vitis vinifera* L.) plants. *Plant Physiology and Biochemistry*, 43: 693-699.
- Bertamini, M., Muthuchelian, K., Rubinigg, M., Zorer, R., Velasco, R., Nedunchezian, N., 2006. Low-night temperature increased the photoinhibition of photosynthesis in grapevine (*Vitis vinifera* L. cv. Riesling) leaves. *Environmental and Experimental Botany*, 57: 25-31.
- Bertin, P., Kinet, J.M., Bouharmont, J., 1996. Evaluation of chilling sensitivity in different rice varieties. Relationship between screening procedures applied during germination and vegetative growth. *Euphytica*, 89: 201-210.
- Blennow, A., Engelson, S.B., Nielsen, T.H., Baunsgaard, L., Mikkelsen, R., 2002. Starch phosphorylation: a new front line in starch research. *Trends in Plant Science*, 7(10): 445-450.
- Bloom, A.J., Zwieniecki, M.A., Passioura, J.B., Randall, L.B., Holbrook, N.M., St Clair, D.A. 2004. Water relations under root chilling in a sensitive and tolerant tomato species. *Plant, Cell and Environment*, 27: 971-979.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254.
- Breazeale, U.D., Buchanan, B.B., Wolosiuk, R.A., 1978. Chloroplast sedoheptulose-1,7-bisphosphatase: evidence for regulation by the ferredoxin/thioredoxin system. *Z Naturforsch Teil*, 33C: 521-528.
- Britz, S.J., 1990. Regulation of photosynthate partitioning into starch in soybean leaves. *Plant Physiology*, 94: 350-356.
- Brüggeman, W., 1992a. Effects of long-term chilling under low irradiance on photosynthesis parameters of four Fabaceae species of different chilling tolerance. *Photosynthetica*, 27(4): 619-626.
- Brüggeman, W., 1992b. Low-temperature limitations of photosynthesis in three tropical *Vigna* species: A chlorophyll fluorescence study. *Photosynthesis Research*, 34: 301-310.

- Brüggeman, W., Van der Kooij, T.A.W., Hasselt, P.R., 1992. Long-term chilling of young tomato plants under low light and subsequent recovery. II. Chlorophyll fluorescence, carbon metabolism and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Planta*, 186: 179-187.
- Brychkova, G., Alikulov, Z., Fluhr, R., Sagi, M., 2008. A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the *Atxhd1* *Arabidopsis* mutant. *Plant Journal*, 54: 496-509.
- Buchanan, B.B., Wolosiuk, R.A., 1998. Photosynthesis: Carbon reactions. In: Taiz, L., Zeiger, E. (eds). *Plant Physiology*. Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts, USA.
- Buchanan, B.B., 1991. Regulation of CO₂ assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. *Archives of Biochemistry and Biophysics*, 288: 1-9.
- Cadet, F., Meunier, J.C., 1988. Spinach (*Spinacia oleracea*) chloroplast sedoheptulose-1,7-bisphosphatase. Activation and deactivation, and immunological relationship to fructose-1,6-bisphosphatase. *Biochemical Journal*, 253: 243-248.
- Castle, M.L., Crush, J.R., Rowath, J.S., 2006. The effect of root and shoot temperature of 8°C or 24°C on the uptake and distribution of nitrogen in white clover (*Trifolium repens* L.) *Australian Journal of Agricultural Research*, 57:577-581.
- Castrillo, M., 1992. Sucrose metabolism in bean plants under water deficit. *Journal of Experimental Botany*, 43(257): 1557-1561.
- Caulfield, F., Bunce, J.A., 1988. Comparative responses of photosynthesis to growth temperature in soybean (*Glycine max* [L.] Merrill) cultivars. *Canadian Journal of Plant Science*, 68: 419-425.
- Ceccarelli, S., Grando, S., 1996. Drought as a challenge for the plant breeder. *Plant Growth Regulation*, 20: 149-155.
- Cerff, R., 1982. Separation and purification of NAD- and NADP-linked glyceraldehydes-3-phosphate dehydrogenases from higher plants. In: Edelman M (ed) *Methods in chloroplast Molecular Biology*. Elsevier Biochem Press, Amsterdam. pp. 683-694.

- Chatterton, N.J., Silviu, J.E., 1979. Photosynthate Partitioning into Starch in Soybean Leaves: I. Effects of Photoperiod versus Photosynthetic Period Duration. *Plant Physiology*, 64(5): 749–753.
- Chaves, M.M., 1991. Effects of water deficits on carbon assimilation. *Journal of Experimental Botany*, 42(234): 1-16.
- Chiadmi, M., Navaza, A., Miginiac-Maslow, M., Jacquot, J.P., Cherfils, J., 1999. Redox signalling in the chloroplast structure of oxidized pea fructose-1,6-bisphosphate phosphatase. *EMBO Journal*, 18: 6809-6815.
- Chueca, A., Sahrawy, M., Pagano, E.A., Gorgé, J.L., 2002. Chloroplast fructose-1,6-bisphosphatase: structure and function. *Photosynthesis Research*, 74: 235-249.
- Cochard, H., Coll, L., Le Roux, X., Améglio, T., 2002. Unraveling the effects of plant hydraulics on stomatal closure during water stress in walnut. *Plant Physiology*, 128: 282-290.
- Crafts-Brandner, S.J., Klein, R.R., Klein, P., Hölzer, R., Feller, U., 1996. Coordination of protein and mRNA abundances of stromal enzymes and mRNA abundances of the Clp protease subunits during senescence of *Phaseolus vulgaris* (L.) leaves. *Planta* 200: 312-318.
- De Beer, G.P., Pretorius, A.J., 2002. Report of the National Soybean Genotype Trials 2001/2002. Grain Crops Institute, Agricultural Research Council, Potchefstroom.
- Dennis, D.T., Blakeley, S.D., 2000. Biochemistry and molecular biology of plants. In: Buchanan, B., Gruissem, W., Jones, R. (eds) American Society of Plant Physiologists. pp. 630-675.
- Doesthali, V., Salunke, C., Akmanchi, A., 2005. Relationship of LAI and biomass of soybean with agroclimatic indices. *Journal of Agrometeorology*, 7(2): 249-254.
- Doehlert, D.C., Huber, S.C., 1983. Regulation of spinach leaf sucrose phosphate synthase by glucose-6-phosphate, inorganic phosphate and pH. *Plant Physiology*, 73: 989-994.
- Duke, S.H., Schrader, L.E., Henson, C.A., Servaites, J.C., Vogelzang, R.D., Pendleton, J.W., 1979. Low root temperature effects on soybean nitrogen metabolism and photosynthesis. *Plant Physiology*, 63: 956-962.

- Duxbury, M.R., Birch, E.B., Parsons, M.J., 1990. Introduction and environmental requirements, Co-ordinated Extension, Natal Soybeans 1, University of Natal, Pietermaritzburg.
- Ellis, R.J., 1979. The most abundant protein in the world. Trends in Biochemical Science, 4: 241-244
- Enami, L., Kitamura, M., Tomo, T., Isokawa, Y., Ohta, H., Katoh, S., 1994. Is the primary cause of thermal inactivation of oxygen evolution in spinach PS II membranes release of the extrinsic 33 kDa protein or of Mn? Biochimica et Biophysica Acta, 1186:52-58.
- Erickson, R.O., Michelini, F.J., 1957. The plastochron index. American Journal Of Botany, 44: 297-305.
- Farquhar, G.D., von Caemmerer, S., Berry, J.A., 1980. A biochemical model of photosynthetic CO₂ assimilation in leaves of C₃ species. Planta, 149: 78-90.
- Farquhar, G.D., Sharkey, T.D., 1982. Stomatal conductance and photosynthesis. Annual Review of Plant Physiology, 33: 317-345.
- Farquhar, G.D., von Caemmerer, S., 1982. Modelling of photosynthetic responses to environmental conditions. In: Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H. (eds) Physiological Plant Ecology. II. Encyclopedia of Plant Physiology, New Series, Vol 12B, Springer-Verlag, Berlin. pp. 548-577.
- Fennell, A., Li, P.H., Markhart III, A.H., 1990. Influence of air temperature on water relations and freezing tolerance of spinach (*Spinacia oleracea*). Physiologia Plantarum, 78: 51-56.
- Fisahn, J., KOssmann, J., Matzke, G., Fuss, H., Bilger, W., Willmitzer, L., 1995. Chlorophyll fluorescence quenching and violaxanthin deepoxidation of FBPase antisense plants at low light intensities and low temperatures. Physiologia Plantarum, 95: 1-10.
- Flexas, J., Badger, M., Chow, W.S., Medrano, H., Osmond, C.B., 1999. Analysis of the relative increase in photosynthetic O₂ uptake when photosynthesis in grapevine leaves is inhibited following low night temperatures and/or water stress. Plant Physiology, 121: 675-684.

- Force, L., Critchley, C., Van Rensen, J.J.S., 2003. New fluorescence parameters for monitoring photosynthesis in plants. *Photosynthesis. Research*, 78: 17-33.
- Fracheboud, Y., Haldimann, P., Leipner, J., Stamp, P., 1999. Chlorophyll fluorescence as a selection tool for cold tolerance of photosynthesis in maize (*Zea mays* L.). *Journal of Experimental Botany*, 50: 1533-1540.
- Frederick, J.R., Alm, D.M., Hesketh, J.D., 1989. Leaf photosynthetic rates, stomatal resistances and internal CO₂ concentrations of soybean cultivars under drought stress. *Photosynthetica*, 23(4): 575-584.
- Funatsuki, H., Kurosaki, H., Murakami, T., Matsuba, S., Kawaguchi, K., Yumoto, S., Sato, Y., 2003. Deficiency of a cytosolic ascorbate peroxidase associated with chilling tolerance in soybean. *Theories for Applied Genetics*, 106: 494-502.
- Galtier, N., Foyer, C.H., Murkie, E., Alfred, R., Quick, P., Voelker, T.A., Thepenier, C., Lasceve, G., Betsche, T., 1995. Effect of light and atmospheric carbon dioxide enrichment on photosynthesis and carbon partitioning in the leaves of tomato (*Lycopersicon esculentum* L.) plants overexpressing sucrose phosphate synthase. *Journal of Experimental Botany*, 46: 1335-1344.
- Gartska, M., Drozak, A., Rosiak, M., Venema, J.H., Kierdaszuk, B., Simeonova, E., Van Hasselt, P.R., Dobrucki, J., Mostowska, A., 2005. Light-dependent reversal of dark-chilling induced changes in chloroplast structure and arrangement of chlorophyll-protein complexes in bean thylakoid membranes. *Biochimica et Biophysica Acta*, 1710: 13-23.
- Gass, T., Schori, A., Fossati, A., Soldati, A., Stamp, P., 1996. Cold tolerance of soybean (*Glycine max* (L.) Merr.) during the reproductive phase. *European Journal of Agronomy*, 5: 71-88.
- Geiger, D.R., Servaites, J.C., 1994. Diurnal regulation of photosynthetic carbon metabolism in C₃ plants. *Annual Review of Plant Physiology*, 45: 235-256.
- Gepstein, S., 1988. Photosynthesis. In: Nooden, L.D., Leopold, A.C. (Eds), *Senescence and aging in plants*. Academic Press, San Diego, pp. 85-109.
- Govindachary, S., Bukhov, N.G., Joly, D., Carpentier, R., 2004. Photosystem II inhibition by moderate light under low temperature in intact leaves of chilling-sensitive and -tolerant plants. *Physiologia Plantarum*, 121: 322-333.

- Gutteridge, S., Parry, M.A.J., Schmidt, C.N.G., 1982. The reactions between active and inactive forms of wheat ribulose biphosphate carboxylase and effectors. *European Journal of Biochemistry*, 126: 597-602.
- Guy, C.L., Huber, J.L.A., Huber, S.C., 1992. Sucrose Phosphate Synthase and Sucrose Accumulation at Low Temperature. *Plant Physiology*, 100: 502-508.
- Guy, S., Berger, M., Planchon, C., 1997. Response to low temperature in dinitrogen fixing soybeans. *Plant Science*, 123: 67-75.
- Haldimann, P., Strasser, R.J., 1999. Effects of anaerobiosis as probed by the polyphasic chlorophyll a fluorescence rise kinetic in pea (*Pisum sativum* L.). *Photosynthesis Research*, 62: 67-83.
- Hällgren, J-E., Öquist, G., 1990. Adaptations to low temperatures. In: Alscher, R.G., Cumming, J.R. (eds). *Stress responses in plants: adaptation and acclimation mechanisms*. Wiley-Liss, Inc. pp. 265-293.
- Hankamer, B., Barber, J., 1997. Structure and membrane organization of Photosystem II in green plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 48: 641-671.
- Harper, J.E., 1987. Nitrogen metabolism. In: Wilcox, J.R. (eds) *Soybeans: Improvement, Production, and Uses; Second Edition*, American Society of Agronomy. Madison, Wisconsin. pp. 497-533.
- Harrison, E.P., Willingham, N.M., Lloyd, J.C., Raines, C.A., 1998. Reduced sedoheptulose-1,7-bisphosphatase levels in transgenic tobacco lead to decreased photosynthetic capacity and altered carbohydrate accumulation. *Planta*, 204: 27-36.
- Harrison, E.P., Olcer, H., Lloyd, J.C., Long, S.P., Raines, C.A., 2001. Small decreases in SBPase cause a linear decline in the apparent RuBP regeneration rate, but do not affect Rubisco carboxylation capacity. *Journal of Experimental Botany*, 52(362): 1779-1784.
- Hatch, D.J., Macduff, J.H., 1991. Concurrent rates of N₂ fixation, nitrate and ammonium uptake by white clover in response to growth and different root temperatures. *Annals of Botany*, 67: 265-274.

- Helmerhorst, E., Stokes, G.B., 1980. Microsentrifuge desalting: a rapid, quantitative method for desalting small amount of protein. *Analytical Biochemistry*, 104: 130-135.
- Higuchi, M., Noguchi, T., Sonoike, K., 2003. Over-reduced states of the Mn-cluster in cucumber leaves induced by dark-chilling treatment. *Biochimica et Biophysica Acta*, 1604: 151-158.
- Hinze, A., Karg, D., Van Zyl, M., Mohamed, N., Steyn, N.P., 2004. The acceptability of different types of soymilk available in Cape Town in consumers from high and low socio-economic areas. *International Journal of Consumer Studies*, 28(1): 40-48.
- Hirotsu, N., Makino, A., Yokota, S., Mae, T., 2005. The Photosynthetic Properties of Rice Leaves Treated with Low Temperatures and High Irradiance. *Plant and Cell Physiology*, 46: 1377-1383.
- Hjelm, U., Ögren, E., 2003. Is photosynthetic acclimation to low temperature control-led by capacities for storage and growth at low temperature? Results from comparative studies of grasses and trees. *Physiologia Plantarum*, 119: 113-120.
- Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil. *Californian Agricultural Experimental Station Circular*, 347.
- Holaday, A.S., Martindale, W., Alred, R., Brooks, A.L., Leegood, R.C., 1992. Changes in activities of enzymes of carbon metabolism in leaves during exposure of plants to low temperature. *Plant Physiology*, 98: 1105-1114.
- Huber, S.C., 2007. Exploring the role of protein phosphorylation in plants: from signaling to metabolism. *Biochemical Society Transactions*, 35(1): 28-32.
- Huber, S.C., Huber, J.L., 1992. Role of sucrose-phosphate synthase in sucrose metabolism in leaves. *Plant Physiology*, 99: 1275-1278.
- Huber, S.C., Huber, J.L., 1996. Role and regulation of sucrose-phosphate synthase in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47: 431-444.
- Huber, S.C., Israel, D.W., 1982. Biochemical basis for partitioning of photosynthetically fixed carbon between starch and sucrose in soybean (*Glycine max* Merr.) leaves. *Plant Physiology*, 69: 691-696.

- Huber, S.C., Huber, J.A., Hanson, K.R., 1990. Regulation of the partitioning of products of photosynthesis. In: Perspectives in biochemical and genetic regulation of photosynthesis. Zelitch, I. ed. Liss, New York. pp. 85-101.
- Huber, S.C., Nielsen, T.H., Huber, J.L.A., Pharr, D.M., 1989. Variation among species in light activation of sucrose-phosphate synthase. *Plant and Cell Physiology*, 30: 277-285.
- Hudson, G.S., Evans, J.R., Von Caemmerer, S., Arvidson, Y.B.C., Andrews, T.J., 1992. Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. *Plant Physiology*, 98: 294-302.
- Huner, N.P.A., Öquist, G., Hurry, V.H., Krol, M., Falk, S., Griffith, M., 1993. Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. *Photosynthesis Research*, 37: 19-39.
- Hume, D.J., Jackson, A.K.H., 1981. Frost tolerance in soybeans. *Crop Science*, 21: 689-692.
- Hume, D.J., Shanmugasundaram, S., Beversdorf, W.D., 1985. Soybean (*Glycine max* L. Merrill). In: Grain legume crops, Summerfield, R.J. & Roberts, E.H. (eds), Collins Professional and Technical Books, London, pp. 391-432.
- Hurry, V.M., Huner, N.P.A., Selstam, E., Gardeström, P., Öquist, G., 1998. Photosynthesis at low growth temperatures. In: Raghavendra, A.S. (ed.) *Photosynthesis: A comprehensive treatise*. Cambridge University Press, New York, USA. pp. 238-249.
- Hurry, V.M., Malmberg, G., Gardeström, P., Öquist, G., 1994. Effects of short-term shift low temperature and of long-term hardening on photosynthesis and ribulose-1,5-bisphosphate carboxylase/oxygenase and sucrose phosphate synthase activity in leaves of winter rye (*Secale cereale* L.). *Plant Physiology*, 106: 983-990.
- Hurry, V.M., Strand, A., Tobiaeson, M., Gardeström, P., Öquist, G., 1995. Cold hardening of spring winter wheat and rape results in differential effects on growth, carbon metabolism, and carbohydrate content. *Plant Physiology*, 109: 697-706.

- Hutchison, R.S., Groom, Q., Ort, D.R., 2000. Differential effects of chilling-induced photooxidation on the redox regulation of photosynthetic enzymes. *Biochemistry*, 39: 6679-6688.
- Isopp, H., Frehner, M., Long, S.P., Nösberger, J., 2000. Sucrose-phosphate synthase responds differently to source-sink relations and to photosynthetic rates: *Lolium perenne* L. growing at elevated p_{CO_2} in the field. *Plant, Cell and Environment*, 23: 597-607.
- Jamadagni, B.M., Patil, R.B., Birari, S.P., 1994. Plastochron index in relation to water stress in cowpea. *Biologia Plantarum*, 37(1): 139-142.
- Jensen, R.G., Bahr, J.T., 1977. Ribulose 1,5-bisphosphate carboxylase-oxygenase. *Annual Review of Plant Physiology*, 28: 379-400.
- Jensen, P.E., Bassi, R., Boekema, E.J., Dekker, J.P., Jansson, S., Leister, D., Robinson, C., Scheller, H.V., 2007. Structure, function and regulation of plant photosystem I. *Biochimica et Biophysica Acta*, 1767: 335-352.
- Jones, M.G.K., Outlaw, W.H., Lowry, O.H., 1977. Enzymatic assay of 10^{-7} to 10^{-4} moles of sucrose in plant tissues. *Plant Physiology*, 60: 379-383.
- Jones, P.G., Lloyd, J.C., Raines, C.A., 1996. Glucose feeding of intact wheat plants represses the expression of a number of Calvin cycle genes. *Plant, Cell and Environment*, 19: 231-236.
- Jones, T.L., Ort, D.R., 1997. Circadian regulation of sucrose phosphate synthase activity in tomato by protein phosphatase activity. *Plant Physiology*, 113: 1167-1175.
- Jones, T.L., Tucker, D.E., Ort, D.R., 1998. Chilling delays circadian pattern of sucrose phosphate synthase and nitrate reductase activity in tomato. *Plant Physiology*, 118: 149-158.
- Kautsky, H., Hirsch, A., 1931. Neue versuche zur kohlenensäure assimilation. *Naturwissenschaften*, 19: 964.
- Kautsky, H., Appel, W., Amann, H., 1960. Chlorophyllfluoreszenz und Kohlenensäureassimilation. XIII. Die Fluoreszenzkurve und die Photochemie der Pflanze. *Biochemische Zeitschrift*, 332: 227-292.
- Kelly, G.J., 2001. Photosynthesis: Carbon Metabolism from DNA to Deoxyribose. *Progress in Botany*, 62: 238-265.

- Kemp, K., 2002. Soybean: To crush or not to crush. SA Grain, April Issue: 130-131.
- Kerr, P.S., Rufty jr, T.W., Huber, S.C., 1985. Endogenous rhythms in photosynthesis, sucrose phosphate synthase activity, and stomatal resistance in leaves of soybean (*Glycine max* [L.] Merr.). Plant Physiology, 77: 275-280.
- Keys, A.J., Parry, M.A.J., 1990. Ribulose biphosphate carboxylase/oxygenase and carbonic anhydrase. Methods in Plant Biochemistry, 3: 1-15.
- Kingston-Smith, A.H., Foyer, C.H., 2000. Bundle sheath proteins are more sensitive to oxidative damage than those of the mesophyll in maize leaves exposed to paraquat or low temperature. Journal of Experimental Botany, 51(342): 123-130.
- Kingston-Smith, A.H., Bollard, A.L., Minchin, F.R., 2005. Stress-induced changes in protease composition are determined by nitrogen supply in non-nodulating white clover. Journal of Experimental Botany, 56: 745-753.
- Kirschbaum, M.U.F., 1994. The sensitivity of C₃ photosynthesis to increasing CO₂ concentration: a theoretical analysis of its dependence on temperature and background CO₂ concentration. Plant, Cell and Environment, 17: 747-754.
- Klein, R.R., Crafts-Brander, S.J., Salvucci, M.E., 1993. Cloning and developmental expression of the sucrose-phosphate-synthase gene from spinach. Planta, 190: 498-510.
- Kossmann, J., Sonnewald, U., Willmitzer, L., 1994. Reduction of the chloroplastic fructose-1,6-bisphosphatase in transgenic potato plants impairs photosynthesis and plant growth. Plant Journal, 6: 637-650.
- Kolbe, A., Tiessen, A., Schluepmann, H., Paul, M., Ulrich, S., Geigenberger, P., 2005. Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. Proceedings of the National Academy of Sciences of the United States of America, 102(31): 11118-11123.
- Kościelniak, J., Biesaga- Kościelniak, J., 2006. Photosynthesis and non-photochemical excitation quenching components of chlorophyll excitation in maize and field beans during chilling at different photon flux density. Photosynthetica, 44(2): 174-180.

- Kosugi, Y., Shibata, S., Kobashi, S., 2003. Parameterization of the CO₂ and H₂O gas exchange of several temperate deciduous broad-leaved trees at the leaf scale considering seasonal changes. *Plant, Cell and Environment*, 26: 285-301.
- Kratsch, H.A., Wise, R.R., 2000. The ultrastructure of chilling stress. *Plant, Cell and Environment*, 23: 337-350.
- Krause, G.H., Weis, E., 1991. Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Physiology and Plant Molecular Biology*, 42: 313-349.
- Krause, K.P., Stitt, M., 1992. Sucrose-6-phosphate levels in spinach leaves and their effects on sucrose-phosphate synthase. *Phytochemistry*, 31(4): 1143-1146.
- Krüger, G.H.J., Tsimilli-Michael, M., Strasser, R.J., 1997. Light stress provokes plastic and elastic modifications in structure and function of photosystem II in camellia leaves. *Physiologia Plantarum*, 101: 265-277.
- Kubien, D.S., Von Caemmerer, S., Furbank, R.T., Sage, R.W., 2003. C₄ Photosynthesis at Low Temperature. A Study Using Transgenic Plants with Reduced Amounts of Rubisco. *Plant Physiology*, 132: 1-9.
- Kung, S.D., 1976. Tobacco fraction 1 protein: A unique genetic marker. *Science*, 191: 429-434.
- Labate, C.A., Leegood, R.C., 1990. Factors influencing the capacity for photosynthetic carbon assimilation in barley leaves at low temperatures. *Planta*, 182: 492-500.
- Labate, C.A., Adcock, M.D., Leegood, R.C., 1990. Effects of temperature on the regulation of photosynthetic carbon assimilation in leaves of maize and barley. *Planta*, 181: 547-554.
- Ladrera, R., Marino, D., Larrainzar, E., González, E.M., Arrese-Igor, C., 2007. Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. *Plant Physiology* 145: 539-546.
- Laing, W.A., Stitt, M., Heldt, H.W., 1981. Control of CO₂ fixation . Changes in the activity of ribulose phosphate kinase and fructose- and sedoheptulose-bisphosphatase in chloroplasts. *Biochimica et Biophysica Acta*, 637: 348-359.
- Latzko, E., Gibbs, M., 1972. Measurement of intermediates of the photosynthetic carbon reduction cycle, using enzymatic methods. *Methods in Enzymology*, 24: 261-268.

- Lavorel, J., Etienne, A.L., 1977. *In vivo* chlorophyll fluorescence. Topics in Photosynthesis, 2: 203-268.
- Lawn, R.J., Hume, D.J., 1985. Response of tropical and temperate soybean genotypes to temperature during early reproductive growth. Crop Science, 25: 137-142.
- Lazár, D. and Ilík, P., 1997. High-temperature induced chlorophyll fluorescence changes in barley leaves: Comparison of the critical temperatures determined from fluorescence induction and from fluorescence temperature curve. Plant Science, 124: 159-164.
- Lee, S.H., Singh, A.S., Chung, G.C., Ahn, S.J., Noh, E.K., Steudle, E., 2004. Exposure of roots of cucumber (*Cucumis sativus*) to low temperature severely reduces root pressure, hydraulic conductivity and active transport of nutrients. Physiologia Plantarum, 120: 413-420.
- Leegood, R.C., 1993. Carbon metabolism. In: Photosynthesis and Production in a changing environment: a field and laboratory manual. D.O. Hall et al. eds. Chapman & Hall, London, pp. 247-267.
- Lefebvre, S., Zakhleniuk, O.V., Slee, N.J., Lloyd, J.C., Raines, C.A., 2003. Sedoheptulose-1,7-bisphosphatase, an important enzyme for carbon assimilation and accumulation. Comparative Biochemistry and Physiology, Part A (134) S146.
- Lefebvre, S., Lawson, T., Zakhleniuk, O.V., Lloyd, J.C., Raines, C.A., 2005. Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. Plant Physiology 138: 451-460.
- Legros, T., Smith, D.I., 1994. Root zone temperature sensitivity of nitrogen fixing and nitrate-supplied soybean (*Glycine max* (L.) Merr. cv Maple Arrow) and lupin (*Lupinus albus* L. cv Ultra) plants. Environmental and Experimental Botany, 34(2): 117-127.
- Lindahl, M., Tabak, S., Cseke, L., Pichersky, E., Andersson, B., Adam, Z., 1996. Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplasts of higher plants. Journal of Biological Chemistry, 271: 29329-29334.

- Lorimer, G.H., Badger, M.R., Andrews, T.J., 1976. The activation of rubulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism, and physiological implications. *Biochemistry*, 15: 529-536.
- Ma, W., Wei, L., Wang, Q., Shi, D., Chen, H., 2007. Increased activity of the non-regulated enzymes fructose-1,6-bisphosphate aldolase and triosephosphate isomerase in *Anabaena* sp. strain PCC 7120 increases photosynthetic yield. *Journal of Applied Phycology*, 19: 207-213.
- Makino, A., Nakano, H., Mae, T., 1994. Effects of growth temperature on the responses of ribulose-1,5-bisphosphatase carboxylase, electron transport components, and sucrose synthesis enzymes to leaf nitrogen in rice, and their relationship to photosynthesis. *Plant Physiology*, 105: 1231-1238.
- Mann, H.B., Whitney, D.R., 1947. On a test of whether one of two random variables is stochastically larger than the other. *Annals of Mathematical Statistics*, 18: 50-60.
- Markhart, A.H., III, 1986. Chilling injury: A review of possible causes. *Horticultural Science* 21: 1329-1333.
- Martin, W., Schnarrenberger, C., 1997. The evolution of the Calvin cycle from prokaryotic to eukaryotic chromosomes: A case study of functional redundancy in ancient pathways through endosymbiosis. *Current Genetics*, 32: 1-18.
- Martino-Catt, S., Ort, D.R., 1992. Low temperature interrupts circadian regulation of transcriptional activity in chilling sensitive plants. *Proceedings of the National Academy of Sciences of the United States of America*, 89: 3731-3735.
- McClure, P.R., Israel, D.W., 1979. Transport of nitrogen in the xylem of soybean plants. *Plant Physiology*, 64: 411-416.
- Medrano, H., Escalona, J.M., Bota, J., Gulías, J., Flexas, J., 2002. Regulation of photosynthesis of C_3 plants in response to progressive drought: stomatal conductance as a reference parameter. *Annals of Botany*, 89: 895-905.
- Melkonian, J., Owens, T.G., Wolfe, D.W., 2004a. Gas exchange and co-regulation of photochemical and nonphotochemical quenching in bean during chilling at ambient and elevated carbon dioxide. *Photosynthesis Research*, 79: 71-82.

- Melkonian, J., Yu, L.-X., Setter, T.L., 2004b. Chilling response of maize (*Zea mays* L.) seedlings: root hydrolic conductance, abscisic acid, and stomatal conductance. *Journal of Experimental Botany*, 55(403): 1751-1760.
- Messina, M., 1999. Legumes and soybeans: overview of their nutritional profiles and health effects. *American Journal of Clinical Nutrition*, 70: 439-450.
- Miao, M., Xu, X., Chen, X., Xue, L., Cao, B., 2006. Cucumber carbohydrate metabolism and translocation under chilling night temperature. *Journal of Plant Physiology*, 164(5): 621-628.
- Michels, A.K., Wedel, N., Kroth, P.G., 2005. Diatom plastids possess a phosphoribulosekinase with an altered regulation and no oxidative pentose phosphate pathway. *Plant Physiology*, 137: 911-920.
- Murata, N., Takahashi, S., Nishiyama, Y., Allakhverdiev, S.I., 2007. Photoinhibition of photosystem II under environmental conditions. *Biochimica et Biophysica Acta*, 1767: 414-421.
- Musser, R.L., Thomas, S.A., Wise, R.R., Peeler, T.C., 1984. Chloroplast ultrastructure, chlorophyll fluorescence, and pigment composition in chilling-stressed soybeans. *Plant Physiology*, 74: 749-754.
- Musser, R.L., Kramer, P.J., Thomas, J.F., 1986. Periods of shoot chilling sensitivity in soybean flower development, and compensation in yield after chilling. *Annals of Botany*, 57: 317-329.
- Neuhaus, H.E., Quick, W.P., Siegl, G., Stitt, M., 1990. Control of photosynthate partitioning in spinach leaves. *Planta*, 181: 583-592.
- Neuner, G., Larcher, W., 1990. Determination of differences in chilling susceptibility of two soybean varieties by means of *in vivo* chlorophyll fluorescence measurement. *Journal of Agronomy and Crop Science*, 164: 73-80.
- Nie, G.Y., Baker, N.R., 1991. Modifications to thylakoid composition during development of maize leaves at low growth temperatures. *Plant Physiology*, 95: 184-191.
- Nishiyama, Y., Yamamoto, H., Allakhverdiev, S.J., Inaba, M., Yokota, A., Murata, N., 2001. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. *EMBO Journal* 20: 5587-5594.

- Nishizawa, A.N., Buchanan, B.B., 1981. Enzyme regulation in C₄ photosynthesis. Purification and properties of thioredoxin-linked fructose biphosphatase and sedoheptulose biphosphatase from corn leaves. *Journal of Biological Chemistry*, 256: 6119-6126.
- Niu, S.L., Li, L.H., Jiang, G.M., Gao, L.M., Li, Y.G., Peng, Y., Liu, M.Z., 2004. Gas exchange and chlorophyll fluorescence response to simulated rainfall in *Hedysarum fruticosum* var. *monolicum*. *Photosynthetica*, 42(1): 1-6.
- Noël, G.M., Pontis, H.G., 2000. Involvement of sucrose synthase in sucrose synthesis during mobilization of fructans in dormant Jerusalem artichoke tubers. *Plant Science*, 159: 191-195.
- Ocón, E., Hermoso, R., Chueca, A., Lázaro, J.J., 2001. Cross-linking between chloroplast fructose-1,6-bisphosphatase and thioredoxin *f*. *Physiologia Plantarum*, 113: 452-460.
- Ogren, W.L., 1984. Photorespiration: Pathways, regulation and modification. *Annual Review of Plant Physiology*, 35: 415-442.
- Ölçer, H., Lloyd, J.C., Raines, C.A., 2001. Photosynthetic capacity is differentially affected by reductions in sedoheptulose-1,7-bisphosphatase activity during leaf development in transgenic tobacco plants. *Plant Physiology*, 125: 982-989.
- Ohnishi, N., Allakhverdiev, S.I., Takahashi, S., Higashi, S., Watanebe, M., Nishiyama, Y., Murata, N., 2005. Two-step mechanism of photodamage to photosystem II: step 1 occurs at the oxygen-evolving complex and step 2 occurs at the photochemical reaction center. *Biochemistry*, 44: 8494-8499.
- Öquist, G., Hurry, V.M., Huner, N.P.A., 1993. Low-temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye. *Plant Physiology*, 101: 245-250.
- Oukarroum, A., El Madidi, S., Schansker, G., Strasser, R.J., 2007. Probing the responses of barley cultivars (*Hordeum vulgare* L.) by chlorophyll a fluorescence OLKJIP under drought stress and re-watering. *Environmental and Experimental Botany*, 60: 438-446.

- Papageorgiou, J., 1975. Chlorophyll fluorescence: an intrinsic probe of photosynthesis. In: Govindjee (Ed), *Bioenergetics of Photosynthesis*, Academic Press, New York, pp. 319-371.
- Parvanova, D., Popova, A., Zaharieva, I., Lambrev, P., Konstantinova, T., Taneva, S., Atanasov, A., Goltsev, V., Djilianov, D., 2004. Low temperature tolerance of tobacco plants transformed to accumulate proline, fructans or glycine betaine. Variable chlorophyll fluorescence evidence. *Photosynthetica*, 42:179-185.
- Peoples, M.B., Faizah, A.W., Rerkasem, B., Herridge, D.F., 1989. Methods for evaluating nitrogen fixation by nodulated legumes in the field. ACIAR monograph no. 11, Australian Centre for International Agricultural Research, Canberra, Australia.
- Pietrini, F., Iannelli, M.A., Battistelli, A., Moscatello, S., Loreto, F., Massacci, A., 1999. Effects on photosynthesis, carbohydrate accumulation and regrowth induced by temperature increase in maize genotypes with different sensitivity to low temperature. *Australian Journal of Plant Physiology*, 26: 367-373.
- Portis jr, A.R., 1992. Regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. *Annual Review of Plant Physiology and Plant Molecular Biology*, 43: 415-437.
- Preis, J., 1982. Regulation of the biosynthesis and degradation of starch. *Annual Review of Plant Physiology*, 33: 431-454.
- Prins, A., Van Heerden, P.D.R., Olmos, E., Kunert, K.J., Foyer, C.H., 2008. Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. *Journal of Experimental Botany*, 59: 1935-1950.
- Raines, C.A., Lloyd, J.C., Dyer, T.A., 1991. Molecular biology of the C₃ photosynthetic carbon reduction cycle. *Photosynthesis Research*, 27: 1-14.
- Raines, C.A., Lloyd, J.C., Dyer, T.A., 1999. New insights into the structure and function of sedoheptulose-1,7-bisphosphatase; an important but neglected Calvin cycle enzyme. *Journal of Experimental Botany*, 50(330): 1-8.

- Raines, C.A., Harrison, E.P., Ölçer, H., Lloyd, J.C., 2000. Investigating the role of the thiol-regulated enzyme sedoheptulose-1,7-bisphosphatase in the control of photosynthesis. *Physiologia Plantarum*, 110: 303-308.
- Raines, C.A., 2003. The Calvin cycle revisited. *Photosynthesis Research*, 75: 1-10.
- Raines, C.A., 2006. Transgenic approaches to manipulate the environmental responses of the C₃ carbon fixation cycle. *Plant, Cell and Environment*, 29: 331-339.
- Raison, J.K., Orr, G.R., 1986. Phase transition in thylakoid polar lipids of chilling sensitive plants. A comparison of detection methods. *Plant Physiology*, 80: 638-645.
- Raison, J.K., Roberts, J.K.M., Berry, J.A., 1982. Correlations between the thermal stability of chloroplast (thylakoid) membranes and the composition and fluidity of their polar lipids upon acclimation of the higher plant, *Nerium oleander*, to growth temperature. *Biochimica et Biophysica Acta*, 688: 218-228.
- Rao, I.M., Fredeen, A.L., Terry, N., 1993. Influence of phosphorus limitation on photosynthesis, carbon allocation and partitioning in sugar beet and soybean grown with a short photoperiod. *Plant Physiology and Biochemistry*, 31(2): 223-231.
- Robinson, J.M., 1996. Leaflet photosynthesis rate and carbon metabolite accumulation patterns in nitrogen-limited, vegetative soybean plants. *Photosynthesis Research*, 50: 133-148.
- Roitsch, T., 1999. Source-sink regulation by sugars and stress. *Current Opinions in Plant Biology*, 2: 198-206.
- Routaboul, J.M., Fischer, S.F., Browse, J., 2000. Treinoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiology*, 124: 1697-1705.
- Roy, H., 1989. Rubisco assembly: a model system for studying the mechanism of chaperonin action. *Plant Cell*, 1: 1035-1042.
- Ruelland, E., Migonac-Maslow, M., 1999. Regulation of chloroplast enzyme activities by thioredoxins: activation or relief from inhibition? *Trends in Plant Science*, 4(4): 136-141.

- Sahrawy, M., Chueca, A., Hermoso, R., Lázaro, J.J., López Gorgé, J., 1990. In-vivo and in-vitro synthesis of photosynthetic fructose-1,6-bisphosphatase from pea (*Pisum sativum* L.). *Planta*, 182: 319-324.
- Sahrawy, M., Ávila, C., Chueca, A., Cánovas, F.M., López-Gorgé, J., 2004. Increased sucrose level and altered nitrogen metabolism in *Arabidopsis thaliana* transgenic plants expressing antisense chloroplastic fructose-1,6-bisphosphatase. *Journal of Experimental Botany*, 55(408): 2495-2503.
- Sakamoto, W., 2006. Protein degradation machinery in plastids. *Annual Review of Plant Biology*, 57: 599-621.
- Sassenrath, G.F., Ort, D.R., Portis jr, A.R., 1990. Impaired reductive activation of stromal bisphosphatases in tomato leaves following low-temperature exposure to high light. *Archives of Biochemistry and Biophysics*, 282: 302-308.
- Savitch, L.V., Massacci, A., Gray, G.R., Huner, N.P.A., 2000. Acclimation to low temperature or high light mitigates sensitivity to photoinhibition: roles of the Calvin cycle and the Mehler reaction. *Australian Journal of Plant Physiology*, 27: 253-264.
- Schaffer, A.A., Nerson, H., Zamski, E., 1991. Premature leaf chlorosis in cucumber associated with high starch accumulation. *Journal of Plant Physiology*, 138: 186-190.
- Scheller, H.V., Haldrup, A., 2005. Photoinhibition of photosystem I. *Planta*, 221: 5-8.
- Schenk, G., Duggleby, R.G., Nixon, P.F., 1998. Properties and functions of the thaimin diphosphate dependant enzyme transketolase. *International Journal of Biochemistry and Cellular Biology*, 30: 1297-1318.
- Schimkat, D., Heineke, D., Heldt, H.W., 1990. Regulation of sedoheptulose-1,7-bisphosphatase by sedoheptulose-7-phosphate and glycerate and of fructose-1,6-bisphosphatase by glycerate in spinach chloroplasts. *Planta*, 181: 97-103.
- Schnarrenberger, C., Krüger, I., 1986. Distinction between cytosol and chloroplast fructose bisphosphate aldolases from pea, wheat and corn leaves. *Plant Physiology*, 80: 301-304.

- Schreiber, U., Neubauer, C., 1987. The polyphasic rise of chlorophyll fluorescence upon onset of strong continuous illumination. II. Partial control by photosystem II donor side and possible ways of interpretation. *Z. Naturforsch.*, 42c: 1255-1264.
- Schürmann, P., Wolosiuk, R.A., 1978. Studies on regulatory properties of chloroplast fructose-1,6-bisphosphatase. *Biochimica et Biophysica Acta*, 522: 130-138.
- Sedigh, M., Jolliff, G.D., Orf, J.H., 1989. Night temperature effects on soybean phenology. *Crop Science*, 29: 400-406.
- Serraj, R., Sinclair, T.R., 1996. Processes contributing to N₂-fixation insensitivity to drought in the soybean cultivar Jackson. *Crop Science*, 36: 961-968.
- Sevostyanova, I.A., Solovjeva, O.N., Kochetov, G.A., 2006. Two methods for determination of transketolase activity. *Biochemistry (Moscow)*, 71(5): 560-562.
- Shapiro, S.S., Wilk, M.B., Chen, H.J., 1968. A comparative study of various tests of normality. *Journal of American Statistical Association*, 63: 1343-1372.
- Shen, J.R., Terashima, I., Katoh, S., 1990. Cause for dark chilling-induced inactivation of photosynthetic oxygen-evolving system in cucumber leaves. *Plant Physiology*, 93: 1354-1357.
- Shen, W., Nada, K., Tachibana, S., 2000. Involvement of polyamines in the chilling tolerance of cucumber cultivars. *Plant Physiology*, 124: 431-439.
- Shih, M.C., Lazar, G., Goodman, H.M., 1986. Evidence in favor of chloroplasts: primary structure and evolution of tobacco glyceraldehydes-3-phosphate dehydrogenase. *Cell*, 47: 73-80.
- Signora L., Galtier, N., Skøt, L., Lucas, H., Foyer, C.H., 1998. Over-expression of sucrose phosphate synthase in *Arabidopsis thaliana* results in increased foliar sucrose/starch ratios and favours decreased foliar carbohydrate accumulation in plants after prolonged growth with CO₂ enrichment. *Journal of Experimental Botany*, 49(321): 669-680.
- Sijtsema, S.J., Linnemann, A.R., Gaasbeek, T., Dagevos, H., Jongen, W.M.F., 2002. Variables Influencing Food Perception Reviewed for Consumer-Oriented Product Development. *Critical Reviews in Food Science and Nutrition*, 42(6): 565 - 581.

- Singh, B.S., Haley, L., Nightengale, J., Kang, W.H., Haigler, C.H., Holaday, S., 2005. Long-term night chilling of cotton (*Gossypium hirsutum*) does not result in reduced CO₂ assimilation. *Functional Plant Biology*, 32: 655-666.
- Skarzynski, T., Wonacott, A.J., 1988. Co-enzyme-induced conformational changes in glyceraldehydes-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. *Journal of Molecular Biology*, 203: 1097-1118.
- Smith, A.M., Zeeman, S.C., Smith, S.M., 2005. Starch degradation. *Annual Review of Plant Biology*, 56: 73-98.
- Smith, J.M.B., 1994. Crop, pasture and timber yield index. Cedara Report, N/A/94/4. Natal Agricultural Research Institute, Cedara, pp. 82.
- Snyder, F.W., Bunce, J.A., 1983. Use of the Plastochron Index to Evaluate Effects of Light, Temperature and Nitrogen on Growth of Soya Bean (*Glycine max* L. Merr.). *Annals of Botany*, 52: 895-903.
- Sonoike, K., 1996. Degradation of PsbB gene product, the reaction centre subunit of photosystem I, is caused during photoinhibition of photosystem I: possible involvement of active oxygen species. *Plant Science*, 115: 157-164.
- Souza, R.P., Machado, E.C., Silva, J.A.B., Lagôa, A.M.M.A., Silveira, J.A.G., 2004. Photosynthetic gas exchange, chlorophyll fluorescence and some associated metabolic changes in cowpea (*Vigna unguiculata*) during water stress and recovery. *Environmental and Experimental Botany*, 51: 45-56.
- Spreitzer, R.J., Salvucci, M.E., 2002. Rubisco: Structure, regulatory interactions and possibilities for a better enzyme. *Annual Review of Plant Biology*, 53: 449-475.
- Srivastava, A., Greppin, H., Strasser, R.J., 1995. Acclimation of land plants to diurnal changes in temperature and light. From "From light to biosphere". In: Mathis P (Ed.), *Photosynthesis: Kluwer Academic Publishers, The Netherlands*, Vol. IV, pp. 909-912.
- Sthapit, B.R., Witcombe, J.R., Wilson, J.M., 1995. Methods of selection for chilling tolerance in Nepalese rice by chlorophyll fluorescence analysis. *Crop Science*, 35: 90-94.
- Stitt, M., Cseke, C., Buchanan, B.B., 1984. Regulation of the fructose-2,6-bisphosphate concentration in leaves. *European Journal of Biochemistry*, 143: 89-93.

- Stitt, M., Wilke, I., Feil, R., Heldt, H.W., 1988. Coarse control of sucrose-phosphate synthase in leaves: Alterations of the kinetic properties in response to the rate of photosynthesis and the accumulation of sucrose. *Planta*, 174: 217-230.
- Stitt, M., Schulze, D., 1994. Does Rubisco control the rate of photosynthesis and plant growth? An exercise in molecular ecophysiology. *Plant, Cell and Environment*, 17: 465-487.
- Strand, Å., Zrenner, R., Trevanion, S., Stitt, M., Gustafsson, P., Gardestrom, P., 2000. Decreased expression of two key enzymes in the sucrose biosynthesis pathway, cytosolic fructose-1,6-bisphosphatase and sucrose phosphate synthase, has remarkably different consequences for photosynthetic carbon metabolism in transgenic *Arabidopsis thaliana*. *Plant Journal* 23: 759-770.
- Strand, Å., Foyer, C.H., Gustafsson, P., Gardeström, P., Hurry, V., 2003. Altering flux through the sucrose biosynthesis pathway in transgenic *Arabidopsis thaliana* modifies photosynthetic acclimation at low temperatures and the development of freezing tolerance. *Plant Cell and Environment*, 26: 523-535.
- Strasser, B.J., Strasser, R.J., 1995. Measuring fast fluorescence transients to address environmental questions: The JIP-test. In: Mathis, P. (Ed.), *Photosynthesis: From light to biosphere*. Kluwer Academic Publishers, The Netherlands, Vol. V, pp. 977-980.
- Strasser, R.J., Govindjee, 1992. The F_0 and the O-J-I-P fluorescence rise in higher plants and algae. In: Argyroudi-Akoyunoglou, J.H. (Ed.), *Regulation of chloroplast biogenesis*. Plenum Press, New York, pp. 423-426.
- Strasser, R.J., Srivastava, A., Govindjee, 1995. Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria. *Photochemistry and Photobiology*, 61:32-42.
- Strasser, R.J., Eggenberg, P., Strasser, B.J., 1996. How to work without stress but with fluorescence. *Bulletin of the Royal Society of Liege*, 65: 330-349.
- Strasser, R.J., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescent transient as a tool to characterise and screen photosynthetic samples. In: Yunus, M., Pathre, U., Mohanty, P. (Eds.), *Probing Photosynthesis: mechanisms, regulation and adaptation*. Taylor and Francis, London, pp. 445-483.

- Strasser, R.J., Srivastava, A., Tsimilli-Michael, M., 2004. Analysis of the chlorophyll a fluorescence transient. From "Advances in Photosynthesis and Respiration, Vol. 19; Chlorophyll Fluorescence a Signature of Photosynthesis". In: Papageorgiou G and Govindjee (eds), Kluwer Academic Publishers, the Netherlands, pp. 321-362.
- Strasser, R.J., Tsimilli-Michael, M., 2001. Stress in plants, from daily rhythm to global changes, detected and quantified by the JIP-test. *Chimie Nouvelle (SRC)*, 75: 3321-3326.
- Svenning, M.M., Juntilla, O., Macduff, J.H., 1996. Differential rates of inhibition of N₂ fixation by sustained low concentrations of NH₄⁺ and NO₃⁻ in northern ecotypes of white clover (*Trifolium repens* L.) *Journal of Experimental Botany*, 47: 729-738.
- Tabaei-Aghdaei, S.R., Pearce, R.S., Harrison, P., 2003. Sugars regulate cold-induced gene expression and freezing-tolerance in barley cell cultures. *Journal of Experimental Botany*, 54(387): 1565-1575.
- Tambussi, E.A., Bartoli, C.G., Guiamet, J.J., Beltrano, J., Araus, J.L., 2004. Oxidative stress and photodamage at low temperatures in soybean (*Glycine max* L. Merr.) leaves. *Plant Science*, 167:19-26.
- Tamoi, M., Nagaoka, M., Miyagawa, Y., Shigeoka, S., 2006. Contribution of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase to the photosynthetic rate and carbon flow in the Calvin cycle in transgenic plants. *Plant and Cell Physiology*, 47(3): 380-390.
- Terashima, I., Huang, L.K., Osmond, C.B., 1989. Effects of leaf chilling on thylakoid functions, measured at room temperature, in *Cucumis sativus* L. and *Oryza sativa* L. *Plant and Cell Physiology*, 30: 841-850.
- Terzaghi, W.B., Fork, D.C., Berry, J.A., Field, C.B., 1989. Low and high temperature limits to PSII. *Plant Physiology*, 91: 1494-1500.
- Thibaud, M.C., Gineste, S., Nussaume, L., Robaglia, C., 2004. Sucrose increases pathogenesis-related PR-2 gene expression in *Arabidopsis thaliana* through an SA dependent but NPR1-independent signalling pathway. *Plant Physiology and Biochemistry*, 42(1): 81-88.

- Tognetti, J.A., Salerno, G.L., Crespi, M.D., Pontis, H.G., 1990. Sucrose and fructan metabolism of different wheat cultivars at chilling temperatures. *Physiologia Plantarum*, 78: 554-559.
- Toroser, D., Huber, S.C., 1997. Protein phosphorylation as a mechanism for osmotic-stress activation of sucrose-phosphate synthase. *Plant Physiology*, 114: 947-955.
- Tsimilli-Michael, M., Pêcheux, M., Strasser, R.J., 1998. Vitality and stress adaptation of the symbionts of coral reef and temperate foraminifers probed *in hospite* by the fluorescence kinetics O-J-I-P. *Arch Sci Genève*, 51: 1-36.
- Tsimilli-Michael, M., Pêcheux, M., Strasser, R.J., 1999. Light and heat stress adaptation of the symbionts of coral reef and temperate foraminifers probed *in hospite* by the chlorophyll a fluorescence kinetics O-J-I-P. *Naturforsch*, 54C: 671-680.
- Vadez, V., Sinclair, T.R., Serraj, R., Purcell, L.C., 2000. Manganese application alleviates the water deficit-induced decline of N₂-fixation. *Plant, Cell and Environment*, 23: 497-505.
- Van der Drift, C., Dewindt, F.E., Vogels, G.D., 1970. Allantoate hydrolysis by allantoate amidohydrolase. *Archives of Biochemistry and Biophysics*, 136: 273-279.
- Van der Vyver, C., Schneidereit, J., Driscoll, S., Turner, J., Turner, J., Kunert, K., Foyer, C.H., 2003. Oryzacystatin I expression in transformed tobacco produces a conditional growth phenotype and enhances chilling tolerance. *Plant Biotechnology Journal*, 1: 101-112.
- Van Heerden, P.D.R. and Krüger, G.H.J., 2000. Photosynthetic limitation in soybean during cold stress. *South African Journal of Science*, 96: 201-206.
- Van Heerden, P.D.R., Krüger, G.H.J., Loveland, J.E., Parry, M.A.J., Foyer, C.H., 2003a. Dark chilling imposes metabolic restrictions on photosynthesis in soybean. *Plant, Cell and Environment*, 26: 323-327.
- Van Heerden, P.D.R., De Villiers, M.F., Van Staden, J., Krüger, G.H.J., 2003b. Dark chilling increases glucose-6-phosphate dehydrogenase activity in soybean leaves. *Physiologia Plantarum*, 119: 221-230.
- Van Heerden, P.D.R., Tsimilli-Michael, M., Krüger, G.H.J., Strasser, R.J., 2003c. Dark chilling effects on soybean genotypes during vegetative development: parallel

- studies of CO₂ assimilation, chlorophyll a fluorescence kinetics O-J-I-P and nitrogen fixation. *Physiologia Plantarum*, 117: 476-491.
- Van Heerden, P.D.R., Strasser, R.J., Krüger, G.H.J., 2004a. Reduction of dark chilling stress in N₂-fixing soybean by nitrate as indicated by chlorophyll a fluorescence kinetics. *Physiologia Plantarum*, 121: 239-249.
- Van Heerden, P.D.R., Viljoen, M.M., De Villiers, M.F., Krüger, G.H.J., 2004b. Limitation of photosynthetic carbon metabolism by dark chilling in temperate and tropical soybean genotypes. *Plant Physiology and Biochemistry*, 42: 117-124.
- Van Heerden, P.D.R., Kiddle, G., Pellny, T.K., Mokwala, P.W., Jordaan, A., Strauss, A.J., de Beer, M., Schlüter, U., Kunert, K.J., Foyer, C.H., 2008. Roles for the regulation of respiration and the oxygen diffusion barrier in soybean in the protection of symbiotic nitrogen fixation from chilling –induced inhibition and shoots from premature senescence. *Plant Physiology*, 148: 316-327.
- Veitch, N.J., Mauger, D.A., Cazzulo, J.J., Lindqvist, Y., Barrett, M.P., 2004. Transketolase from *Leishmania maxicana* has a dual subcellular localization. *Biochemistry Journal*, 382: 759-767.
- Venema, J.H., Posthumus, F., De Vries, M., Van Hasselt, P.R., 1999. Differential response of domestic and wild *Lycopersicon* species to chilling under low light: growth, carbohydrate content, photosynthesis and the xanthophyll cycle. *Physiologia Plantarum*, 105: 81-88.
- Ventrella, A., Catucci, L., Villari, V., Scolaro, L.M., Agostiano, A., 2007. Focus on the aggregation of Photosystem II complexes. *Bioelectrochemistry*, 70: 33-38.
- Vessey, T.L., Quick, W.P., Sharkey, T.D., Stitt, M., 1991. Water stress, carbon dioxide, and light effects on sucrose-phosphate synthase activity in *Phaseolus vulgaris*. *Physiologia Plantarum*, 81: 37-44.
- Vogels, G.D., Van der Drift, C., 1976. Degradation of purines and pyrimidines by microorganisms. *Bacteriological Review*, 40: 403-468.
- Von Caemmerer, S., 2000. *Biochemical Models of Leaf Photosynthesis*. CSIRO Publishing, Collingwood, Australia, pp. 1-165.
- Walker, A.J., Ho, L.C., 1977. Carbon translocation in the tomato: pathways of carbon metabolism in the fruit. *Annals of Botany*, 41: 813-823.

- Walsh, K.B., Layzell, D.B., 1986. Carbon and Nitrogen Assimilation and Partitioning in Soybeans Exposed to Low Root Temperatures. *Plant Physiology*, 80: 249-255.
- Weissbach, A., Horecker, B.L., Hurwitz, J., 1956. The enzymatic formation of phosphoglyceric acid from ribulose diphosphate and carbon dioxide. *Journal of Biological Chemistry*, 218: 795-810.
- Wery, J., Silim, S.N., Knights, E.J., Malhotra, R.S., Cousin, R., 1994. Screening techniques and sources of tolerance to extremes of moisture and air temperature in cool season food legumes. *Euphytica*, 73: 73-83.
- Wilcox, J.R., 1987. Soybeans: Food, Feed and Future. In: *Soybeans: Improvement, Production and Uses*. 2nd edition. American Society of Agronomy, Inc. Publishers, Madison, Wisconsin, USA, pp. 165-172.
- Willingham, N.M., Lloyd, J.C., Raines, C.A., 1994. Molecular cloning of the *Arabidopsis thaliana* sedoheptulose-1,7-bisphosphatase gene and expression studies in wheat and *Arabidopsis thaliana*. *Plant Molecular Biology*, 26: 1191-1200.
- Winkler, R.D., Blevins, D.G., Palacco, J.C., Randall, D.D., 1987. Ureide Catabolism of Soybeans : II. Pathway of Catabolism in Intact Leaf Tissue. *Plant Physiology*, 83: 585-591.
- Winter, H., Huber, S.C., 2000. Regulation of sucrose metabolism in higher plants: Localization and regulation of activity of key enzymes. *Critical Reviews in Biochemistry and Molecular Biology*, 35(4): 253-289.
- Wintermans, J.F.G.M., De Mots, A., 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. *Biochimica et Biophysica Acta*, 109: 448-453.
- Yamaguchi-Shinozaki, K., Shinozaki, K., 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology*, 57: 781-803.
- Yamori, W., Noguchi, K., Terashima, I., 2005. Temperature acclimation of photosynthesis in spinach leaves: analysis of photosynthetic components and temperature dependencies of photosynthetic partial reactions. *Plant, Cell and Environment*, 28: 536-547.

- Yamori, W., Suzuki, K., Noguchi, K., Nakai, M., Terashima, I., 2006. Effects of Rubisco kinetics and Rubisco activation state on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. *Plant, Cell and Environment*, 29: 1659-1670.
- Young, E.G., Conway, C.F., 1942. On the estimation of allantoin by the Rimini-Schryver reaction. *Journal of Biological Chemistry*, 142: 839-853.
- Young, V.R., Puig, M., Queiroz, E., Scrimshaw, N.S., Rand, W.M., 1984. Evaluation of the protein quality of an isolated soya bean protein in young men: relative nitrogen requirement and effects of methionone supplementation. *American Journal of Clinical Nutrition*, 39: 16-24.
- Yourstone, K.S., Wallace, D.H., 1990. Application of plastochron index to common bean growth in controlled environments. *Journal of America Society for Horticultural Science*, 115(5): 820-823.
- Zhou, Y-H., Huang, L-F., Du, Y-S., Yu, J-Q., 2004a. Greenhouse and field cucumber genotypes use different mechanisms to protect against dark chilling. *Functional Plant Biology*, 31: 1215-1223.
- Zhou, Y.H., Yu, J.Q., Huang, L.F., Nogués, S., 2004b. The relationship between CO₂ assimilation, photosynthetic electron transport and water-water cycle in chill-exposed cucumber leaves under low light and subsequent recovery. *Plant, Cell and Environment*, 27: 1503-1514.
- Zhu, S.Q., Yu, C.M., Liu, X.Y., Ji, B.H., Jiao, D.M., 2007. Changes in unsaturated levels of fatty acids in thylakoid membrane lipids during chilling-induced resistance in rice. *Journal of Integrative Plant Biology*, 49(4): 463-471.
- Zrenner, R., Stitt, M., 1991. Comparison of effect of rapidly and gradually developing water-stress on carbohydrate metabolism in spinach leaves. *Plant, Cell and Environment*, 14: 939-946.
- Zúñiga-Feest, A., Ort, D.R., Gutiérrez, A., Gidekel, M., Bravo, L.A., Corcuera, L.J., 2005. Light regulation of sucrose-phosphate synthase activity in the freezing-tolerant grass *Deschampsia antarctica*. *Photosynthesis Research*, 83: 75-86.

Appendix I

Summary of JIP-test formulae using data extracted from the OJIP transients:

Extracted and technical fluorescence parameters	
F_0	= $F_{50\mu s}$, fluorescence intensity at $50\mu s$
$F_{100\mu s}$	= fluorescence intensity at $100\mu s$
$F_{300\mu s}$	= fluorescence intensity at $300\mu s$
F_J	= fluorescence intensity at the J-step (at 2ms)
F_I	= fluorescence intensity at the I-step (at 30ms)
F_M	= maximal fluorescence intensity
t_{F_M}	= time to reach F_M , in ms
V_J	= $(F_{2ms} - F_0) / (F_M - F_0)$
$(dV/dt)_0 = M_0$	= $4 \cdot (F_{300} - F_0) / (F_M - F_0)$
Quantum efficiencies or flux ratios	
$\phi_{P_0} = TR_0 / ABS$	= $[1 - (F_0 / F_M)] = F_V / F_M$
$\phi_{E_0} = ET_0 / ABS$	= $[1 - (F_0 / F_M)] \cdot \psi_0$
$\psi_0 = ET_0 / TR_0$	= $(1 - V_J)$
Specific fluxes or specific activities	
ABS / RC	= $M_0 \cdot (1 / V_J) \cdot (1 / \phi_{P_0})$
TR_0 / RC	= $M_0 \cdot (1 / V_J)$
ET_0 / RC	= $M_0 \cdot (1 / V_J) \cdot \psi_0$
DI_0 / RC	= $(ABS / RC) - (TR_0 / RC)$
Phenomenological fluxes or phenomenological activities	
ABS / CS	= $ABS / CS_{Chl} = Chl / CS$ or $ABS / CS_0 = F_0$ or $ABS / CS_M = F_M$
TR_0 / CS	= $\phi_{P_0} \cdot (ABS / CS)$
ET_0 / CS	= $\phi_{P_0} \cdot \psi_0 \cdot (ABS / CS)$
DI_0 / CS	= $(ABS / CS) - (TR_0 / CS)$
Density of reaction centres	
RC / CS	= $\phi_{P_0} \cdot (V_J / M_0) \cdot ABS / CS$
Performance indexes	
PI_{ABS}	= $(RC / ABS) \cdot [\phi_{P_0} / (1 - \phi_{P_0})] \cdot [\psi_0 / (1 - \psi_0)]$
PI_{CS}	= $(RC / CS) \cdot [\phi_{P_0} / (1 - \phi_{P_0})] \cdot [\psi_0 / (1 - \psi_0)]$

Appendix II

The performance index

The performance index on absorption basis, PI_{ABS} , was introduced as:

$$PI_{ABS} = \frac{\gamma_{RC}}{1 - \gamma_{RC}} \cdot \frac{\phi_{Po}}{1 - \phi_{Po}} \cdot \frac{\psi_o}{1 - \psi_o} \quad (\text{Eq. 1})$$

where $\gamma_{RC} = \text{Chl}_{RC}/\text{Chl}_{total}$ is the fraction of reaction center chlorophylls relatively to the total chlorophyll content. Since $\text{Chl}_{total} = \text{Chl}_{antenna} + \text{Chl}_{RC}$, we get $\gamma_{RC}/(1 - \gamma_{RC}) = \text{Chl}_{RC}/\text{Chl}_{antenna} = \text{RC}/\text{ABS}$. This expression can be de-convoluted into two JIP-test parameters and estimated from the original fluorescence measurements as $\text{RC}/\text{ABS} = \text{RC}/\text{TR}_o \cdot \text{TR}_o/\text{ABS} = [(F_{2ms} - F_{50\mu s})/4(F_{300\mu s} - F_{50\mu s})] \cdot F_V/F_M$. The factor 4 is used to express the initial fluorescence rise per 1 ms. The expression RC/ABS shows the contribution to the PI_{ABS} due to the RC density on a chlorophyll basis. The contribution of the light reactions for primary photochemistry are estimated according to the JIP-test as $[\phi_{Po}/(1 - \phi_{Po})] = \text{TR}_o/\text{DI}_o = k_P/k_N = F_V/F_o$. The contribution of the dark reactions are derived as $[\psi_o/(1 - \psi_o)] = \text{ET}_o/(\text{TR}_o - \text{ET}_o) = (F_M - F_{2ms})/(F_{2ms} - F_{50\mu s}) = (\text{RC}/\text{CS})/(\text{ABS}/\text{CS})$, derived from data extracted from the fluorescence measurements (see Appendix 1). Since RC/ABS is proportional to the molar ratio, and the PI_{ABS} can be expressed in relative units, the proportionality factor can be omitted. Hence:

$$PI_{ABS} = \frac{\text{RC}}{\text{ABS}} \cdot \frac{\phi_{Po}}{1 - \phi_{Po}} \cdot \frac{\psi_o}{1 - \psi_o} \quad (\text{Eq. 2})$$

Multiplying by the flux ABS/CS , so that a factor for the chlorophyll concentration per leaf cross section is integrated, leads to the definition of the performance index on cross section basis, PI_{CS} :

$$PI_{CS} = \frac{\text{RC}}{\text{CS}} \cdot \frac{\phi_{Po}}{1 - \phi_{Po}} \cdot \frac{\psi_o}{1 - \psi_o} = \frac{\text{ABS}}{\text{CS}} \cdot PI_{ABS} \quad (\text{Eq. 3})$$

Substitution of the biophysical by the experimental parameters (see Appendix 1) results in:

$$PI_{\text{ABS}} = \frac{1 - (F_0 / F_M)}{M_0 / V_J} \cdot \frac{F_M - F_0}{F_0} \cdot \frac{1 - V_J}{V_J} \quad (\text{Eq. 4})$$

$$PI_{\text{CS}} = \frac{\text{ABS}}{\text{CS}} \cdot \frac{1 - (F_0 / F_M)}{M_0 / V_J} \cdot \frac{F_M - F_0}{F_0} \cdot \frac{1 - V_J}{V_J} \quad (\text{Eq. 5})$$