BIOCHEMICAL AND BIOPHYSICAL INDICATORS OF CHILLING TOLERANCE IN SOME SPECIES OF *EUCALYPTUS*.

by

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Submitted in partial fulfillment of the academic requirements for the degree of Master of Science in the Department of Biology, University of Natal.

Durban

1993

PREFACE

The experimental work described in this thesis was conducted in the FRD/UND Photosynthetic Nitrogen Metabolism Research Unit at the Biology Department, University of Natal, Durban, under the supervision of Dr Alan Amory.

All the work presented in this study was original research conducted by the author and has not been submitted in any form to another University. Where use was made of the work of other individuals it has been acknowledged in the text.

ACKNOWLEDGEMENTS

I would like to acknowledge the financial support of the Foundation for Research and Development, the Photosynthetic Nitrogen Metabolism Research Unit and the University of Natal, Durban, towards this work.

I would like to thank Dr Alan Amory for his supervision and input into this study, especially during the final stages. Thanks also to Dr Paula Watt for proof-reading and commenting on the script. My appreciation goes to Professor Trofsky, Department of Mathematical Statistics, UND, for the time spent writing a program for the analysis of some of the data generated by this study.

Thanks to Mondi Forests, Hilton who supplied the *Eucalyptus* material and general information utilised in this study.

My family has been very supportive during the whole of my Masters degree. My parents deserve a big thankyou, for all their care and help. Thanks especially to my mother, Cherrie Stewart, for reading parts of the script. My grandparents, Ena and Roy Spencer, have always been there and I thank them for all their prayers.

Thank you, Ben, for all the late nights you spent waiting in the lab for me to finish experiments and for all the emergency burgers you brought, together with loads of encouragement and motivation.

I would like to thank all my friends for their support, encouragement and enthusiasm. I would like to make special mention of Mornay, Jacqui, Janine, Ryan and Huw.

ABSTRACT

Although *Eucalyptus* species are indigenous to Australia, they have been successfully cultivated in South Africa, where they are mainly used in the mining and paper industries. With the explosion in these industries it has been necessary to increase *Eucalyptus* plantations, often into areas which experience frost and chilling temperatures. To combat this, high yielding *Eucalyptus* species able to cope with these conditions would be desirable. The ability to rank species according to their chilling tolerance will enhance decisions as to the suitability of species for use in the field. To this aim, two biochemical and two biophysical parameters were chosen to investigate and characterise *Eucalyptus nitens, E. smithii, E. macarthurii, E. grandis* and *E. grandis* x *nitens* (GN1026). Ranking of these species in terms of chilling tolerance did not appear possible using the data from the biochemical parameters (proline concentration and glutathione reductase activity), but the biophysical parameters (fluorescence characteristics and onset of temperature of melt) gave results comparable to those obtained in field trials using these species. Fluorescence characteristics were particularly useful in assessing the chilling tolerance of the species in question. Fluorescence is the recommended technique for further studies as it is relatively inexpensive, rapid, does not require destructive sampling and can be used in both the laboratory and field.

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LIST OF ABBREVIATIONS

centimetre	cm
degrees Celsius	°C
gram	g
hydrogen ion	H^+
hydrogen peroxide	H_2O_2
kiloDalton	kDa
Michaelis-Menten value	K_m
microEinsteins	μE
microgram	μg
micromole	μmol
millilitre	ml
millimolar	mM
metre (distance)	m
molar (concentration)	M
molecular oxygen	O_2
oxidised nicotinamide adenine diphosphate	$NADP^+$
percentage	%
reduced nicotinamide adenine diphosphate	NADPH
revolutions per minute	rpm
second (time)	s
superoxide	O_2^-
ultra-violet (light)	uV
volume by volume	v/v
volume by weight	v/w

CHAPTER 1
INTRODUCTION

The genus *Eucalyptus* was introduced into South Africa in the mid-1800's (Penfold and Willis, 1961). Initially, the most cultivated cultivated species was *E. grandis*, then known as *E. saligna*. Today this species is still one of the major sources of eucalypt bulk sawn timber (von Gadow *et al.*, 1987). The genus is also utilized for mining timber, pulpwood and poles (van Gadow *et al.*, 1987), and for the period 1989/1990, 40% of all plantation area in Southern Africa was under *Eucalytus* species (South African Forestry Facts, 1991), 73% of which was privately owned. Of newly afforested land, 53% was planted with *Eucalyptus* species and in the Annual Report of Forestry Costs in South Africa (1991), the gross profit margin per hectare planted with gum was R600. In Natal, the real rate of return on capital employed in 1990 was 6.4%, about 5% higher than in the Transvaal and the SE Transvaal (Annual Report of Forestry Costs in South Africa, 1991).

With the amount of land being owned by forestry companies increasing each year (AMIC Annual Report, 1991; SAPPI Forests Annual Report, 1992), research in the field of *Eucalyptus* growth and management is becoming increasingly more important. As those *Eucalyptus* species which yield high quality wood cannot be cultivated in areas where large temperature differences are experienced (B. Herman, *pers. comm.* Mondi Forests, Research and Development, Natal Office, P.O. Box 39, Pietermaritzberg, 3200), it is of interest to rapidly identify those tolerant of temperatures extremes, particularly of chilling temperatures. An inital step in this process is the establishment of a protocol that is able to determine the cold tolerance of seedlings of *Eucalyptus* species or clones. This is a primary aim of this project.

In this chapter, the characteristics of the *Eucalyptus* species studied in the investigation are discussed. This is followed by an overview of freezing and chilling stress, those factors that influence this type of stress and those that enable the plant to deal with such stress conditions. Finally, the objectives of the study are described.

1.1 *Eucalyptus* species and their habitats

A number of different *Eucalyptus* species are grown in South Africa and include *E. nitens, E. macarthurii. E. smithii, E. grandis* and the hybrid between *E. grandis* and *E. nitens*, GN1026. *E. grandis* naturally occurs in a subtropical to warmer-temperate climate which is humid throughout the year (Poynton, 1979). Maximum and minimum temperatures for the warmest and

coolest months vary from 29 to 32°C and from 5 to 6°C, respectively, with winter frost only occuring in valleys and at high elevations. The rainfall averages 1000 to 1800 mm a year and is heaviest in summer (Poynton, 1979). This species is generally used for house construction and general carpentry, but not for paper making in Australia, although it has been put to this use in other countries (Poynton, 1979), including South Africa, where the potential uses of *E. grandis* are immense (B. Herman, *pers. comm.*). In 1979 it was already the most popular *Eucalyptus* species planted, and it was benefitting the timber industry, which has expanded greatly since then (see above).

E. macarthurii has a very limited natural distribution in Australia. Its latitudinal range is from 33.5 to 35°S and is found between 700 and 900 m above sea level (Poynton, 1979). The climate in temperate and fairly humid, with a heavy rainfall in summer - average rainfall is 750 to 1150 mm a year, distributed over 90 to 100 days. The maximum and minimum temperatures in these areas are between 26 and 3°C, with frost occuring on up to 40 nights of the year and light snow falling at higher elevations during winter. This species has pale wood of medium strength and density which is not durable, and as such, not suitable for timber. In South Africa this species has been used for pulpwood and mining timber and is able to grow in areas of severe frost (Poynton, 1979).

E. nitens has a distribution between 30 to 38°S and between 600 and 1200 m above sea level. The climate under which it grows best is temperate or cooler-temperate and humid. Mean maximum and minimum temperatures are 21 to 24°C and -2 to 2°C (Poynton, 1979). Winter frost occurs on up to 150 nights a year, and light to heavy snow falls may be experienced at higher elevations. Rainfall varies between 760 and 1270 mm a year and this is spread over 90 to 140 days of the year. This tree has been used in both the timber and paper industry and is more resistant to frost and snow than most other species used for these purposes (Poynton, 1979). In South Africa, this species has performed well, particularly in the Transvaal, and is expected to be grown on an increased scale in the future.

E. smithii has a limited distribution along from the coastal belt to the mountains on the eastern, central and southern areas of New South Wales and in eastern Victoria. The climate is temperate to cooler-temperate and humid (Poynton, 1979). Mean maximum and minimum temperatures are 22 to 28°C and 2 to 9°C respectively, with frost occuring on up to 40 days of the year at higher elevations. Rainfall is between 750 and 1250 mm a year, and it is well distributed. This species is used for primarily for paper pulp and for limited constructional work.

Progeny crosses involving *E. nitens* are morphologically intermediate with respect to both parents (Tibbits, 1986), thus GN1026 can be considered to have characteristics of both *E. nitens* and *E. grandis*.

1.2 Variation in frost tolerance

Eucalypts have been found to vary in frost tolerance between -3 and -20°C (Davidson and Reid, 1987; Tibbits and Reid, 1987). As with other plants, levels of frost tolerance vary with genetic factors which appear to be activated by environmental conditions such as day length and low external temperatures (Tibbits, 1986). Frost hardening is a relatively slow process in many species of eucalypts (Tibbits and Reid, 1987; Hallam and Reid, 1989). The reaction of any one species to frost temperature will be site dependent to a large extent, and will reflect its ability to harden in response to seasonal-diurnal changes in temperature at that site (Hallam *et al.*, 1989). Eucalypts have been placed into groups, depending on their frost tolerance levels. Most species are unable to withstand temperatures of -18 to -20°C for long periods of time, but those that are able to do so are slow growers. Some of the faster growers with favourable wood qualities, including *E. grandis*, possess low levels of frost tolerance and are unlikely to survive temperatures below -9°C for extended periods. A third group, including *E. nitens*, contains those species which have moderate growth rates and a degree of frost tolerance (Tibbits, 1986).

1.3 Mechanisms involved in cold and freezing stress.

The ability of some plant varieties to tolerate cold and freezing stress has been the subject of research for some time (Guy, 1990). The aim of such work has been to elucidate the mechanisms during the freeze/thaw cycle that leads to cell death, and to understand the biochemical and physiological changes that occur during cold acclimation and that induce frost tolerance. Cold stress usually implies temperatures below 10°C, while freezing or frost stress involves temperatures below 0°C. As *Eucalyptus* plants are required to grow in day temperatures which can reach 10°C and in night temperatures of approximately -10°C, it was necessary to consider the effects of both cold and frost stress in this study. This review of the literature will summarise the physical processes and metabolic events known to occur during freezing and chilling. Such information was used to define the objectives of this project.

1.3.1 Freezing stress

Plants adapted to withstand freezing temperatures are often in a dormant state with a minimum of active tissue (Blum, 1988). This implies that active tissue is unable to withstand below zero temperatures. Although this is on the whole true, resistance of active tissue to low temperatures is possible, albeit to a limited extent (Blum, 1988). Freezing resistance in a plant can be defined as the ability to conserve life so that regrowth is possible after freezing.

Plant water may supercool to very low temperatures without the formation of ice crystals when there is no source of ice nucleation present. Once ice formation has been initiated, freezing can occur both intra- and extracellularly. It appears that ice formation rather than low temperature harms plants (Blum, 1988). Depending on the rate at which the cell is cooled, intracellular freezing may occur either by internal nucleation of ice crystals or be due to penetration of external ice into the cell (Mazur, 1969). Although intracellular freezing disrupts the cell (Öquist and Martin, 1986), intracellular ice does not readily form as the plasma membrane is an effective barrier against intrusion of extracellular ice and the cell usually lacks efficient ice nucleators (Levitt, 1972; Blum, 1988).

The presence of ice crystals outside the cell is termed extracellular freezing. In this case, ice nucleation usually commences on the cell walls and xylem elements (Olien, 1967; Jeffree *et al.*, 1987). Extracellular ice formation causes cell dehydration until a thermodynamic equilibrium is reached between the fluid cell water and the extracellular ice (Mazur, 1963, 1969; Levitt, 1980; Öquist and Martin, 1986) with the amount of water lost by the cell determining its rate of dehydration (Blum, 1988). The formation of this equilibrium results in further strains on the plant cell such as concentration of the contents of the cell as water is lost to the exterior and, as a result, the protoplast adheres to the cell wall. Both the protoplast and cell wall eventually collapse as freezing removes more water from the cell (Levitt, 1980). Prevention of cellular dehydration could be reduced by an increase in internal cell osmolality (Blum, 1988).

The rate at which ice crystals form in plant cells depends on a variety of factors, including the amount of osmotically available water outside the cell relative to the amount of intracellular water (Wenkert, 1980), the rate of cooling (Mazur, 1969), the extent to which ice crystals are prevented from entering the cell by the plasma membrane (Dowgert and Steponkus, 1984) and the solute concentration of the cell (Wenkert, 1980). In whole plants the spread of ice crystals can be slowed down, or prevented, by the presence of physical barriers such as nodes. Thus buds can be supercooled while the rest of the plant contains ice crystals (Blum, 1988). The formation of ice crystals in large spaces in organs, for example in the space between the epidermis and the mesophyll of a leaf, can act as a protective mechanism as ice is concentrated into areas where little damage can occur (Blum, 1988).

Work by Steponkus (1984) on protoplasts has led to a better understanding of the phenomenon of the freeze-thaw cycle which is said to have a large role in freezing damage even though there is divided opinion as to its applicability (Li, 1984). Injury caused by the freeze-thaw cycle, for example expansion-induced lysis, where cells expand due to an influx of water on thawing, depends on temperature, cooling rate and whether the plant is hardened or not (Blum, 1988).

Using protoplasts Blum (1988) found that, as well as causing injury through dehydration, low temperatures may have a disruptive effect on the stability of the lipid and protein bilayer of membranes. In turn this damage will affect the degree of supercooling in a cell, the temperature at which ice will form, the rate of injury of a cell and the capacity for surviving thawing.

1.3.2 Freezing tolerance

Many plants are able to become freeze-tolerant or "hardened" in response to a cold or freezing environment. This response involves physiological, chemical and physical processes and reactions at all levels in the plant (Öquist and Martin, 1986; Blum, 1988; Guy, 1990). This is the positive effect of exposure of the plant to stress once exposed to low temperatures, and is a reversible process (Blum, 1988; Guy, 1990). Temperature is thought to be the most important factor in inducing acclimation, but other factors such as light intensity, photoperiod, nutrients and plant-water status also play a role (Blum, 1988; Guy, 1990). The hardening process is thought to be partially responsible for shifting plant metabolism toward a more freeze tolerant dormant state (Li and Sakai, 1982).

Plants appear to have developed mechanisms which enable them to withstand freezing temperatures and develop "hardiness". During hardening the cellular solute concentration, sugars, amino acids and other organic acids accumulate in most freezing tolerant plants. This increase in cellular concentration or decrease in osmotic potential, decreases the cellular freezing temperature and permits a smaller loss of water to external ice formation (Blum, 1988). The amount of bound water in the cell influences the freezing tolerance of plants as bound water does not participate in the cell's osmometric response, and lower temperatures are required to remove bound water from a cell (Williams, 1981). Although increases in solute concentration have an effect on freezing tolerance, this effect is usually limited to a 4°C depression in the freezing point (Levitt, 1980; Williams, 1981).

Hardened cells show reduced plasma membrane injuries as a result of cell expansion and contraction during the freeze/thaw cycle. This was accounted for by an increase in the reservoir of membrane materials in hardened cells (Steponkus, 1984), or by a release and reincorporation of lipid material on contraction and expansion of the membrane, respectively (Williams, 1981). Membranes in hardened cells have been seen as more stable and fluid, possibly due to a high rate of membrane lipid unsaturation (Blum, 1988). For example, the ratio of unsaturated to saturated fatty acids (*viz.* monogalactosyldiacylglycerol - MGDG), in thylakoid membranes of pine needles decreased as winter progressed in acclimated plants (Öquist and Martin, 1986). Stability would allow further supercooling of the intracellular solution as there is less mechanical disruption of the

membrane and the freezing temperature of the cell can be reduced (Levitt, 1980; Pomeroy *et al.*, 1983).

A good correlation has been found between the level of MGDG unsaturation and the rate of electron transport, indicating a relationship between lipid structure and plastoquinone, which links photosystem II and the cytochrome f-b_6 complex (Öquist and Martin, 1986). Even in frost tolerant species, photosynthesis is to a large extent inhibited by freezing temperatures directly, or indirectly through cell dehydration at freezing temperatures. Excess light has a photoinhibitory effect at low temperatures, but this effect is eventually overridden by the effect of temperature itself (Öquist and Martin, 1986). Plants adapted to low temperatures are able to enter a state of dormancy and to recover from inhibition of photosynthesis to a greater extent than frost sensitive plants.

As photosynthesis is inhibited at freezing temperatures, high light intensity will result in rapid photoinhibition as leaves cannot dissipate excess energy under these circumstances (Jankhe *et al.*, 1991). As molecular oxygen has a role in photoinhibition, the removal of this compound will be an important aspect of tolerance in frost tolerant plants (Jankhe *et al.*, 1991). Frost tolerant plants have been found to contain higher amounts of glutathione and glutathione reductase on hardening (de Kok and Oosterhuis, 1983). It has been proposed that these compounds regulate the thiol/disulphide ratio in proteins and may therefore protect cell membranes against peroxide and free radicals that form as a result of photoinhibition due to frost inhibited photosynthesis (Halliwell and Foyer, 1978; de Kok and Oosterhuis, 1983).

Under low temperature conditions metabolic changes such as alterations in membrane lipid composition (Lynch and Thompson, 1984) and changes in RNA and protein content (Marmiroli *et al.*, 1986; Catavelli and Bartels, 1989) occur. Evidence suggests a possible molecular basis for these metabolic changes. A number of enzymes from plants at low temperatures show changes in activity, freeze stability and isozyme variation, compared to plants kept at warmer temperatures (Guy, 1990). Peroxidase, lactate dehydrogenase, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase activities increased during acclimation (Guy, 1990), while wheat invertase changed from a low to a higher molecular weight form (Roberts, 1978). Under low temperature new isozymic variants for some enzymes, including ATPases, esterases and peroxidases, were observed in frost tolerant plants (Krasnuk *et al.*, 1976). The freeze stability of many of these enzymes was increased under these conditions. Under freezing temperatures in cold tolerant plants, ribulose diphosphate carboxylase-oxygenase (Rubisco) undergoes a stable conformational change and its kinetic properties and cryostabilities are altered (Huner *et al.*, 1981). Very few of these observations have been explained, but in general it is presumed that these changes enable enzymes to survive freezing temperatures (Guy, 1990).

The induction of freezing tolerance has been linked to the accumulation of soluble proteins in many plants (Siminovitch and Briggs, 1953; Levitt, 1972). Although it is doubted that soluble proteins accumulate due to freezing temperatures (Guy, 1990), it has been proposed that cold acclimation requires the synthesis of new proteins during the development of maximal freezing tolerance (Weiser, 1970). This hypothesis has been well supported as numerous authors have found that many cold-acclimated plants contain new protein species (Uemura and Yoshida, 1984; Marmiroli *et al.*, 1986; Sarhan and Perras, 1987). It has also been found that cold-acclimated tissue can synthesize protein faster than nonacclimated tissue and that new proteins appear rapidly (within one day) after exposure to low temperatures (Marmiroli *et al.*, 1986). Weiser (1970) also proposed that in freezing tolerant plants there was a transcriptional activation of a set of genes not normally expressed. This part of his theory has also been substantiated as newly translatable mRNAs were induced in leaves exposed to low temperatures in many cold tolerant plants (Johnson-Flanagan and Singh, 1987; Tseng and Li, 1987).

Exposure to low temperatures often results in the accumulation of low molecular weight compounds with cryoprotectant properties e.g. disaccharide and trisaccharide sugars (Levitt, 1972; Yelenosky, 1979; Salerno and Pontis, 1982). Such compounds help sustain vicinal water around proteins by decreasing protein-solvent interaction (Yancey *et al.*, 1982) and stabilize membranes through interactions with the polar head groups of phospholipids (Marmiroli *et al.*, 1986; Anchoroguyit *et al.*, 1987). Examples of other cryoprotective compounds are carbohydrates (sucrose, raffinose and sorbitol), proline, polyamines, antioxidants (ascorbate, glutathione) and glycinebetaine. These compounds appear to have roles as osmolytes and cryoprotectants, and the enzymes present that are concerned with these compounds would seem to be possible targets for upregulation at freezing temperatures (Guy, 1990).

1.3.3 Freezing avoidance

Another means of coping with freezing temperatures is freezing avoidance. This is the phenomenon of supercooling, which is controlled by lack of sources of nucleation or by attributes of the plant that involve the "stability of the system" (Blum, 1988). Supercooling has been found to be effective down to temperatures of -47°C. Levitt (1980) proposed six conditions for supercooling: 1. small cell size, 2. little or no intercellular spaces for nucleation, 3. a relatively low moisture content, 4. absence of internal nucleators, 5. barriers against external nucleators and 6. presence of antinucleators. Supercooling is only an attribute of parts of the plant (for example the buds, woody parenchyma and flowers) (Blum, 1988). A disadvantage of supercooling as a freezing-avoidance mechanism is that if supercooled tissues freeze, the results are lethal (Levitt, 1980).

1.3.4 Chilling stress

Tropical or subtropical plants are usually more susceptible to chilling, although temperate zone plants may also be sensitive (Lyons,1973). In general any temperature below 10°C can produce signs of chilling stress, with the injury usually increasing with the degree of chilling (Levitt, 1980), and varying with the chill-sensitivity of the plant undergoing the stress (Lyons, 1973). Three kinds of chilling stress injury may occur: 1. direct, 2. indirect and 3. secondary stress injury.

1.3.4.1 Direct Injury

Direct injury occurs very rapidly (after a few minutes to a few hours exposure) (Levitt, 1980). Tissues exposed to temperatures below 10°C have shown halted cytoplasmic streaming and pseudoplasmolysis (Levitt, 1980). This has been explained by a sudden increase in permeability resulting in leakage of cell solutes (Lieberman *et al.*, 1958). Solute leakage was observed to occur at both a slow and fast rate. These rates coincided with different phases of solute leakage. During the second phase of rapid leakage, most of the electrolyte was lost from the tissue (Levitt, 1980) and obvious lesions appeared. Lipid phase separation in the membrane after exposure to cold temperatures is a possible explanation of ion leakage in the blue-green alga *Anacystis nidulans* (Ono and Murata, 1981). Applicability of the behavior of this blue-green alga to that of higher plants is dubious, but various authors have expressed the view that there may be some correlation (Graham and Patterson, 1982). Passive ion leakage would occur if chilling in some way prevented the production or utilization of ATP necessary for the maintenance of ion gradients across the cell membrane (Penning de Vreis, 1975).

1.3.4.2 Indirect Injury

Levitt (1980) identifies solute leakage as the main cause of indirect injury. For this to occur, light is necessary and it is possible that a decrease in active ion uptake results in more permeable membranes. Evidence has shown that there is a disruption of an active ion uptake process in chilling sensitive plants (Lyons, 1973; Nordin, 1977), possibly indicating that slow, indirect injury is due to a metabolic disturbance (Levitt, 1980). This damage may be irreversible (Terashima *et al.*, 1991a).

Many kinds of metabolic disturbances have been proposed to lead to chilling injury. At low temperatures it is possible for the rate of respiration to exceed the photosynthetic rate. Damaged chloroplast thylakoids would result in a decrease in photosynthesis as they are less able to maintain a high energy state (Garber, 1977; Melcarek and Brown, 1977), giving rise to the concept of "starvation" of the plant at low temperatures, or the idea that carbohydrate reserves would be utilised more rapidly than they were produced (Levitt, 1980). Although most chilling damage appeared to occur before all plant reserves were utilized, it has been found that in chilling sensitive

plants translocation was inhibited and only recovered slowly after exposure to low temperatures. This may account for a decrease in photosynthesis and result in root starvation (Crawford and Huxter, 1977).

Chilling temperatures may inhibit the aerobic and not the anaerobic phases of respiration (Levitt, 1980). Numerous workers have supported this hypothesis by showing that chilling inhibits aerobic respiration to a greater extent than it does anaerobic respiration in chilling sensitive plants (Murata, 1969; Lyons, 1973), although the phase of aerobic respiration inhibited is unknown. As anaerobic respiration continues at chilling temperatures without its aerobic counterpart, toxic intermediates and end products, such as acetaldehyde and ethanol, are produced which would explain chilling injury (Murata, 1969; Lyons, 1973). Peroxidases could also be produced, as there would be higher levels of oxygen in the tissue due to inhibited aerobic respiration, and these would oxidise substrates (Lyons, 1973). This oxidative stress is thought to influence chilling injury (Hariyadi and Parkin, 1993).

Perhaps one of the most possible explanations of chilling-induced injury is the occurrence of biochemical lesions. These are abnormalities in metabolism which result in deficiencies of essential intermediary metabolites (for example, ATP) (Levitt, 1980). Although evidence for the occurrence of biochemical lesions is indirect, effects such as cessation of cytoplasmic streaming, inhibition of ion uptake leading to ion leakage, and the inhibition of protein synthesis can be explained by them (Levitt, 1980).

1.3.4.3 Secondary Stress Injury

Death of plants after exposure to chilling temperatures can also be caused by secondary water stress. When chilled, roots do not appear to be able to absorb water rapidly enough to replace that lost by transpiration (Levitt, 1980). Chilling sensitive plants were less able to absorb water through their roots than tolerant plants and it has been observed that decreased absorption is due to a decrease in permeability of the root cell (Kaufmann, 1975).

1.3.5 Chilling acclimation.

Acclimation to cold temperatures, as for freezing temperatures, results in a lowering of the temperature at which the plant is damaged or killed. It can be seen as a gradual adaption to low temperature and involves the synthesis of components such as proteins, lipids, enzymes, metabolites and membranes which either confer cold tolerance or protection from the cold (Graham and Patterson, 1982).

During acclimation, increases in RNA, membrane-bound and soluble proteins were observed (Cabané *et al.*, 1993; Jarillo *et al.*, 1993; Werentilnyk *et al.*, 1993). Tolerance to cold conditions has been found to induce an increase in the number of translatable mRNAs in alfalfa (Castonguay *et al.*, 1993). This suggests a role in desiccation tolerance which follows extracellular freezing. Some of these mRNA encode proteins with a high sequence homology with members of the heat-shock protein (HSP 70) family (Cabané *et al.*, 1993). The mRNA may also encode polypeptides with an antinucleating role (Gilmour *et al.*, 1992; Houde *et al.*, 1992). Increases in RNA and various proteins could result in the presence of isozymes which are active at cold temperatures and a higher content of enzyme protein (Levitt, 1980). Support for this has been provided as structural and kinetic properties of Rubisco change when plants are cold hardened (Graham and Patterson, 1982). Decreases in thermostability, activation energy and specific activity of malate dehydrogenase in cold tolerant plants were observed (Simon, 1979). These may involve modification of regulatory processes rather than changes in amino acid sequence of the enzyme (Graham and Patterson, 1982).

In most plant membranes at cold temperatures, there is an increase in lipids, especially phospholipids, and an increase in the degree of unsaturation of fatty acid chains within those membranes (Clarkson *et al.*, 1980). It is commonly believed that the changes in these lipids are required to maintain the fluidity of plant membranes within those limits required for the efficient functioning of membranes at low temperatures (Berry and Raison, 1982). Increases in unsaturation of fatty acids results in a decrease in the phase transition temperature which ensures membrane fluidity and high permeability to water at low temperatures (Levitt, 1980). There appears to be a relationship between the amount of linolenic acid (a predominant unsaturated fatty acid in plants) and the degree of cold hardiness in many plants (St John *et al.*, 1979; Levitt, 1980). The availability of oxygen may be important in chill hardening as this is the rate limiting factor for the desaturation of fatty acids (Harris and James, 1969).

It seems that membrane phase changes are not observed in chilling-resistant species and it is generally conceded that, although the bulk membrane lipids of chilling-sensitive plants do not undergo gel-to-liquid crystalline phase changes, some minor lipid fractions do undergo such a transition (Lyons, 1973). There is evidence that the acclimation of woody species generally results in an increase in phospholipids (Yoshida and Sakai, 1973). This is probably associated with a general proliferation of cell membranes (Graham and Patterson, 1982), a factor which appears to show good correlation with cold hardiness. Although this does not necessarily result in a change in the degree of lipid unsaturation of woody plants, the cold hardening mechanism is thought to be similar in these and herbaceous plants (see Graham and Patterson, 1982).

Although unsaturation of membrane lipids is necessary for chilling tolerance, other factors are more important, possibly because unsaturated fatty acids form lipid peroxides (Levitt, 1980). These peroxidases reduce hydroperoxides from polyunsaturated fatty acids, for example glutathione peroxidase/reductase. It is possible that under normal circumstances this reaction is controlled by the reducing power in active cells but, as the temperature decreases metabolism, less reducing power is produced (Christophersen, 1969). The factors that are thought to play an important role in chilling stress damage are cell-mediated damage and photodynamic damage associated with an inability of the photosynthetic apparatus to function at low temperatures. There is doubt as to whether membrane lipids and/or fluidity is concerned with these factors (see Graham and Patterson, 1982).

Plants adapted to low temperatures are generally characterised by a low optimum for net photosynthesis, a high photosynthetic rate at suboptimal temperatures and a low tolerance for high temperatures (Öquist and Martin, 1986). On return to favourable conditions, they are also able to regain a high rate of net photosynthesis within a short period. Chilling-tolerant plants may be able to maintain high levels of carbohydrate reserves with change with alterations in photosynthetic capacity, rate of growth and degree of cold tolerance through the year (Amundson *et al.*, 1993). Photoinhibition is a decrease in the efficiency of photon utilization by PSII phytochemistry of the plant (Demmig *et al.*, 1988; Adams III *et al.*, 1990; Somersalo and Krause, 1990), and is possibly the result of a variety of processes working either singly or in combination: the antennae of PSII may absorb more light than can be dissipated in an orderly fashion (Demmig *et al.*, 1987; Öquist and Huner, 1991), or there may be an increase in the rate constant for nonradiative dissipation of excitation energy (Demmig *et al.*, 1987). Chilling plants renders them more sensitive to photoinhibition, resulting in a given light level that previously was not excessive becoming so (Greer *et al.*, 1988). Low temperatures may inhibit the *de novo* synthesis necessary for the repair of photodamage (Greer *et al.*, 1986), or they may affect the alternative routes of excess energy dissipation (Krause, 1988). Dissipation of excitation energy via other pathways leads to fluorescence quenching, which may be a strategy to allow for the survival of the photosynthetic structures under stress conditions (Demmig *et al.*, 1988; Somersalo and Krause, 1990a).

Although it appears that photoinhibition at low temperatures is an important cause of decreased photosynthetic rate (Öquist and Huner, 1991), it has also been proposed that plants at low temperatures under conditions which do not lead to photoinhibition, show an inhibition of photosynthesis (Brüggemann *et al.*, 1992), possibly due to the temperature sensitivity of water oxidation in chloroplasts (Barnes and Wilson, 1984). Less chlorophyll is developed under chilling stress and this has been related to halted development of the thylakoid membrane system (Blum, 1988), with possible electron transport inhibitition (see Öquist and Martin, 1986; Jankhe *et al.*,

1991). In lower temperatures, chilling sensitive plants were found to have a lower rate of carboxylation, but it was reported that the activity of glycolate oxidase was not affected (Sawada *et al.*, 1974), suggesting that the Rubisco oxygenase activity was less affected by cold than its carboxylase activity. The rate of acclimation of photosynthesis in plants can vary from a day to a few weeks, depending on factors such as leaf age and nutrient supply or on a lower potential for acclimation (see Öquist and Martin, 1986). Photosynthetic tolerance to chilling temperatures is partly related to specific enzymes and can be seen in the amount of chlorophyll produced under stress (Blum, 1988).

Molecular oxygen has been found to have a role in the photoinhibition of photosynthesis and, therefore, it would be important to remove this compound to confer a measure of resistance against photoinhibition on plants (Jankhe *et al.*, 1991). A mechanism thought to enable this is oxidation/reduction or the SH-exchange reactions of membrane proteins (Levitt, 1980; de Kok and Oosterhuis, 1983). As glutathione is a protein with an attached sulphydryl group, it would be involved in these reactions (de Kok and Oosterhius, 1983). The oxygen radical scavanging system of plants is comprised of superoxide dismutase (SOD), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and ascorbate peroxidase (APX) (Nakano and Asada, 1987; Jankhe *et al.*, 1991). Although little is known about the relationship between chilling-dependent photoinhibition and the activities of these enzymes, it has been reported that cold hardening increases the activity of some of the scavanging enzymes, including GR, and decreases the substrates of these enzymes in chilling-tolerant species (Esterbauer and Grill, 1978; de Kok and Oosterhuis, 1983; Jankhe *et al.*, 1991, Kuroda *et al.*, 1991). An increase in GR can be explained by an increase in glutathione. As more oxygen radicals are found in the system, there is an increase in reduced glutathione (Charles and Halliwell, 1980; Dhindsa, 1991), which may regulate the thiol/disulphide ratio in proteins, resulting in the protection of cell membranes against peroxide and free radicals (de Kok and Oosterhius, 1983). As reduced glutathione acts via GR (Halliwell and Foyer, 1978), there will be an increase in GR activity.

Factors such as proline accumulation and photoperiod shortening are involved in both cold acclimation and freeze hardening. More tolerant plants may be able to maintain high levels of carbohydrate reserves, mainly soluble sugars and starch, which change with alterations in photosynthetic capacity, rate of growth and degree of cold tolerance, e.g. Red Spruce (Amudson *et al.*, 1993). Various structural changes, such as changes in leaf thickness, mesophyll cell size and chlorophyll concentration also occur during cold acclimation (Öquist and Martin, 1986). Frost-hardening may decrease the reduction state of Q_A, the primary, stable quinone acceptor of PSII. This has been linked to increased availability of phosphate and resulted in decreased sensitivity of rye to photoinhibition (Hurry *et al.*, 1993). Photoinhibition was decreased in non-

hardened rye when they were fed orthophosphate as photosynthetic capacity was increased and the reduction state of Q_A was lowered (Hurry *et al.*, 1993).

Freezing and cold stress subject the plant to conditions that quickly induce death unless protective mechanisms in the plant are activated. Only those plants which are able to resist or tolerate low temperatures (acclimated plants) have these mechanisms, and it is these plants which are consequently able to survive the stress. Acclimation or hardening appears to be induced by a variety of factors. One of the most obvious is the presence of cryoprotective compounds such as proline, disaccharide or trisaccharide sugars. Lipid membrane unsaturation is also protective as hydroperoxides can be reduced from polyunsaturated fatty acids by glutathione reductase. The antioxidant system has a role to play as this system removes molecular oxygen which is involved in photoinhibition. The photosynthetic processes are able to adapt to low temperatures in resistant or tolerant species, allowing these plants more rapid and complete recovery from the stress than non-adapted plants.

1.3.5.1 Role of Proline

Under stress conditions, the amount of soluble proteins in hardened plants increases (Chen and Li, 1977; Siminovitch and Cloutier, 1982; Bassi and Sharma, 1993; Venkateswarlu and Ramesh, 1993). These soluble proteins appear to be low molecular weight compounds and are called compatible solutes. They accumulate in plant cells in response to stress and have little effect on metabolism, even when in high concentrations (Smirnoff and Cumbes, 1989; Diamantoglou and Rhizopoulou, 1992; Naidu *et al.*, 1992b). Under stress conditions these low molecular weight organic solutes may comprise up to 10% of the tissue dry weight (Stewart and Lee, 1974) and may contribute significantly to the adaptive ability of plants under such conditions (Aspinall and Paleg, 1981; Bassi and Sharma, 1993). Compatible solutes, which are thought to act as cytoplasmic osmotica, can be grouped into three major types: (i) proline and amino acids (ii) betaines, primarily glycinebetaine, and (iii) sugar alcohols such as mannitol, sorbitol and pinitol (Naidu *et al.*, 1991; Smirnoff and Cumbes, 1992; Venkateswarlu and Ramesh, 1993). These compounds are able to protect enzyme structure and function (Paleg *et al.*, 1984; Naidu *et al.*, 1991; Nikolopoulos and Manetas, 1991), regulate osmotic potential (Aspinall and Paleg, 1981), cytosolic pH (Venekamp, 1989) the NAD/NADPH ratio (Alia *et al.*,1991) and stabilize membranes (Cloutier, 1983; Jolivet *et al.*, 1983; Paleg *et al.*, 1984; Koster and Lynch, 1992). Mannitol, and proline to some extent, has been found to be a hydroxyl scavenger (Alia *et al.*, 1991; Smirnoff and Cumbes, 1992).

Although betaines and sugar alcohols are of importance in chilling tolerance of plants, an emphasis was placed on proline in this project. As a result, the affect of amino acids, and particularly the

imino acid proline, will be discussed here. There are three possible reasons for the increase in amino acid content of stressed, particularly cold stressed, plants. Firstly, at low temperatures, the respiratory activity of plants is low (Raison, 1980), resulting in the accumulation of several TCA cycle compounds, which could promote the synthesis of specific amino acids (Boggess *et al.*, 1976). Secondly, a decrease in the rate of protein synthesis at low temperatures may cause an accumulation of amino acids, and thirdly, the entry of these amino acids into the respiratory chain may be inhibited by low temperatures, allowing the amino acid accumulation (Naidu *et al.*, 1991).

Proline has been found to accumulate in response to stress conditions such as cold, heat, water, metal ion and osmotic stress (Chu *et al.*, 1978; Yelenosky, 1979; Ilhai and Dorffling, 1982; Mukherjee and Choudhuri, 1983; Voetberg and Stewart, 1984; Aloni and Rosenshtein, 1984; Blum, 1988; Charest and Phan, 1991; Naidu *et al.*, 1992a; Bassi and Sharma, 1993; Dörffling *et al.*, 1993). Proline accumulation appears to be proportional to the degree of stress the plant is subjected to (Voetberg and Stewart, 1984; Treichel *et al.*, 1984) and is connected with resistance to injury (see Chu *et al.*, 1978). Possible differences may be accounted for by differences in proline synthesis (Joyce *et al.*, 1992). Proline has been associated with freeze hardening and cold tolerance (Dörffling *et al.*, 1993), although the presence of light was found necessary for the accumulation of proline in both cases (Chu *et al.*, 1978; Yelenosky, 1979). Joyce *et al.* (1984) found that light does not appear to be the initiator of proline synthesis as plants are able to accumulate proline in the dark, but continuous or prolonged exposure to light was found to enhance accumulation. It has been suggested that this light stimulation is dependent on the provision of energy-rich compounds from photosynthesis, for example, NADPH (Noguchi *et al.*, 1968; Joyce *et al.*, 1992) or carbohydrates (Stewart *et al.*, 1966; Stewart, 1978; Joyce *et al.*, 1992). Adams and Frank (1980) suggest that two molecules of NAD(P)H are able to produce one molecule of proline from glutamic acid. Tolerant plants appear to accumulate less proline than sensitive plants during the stress period (Aloni and Rosenshtein, 1984). Proline accumulation is thought to be the consequence of a specific metabolic event rather than the consequence of temperature-affected changes in the amino acid pool (Chu *et al.*, 1978), and the process is sensitive to changing climatic conditions (Treichel *et al.*, 1984).

The formation of proline (Figure 1.1) appears to be degradative in higher plants, via ornithine transaminase (Keuh *et al.*, 1984) and is generally associated with conditions of growth inhibition and delays of senescence, implying that proline is released from a protein broken down during the later stages of senescence (Chu *et al.*, 1978). Proline synthesis appears to be subject to feedback regulation (Keuh *et al.*, 1984). Although this enzyme has not been identified, it is thought to be glutamate kinase as preparations of this enzyme have been shown to be proline-sensitive (Keuh *et*

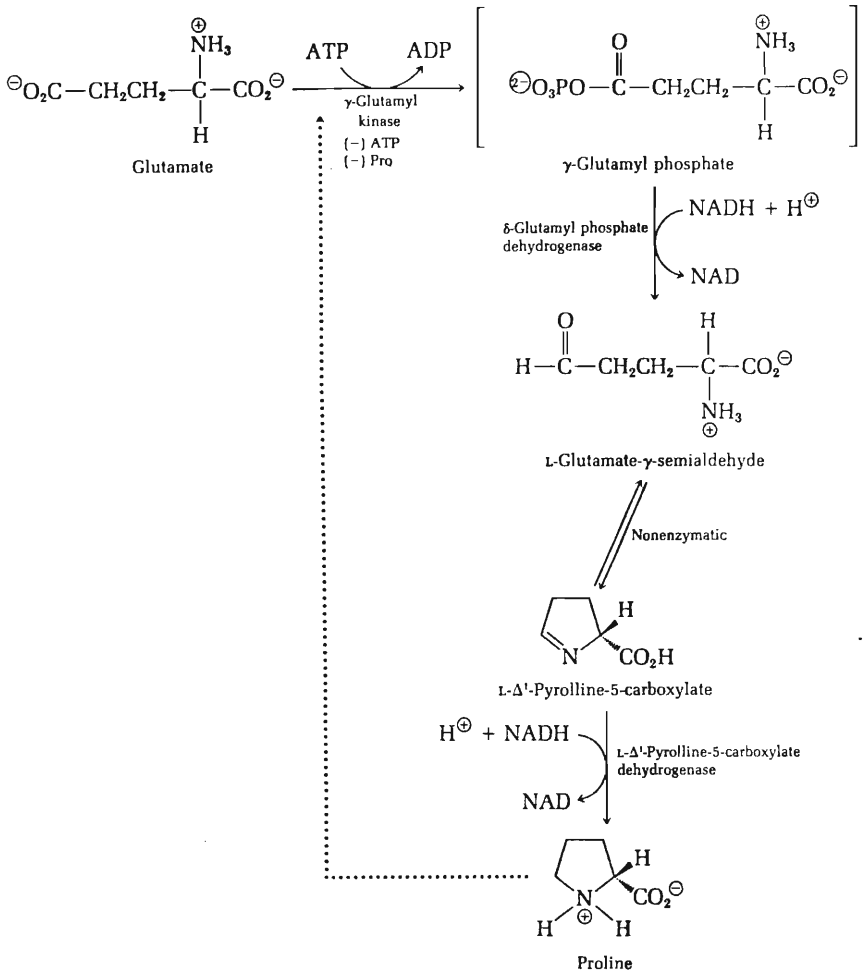

Figure 1.1 Pathway for proline biosynthesis. The first step of the pathway is allosterically inhibited by proline. From Rawn (1983).

al., 1984). It appears that plants differ in the biochemical method through which they accumulate proline - different stress treatments seem to induce proline accumulation differently (Boggess *et al.*, 1976; Chu *et al.*, 1978; Voetberg and Stewart, 1984). The enzymes involved in the synthesis of proline increase their activity under conditions of stress (Argandona and Pahlich, 1991), and this appears to be related to the enhancement of glycolysis under similar conditions. As stress reduces glucose consumption via glycolysis, more glucose is available for the activation of proline synthetic enzymes (Argandona and Pahlich, 1991). Boggess and Stewart (1980) have shown that proline accumulation in barley leaves is caused by *de novo* synthesis of proline and not by an increase in proteolysis as suggested by other authors (see Chu *et al.*, 1978; Keuh *et al.*, 1984). Alternatively, it has been suggested that for proline to accumulate there should be a decrease in the rate of proline oxidation, or an increase in the rate of synthesis from glutamic acid. These factors could occur simultaneously (Dallmier and Stewart, 1992). The first two enzymes in the synthetic pathway of proline are not known, but the third is thought to be pyrroline 5-carboxylate reductase (P5CR) (McNamer and Stewart, 1974; Miler and Stewart, 1976; Rayapati *et al.*, 1989). This is the enzyme that catalyses the production of proline from pyrroline 5-carboxylate (P5C) (Kohl *et al.*, 1991). Boggess *et al.* (1976) found that the synthetic rate of P5C controlled the rate

proline accumulation, although in salt-stressed plants, Laliberté and Hallebust (1989) and Delauney and Verma (1990) found that an increase in P5C activity caused increased proline accumulation. An increase in P5CR activity may also play a role in proline accumulation as this may, firstly, increase the activity of the oxidative portion of the pentose phosphate pathway by producing $NADP^+$, which would then increase proline accumulation (Joyce *et al.*, 1992). Secondly, an increase in P5CR activity may allow the production of proline under stress conditions (Kohl *et al.*, 1991).

Although free proline has been found in leaves, bark and sapwood of trees (*Ceratonia siliqua, Laurus mobilis, Myrtus communis, Pinus halepensis*) (Diamantoglou and Rhizopoulou, 1992), the location of proline synthesis is uncertain (Kohl *et al.*, 1991; Voetberg and Sharp, 1991; Diamantoglou and Rhizopoulou, 1992). Kohl *et al.* (1988) found P5CR to be localized in the cytosol, but Rayapati *et al.* (1989) have put forward evidence for its location in plastids, primarily chloroplasts. They found a stimulation of P5CR activity under conditions such as higher NADPH and salt concentrations. These conditions are found in chloroplasts and their work has been supported by evidence of other workers (Krueger *et al.*, 1986; Kohl e*t al.*, 1988; Alia *et al.*, 1991).

Although the exact role of proline is not known (Duncan and Widholm, 1987), several roles have been attributed to supraoptimal proline levels. It is thought to be a cell-compatible solute as it is very soluble and non-toxic in large quantities and, when in large amounts, proline may contribute to osmotic adjustment (Stewart and Lee, 1974; Hellergren and Li, 1981; Aloni and Rosenshtein, 1984; Blum, 1988; Voetberg and Sharp, 1991).

Proline could act as a storage compound for energy and, as significant amounts of nitrogen and carbon may be temporarily sequestered in the form of proline (Naidu *et al.*, 1992b), could be used during post-stress metabolism (Barnett and Naylor, 1966; Stewart *et al.*, 1966; Aloni and Rosenshtein, 1984; Joyce *et al.*, 1992). For example, proline could be used as a carbohydrate source for the Krebs cycle (Stewart, 1972). Proline may be useful in adjustment of the entire plant under stess conditions (Diamantoglou and Rhizopoulou, 1992).

Another possible role is protein stabilization as proline is able to bind water to proteins and so maintain their hydration (Heber *et al.*, 1971; Ilhai and Dorffling, 1982; Aloni and Rosenshtein, 1984). This is thought to result in cell wall and membrane modification (Heber *et al.*, 1971), which would, to a certain extent, explain why proline is able to protect membranes from disruption when tissue is placed in stress conditions (Blum, 1988). As proline is able to bind water to proteins, it is able to assist in prevention of enzyme denaturation (Paleg *et al.*, 1981).

Proline has been observed to enhance PSII activity of isolated thylakoids and to decrease the loss of photochemical activity of thylakoids exposed to strong light by preventing the photoinhibitory loss of choroplast activity (Alia *et al.*, 1991). Proline is also able to protect components involved in water oxidation. These roles of proline are possible as it is thought that proline is able to reduce the production of, or to scavenge, free radicals, thereby decreasing lipid peroxidation (Smirnoff and Cumbes, 1989; Alia *et al.*, 1992). Under conditions of cold stress this is of importance as oxygen radical production increases (Patterson *et al.*, 1974; Smirnoff and Cumbes, 1989).

Another possible role given to proline is that of a redox shuttle (Kohl *et al.*, 1988). Under unstressed conditions P5CR would transport reducing potential to mitochondria in the form of proline, while under stressed conditions the shuttle would be uncoupled, resulting in an accumulation of proline (Rayapati *et al.*, 1989). This is presumably possible as proline is non-toxic and has biocompatible characteristics, even when in high concentrations in the cell.

1.3.5.2 Role of Glutathione Reductase

Although oxygen is necessary for life, it can become a source of toxins (oxygen free radicals) and endanger the life it enables. For example exposure of membranes to superoxide increases the lipid phase transition temperatures, releases lipid phosphates and increases the accumulation of free fatty acids (Kendall & McKersie, 1989). Free radicals produced from oxygen are able to participate in a range of biological reactions such as catalytic oxidase reactions, electron transport systems (Elstner, 1982), deesterification reactions (Mead, 1976; Serenatna *et al.*, 1985; Katsaras *et al.*, 1986), and they may increase the degradation of certain labile polypeptides and increase the activity of a thylakoid-bound endopeptidase (Casano and Trippi, 1992). In these ways oxygen free radicals play a role in mediating the effects of freezing or chilling injury to membranes in non-acclimated tissue (Kendall and McKersie, 1989).

Oxygen radicals are produced by a variety of conditions, and the effect appears to be exacerbated by stressful environmental conditions (Kendall & McKersie, 1989; Schöner & Krause, 1990). The Mehler reaction (photoreduction of oxygen) produces superoxide anion radicals ($O_2^{\cdot-}$) (Asada *et al.*, 1974; Badger, 1985; Robinson, 1988; Schöner & Krause, 1990). These are then dismutated by the chloroplast to hydrogen peroxide (H_2O_2). Oxygen radicals can be generated by the enzymes peroxidase (Yamazaki and Pietts, 1968), galactose oxidase and aldehyde oxidase (Fridovich, 1970). Electrons may "leak" from intermediate carriers of the mitochondrial electron transport chain and react with oxygen to produce radicals (Rich and Bonner, 1978; Orrenius, 1985) which contribute to the pool of oxygen radicals. Under conditions of stomatal closure, CO_2 fixation is low, resulting in limited $NADP^+$ as an electron acceptor. Oxygen is able to function as an alternative acceptor, resulting in the formation of $O_2^{\cdot-}$ and H_2O_2 (Egneus *et al.*, 1975). Although both of these

compounds are reactive and able to initiate damaging reactions, even more reactive and toxic are singlet oxygen ($^1O_2$) and hydroxyl radicals (OH^-). Both of these radicals are strong oxidising agents (Salin, 1987), especially $^1O_2$, which can attack biological molecules such as amino acids and polyunsaturated fatty acids to produce lipid peroxides which result in membrane damage (Larson, 1988). Free radicals are produced in chloroplasts, mitochondria and peroxisomes (Sandalio *et al.*, 1988), and there is evidence for their production in other organelles (see Sandalio *et al.*, 1988). It has been suggested that PSII is the site for the origin of radical formation, especially when these are involved in photoinhibition (Tsciersch and Ohmann, 1993).

$O_2^{\cdot-}$ can be produced through a number of pathways. It is the result of the first step in univalent oxygen reduction and can also be produced through the univalent oxidation of H_2O_2 (Salin, 1987). Superoxides can be produced non-enzymatically via autooxidation of substrates such as ferredoxins, hydroquinones, thiols or reduced haemoproteins (Mehler, 1951; Fridovich, 1974, 1975, 1976; Elstner, 1982; Badger, 1985; Asada and Takahashi, 1987), or enzymatically via some flavoprotein dehydrogenases (Salin, 1987). Superoxide generation has been shown in neutrophils, monocytes and macrophages (Halliwell and Gutteridge, 1985), as well as in mitochondria (Boveris, 1984), chloroplasts (Asada, 1984), microsomes (Kuthan and Ullrich, 1982), nuclei (Patton *et al.*, 1980) and in glyoxisomes, where $O_2^{\cdot-}$ is generated as by or end products of oxygen reduction by glycolate oxidase and urate oxidase (Sandalio *et al.*, 1988). This radical is an oxidising agent (Fee & Valentine, 1977) as it has been implicated in lipid peroxidation, viral inactivation, membrane damage, cellular toxicity and single-stranded breaks in DNA (Fridovich, 1986). These effects are likely to be indirect, caused by OH^-, as this more powerful oxidant is generated by the superoxide radical. $O_2^{\cdot-}$ is able to cause direct damage such as catalase inactivation, NAD(P)H and epinephrine oxidation and it can decrease the plating efficiency of Chinese hamster cell lines (Fridovich, 1986). It is also a good reductant (Green & Hill, 1984). Superoxide is finally metabolised to water in a cycle involving ascorbic acid and glutathione (Schöner and Krause, 1990).

H_2O_2 is the most stable oxy-intermediate and is able to act as both an oxidant and mild reductant (Salin, 1987). It is produced via the Mehler reaction, along with superoxide (Mehler, 1951). It can also be formed by dismutation of two superoxide anions (Scandalios, 1993). The non-haem Fe-S center of Photosystem I may produce H_2O_2 (Elstner, 1982), and Mukerjee and Choudhuri (1983) mention glycolate oxidation by glycolate oxidase as a major source of this reactive species. Although it is relatively unreactive, H_2O_2 is able to complex with transition metals, and its toxicity may be enhanced in the presence of metal catalysts, possibly as a result of metal-catalysed hydroxyl radical formation (Salin, 1987). Its mode of toxicity is primarily via oxidation of sulfhydryl groups (Jocelyn, 1972), but the result of these reactions can only be seen when

hydrogen peroxide is present at non-physiological concentrations (Fridovich, 1976). At low concentrations, H_2O_2 inhibits light-activated enzymes in the Calvin cycle by interaction with reduced sulfhydryl groups which are necessary for activity (Kaiser, 1979; Robinson *et al.*, 1980). The catalytic breakdown of H_2O_2 is via the photorespiratory mechanism using catalase (Tolbert, 1971). The non-enzymatic removal of this reactive species involves a reaction with glycolate, resulting in the formation of CO_2 and formate (Grodzinski & Butt, 1976; Oliver, 1979; Cossins *et al.*, 1988).

OH^- can be formed when superoxide and H_2O_2 react in a "Haber-Weiss" reaction (Scandalios, 1993) and is a strong oxidising agent. Although it is highly reactive, it has a short half-life (Salin, 1987). As it will react with any available substrate, it has the potential to do enormous damage (Gamble and Burke, 1984), such as leading to DNA lesions and mutations and often leads to irreparable metabolic dysfunction and cell death (Scandalios, 1993). It has been shown that these radicals are involved in photoinhibition (Tschiersch and Ohmann, 1993).

$^1O_2$ can be formed in a number of chemical, photochemical and biochemical systems that involve free radicals, lipid peroxides or photooxidations (Krinsky, 1979; Murray, 1979; Mishra *et al.*, 1993). In photosynthetic organisms, chlorophyll can act as a photosensitizer: chlorophyll molecules absorb light and form an excited singlet ($^1Chl^*$). This is converted by intersystem crossing to $^3Chl^*$. This state allows transfer of energy from chlorophyll to oxygen, resulting in singlet oxygen (Schöner and Krause, 1990). $^1O_2$ is very reactive, able to add to enes and dienes to form hydroperoxides and endoperoxides (Foote, 1979; Krinsky, 1979), and to cause lipid peroxidation which weakens or alters membrane structure.

To enhance the ability of plants to survive conditions that lead to oxidation, these toxic oxygen radicals need to be efficiently removed. The chloroplastic scavenger system that has been shown to operate, even under non-stress conditions, is comprised of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and superoxide dismutase (SOD) (see Figure 1.2) (Foyer and Halliwell, 1976; Esterbauer and Grill, 1978; de Kok and Oosterhuis, 1983; Guy and Carter, 1984; Guy *et al.*, 1984; Nagawara and Sagisaka, 1984; Sagisaka, 1985; Asada and Takahashi, 1987). Without this enzymatic defence system, plants could not efficiently convert solar energy to chemical energy (Scandalios, 1993). Numerous authors have found this system to be operational under stress conditions, for example under cold stress (Schöner and Krause, 1990; Jankhe *et al.*, 1991; Anderson *et al.*, 1992; Cakmak and Marschner, 1992), drought stress (Gamble and Burke, 1984; Smirnoff and Colombé, 1988), exposure to air pollutants (Tanaka *et al.*, 1985) and magnesium deficiencies (Cakmak and Marschner, 1992). When plants are exposed to high light, or even

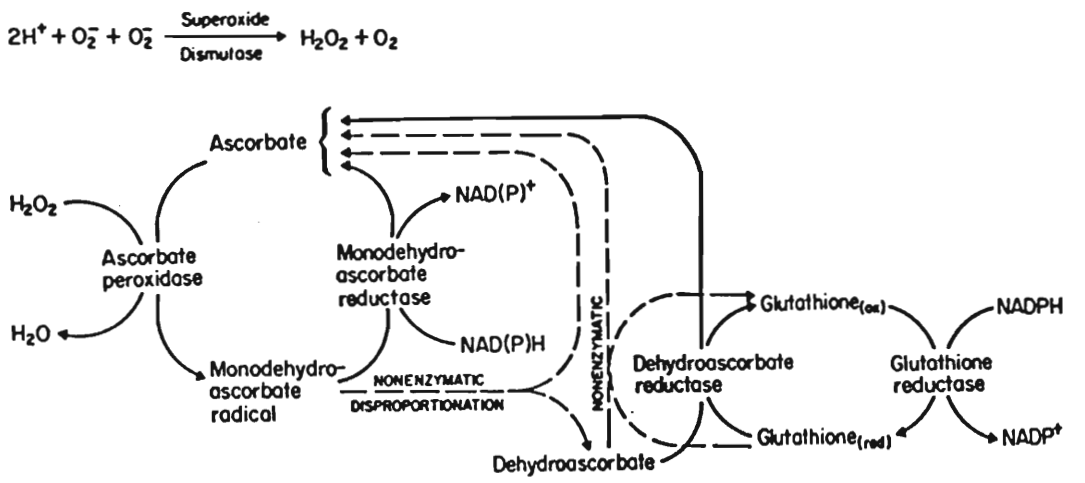

Figure 1.1 The chloroplastic oxide scavenging pathway. Solid lines indicate enzyme-catalysed and dashed lines non-enzyme-catalysed reactions are indicated. From Jahnke *et al.* (1991).

moderate light intensity (Kyle *et al.*, 1987; Somersalo and Krause, 1988, 1989), simultaneously with environmental stress that impairs absorption of light energy for CO_2 fixation, there is a higher potential for oxygen activation (Hodgson and Raison, 1991). This could be counteracted by enhanced levels of protective enzymes (Schöner and Krause, 1990), especially when the plant is exposed to stress conditions for periods of time (Esterbauer and Grill, 1978; de Kok and Oosterhuis, 1983; Guy and Carter, 1984; Schöner and Krause, 1990). These can be accounted for by the induction of enzyme biosynthesis brought about by synergism between low temperature and light (Schöner and Krause, 1990). Although the enzymes of this scavenging pathway do not always increase under stress conditions, it appears that pre-stress levels are at least maintained (Walker and McKersie, 1993).

There are three lines of evidence for the existence of this scavenging pathway. The first is that intact chloroplasts are able to photoreduce H_2O_2 rapidly with the evolution of O_2 (Nakano & Asada, 1981; Jablonski & Anderson, 1982; Anderson *et al.*, 1983). This occurs to a lesser extent, or not at all, in the dark as ascorbate is lost with concomitant inactivation of APX (Anderson *et al.*, 1983; Asada & Badger, 1984; Nakano & Asada, 1987). Secondly, ruptured chloroplasts have little photosynthetic activity, but this is recovered when GSH and DHA are added (Nakano & Asada, 1981; Jablonski & Anderson, 1982). Thirdly, all the enzymes necessary for this pathway have been isolated, purified and shown to occur in chloroplasts (Foyer & Halliwell, 1977; Nakano & Asada, 1981; Jablonski & Anderson, 1982; Anderson *et al.*, 1983; Bielawski & Joy, 1986; Gillham & Dodge, 1986; Nakano & Asada, 1987). Furthermore, calculations based on enzyme rates and substrate concentrations support the conclusion that the H_2O_2 scavenging system can

operate at the rates necessary to detoxify the H_2O_2 generated in the light by the Mehler reaction (Smith *et al.*, 1989).

Although each of these enzymes have an important role to play in scavenging free radicals, GR is of particular interest in this project as it has been found to be of importance in protecting plants exposed to chilling stress (see Jankhe *et al.*, 1991; Kuroda *et al.*, 1991). As such, this enzyme will be more extensively discussed in the following survey of these enzymes.

SOD is involved in the removal of $O_2^{\cdot-}$ to produce H_2O_2 and O_2, while the ascorbate cycle which contains APX, MDHAR, DHAR and GR, is involved in the removal of H_2O_2 to produce H_2O and O_2 (Cakmak & Marschner, 1992). Superoxide dismutases are a group of metalloenzymes which catalyse the disproportionation of superoxides. This involves the alternate reduction and oxidation of the metal associated with the enzyme (Salin, 1987). There are three different types of SODs: a Cu-Zn, a Mn- and a Fe-containing SOD (Tsang *et al.*, 1993). Although the basis for these different types has not yet been established, it appears that there are multiple genes for SODs in most plants (Scandalios, 1993). The most abundant SOD is the Cu-Zn form, which is primarily associated with the chloroplast, but also with other organelles, for example the mitochondrion (Lumsden *et al.*, 1977; Asada *et al.*, 1977). The Fe-containing SOD is found in representatives of a few families (Bridges & Salin, 1981; Sevilla *et al.*, 1984) and is also associated with the chloroplast, although not with the mitochondrion (Salin and Bridges, 1981). As a result, cellular organelles have protection against superoxide anion damage. In the soybean root, Cu-Zn SOD activity is induced by copper ions via the synthesis of cytosolic Cu-Zn SOD (Chongpraditnun *et al.*, 1992). This may be the result of either the direct effect of copper on the SOD gene, or it may be indirectly due to an increase in superoxide anion levels as copper is known to cause oxidising conditions under which free radicals are produced (Carri *et al.*, 1991). The way in which SOD genes are regulated to respond and protect cell against oxidative damage has not yet been fully made clear (Scandalios, 1993).

SOD has been found to increase in plants exposed to chilling conditions (Guy & Carter, 1984; Schöner & Krause, 1990), and chilling tolerant maize has been reported to contain almost twice the SOD activity of non-tolerant maize (Jankhe *et al.*, 1991). This is because the percentage of total electron flow to oxygen increases with chilling stress and more superoxide anion radicals, together with hydrogen peroxide, are produced. Once radicals have been removed by SOD and are in the form of hydrogen peroxide, they are detoxified by specific peroxidases which oxidise ascorbate (Polle *et al.*, 1992). Chilling was found to increase the mRNA for Fe SOD, but not that of the other forms of this enzyme. After chilling levels of mRNA for Cu-Zn and Mn SODs increased (Tsang *et al.*, 1993).

APX is one of the scavenging enzymes that increases in a plant exposed to chilling conditions (Jankhe *et al.*, 1991; Walker and McKersie, 1993). This is especially so during the first stage of acclimation, where the enzyme may participate in detoxification of H_2O_2 (Kuroda *et al.*, 1991) and $O_2^{\cdot-}$ (Foyer and Halliwell, 1976). Enhancement of the peroxide scavenging system during cold acclimation appears to occur in two stages: firstly the enzymatic activities involved in degradation of peroxides increases, and secondly, an alternative enzyme system develops for the detoxification of peroxides, coupled with the pentose-phosphate cycle (Kuroda *et al.*, 1991). Peroxides are known to carry out oxidative reactions on acceptor molecules while reducing peroxidic substrates (Larson, 1988). These enzymes, which are associated with the cell wall, contain a haem-group and catalyse H_2O_2 removal. They generate phenoxy compounds from cinnamic acid which polymerise in a lignification process (Salin, 1987). Ascorbate peroxidase catalyses the formation of dehydroascorbate from ascorbate (Foyer and Halliwell, 1976; Groden and Beck, 1979) in the ascorbate cycle, which is important in peroxide decompostion (Salin, 1987).

In this cycle, ascorbate is peroxidised, forming ascorbate free radicals which form monodehydroascorbate (MDHA) radicals via enzymatic reduction (Halliwell, 1982; Kuroda *et al.*, 1991; Polle *et al.*, 1992). The latter are reduced by MDHAR, with NADPH as reductant, to form reduced ascorbate. MDHA radicals are able to form dehydroascorbate (DHA) and ascorbate via non-enzymatic disproportionation (Hossain *et al.*, 1984; Jankhe *et al.*, 1991) or dismutation (Polle *et al.*, 1992). DHA is reduced by DHAR to glutathione disulphide (GSSG), which is reduced by GR with NADPH as a cofactor to produce reduced glutathione (GSH) (Polle *et al.*, 1992). In non-photosynthetic tissue, DHA is reduced to ascorbate via glucose-6-phosphate dehydrogenase (G6PD), GR and DHAR (Sagisaka and Asada, 1981; Sagisaka, 1982; Nakagawara and Sagisaka, 1984). The reducing power for this pathway is supplied directly from light-driven electron transport and indirectly from secondary enzymatic activities, for example glucose-6-phosphate dehydrogenase and NAD-malate dehydrogenase (Anderson *et al.*, 1992; Polle *et al.*, 1992).

GR, also known as glutathione peroxidase, was first discovered in 1951 (Conn and Vennesland, 1951; Mapson and Goddard, 1951). It is located primarily in the chloroplast of higher plants, although it has also been found in the mitochondrion and cytoplasm, where there may also be a H_2O_2 scavenging pathway (Young and Conn, 1956; Foyer and Halliwell, 1976, Jablonski and Anderson, 1981; Smith *et al.*, 1989; Creissen *et al.*, 1991). GR has been found in photosynthetic as well as non-photosynthetic tissue, such as roots (Bielawski and Joy, 1986; Gillham and Dodge, 1986; Dalton *et al.*, 1987). Bielawski and Joy (1986) found that the GR isozyme from pea roots had a higher affinity for GSSG and NADPH, was more sensitive to Zn^{2+}, Cu^{2+} and Fe^{2+} inhibition and more resistant to heat inactivation than that of pea leaves. Edwards *et al.* (1990) reports that 77%, 20% and 3% of GR in pea is associated with the chloroplast, cytoplasm and mitochondria,

respectively, while Esterbauer and Grill (1978) found that most of the GR in spruce needles was present in the soluble parts of the cytoplasm.

GR is possibly the key enzyme of the ascorbate cycle, reducing GSSG to GSH and utilising NADPH as the reducing cofactor for the reaction (Foyer and Halliwell, 1976). The GSSH/GSH ratio is of significance as it is thought to prevent protein and membrane oxidation stress during low temperature (Alscher, 1989) and other stress conditions (Smith *et al.*, 1990). A possible reason for this is that GSH is able to serve as a co-substrate for peroxidases or as a substrate for oxyradicals (Rennenberg, 1982; Ziegler, 1985; Salin, 1987).

GR is a heterotetramer composed of subunits of 60 and 32 kDa (Connell and Mullet, 1986; Tanaka *et al.*, 1988; Smith *et al.*, 1989), and although there have been reports of subunits of lower molecular weights (Kalk-Torres *et al.*, 1984), these are thought to be due to partial proteolytic enzyme digestion (Connell and Mullet, 1986). Connell and Mullet (1986) report that GR activity was associated with the 60 kDa subunit. Although the smaller subunit does not a appear to have a catalytic function, its function is as yet unknown (Smith *et al.*, 1989). GR purified from pea leaves has been reported to have a native molecular weight of about 145 kDa, with two subunits of about 72 kDa each (Halliwell and Foyer, 1978). Kalk-Torres *et al.* (1984) found GR from pea to have a molecular weight of 156 kDa, with two subunits of 41 and 42 kDa each. These researchers suggest that GR is a $\propto_2 ß_2$ tetramer. Edwards *et al.* (1990), on the other hand, have located a single band corresponding to total GR with an apparent molecular weight of 55 kDa. On two dimensional gels this was resolved to eight spots varying in isoelectric points (pI) from 5.2 to 6.5. Work by Madamanchi *et al.* (1992) showed that purified GR from pea seedlings could be resolved into six isoforms by chromatofocusing. Five of these were attributed to the chloroplast, with pI values between 5.6 and 6.3, while the last isoform was said to be extraplastidic with a pI value of 4.9. Both of these forms had native molecular weights of 114 kDa, and the plastidic form was a homodimer with a subunit of 55 kDa. The extraplastidic form was shown, via Western blots, to have two polypeptides, one of 55 and the other of 36 kDa. The considerable variation seen in size and form of GR from the same species may be due to post-translational processing of a single gene product (Faye *et al.*, 1986), or that the different forms are regulated in response to environmental stress (Madamanchi *et al.*, 1992). When the cDNAs of pea GR were cloned, there appeared to be a nuclear encoded sequence which coded for a polypeptide of 59 kDa (Creissen *et al.*, 1991), and when the linear amino acid sequence of pea GR was compared to the amino acid sequence of GR from other plant and animal species, they all were of a similar size with a high degree of homology and conservation of amino acid sequence in the region responsible for forming the redox-active disulphide bridge.

Multiple forms of GR have also been found in other plants, for example Guy and Carter (1984) found that at 5°C hardened spinach plants had two GR isozymes not present in non-hardened plants. Mahan and Burke (1987) found GR purified from corn mesophyll chloroplasts to have a specific activity of 26 μmol NADPH oxidised (mg protein)$^{-1}$ min^{-1} and a native molecular weight of 90$\pm$30 kDa. This GR was found to have four polypeptides of 65, 63, 34 and 32 kDa, and it was suggested that corn GR is a heterotetramer. Scots pine yielded GR with specific activity of 3.65 μmol (mg protein)$^{-1}$ which was lower than that for spinach (Halliwell and Foyer, 1978), but higher than that for other species (Kalk-Torres *et al.*, 1984; Connell and Mullet, 1986; Mahan and Burke, 1987). The two polypeptides from pine GR had estimated molecular weights of 59 kDa each (Wingsle, 1989). GR from two legumes had a native molecular weight of about 140 kDa, with two subunits from each legume - alfalfa showed subunits of 57 and 36 kDa and saingoin subunits of 57 and 37 kDa (Kidambi *et al.*, 1990).

The K_m of pine GR for GSSG at 25°C was found to be 28$\pm$4 μM by Wingsle (1989). This agreed with the K_ms reported for other species (Kalk-Torres *et al.*, 1984). Smith *et al.* (1989) reported a GR K_m for GSSG that varied between 10 and 60 μM, depending on the species examined. The K_m for GSSG of this enzyme has been found to vary with temperature and pH (Turner and Pollock, 1993). Generally the GR K_m for NADPH lies between 2 and 10 μM (Smith *et al.*, 1989). The pine GR K_m for NADPH was reported to be 1$\pm$1 μM at both 25°C and 3°C (Wingsle, 1989). Researchers have reported various K_ms for pea NADPH, for example 1.7 μM (Kalk-Torres *et al.*, 1984; Mahan and Burke, 1987), 3 μM (Connell and Mullet, 1986) and 4-4.8 μM (Madamanchi *et al.*, 1992). GR K_m for NADH was reported to be 17 μM (Connell and Mullet, 1986). Guy and Carter (1984) showed that the K_ms for GR in hardened spinach decreased with decreasing assay temperature, which suggested that structural and functional changes increase enzyme efficiency at low temperatures and make them more stable at freezing temperatures (Huner and Macdowell, 1979; Guy and Carter, 1984). Mahan *et al.* (1990) found that GR K_ms from spinach, corn and cucumber are temperature dependent and this dependency was species dependent. In maize, Turner and Pollock (1993) found that the apparent K_m of glutathione reductase increased with increasing temperature and was also affected by pH and varying temperatures in combination. There appears to be six-fold variation between 15 and 34°C for GR K_ms (Mahan *et al.*, 1987). This variation may be due to the presence of isozymes (Guy and Carter, 1984) and highlights the danger of measuring K_ms in unfractionated cell homogenates, as variation may be caused by environmental influence on the predominant isozyme, or it may be due to relative amounts of isozymes with different K_ms (Smith *et al.*, 1989). Turner and Pollock (1993) conclude that, although the apparent K_m of GR is temperature dependant *in vitro*, the effects on metabolism might be moderated or exaggerated by changes in substrate level as temperature rose or fell *in vivo*.

GR is inhibited by high concentrations of $NADP^+$ and GSH (Wingsle, 1989). This probably has a role in regulating the enzyme (Jablonski and Anderson, 1978). $NADP^+$ inhibitition is non-competetive with respect to GSSG, but competetive with respect to NADPH. The inhibition by GSH is non-competetive with respect to both GSSG and NADPH (Halliwell and Foyer, 1978; Wingsle, 1989). When incubated with substrate, GR undergoes redox interconversion reactions. With GSSG, GR is oxidised, resulting in a more stable form of the enzyme (Kalk-Torres *et al.*, 1984; Bielawski and Joy, 1986; Mahan and Burke, 1987). With NADPH, GR undergoes irreversible inactivation (Kalk-Torres *et al.*, 1984), but in yeast cells this reduction is reversible (Pinto *et al.*, 1985). GR is also inactivated by sulfhydryl-modifying reagents (Kalk-Torres *et al.*, 1984).

Under oxidative stress conditions, for example chilling, both synthesis and activity of GR has been shown to increase (Tanaka *et al.*, 1988; Jankhe *et al.*, 1991; Anderson *et al.*, 1992; Doulis *et al.*, 1993). The increase in GR may be due to the production of new isozymes with different kinetic properties (Guy and Carter, 1984; Burke, 1990; Mahan *et al.*, 1990) and is probably caused by the involvement of GR in the ascorbate scavenging pathway. GR is a constitutive, non-regulated enzyme which maintains the GSH/GSSG ratio in favour of GSH (Kalk-Torres *et al.*, 1984), whose function is to protect enzymes and membranes against oxidation by free radicals in the aqueous phase (Barclay, 1988). The increase in GR under conditions of chilling stress is not always seen in plants. For example, in plants grown in a growth chamber at 5°C there was an increase in GR and GSH, but under field conditions, this increase was not seen (Smith *et al.*, 1989). Other authors have found that oxidative stress caused little or no increase in GR and GSH (Tanaka, 1985; Rao, 1992; Wingsle *et al.*, 1992). Guy *et al.* (1984) concluded that increases in GR and the GSH/GSSG ratio had no direct involvement in leaf cold hardiness in citrus leaves. These findings led to the conclusion that interactions between photoperiod, temperature and light may be more important than any single factor (Smith *et al.*, 1989).

Under stress conditions, especially low temperature, oxygen interactions may be detrimental as there is a decreased chloroplastic ability to remove oxygen intermediates as scavenging enzymes lose their activity (Jankhe *et al.*, 1991). Plants have other means of scavenging oxyradicals, as will be briefly discussed below. Catalase, an efficient metalloenzyme, converts H_2O_2 to water and oxygen (Larson, 1988). Although it is abundant in plant tissue, catalase is primarily associated with the peroxisome (Halliwell, 1982) and not the chloroplast (van Ginkel and Brown, 1978).

Glutathione is a multifunctional thiol tripeptide widely distributed in pro- and eukaryotes (Creissen *et al.*, 1991). It appears to be located primarily in the chloroplast, but is also found in the cytosol. It is involved in a number of biological reactions such as protein disulphide reduction reactions,

the detoxification of xenobiotics such as herbicides or gaseous pollutants, the prevention of lipid peroxidation and the regulation of gene expression involved in responses to environmental stress or pathogen attack (Wingate *et al.*, 1988; Alscher, 1989; Lawton *et al.*, 1990; Edwards *et al.*, 1991). It has been found to increase in response to stress, including chill-stress, conditions (Badiani *et al.*, 1993; Doulis *et al.*, 1993; Walker and Mckersie, 1993). The reduced form performs the majority of these protective functions, and the mechanism whereby GSH is thought to protect plants against oxyradicals caused by cold stress can be explained by the thiol:disulphide theory of Levitt (1962). Frost resistance is thought to arise from thiol oxidation prevention, from prevention of thiol:disulphide interchange and the formation of intermolecular disulphides. For this to be feasible, a pool of glutathione for the oxidant scavenging or reducing system, an active GR to regenerate GSH, a supply of NADPH, and the operation of the system at low temperatures are requirements (Smith *et al.*, 1989). Homoglutathione and hydroxymethylglutathione have been isolated and found to be involved in scavenging of hydrogen peroxide, but oxidised glutathione appears to be a better substrate for glutathione reductase (Zopes *et al.*, 1993). A clear role for hydroxymethylglutathione has not yet been illucidated (Zopes *et al.*, 1993).

Ascorbic acid is another molecule involved in the non-enzymatic removal of oxyradicals (Asada and Takahashi, 1987) and is known to accumulate under stress conditions (Schöner and Krause, 1990; Badiani *et al.*, 1993; Mishra *et al.*, 1993). It is found in high concentrations in many cell environments, for example in the chloroplast (Schöner and Krause, 1990). This vitamin reduces superoxide radicals to water and dehydroascorbic acid, and is also able to react with singlet oxygen (Larson, 1988). It is able to donate a hydrogen atom to tocopherol-derived phenol radicals and so regenerate its activity. Vitamin C is the chain-breaking scavenger for peroxy-radicals and is able to act synergistically with vitamin E (see Larson, 1988).

The tocopherols are naturally occuring compounds with activity of vitamin E. Their mode of action is to inactivate two equivalents of chain-carrying peroxy-radicals (Larson, 1988). The most biologically active of the tocopherols is α-tocopherol, which is an efficient singlet oxygen quencher (Larson, 1988). It has been observed to decrease in both chilling-tolerant and sensitive tomato plants under low temperature conditions, but the decrease in the sensitive variety was more extensive (Walker and McKersie, 1993).

Flavenoids or phenolics, which are common in leaves, flowering tissues, pollens, stems and barks are also considered to have a protective effect against oxyradicals (Larson, 1988). Most phenolics and flavenoids are thought to protect plants by preventing ascorbic acid destruction and they are able to inhibit lipid autooxidation by donating a H atom to peroxy radicals derived from the autooxidation of fatty acid derivatives (Torel *et al.*, 1986). Flavenoids are a family of therapeutic

agents with properties of vitamin P. They are thought to protect plants against vascular disorders by decreasing permeability and fragility of capillaries (Torel *et al.*, 1986). This is related to their antioxidant function as vascular disorders may be caused by oxidative damage of cell membranes. Flavenoids are thought to inhibit superoxide promoted redox reactions within the chloroplast (Takahama, 1983) and they have been known to act as light filters for organelle protection against uV damage (Caldwell *et al.*, 1983). They are able to slow down the formation of conjugated dienes during autoxidation of linoleic acid and methyl linolenate. Their efficiency is related to their concentration and the degree of unsaturation of fatty acids in the tissue (Torel *et al.*, 1986).

Several alkaloids are able to inhibit superoxide radical damage, for example strychnine and brucine (Larson, 1988). These are physical quenchers and are not chemically destroyed by the quenching process, and so are able to inactivate many molecules of superoxide per alkaloid molecule (see Larson, 1988).

Certain chlorophyll derivatives, such as pheophytin, are known to inhibit autooxidation in the dark (Larson, 1988) and there are specific changes in the chlorophyll:carotenoid ratio in cold acclimating spinach leaves that protect against high light stress under cold conditions (Schöner and Krause, 1990; Mishra *et al.*, 1993). ß-carotene is able to protect membranes against superoxide radical peroxidation (Larson, 1988) and was found to increase in leaves of *Triticum durum* and *Thuja plicata* during low temperature conditions (Badiani *et al.*, 1993; Weger *et al.*, 1993) but not in *Pinus sylvestris* L. (Wingsle *et al.*, 1992). Carotenes or carotenoids are seen as having an important role in protection against active, toxic species (Mishra *et al.*, 1993; Badiani *et al.*, 1993) and their accumulation may function to decrease the light intensity reaching the photsynthetic apparatus (Weger *et al.*, 1993). An increase in these compounds under low, non-chilling temperatures may function to anticipate a possible enhanced requirement of pigments and antioxidants in case of cold-induced oxidative stress and damage (Badiani *et al.*, 1993). Changes in the xanthophyll cycle may have a role in protection against oxyradical damage (Demmig *et al.*, 1987; Wingsle *et al.*, 1992).

1.3.5.3 Role of Fluorescence

Light is absorbed by plant photosynthetic pigments. The excitation energy is then tranferred to the reaction centers of photosystem (PS) I and II. Once in these centers, energy drives photochemical reactions necessary for photosynthetic energy conversion. There are three reactions that compete with photochemical reactions for energy. These are thermal deactivation, excitation energy transfer to non-fluorometric pigments and fluorescence (Krause and Weis, 1991). These

reactions will be discussed in more detail later as it is thought they have protective roles against photoinhibition. As the events of photosynthetic electron transport in PSII are of relevance to the following discussion, they shall be described here before a more detailed look is taken at photoinhibiton and chlorophyll fluorescence.

In the primary photochemical reaction, one electron is transferred from the pigment P_{680} in the first excited singlet state (P^*_{680}) to pheophytin *a* (Krause, 1988). From there, the electron is transferred to a primary quinone-type acceptor, Q_A. The charge separation creates a highly active oxidant, P^+_{680}, which receives an electron from the secondary donor Z. Z has been identified as a tyrosine residue of the D1 protein (Krause and Weis, 1991). In the oxidised state, Z^+, this donor is reduced by an electron from the water oxidation system. Finally, the electron is transmitted to the quinone Q_B. After this quinone has received two electrons from the stroma side of the thylakoid membrane, it merges into the plastoquinone/plastohydroquinone (PQ) pool. This may be represented as follows:

$$
\begin{array}{l}
Z\ P_{680}\ Pheo\ Q_A\ Q_B \\
\qquad \downarrow h\nu \\
Z\ P^*_{680}\ Pheo\ Q_A\ Q_B \\
\qquad \downarrow \\
Z\ P^+_{680}\ Pheo^-\ Q_A\ Q_B \\
\qquad \downarrow \\
Z\ P^+_{680}\ Pheo\ Q^-_A\ Q_B \\
\qquad \downarrow \\
Z^+\ P_{680}\ Pheo\ Q^-_A\ Q_B \\
\qquad \downarrow \\
Z\ P_{680}\ Pheo\ Q^-_A\ Q_B \\
\qquad \downarrow e^- \\
Z\ P_{680}\ Pheo\ Q_A\ Q^-_B
\end{array}
$$

There are a number of reactions that are able to contribute to the photosynthetic electron flow. The most important of these is the use of the products of the photosynthetic electron flow for carbon dioxide fixation. If this reaction is enhanced there will be an increase in the photosynthetic transport carriers and in the biochemical capacity of the the electron flow (Demmig-Adams and Adams III, 1992). Oxygen fixation by Rubisco, with the formation of phosphoglycolic acid as the primary step of photorespiration (Baker, 1991) is able to contribute to the electron flow, as is the reduction of other compounds such as nitrogen (Chapin *et al.*, 1987) and sulpher (Adams *et al.*, 1989), although the latter do not contribute to a large extent. Lastly, there is the potential for direct reduction of oxygen by PSI in the Mehler reaction, which results in superoxide and hydrogen peroxide production. These are potentially harmful, but the Mehler reaction is a dissipative pathway, especially when coupled to the ascorbate peroxidase reaction (Radmer and Kok, 1976). These reactions may have an important role in the build up of the transthylakoid pH

gradient (see below), especially if photosynthetic rates are low (Demming-Adams and Adams III, 1992).

Photoinhibition is a common phenomonen in both the field and under experimental conditions (Demming and Björkman, 1987; Krause, 1988; Adams III *et al.*, 1990). It can be observed under conditions of high light or under moderate light when combined with an environmental stress. Light stress results when more light is absorbed than used in photosynthesis (Björkman, 1968). Stress (e.g. water or chilling stress) may heighten light intensity or reduce photosynthesis, so increasing the light intensity/photosynthesis ratio (Demmig *et al.*, 1988; Demming-Adams and Adams III, 1992). Photoinhibition is primarily based on an inactivation of the electron transport system of thylakoids (Osmond, 1981). Chilling cucumber leaves (a chilling sensitive species) resulted in uncoupling of the thylakoids by the dissociation of coupling factor 1 (CF_1) which is membrane-associated (Terashima *et al.*, 1991a). When CF_1 reassociated at warmer temperatures, thylakoids were recoupled. Chilling of leaves in the light may induce uncoupling of thylakoids, suppressing the energisation of these membranes and thereby affect the pH gradient across them (Terashima *et al.*, 1991b). Plants have recovery or repair and protective mechanisms which influence the severity of photoinhibition, but its extent also varies with the plant's physiological state and with external conditions. When these protective mechanisms are not able to cope with the excess light, the photosynthetic apparatus may be damaged (Demmig-Adams and Adams III, 1992). It is not a uniform phenomena and mechanisms involved may contribute to different extents, depending on the plant's conditions (Krause, 1988). Photoinhibition of photosynthesis (in PSII) *in vivo* has been shown to be enhanced by the presence of oxygen when energy turnover by photorespiration and photosynthetic carbon metabolism are low (van Wijk and Krause, 1991)

Photoinhibition's dominant effect is to change the reaction centers of PSII, resulting in a decrease in the primary photochemical efficiency. These reaction centers are still able to trap energy, but it is converted into heat. Centers in this state are not able to initiate photosynthetic electron transport, nor are they able to contribute to variable fluorescence (Briantais *et al.*, 1992). Thus, photoinhibition may result from a decrease in the rate constant for photochemistry of PSII, or from an increase in the rate constant for non-radiative dissipation of excitation energy (Demmig *et al.*, 1987). These processes may be caused by a lack of chorophyll photo-oxidation, as seen in isolated chloroplasts, or inhibition of the PSII or PSI electron transport, or both (Hodgson and Raison, 1989). Excess light may affect carbon dioxide assimilation, but this cannot be explained by electron transport inhibition (Krause and Cornic, 1987; Giersch and Robinson, 1987). Photoinhibition is able to affect light-induced alkalization of the chloroplast stroma, and it is thought that this impairs light-activation of the carbon reduction cycle (Krause, 1988). The relation between closure of PSII centers and photoinhibition may be indirect, that is, the redox state

of PSII centers may reflect the balance between absorbed light and light dissipated through electron transfer (Ögren, 1991), but it may also be causal as a link between photoinhibition and Q_B damage has been found (Ohad *et al.*, 1984). PSI is also affected by photoinhibition (Krause *et al.*, 1988), but little *in vivo* work has been done in this area.

Net photoinhibition appears to be the difference between inactivation and repair of PSII. The portion of this reaction center that is likely to undergo degradation and synthesis, as a result of excess light, is the D1 protein (Bradbury and Baker, 1986; Kyle, 1987; Greer *et al.*, 1991). The D1 protein is part of the Q_B-binding protein, which may be the initial site of photoinhibition (Kyle, 1987; Ohad *et al.*, 1988). Excess light may induce structural changes to this protein which render it inactive for electron transfer and make it susceptible to a membrane-bound protease (Ohad *et al.*, 1985). Light-dependent degradation of this protein is accelerated after inhibition of the water splitting system (Jegerschöld *et al.*, 1990). It has been observed that photoinhibitory treatment induces only a limited decrease in the rate of Q^-_A reoxidation, implying that damage to the Q_B niche is a transitory state in the process of PSII inactivation (see Briantais *et al.*, 1992). This is presumably followed by damage to another site(s) within the PSII reaction center.

The susceptibility of PSII to photoinhibition at a given light intensity depends on the absorption cross section. $PSII_\alpha$ centers are more susceptible to photoinhibition than PSI_β centers as the former have larger antennae (Cleland *et al.*, 1986; Black *et al.*, 1986). External growth temperature also influences damage to PSII. This photosystem is sensitive to temperature, but is able to adjust to external growth conditions to some extent (Havaux, 1993). Adjustment may be related to adaptive changes in the surroundings of the chloroplastic membrane such as accumulation of thermoprotective compounds or changes in metal or proton cation concentrations (Williams and Gounaris, 1992).

Photoinhibited thylakoids show impaired electron transport from P_{680} to Q_A (see eqn 1). Krause (1988) postulates that this may be due to changes occuring when P^+_{680} traps an electron, resulting in PSII inactivation. Recovery of photoinhibition would then involve restoration of the altered reaction center to a photochemically active state (Cleland and Melis, 1987). These authors maintain that the rate of photoinhibition is independent of the redox state of Q_B. The primary sites of photoinhibition are still disputed (Neuner and Larcher, 1991), resulting in any models of molecular reactions leading to PSII inactivation being working hypotheses (Kyle, 1987; Krause, 1988).

Photoinhibition can be seen as a stable, long-term down regulation of photochemistry which occurs in plants under high light conditions in order to prevent more severe photodynamic damage of the

thylakoid membranes (Ottander and Öquist, 1991; Öquist *et al.*, 1992). There appear to be two different mechanisms or types of photoinhibition (Hodgson and Raison, 1989; Ögren and Evans, 1992). The first mechanism is irreversible, with centers losing the ability to function as energy traps, and is characterised by high F_0 (Demmig and Björkman, 1987). This has been discussed above. The second involves a differential F_0 response and is reversible due to repair or restoration processes. It may have a regulatory or protective role (Somersalo and Krause, 1989). The end result of this is to avoid accumulation of excitation energy at closed PSII centers by increasing thermal dissipation of energy (Somersalo and Krause, 1989; 1990a,b; Ögren and Evans, 1992). This type appears to occur under chilling conditions, although slight differences between reversible and chilling-induced photoinhibition may be noted (Mäenpää *et al.*, 1988). Somersalo and Krause (1989) suggest that under conditions of weak light, photoinhibition with high F_0 may recover completely. They suggest that the first photoinhibition is typified by high F_0, which can recover at low temperature and light, and the second by low F_v and requires higher temperatures and certain metabolic activities to recover.

Although both of these may be regarded as photoinhibition, some workers regard only irreversible photoinhibition as true photoinhibition (Ögren and Evans, 1992). This has not been universally accepted as neither of these states can be discriminated against on a mechanistic basis. Turnover of D1 protein appears to be continuous and could occur as rapidly as the recovery from moderate photosynthesis (Ohad *et al.*, 1984). Both of these states share some characteristics, for example, in *Eucalyptus* there is an equivalent pattern of changed F_0, F_M and quantum yield in both (Ögren and Evans, 1992), and Ögren (1991) found the same relationship between fluorescence quenching during stress treatment and subsequent photoinhibition, regardless of the severity of photoinhibition. Both involve potential loss of photosynthesis and may involve intermediate to high light intensity ranges (Ögren and Evans, 1992).

Chilling-induced photoinhibition does not appear to involve damage to PSII (Mäenpää *et al.*, 1988) and is usually reversible, although it may be irreversible, depending on the conditions (Báló *et al.*, 1991). The reversibility of chilling-induced photoinhibition may be explained by photoinhibition on the donor side of PSII, with a slowing down of electron donation from water increasing the plant's susceptibility (Callahan *et al.*, 1986). Chilling-induced photoinhibition may be caused by rate limitations imposed at chilling temperatures which are unique to chilling sensitive plants, or by an inability to effectively regulate the balance of light energy between the two photosystems at low temperature (Moll and Steinback, 1986). The latter is hypothesised to involve reversible protein phosphorylation of LHCII. Phosphorylation is achieved by light-stimulating activity of one or more thylakoid membrane bound kinases (Moll and Steinback, 1986). The process can be reversed via a membrane bound phosphoprotein phosphatase. Light stimulation of

protein kinase activity is thought to be due to kinase activation via reduction of the PQ pool (Allen *et al.*, 1981). The relative degree of LHCII phosphorylation is thought to mediate the interaction between PSII and LHCII (Barber, 1982), and so regulate the balance of excitation energy arriving at the two reaction centers. This provides a mechanism that controls the relative turnover of the photosystems (Moll and Steinback, 1986). At chilling temperatures, there may be inhibition of thylakoid protein phosphorylation, resulting in a lack of regulation of excitation energy distribution between PSI and PSII. Reduced pools of PQ will increase, decreasing electron carriers on the reducing side of PSII and increasing the damage to PSII directly, or indirectly via effects on the 32 kDa Q_B proteins (D1 protein) (Moll and Steinback, 1986).

Two possible causes for decreased photosynthesis in chilling plants are a direct effect on the leaf or an indirect effect through decreased water uptake (Taylor and Rowley, 1971). Although a decrease in rate and extent of stomatal opening may be another factor, it is not likely to be a primary cause of decreased photosynthesis (van Hasselt and van Berlo, 1980).

Chilling-induced photoinhibition may be related to a disproportionate decrease in photosynthetic activity in plants under chilling temperatures (Hodgson and Raison, 1989), although it does not result in the loss of photosynthetic pigments (Powles, 1984). Under chilling conditions plants may be more susceptible to photoinhibition because the antennae absorb more light than can be orderly dissipated (Osmond, 1981), *de novo* protein synthesis necessary for repair of photodamage may be inhibited (Greer *et al.*, 1986; 1991), or low temperatures may inhibit alternative ways of dissipating excessive excitation energy (Krause, 1988). Chilling-tolerant plants may have a higher capacity for light-saturated photosynthesis which is responsible for maintaining a higher proportion of oxidised to reduced Q_A (i.e. hardened or tolerant plants are able to keep a larger fraction of PSII reaction centres in an open configuration) (Öquist and Huner, 1993).

The site of chilling-induced photoinhibition appears to be on the reducing side of PSI. It could be at the terminal components of thylakoid electron transport (ferredoxin-FeS complex, ferredoxin-NADP reductase) and/or in the Calvin cycle reactions (Hodgson and Raison, 1989). Hetherington *et al.* (1989) agree with the possibility of damage to PSI, but say that there may also be damage to PSII. The temperature for initiation of photoinhibition coincides with that of a phase change in the polar lipids of thylakoids (Raison and Orr, 1986; Hodgson *et al.*, 1987) and thylakoid membranes (Havaux and Lannoye, 1983). This phase change adversely affects membrane associated changes (Garber, 1977), as well as activation energy of intrinsic membrane associated reactions (Wolfe, 1978), and is thought to be the basis for the dysfunction which causes photoinhibion at chilling temperatures (Hodgson *et al.*, 1987).

Chilling-induced inhibition is most often seen in chilling-sensitive plants (Hodgson and Raison, 1989), although it is not directly related to chilling sensitivity (Hetherington *et al.*, 1989). Those plants tolerant of chilling may be able to tolerate or avoid damage by fixing carbon dioxide at low temperatures (Hurry and Huner, 1991). This would be protection only if this photoinhibition is regulated by the rate of carbon metabolism in plant tissue (Osmond, 1981). For plants to be cold tolerant, it appears that growth and development under such conditions is a prerequisite, as is the case for cold tolerant winter wheat and rye (Hurry and Huner, 1991) and spinach (Boese and Huner, 1992). Tolerance appears to be linked to a high level of cell organisation, and perhaps not only with thylakoid membrane chilling resistance (Lapointe *et al.*, 1991). It has also been shown that hardened leaves have fewer PSII reaction centers and produced slower Q^-_A reoxidation kinetics (Briantais *et al.*, 1992). Hardened rye and winter wheat leaves were found to have a higher proportion of oxidised to reduced primary, stable quinone receptor (Q_A) compared to sensitive leaves. A higher proportion of oxidised Q_A was also correlated with an increased capacity for photosynthesis, suggesting that low temperature modulation of photosynthetic apparatus may be important during freezing tolerance induction (Öquist *et al.*, 1993)

Recovery from photoinhibition, which is the relaxation of q_I (see below) is thought to proceed via the PSII repair cycle (Krause and Weis, 1991). This involves a transformation of damaged PSII∝ to PSIIß units in which the reaction center has been reconstructed by D1 degradation and replacemment with newly synthesised D1 protein (Guenther and Malis, 1990). This has been shown by numerous workers. The quinone Q_B, particularly the D1 protein of the Q_B-binding site, is involved in recovery from photoinhibition as *de novo* synthesis of the protein is required for this process in *Chlamydomonas* (Ohad *et al.*, 1984; see also Reisman *et al.*, 1986; Reisman and Ohad, 1986; van Wijk and Krause, 1991). This is substantiated by Öquist *et al.* (1992), who maintain that protein synthesis is required for recovery from photoinhibition as replacement of photoinhibited PSII center components, especially the D1 protein, is necessary. Chow *et al.* (1989) agree that D1 protein recovery may play a role in recovery from photoinhibition, but point out that as photoinhibition includes more than one phase, D1 protein inactivation and subsequent resynthesis may not be the first phase of recovery. For fast recovery from photoinhibition, there may be a pool of D1 protein (Kyle, 1987) or a pool of stable m-RNA which synthesises this protein (Öquist *et al.*, 1987), present in the cell. Guenther and Malis (1990), on the other hand, regard replacement of D1 protein as slow recovery, and fast recovery is thought to be a partial reversal of photoinhibited centers to the active state without D1 protein replacement (Krause and Weis, 1991). Recovery could be linked to moderate light intensity (Greer *et al.*, 1989; Somersalo and Krause, 1990a), as Q_B resynthesis may occur continuously during the light. This has been hypothesized because recovery often occurs immediately on transition from high to moderate or low light intensities (Skogen *et al.*, 1986). This has been confirmed as photoinhibition only

appears to occur when the rate of Q_B inactivation or degradation exceeds the repair rate (Krause, 1988). It is possible that this hypothesis is not valid under all circumstances (Krause and Weis, 1991).

Recovery from photoinhibition appears to be temperature dependent. This is manifested through temperature effects on the repair processes of PSII, for example, at 5°C repair is largely inhibited, resulting in greater photoinhibition (Greer *et al.*, 1991). Low temperature affects recovery at three different stages. Firstly, the breakdown of the D1 protein of PSII Q_B-binding protein is retarded (Gong and Nilsen, 1989), secondly, the removal of D1 products and the insertion of newly synthesised D1 protein may be affected (Kyle, 1987; Guenther and Melis, 1990), and thirdly, low temperature may result in an increased number of photoinhibited PSII reaction centers accumulating. These are still able to trap energy, but this is converted into heat, perhaps acting as a protection system (Demmig *et al.*, 1987; Björkman, 1989). Once the photoinhibitory stress is removed, temperature dependent recovery is consistent with temperature dependent steady state photoinhibition (Greer *et al.*, 1991). At steady state, it is thought that the photoinhibitory rate is matched by the continual recovery rate, that is, breakdown, *de novo* synthesis and insertion of reaction center D1 protein (Krause, 1988; Guenther and Malis, 1990). Recovery rate also appears to depend on degree of hardening as cold-hardened spinach were able to recover more rapidly, and had a higher capacity for recovery, than sensitive plants (Boese and Huner, 1992).

There appear to be protective mechanisms against photoinhibitory damage in the plant at four levels. The first is energy consuming carbon metabolism, including respiration (Krause and Cornic, 1987). The second involves those reactions which scavenge or prevent radical or reactive species formation. The systems which scavenge carotenoid, chlorophyll radicals, superoxide oxygen anions, hydroxyl ions and hydrogen peroxide (the SOD-ascorbate peroxidase systems) are examples here (Asada and Takahashi, 1987). Thirdly, plants are able to change parameters such as carbon metabolism, the light-harvesting and oxygen radical scavenging systems during the process of long-term light acclimation, resulting in increased tolerance of high light (Krause, 1988). Lastly, it is possible to increase the rate constants of thermal deactivation of excited pigments, and thereby promote non-destructive thermal dissipation of excess excitation energy. There are two processes whereby this may occur: (1) Thermal deactivation increases may be related to reversible "energy dependent" fluorescence quenching (q_E). This process allows the photosynthetic apparatus to respond within seconds to minutes to changes in the light conditions. (2) The increases may be related to reversible photoinhibition fluorescence quenching (q_I) (Demmig and Björkman, 1987). This process allows a longer response - within minutes to hours (Krause, 1988). Before these processes are looked at further, it is appropriate that chlorophyll fluorescence is examined.

Fluorescence emmission is one of several first order reactions of deactivation of excited chlorophyll molecules (Butler, 1978). In PSII, these reactions are fluorescence, with a rate constant of k_F, radiationless (thermal) deactivation (k_D), transfer of excitation energy to PSI (k_T) and photochemical reactions (k_P). Most fluorescence emanates from PSII, with a minor contribution from PSI. Fluorescence yield of PSII is related to the fraction of 'open' reaction centers (when Q_A is in the oxidised state) and the absorbed light flux (Krause and Weis, 1991). Maximum fluorescence yield is about 3% of absorbed light, and minimum fluorescence is about 0.6%, due to competition with phytochemistry.

The typical fluorescence induction signal of chloroplasts *in vivo* in continuous light is called the

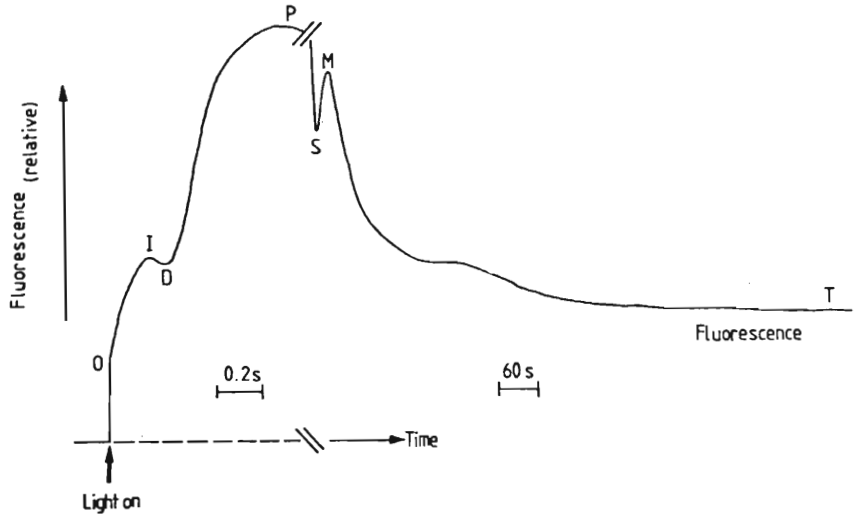

Figure 1.3 Kinetics of 680 nm fluorescence emission, demonstrating the Kautsky effect. From Hipkins and Baker (1986). See text for explanation of symbols.

Kautsky effect (Krause and Weis, 1991) (see Figure 1.3). A typical induction signal is a relatively fast rise from F_O (O) to F_I (the point of inflection, I), followed by a plateau or dip (D) and a slower rise to the peak (P) or fluorescence maximum (F_M) (Krause and Weis, 1991). When the photosynthetic apparatus is dark adapted, Q_A is normally fully oxidised. Continuous illumination causes a fluorescence rise from F_O as a result of Q_A reduction. Fluorescence at F_0 is emission by Chl a antenna molecules (Krause, 1988). Fluorescence rise kinetics depends on PSII cooperativity and heterogeneity, the PQ pool size and its reoxidation rate, electron transport rate beyond PSI, including carbon metabolism, and the rate of electron donation to P^+_{680} (Krause and Weis, 1991). In relatively low actinic light, the rise from F_0 to F_I may reflect reduction of Q_A centers that are loosely or are unconnected to Q_B and PQ (Melis, 1985; Chylla and Whitmarsh, 1989; Cao and Govindjee, 1990). Under low light conditions, the rise from F_I to F_M represents closure of active reaction centers concommitant with PQ reduction. When there is an increase in actinic light

intensity, the rate of Q_A reduction may be faster than Q_A reoxidation, resulting in full reduction of Q_A. At this stage F_M is reached (Krause and Weis, 1991). During the rise from F_0 to F_M, variable fluorescence, the origin of which is still controversial (Krause and Weis, 1991), is not proportional to the redox state of Q_A. Instead, the slope of the rise curve is influenced by PSII heterogeneity as electron transfer is possible between $PSII_\alpha$, but not $PSII_\beta$ units (Krause and Weis, 1991). F_V was assumed to arise owing to back-transfer of excitation energy from closed reaction centers (Butler, 1978), but Klimov and Krasnovskii (1981) postulated that it results from recombination of P^+_{680} and $Pheo^-$ in closed reaction centers and arises from emission by P^*_{680} after charge recombination. Breton (1982) attributed emission to $Pheo^*$. There is strong evidence against this theory as emission from the recombination of P^+_{680} and $Pheo^-$ occurs *in vitro*, but this cannot be variable fluorescence as there is no indication of such long lifetimes (5 - 35 ns of emission) occuring *in vivo* in intact thylakoids. Most of the evidence supports the assumption that Chl fluorescence *in vivo* (both F_M and F_V) emanates from the antenna system (Krause and Weis, 1992). van Dorssen *et al* (1987) found that at low temperatures, emission was from the cP47 core antenna complex.

Fluorescence rise is also related to the reduction of the electron carriers Q_A, Q_B and PQ - the true F_M can only be reached when the PQ pool is reduced, as PQ quenches fluorescence in the oxidised form (Krause and Weis, 1991). This depends on electron transfer via PSI and on the final consumption of reducing equivalents in carbon and other metabolic reactions, and on the transthylakoid pH gradient. In high light conditions, the two rise phases overlap (F_0 to F_I; F_I to F_M). A rise to a high F_I^1 level is followed by a dip (F_D) and a biphasic rise from F_M to F_I^2. At the F_I^1 level, all Q_A is reduced and an initial limit or electron donation to P^+_{680} is assumed. Accumulation of P^+_{680} quenches fluorescence. The rise from F_D to F_I^2 would overcome this limitation. The slower rise phase (F_M to F_I^2) reflects PQ reduction, and may be prevented by electron acceptors that keep PQ oxidised (Krause and Weis, 1991).

Photoinhibition is related to lower F_V values. This is 'photoinhibitory' quenching (q_I) which is frequently expressed as a decrease in the F_V/F_M ratio (Krause, 1988). This has been linearly related to a decrease in optimal photon yield of photosynthesis (Björkman, 1987; Demmig and Björkman, 1987). Recovery in the F_V/F_M ratio is correlated with recovery from photoinhibition.

Quenching generally denotes all processes that lower the fluorescence yield below its maximum. Resolution of quenching components offer information on the functional state of the photosynthetic apparatus, especially on PSII efficiency. Non-photochemical quenching, for example, is regarded as a measure of thylakoid energisation (Terashima *et al.*, 1991a). A decrease of this quenching would indicate an uncoupling of thylakoids. A disappearance of non-photochemical quenching in

chilled leaves would then be related to the thylakoid membrane and not an inactivation of the capacity for fixation of carbon dioxide or an abscence of endogenous electron acceptors (Terashima *et al.*, 1991b). There are two mechanisms whereby fluorescence yield can be lowered: photochemical and non-photochemical quenching (Krause and Weis, 1991; Öquist *et al.*, 1992). Photochemical quenching involves lowering fluorescence yield by the photochemical reaction of other pathways of de-excitation. It depends on the oxidation state of Q_A and the coefficient of photochemical quenching (q_P) denotes the proportion of excitons captured by open traps being converted to chemical energy in the PSII reaction center (Krause and Weis, 1991).

Non-photochemical quenching is unrelated to the redox state of Q_A. It may be caused *in vivo* by three major mechanisms: (1) "energy-dependent" quenching (q_E) caused by intrathylakoid acidification during light-driven proton translocation across the membrane, (2) quenching related to "state 1 - state 2" transitions (q_T) regulated by phosphorylation of light harvesting complex II (LHCII) and (3) "photoinhibitory" quenching (q_I) related to photoinhibition of photosynthesis.

1.3.5.3.1 Mechanism of energy-dependent quenching (q_E)

This is related to light induced increase of intrathylakoid proton (H^+) concentration (Briantais *et al.*, 1979), and is based on an increased rate constant of thermal deactivation (Krause *et al.*, 1983). Excess light results in a build up of transthylakoid delta pH due to limited proton use in photophosphorylation. An increased electron transfer from PSI to oxygen in the Mehler reaction under limited carbon metabolism is important in this process (Krause, 1988), and may explain the protective effect of low concentrations of oxygen (Krause *et al.*, 1985; Krause and Cornic, 1987). Transmembrane delta pH results in energisation of thylakoids and may lead to quenching of about 90% of F_V. The extent of the delta pH depends on the intrathylakoid proton concentration (Laasch, 1987). The delta pH build up would serve to avoid overreduction of PSII by triggering non-radiative dissipation of excitation energy (Weis and Berry, 1987; Horton and Hague, 1988; Krause *et al.*, 1988). There is a negative correlation between q_E and photon yield of photosynthetic carbon dioxide dependent oxygen evolution (Krause *et al.*, 1988; Weis and Berry, 1987; Horton and Hague, 1988). This may indicate a control mechanism in the thylakoid membrane which dynamically adjusts thermal dissipation of excitement energy to the photon requirement of energy consuming carbon metabolism (Krause, 1988). The molecular mechanism for this process is not fully understood. It is known that there is a requirement for $\triangle$pH, but other factors are also involved. q_E has been found to precede photoinhibition, indicating that photoinhibition may take place in PSII centres down-regulated by light-induced acidification of the lumen (van Wijk and van Hasselt, 1993). q_E consists of two components. The first is characterised by a decrease in F_0 and appears to involve zeaxanthin. Zeaxanthin may increase or

enhance q_E at intermediate delta pH levels, but the maximum level of q_E is not altered (Rees *et al.*, 1989). The second component in independent of zeaxanthin.

The origin of q_E is controversial. Weis and Berry (1987) proposed a model, indicating an origin in the reaction center, where two states of PSII reaction centers were assumed. $(PSII)_o$ denotes a state with high photochemical efficiency, while $(PSII)_e$ is a high energy state with a high rate constant of thermal deactivation and low photochemical efficiency. The latter does not contribute to F_V and is responsible for quenching fluorescence. In the first state, there is efficient electron transport to Q_A. As the proton gradient increases, an increasing number of centers are converted to the $(PSII)_e$ state. Once in this state, the system is protected by increasing non-destructive thermal deactivation of excited centers. An alternative model by Genty *et al.* (1989; 1990) proposes that quenching occurs in the antennae. Quenching, according to these authors, is related to a decrease in PSII efficiency and the F_V/F_M ratio in the quenching state is taken as a measure of the quantum efficiency of open centers.

1.3.5.3.2 State-transition related quenching (q_T)

Phosphorylation of part of LHCII leads to a transition from a "state 1" to a "state 2". This may result in a detachment of the LHCII from the core antennae of PSII. The LHCII may also detach from the core antennae because of physical changes to the thylakoid membrane, resulting in an increase in the $PSII_ß$ fraction (Krause and Weis, 1991). This could cause a decrease in the absorption cross section of PSII relative to PSI and fluorescence emission will decrease (Krause and Weis, 1991). Phosphorylation is controlled by the redox state of the intersystem electron chain. Once in "state 2", light energy absorbed by phosphorylated LHCII may be transferred to PSI, although this is still debated (Williams and Allen, 1987). When the ß-fraction of PSII increased by exposure of the plant to moderately high temperatures, there was an enhanced phosphorylation of PSI activity (see Krause and Weis, 1991). State regulation and spillover to PSI may depend on a dynamic interaction between the physical state of the membrane and metabolically controlled enzymatic phosphorylation of LHCII (Krause and Weis, 1991). State 1/state 2 transitions would optimise quantum yield of photosynthesis at low irradiance by balancing the energy distributed to the two photosystems (Horton, 1985).

1.3.5.3.3 Photoinhibitory quenching (q_I)

This quenching is a result of increased nonphotochemical de-excitation of pigments (Krause and Weis, 1991). A correlation has been found between q_I and zeaxanthin formation at the expense of violaxanthin and ß-carotin (Demmig *et al.*, 1987), possibly pointing to a protective role or the xanthophyll cycle. Zeaxanthin is thought to facilitate increased thermal deactivation in PSII antennae in a reversible fashion, but it is not known whether this specific zanthophyll species could

cause quenching of excited states (Krause and Weis, 1991). A decrease in optimal quantum yield of photosynthesis has been related with q_I, which is expressed as a decrease in the F_V/F_M ratio (Demmig and Björkman, 1987; Demmig-Adams *et al.*, 1990; Somersalo and Krause, 1989), or to the loss of PSII photosynthetic activity of isolated thylakoids (Krause *et al.*, 1990). Photoinhibitory quenching may be initiated by PSII reaction centers. A change of a fraction of these centers to "quenchers" has been postulated (Cleland *et al.*, 1986). These centers would act as energy traps, but, being incapable of normal photochemical reactions, would convert it into heat. Control of PSII by nonphotochemical quenching of excitation energy appeared to have been identical in both cold-hardened and nonhardened wheat and rye cultivars but, irrespective of growth temperature, nonphotochemical quenching exerted a stronger control on PSII photochemistry at lower temperatures (Öquist *et al.*, 1993). Formation of q_I has been directly related to damage and degradation of D1 protein in reaction, but more recent data shows that photoinhibition and concomitant quenching precede the dysfunction and degradation of D1. In *Chlydomonas*, degradation of D1 protein (in low light with chloramphenacol which supresses D1 formation) does not lead to F_M quenching which characterises photoinhibition (Briantais *et al.*, 1988). Molecular oxygen and/or reactive species from oxygen may be involved in formation of q_I (Barnéyi and Krause, 1985). Oxygen seems necessary for D1 degradation (Arntz and Trebst, 1986), but anaerobic conditions enhance q_I - related to an increase in F_0, indicating that photoinhibition under anaerobiosis is caused by a different mechanism to that in the presence of oxygen (Krause *et al.*, 1985).

1.3.5.3.4 Other quenching mechanisms

There are three further mechansims whereby fluorescence may be quenched. These are due to magnesium ions, pigment radicals and oxidised plastoquinone. Fluorescence, especially F_V, of thylakoid suspensions is affected by cation concentration in the reaction medium (Krause and Weis, 1991). This is based on lateral segregation of the two photosystems, at high Mg^{2+} levels, which is related to membrane stacking which controls energy transfer from PSII to PSI. While quenching due to Mg ions is important *in vitro*, it does not appear to be of significance *in vivo* (Krause and Weis, 1991).

When excitation energy is captured by excited states of photosynthetic pigments, for example $Pheo^-$, P^+_{680}, Chl^*, carotenoid radical (Car^+), it is converted into heat and fluorescence is quenched (Krause and Weis, 1991). As these excited states are not very stable, they do not appear to contribute to *in vivo* fluorescence to a large extent.

In an oxidised state, the PQ pool exerts a "static" quenching on fluorescence. This is a minor effect, enhanced by detergents *in vitro* (Krause and Weis, 1991).

Environmental stress affects fluorescence quenching. Photoinhibition of PSII is enhanced by reduced Q_A. At high q_E, thermal deactivation increases the number of open reaction centers in the steady state (Weis and Berry, 1987). When q_E is saturated and the light intensity exceeds light saturation of photosynthesis, Q_A becomes reduced and q_P decreases, but q_I is enhanced (Krause and Weis, 1991). Freezing stress affects the formation of q_E in response to delta pH. Under steady state and moderate light, this results in a decrease in q_P and is correlated with high q_I (Krause and Weis, 1991). Formation of q_I is promoted by conditions that limit energy utilisation in carbon metabolism or affect the D1 protein repair process (Krause and Weis, 1991).

1.3.5.4 Role of Water

This has been discussed in some depth earlier, but a few more points in relation to water detection as a tool for determining chilling or frost tolerance must be made. Studies on water in seeds have suggested that water not able to freeze is tightly associated with hydrophilic surfaces (Vertucci, 1990). This type of water appears to be similar for most of the seed developmental stages studied. Berjak *et al.* (1990) suggest that this water (bound water) stabilises intracellular components, allowing seeds to retain viability when bulk or freezable water is removed rapidly.

This technique provides a measure of the excess heat capacity of a system as a function of temperature (Cramer *et al.*, 1981). It is able to yield information on phase transitions, particularly on phase transitions within biological membranes (Jackson *et al.*, 1973; Cramer *et al.*, 1981). More recently, this technique has been used to study the transitions of water (Vertucci, 1990; Ramlov and Hvidt, 1992). The enthalpy of exothermic and endothermic transitions are used as a basis for estimates of the amount of water freezing and ice melting (Ramlov and Hvidt, 1992). Several states of water have been identified using this technique: Freezing of bulk water only occured when seeds were hydrated above 0.55g H_2O g^{-1} dry weight (Vertucci, 1990), and no freezing was seen below a hydration level of 0.3g H_2O g^{-1} dry weight. These results were confirmed in *Artemia* cysts (Ramlov and Hvidt, 1992). Removal of water by freezing to ice appears to be lethal (Ramlov and Hvidt, 1992). Using this tool, it is possible to determine the amount of water in a piece of tissue and this can be correlated with the extent of freezing that will occur in the tissue.

1.4 Objectives

The objectives of this study were:

(1) To establish techniques to investigate certain parameters - biophysical (*viz.* proline concentration and glutathione reductase activity) and biophysical (*viz.* fluorescence characteristics and onset of temperature of melt) reported to be related to chilling tolerance and/or resistance.

(2) To test the usefulness of these parameters as diagnostic tests by applying them to genotypes well defined for their chilling tolerance characteristics and then ranking them on the basis of the data and comparing the resulting rank order with the "true" ranking determined by the foresters in the field.

CHAPTER 2
MATERIALS AND METHODS

2.1 Plant material and growth conditions.

Four species of *Eucalyptus* (*E. grandis*, *E. nitens*, *E. macarthurii* and *E. smithii)* and one hybrid (GN1026, *E.grandis* x *nitens*) were obtained from Mondi Forests (Research and Development Division, Natal Office, P.O.Box 39, Pietermaritzburg, 3200). *E.nitens*, *E. smithii* and *E.macarthurii* were chosen as representatives of tolerant plants (B. Herman, *pers. comm.*), with *E. nitens* being the most resistant to cold conditions (Poynton, 1979). *E. grandis* and GN1026 are comparitively less tolerant (B. Herman, *pers. comm.*), with *E. grandis* being representative of a chilling sensitive plant (Poynton, 1979). The plants were grown in potting soil under 70% shade cloth and watered every morning and evening. Experiments were conducted on two year old plants, using the second to fifth leaves from the apex.

Chilling tolerance was tested in a controlled environment chamber (CONVIRON) (Controlled Environments, 601 Stutnan Street, P.O. Box 347, Pembina, N.D. 58171, USA or 1461 St. James Street, Winnipeg, Canada, RBH 0W7). Plants were subjected to a 14/10 hour light/dark cycle with a light intensity of 200 μmol m^{-2} s^{-1}. There was an initial acclimation period at 20°C for five days. On day six the temperature was reduced to 4°C at a rate of 2°C per hour. The temperature was maintained at 4°C for five days. During the first eleven days, four parameters, proline content, glutathione reductase (GR) activity, fluorescence and enthalpy, were measured daily. On day ten the temperature was returned to 20°C at 2°C per hour. The plants were allowed to recover at this temperature for a five day period. Each parameter was measured on alternate days during this phase.

2.2 Biochemical Parameters

Eucalyptus is known to contain many tannins, phenols and oils (Poynton, 1979; Tibbits, 1986), which would influence the accuracy of biochemical techniques. To compensate for this, methods were modified for use with *Eucalyptus* tissue. Where relevant, method development is included.

2.2.1 Proline content

Proline was initially assayed using the method of Bates *et al.* (1973). In this method, 0.5 g leaf tissue was homogenised in 10 ml 3% aqueous sulfosalicylic acid. The homogenate was filtered through Whatman #2 paper and 2 ml of the filtrate reacted with 2 ml acid-ninhydrin and 2 ml glacial acetic acid in a test-tube for 60 minutes at 100°C. The reaction was terminated by placing

the tubes in an ice bath. The reaction mixture was extracted with 4 ml toluene and vigorously mixed for 15 to 20 seconds. The chromophore in the toluene phase was read at 520 nm. When a known amount of proline was included in the reaction mixture to test percent recovery, this method was found to be accurate for bean plants (almost 100% - results not shown), but when performed on *Eucalyptus* a low percent recovery was obtained (average of 62.42% - results not shown). The phenols and oils in *Eucalyptus* tissue extract appeared to influence the accuracy of this technique. Consequently, a method was chosen that removed any potential interferring substance(s), that of Tal *et al.* (1979).

Leaf tissue was rapidly frozen in liquid nitrogen and homogenised in 2 ml of a methanol:chloroform:water (12:5:1 v/v) mixture. The homogenate was centrifuged at 10000 rpm in a Beckman GP Centrifuge (Beckman Instruments, Inc., Beckman RIIC Ltd., Glenrothes, Scotland) for 10 minutes and the supernatant collected. The pellet was redissolved in 2 ml methanol:chloroform:water (12:5:1 v/v) and centrifuged as above. The combined supernatants were added to 1 ml chloroform and 1.5 ml water, centrifuged, as above, and the proline content determined using a modified method by Singh *et al.* (1973). The aqueous phases of the supernatants were collected, added to 10 ml water and 375 mg activated chloride resin (Permutit cation exchange resin, 200-Karb, unifunctional sulphonated polystyrene beads <200 mesh, 8% DVB, 0.9 - 1.1 WR) and shaken for 10 minutes. The supernatant was decanted, mixed with 5 ml acid ninhydrin and 5 ml glacial acetic acid and was placed in a boiling waterbath for 45 minutes in tubes with screw caps. After cooling, the chromaphore was extracted with 4 ml toluene and read at 520 nm using a DU 7500 Beckman Spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). A standard curve was constructed each time the assay was run, with proline standards in the range of 0 - 15 $\mu g.ml^{-1}$.

The resin was included to ensure removal of interferring compounds. To test the influence of protein on the assay, the sample was boiled at 100 °C for 5 minutes after homogenisation. Boiling the homogenate did not appear to lessen interference when determining proline content, as the reaction mixture remained cloudy, resulting in poor absorbance readings. Using toluene to extract the chromaphore often resulted in clouded samples. This problem was overcome by replacing toluene with benzene to extract the chromophore (Singh *et al.*, 1973).

2.2.2 Glutathione reductase activity

Procedure 1.

To extract glutathione reductase (GR) from leaf tissue, 1 g of tissue was homogenised with a mortar and pestle with 10 volumes of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5

mM EDTA. The homogenate was filtered through cheesecloth and centrifuged at 2500 rpm in a Beckman GP Centrifuge (Beckman Instruments, Inc., Beckman RIIC Ltd., Glenrothes, Scotland) for 10 minutes. Aliquots (5 ml) were run through a G-25 Sephadex column (1.5 x 25 cm at 5°C) pre-equilibrated with 0.1 M potassium phosphate buffer (pH 7.5). The protein was eluted with 0.1 M potassium phosphate buffer (pH 7.5). As contaminating compounds were still found in the elutant, other experimental procedures were attempted.

Four different extraction techniques were examined. Initially, the grinding technique was optimised. Grinding with an Ultra-turrex (Jahnke & Kunkel, IKA-WERK, W.A. Saver) increased the temperature of the reaction solution and did not improve GR activity or make it comparable to that obtained by other authors (Esterbauer and Grill, 1978; Kalk-Torres, 1984; Connell and Mullett, 1986; Mahan and Burke, 1987; Edwards *et al.*, 1990). To prevent oxidation of the enzyme, the tissue had to be ground as rapidly as possible. To this end, manual grinding in liquid nitrogen was attempted. After grinding, 0.1 M phosphate buffer with 0.5 mM EDTA (pH 7.5) was added. This reduced interference when absorbance was read at 340 nm (i.e. increased GR activity from 0.0002 units per gram fresh weight to 0.009 units per gram fresh weight), but results were still not comparable to those obtained by other authors (see Esterbauer and Grill, 1978 (2.33 - 4.14 units per gram fresh weight); Doulis *et al.*, 1993 (0.26 - 1.91 units per gram fresh weight)).

The lack of GR activity was initially attributed to protease activity. A protease inhibitor cocktail at final concentrations of 0.5 and 1 mM was included in the extraction buffer. The cocktail was made up of phenylmethylsulfonylfluoride, ethaaminocaproic acid and benzamide hydrochloride but did not appear improve GRA, which was very erratic and unpredictable (results not shown), suggesting that proteases were not affecting GRA. It was thought that the loss of activity could be attributed to GR oxidation.

As phenolics are known to play a role in altering the oxidation state of enzymes (Loomis and Battaile, 1966), BSA (0.1%) and iso-citrate ascorbic acid (2 and 5 mM) were included in the extraction buffer to minimise the effect of these compounds. In combination, iso-citrate ascorbic acid and BSA appeared to dampen the noise effect on absorbance, but levels of GR activity were still much lower than those obtained for *Nicotinia* (results not shown), and than those in the literature (Esterbauer and Grill, 1978; Connell and Mullett, 1986; Mahan and Burke, 1987; Edwards *et al.*, 1990). As polyaclar AT has been used to combat the effect of phenolics on enzyme activity (Loomis and Battaile, 1966), it was added to the extraction buffer, and when in combination with isocitrate ascorbic acid, GR activity was approximately 10% that obtained by other authors.

In an effort to eliminate all possible interfering compounds, Sephadex columns (G-50 and G-25) were prepared in Pasteur pipettes. This was to lessen the dilution effects of larger columns as the sample volume was small. The columns did not improve GR activity, and as a result, spun columns, similar to those used in DNA purification, were constructed (Maniatus *et al.*, 1982). These columns were used in an attempt to remove any contaminating compounds. Another advantage of this method is that it is rapid and it was hoped that it would remove any compounds before they had time to affect GR. The elutant from these columns showed greater GR activity, but less than that found in literature (Esterbauer and Grill, 1978). This suggested that the enzyme was being oxidised before the sample was applied to the column.

The method of Smith *et al.* (1989) was initially used to determine activity of glutathione reductase. This method involved the use of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), which is reduced by reduced glutathione to form (2-nitrothiobenzoic acid) (TNB). To a 4.5 ml cuvette were added 1.0 ml 0.2 M potassium phosphate buffer (pH 7.5) with 1 mM EDTA, 0.5 ml 3 mM DTNB in 0.01 M phosphate buffer, 0.25 ml water, 0.1 ml 2 mM NADPH, 0.05 ml glutathione redutase (or sample) and 0.1 ml 20 mM GSSG. The reaction was initialised by addition of oxidised glutathione and the absorbance read at 412 nm using a DU 7500 Beckman Spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA).

Once the extraction process was modified, without marked improvement in GR activity, another GR assay was attempted. Because the reduction of glutathione disulphide via GR is a NADPH dependent reaction, this reaction can be followed by the loss of NADPH from the system. This method (Racker, 1955 in Carlberg and Mannervik, 1985) was used to determine GR activity in *Eucalyptus*. 50 μl 0.2 M potassium phosphate buffer, 10 μl 20 mM GSSG, 35 μl extract and 10 μl 2 mM NADPH were added to a 100 μl cuvette and the absorbance at 340 nm was followed. This method also gave lower activites for *Eucalyptus*, than those obtained in the literature (Esterbauer and Grill, 1978; Kalk-Torres, 1984; Connell and Mullett, 1986; Mahan and Burke, 1987; Edwards *et al.*, 1990), even though an extraction buffer containing iso-citrate ascorbic acid and BSA was used. Both the DTNB and NADPH assays were performed on tobacco, and the GR activity levels obtained corresponded with those in the literature (Esterbauer and Grill, 1978; Edwards *et al.*, 1990; Whittaker, 1990).

Procedure 2.

As GR is found in the chloroplast, an attempt was made to extract *Eucalyptus* chloroplasts. The chloroplasts extraction method used was developed by Coulson *et al.* (1993). Plants were placed in the dark for 12 hours to minimise the presence of starch. Surface sterilised leaves were homogenised in extraction buffer of pH 8.0 (0.35 M sorbitol, 50 mM Tris-HCl , 5 mM EDTA,

0.1% BSA, 0.15 % (v/v) mercaptoethanol, 1 mM $MgCl_2$ and 2 mM iso-ascorbic acid) and filtered through muslin and then miracloth. The filtrate was centrifuged at 2000 rpm in a Beckman GPR Centrifuge (Beckman Instruments, Inc., Beckman RIIC Ltd., Glenrothes, Scotland) at 4°C for 15 minutes. The residue on the muslin was scraped off and washed in wash buffer (pH 8.0) (0.35 M sorbitol, 50 mM Tris-HCl and 0.25 mM EDTA) with isoascorbate (0.056 mM) and citrate (0.056 mM) for 10 minutes. This was filtered through miracloth and centrifuged as above. The pellets were collected and pooled. Wash buffer (1 ml) was added to the combined pellets and the mixture loaded onto a sucrose gradient (20 ml 52% sucrose and 10 ml 30% sucrose) which was run at 50000 rpm in a Beckman L8-55M Ultracentrifuge (Spinco Division, 1050 Page Mill Road, Palo Alto, CA 94304, USA) at 4°C for 30 minutes. The chloroplast band was removed with a Pasteur pipette and washed with 4 ml wash buffer without isoascorbate or citrate. The chloroplasts were centrifuged at 2000 in a Beckman GPR Centrifuge (Beckman Instruments, Inc., Beckman RIIC Ltd., Glenrothes, Scotland) for 15 minutes at 4°C.

The chloroplasts were subjected to a number of treatments in order to disrupt the outer membrane. Firstly, they were sonicated, secondly, they were made into acetone powders, thirdly, they were exposed to a detergent (CHAPS: 3-[(3-chloamidopropyl] dimethlyl-ammonio]-1-propansulfonate) and lastly, ammonium sulphate precipitation was attempted. None of these steps resulted in moderate or high GR activity. The acetone powder step was not feasible as it required large amounts of chloroplasts, while it appeared that chlorophyll was an interfering factor in ammonium sulphate precipitation, possibly being precipitated at the same concentration as GR. Ammonium sulphate precipitation was attempted together with CHAPS, but little GR activity was measured.

Procedure 3: Adopted Procedure.

The final procedure attempted was that of acetone powder formation of the whole leaf sample. This was taken from Esterbauer and Grill (1978) and involved grinding 0.2 g leaf tissue in cold 75% acetone. The homogenate was passed through a Millipore 2.5 mm Micro-filter (Catalogue Number XX1002500 P30390). Very cold 100% acetone (-20°C) was passed through the system and a vacuum pump attached. Dry air was passed over the homogenate to obtain dry acetone powders. Each powder was reconstituted with 4 ml of a buffer containing 67 mM KH_2PO_4 and 67 mM Na_2HPO_4 with 1 mM EDTA (pH 7.4). This mixture was shaken for an hour and centrifuged for 10 minutes at 4000 rpm in a Beckman GPR Centrifuge (Beckman Instruments, Inc., Beckman RIIC Ltd., Glenrothes, Scotland). Supernatant (500 μl) was added to 1 ml solution A (1% (w/v) BSA, 1% (w/v) EDTA, 0.1% (w/v) NADPH and 0.4 M Tris-HCl, pH 7.4). GSSG (100 μl of 24.5 mg.10 ml^{-1}) was added to initiate the reaction, which was followed at 340 nm using a DU 7500 Beckman Spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA)

against a blank without GSSG. This method gave GR activity rates most comparable to literature. The Wetter protein assay was used to determine GR activity per mg protein (Wetter, 1984).

2.3 Biophysical Parameters

2.3.1 Fluorescence

Initially, the ratio of variable to maximum fluorescence, corresponding to photochemical efficiency of PS II (F_V/F_M) (Björkman, 1987) was measured. This was calculated as $(F_M - F_0)/F_M$ where F_0 and F_M are measures of the instantaneous fluorescence and the maximum fluorescence and the maxiumum fluorescence that resulted from the first saturating flash measured in dark-adapted leaves. Chlorophyll *a* fluorescence was measured at 25°C using a pulse-amplitude modulation fluorometer (Model PAM 101; H.Walz, Effeltrich, F.R.G.). An intact leaf, the second to fifth from the apex, was placed in the cuvette and dark-adapted for 20 minutes. This was followed by a weak light beam emitted from a diode to obtain F_0. The actinic light source used was 35 $\mu E.m^{-2}.s^{-1}$ at 650 nm. Maximum fluorescence was obtained by applying a one second pulse of saturating light (Schott KI 1500) [on settings ½ and 3-5, depending on the species under investigation] which completely reduced PS I and PS II electron acceptors. Both the weak and saturating beams were kept constant to enable comparisons between species. Induction kinetics and quenching were determined using a 0.7 second flash pulse length, a 10 second interval between flashes and a 1.2 second flash fade out time.

During a second run of the above experiment, the photochemical and non-photochemical quenching of each plant was determined over a 20 minute period (after the plant had reached steady state). These parameters reflect the removal of electrons from the fluorescence system and their chanelling to photochemical processes or towards non-radiative dissipation (Krause and Weis, 1991). Photochemical and non-photochemical quenching were calculated using the following equations:

$$q_P = 1 - (F_M' - F_0')/(F_M - F_0)$$

and

$$q_N = (F_M' - F)/(F_M' - F_O')$$

where F is the steady state fluorescence levels that results from continuous actinic illumination, and F_M' and F_0' are the fluorescence with Q_A reduced and oxidised, respectively.

2.3.2 Onset of Temperature of Melt

Using a Perkin-Elmer differential scanning calorimeter (DSC 7), the enthalpy changes in each species were measured over the temperature stress period. Leaf discs 3 mm in diameter were

punched out of the leaves and placed in the DSC sample pan. This was sealed shut and submitted to cooling from 30 to -70°C at a rate of 5°C per minute. The enthalpy of melt was measured as the sample was heated back to 30°C, as was the temperature at which the tissue melted (see Vertucci, 1990; Berjak *et al.*, 1992).

2.4 Statistical Analysis

Satistical analyses were performed using SAS/STAT Version 6; Copyright 1990 by SAS Institute Inc., Cary, N.C., USA. A program was written to determine the intra- and interspecific contrasts for all four parameters. The General Linear Models Procedure was used to determine interspecific statistical differences. This utilised analysis of variance (ANOVA) and least squared means. The tests were run for all four parameters at the three different temperatures (20°C, 4°C and 20°C [recovery]). Three samples per species were taken each time an assay was performed.

CHAPTER 3
RESULTS

The primary aim of this work was to identify parameters which would allow for the ranking of a number of *Eucalyptus* species with respect to chilling tolerance. To determine the effects of chilling, two biochemical and two biophysical parameters were used in the study of these plants. The biochemical parameters chosen were the concentration of the imino acid proline (often implicated in protection of membranes and proteins under stress conditions (Duncan and Widholm, 1987)) and glutathione reductase activity (thought to be the rate limiting step of the plant antioxidant pathway involved in quenching the production of activated oxygen species (Jahnke *et al.*, 1991)). Biophysical parameters chosen were fluorescence (a measure of photosynthetic efficiency (Krause and Weis, 1991) and the onset of temperature of melt (which is an indicator of the amount of water in the cell (Vertucci, 1990)). Each parameter will be dealt with separately and the arguements used to rank the species explained. To classify the species for chilling tolerance a ranking system was devised.

3.1 Biochemical Parameters

3.1.1 Proline Content

Soluble proteins often increase in plants under stress conditions. This increase is thought to allow the plant to survive such conditions (Chen and Li, 1977; Simonovitch and Cloutier, 1982). One of the compounds that has been found to increase under stress, particularly cold or chilling stress, is proline. Proline has a variety of functions, such as: stabilizing membranes by retaining the hydration state of proteins, protecting enzyme function, maintaining the osmotic balance in cells and having a possible antioxidant role (Blum, 1988; Alia *et al.*, 1991). As a result, it was hypothesised that those plants tolerant of chilling would show increased levels of proline and thus have protection against chilling damage.

Each of the species was acclimated at 20°C for five days, chilled at 4°C for five days and then allowed to recover at 20°C for five days. For each of the first ten days, and on every second day of the recovery period, proline concentrations were measured for each species. The proline content of *E. nitens* (Figure 3.1 A) did not change during the first 20°C period. On chilling, there was a slight decrease, but the level appeared to recover to a prechilling level while the temperature was still at 4°C. The overall proline levels during the recovery period (days 11 to 15) appeared to have been slightly higher than during the initial 20°C period. The proline concentration measured in *E. smithii* was uniform during the initial 20°C period (Figure 3.1 B). On chilling,

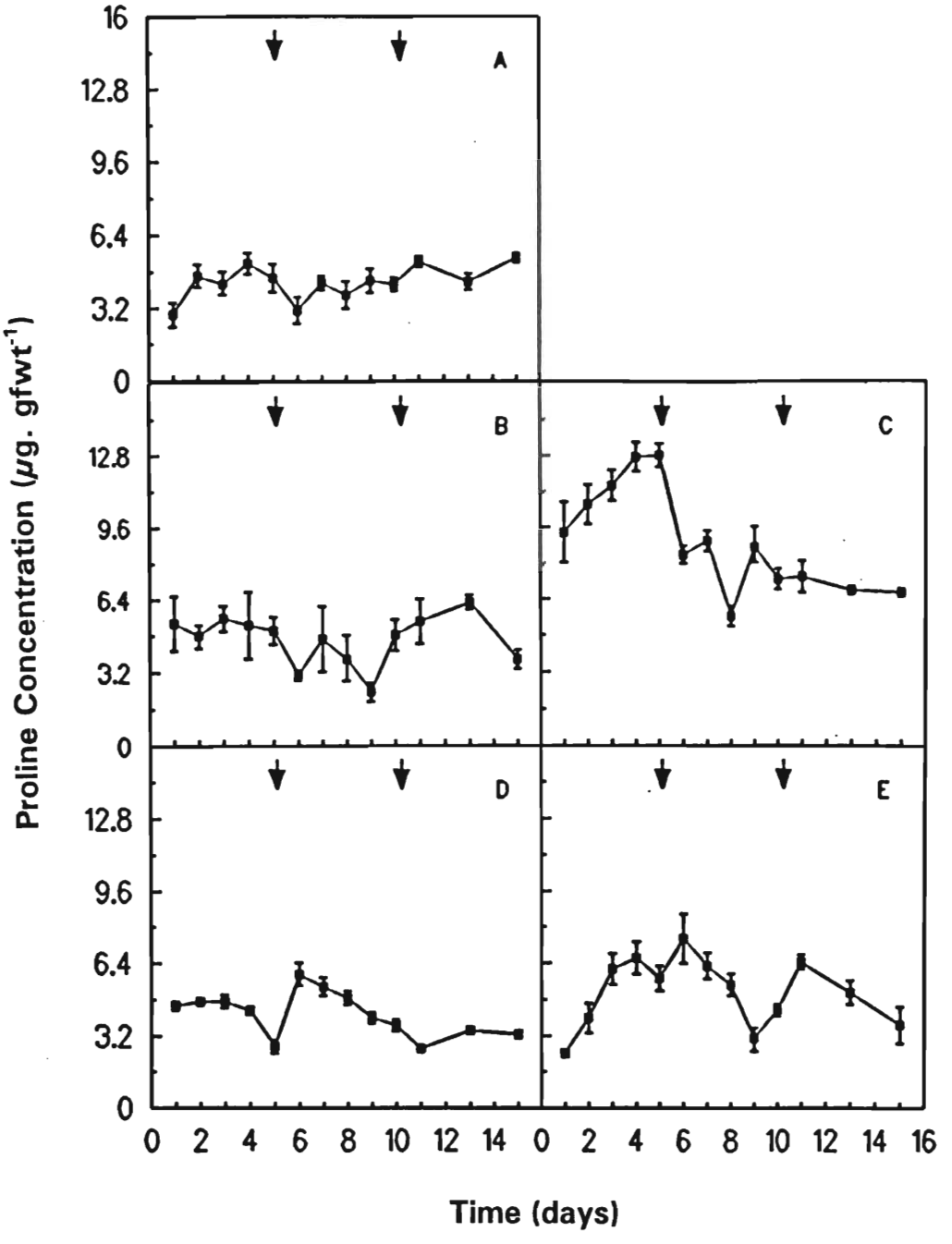

Figure 3.1 The effect of temperature on proline concentration in *Eucalyptus nitens* (A), *E. smithii* (B), *E. macarthurii* (C), GN1026 (D) and *E. grandis* (E) (Experiment 1). The first arrow in each graph represents the change in temperature from 20°C to 4°C, while the second represents the change from 4°C to the 20°C recovery period.

proline concentration tended to decrease in this species and appeared to stay low. When the plants were returned to 20°C, proline concentration in *E. smithii* increased, but then decreased. These data showed little statistical difference over the duration of the experiment. *E. macarthurii* showed a steady increase in proline concentration during the initial 20°C period (Figure 3.1 C). Decreasing the temperature to 4°C resulted in a marked decline in proline concentration. Proline levels decreased even further during chilling, but rose slightly before the temperature was returned

to 20°C. After this, proline concentrations were constant, but approximately half that observed during the initial 20°C period. Proline concentrations in the hybrid GN1026 were constant for the first four days but decreased on the fifth (Figure 3.1 D). On chilling, proline levels increased, but then gradually declined over this five day period. A return to 20°C did not result in a large change in proline levels, although overall these were approximately one and a half times lower than the overall initial proline concentration. *E. grandis* showed a similar pattern of proline concentration over the fifteen days to *E. macarthurii* (Figure 3.1 E). During the initial 20°C period there was a steady increase in proline concentration. There was a marked decline in proline concentration when the plants were subjected to chilling, but levels seemed to recover before the chilling period ended. Once returned to 20°C, there was a steady decline in proline levels.

Statistical analysis (Appendix I) revealed differences between the initial 20°C period and the chilling period and between the initial 20°C period and the 20°C recovery period, indicating that chilling stress had some effect on the proline levels of all species. The hybrid GN1026 and *E. macarthurii* were found to be significantly different from the other species. Data for *E. nitens* were not statistically different from the other species, but during the 4°C chilling period, proline levels in this species increased. This is opposite to the decreased proline levels during this period seen in the other plants. There was a large amount of interaction between all species at all temperature levels. Although this interaction may have been significant, it was too multi-facited to attempt interpretation. No trends were evident in the statistical analysis of this assay.

Change in proline concentration was different in the five species of *Eucalyptus* examined. No two plants appeared to respond in a similar fashion. Therefore, it was decided not to include these data in the system used to rank the species in terms of their chilling tolerance.

3.1.2 Glutathione Reductase Activity

The enzyme glutathione reductase may be the rate-limiting enzyme in the antioxidant pathway found in most plants (Janhke *et al.*, 1991). This enzyme converts oxidised glutathione to reduced glutathione, so maintaining the ratio in the cytoplasm of these two compounds. This ratio affects the oxide scavenging ability of the plant. Chilling-tolerant plants were hypothesised to contain more of this enzyme, valuable because it combats stress conditions, and would therefore show higher GRA levels.

There did not appear to be any significant changes in the levels of GRA in *E. nitens* (Figure 3.2. A), which were low, over the entire experimental period. The overall inital levels of GRA did appear to be slightly higher than the overall 20°C recovery levels, but this was not marked. Chilling did not appear to affect the levels of GRA in this species. Once again, there was no

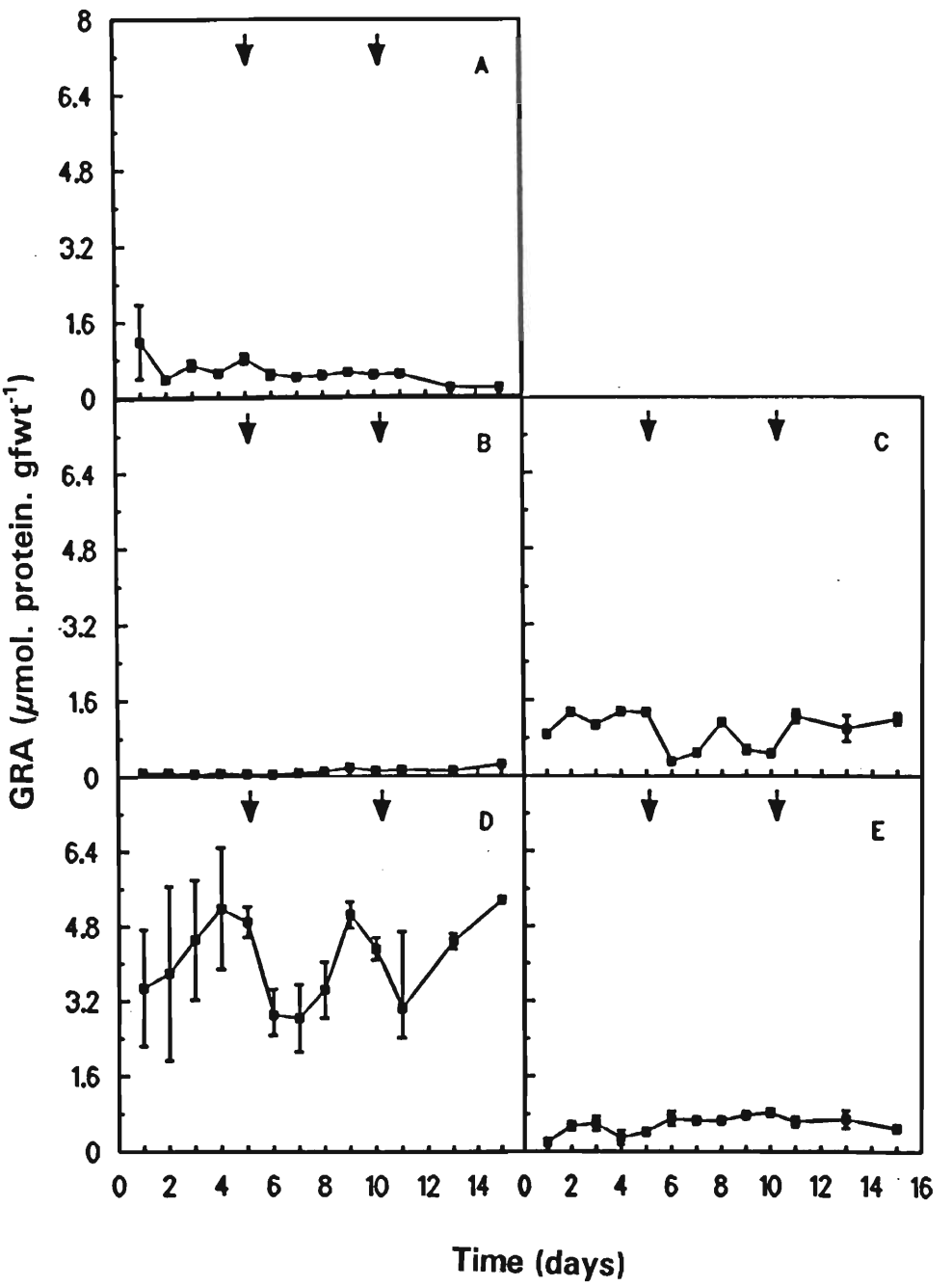

Figure 3.2 The effect of temperature on glutathione reductase activity in *Eucalyptus nitens* (A), *E. smithii* (B), *E. macarthurii* (C), GN1026 (D) and *E. grandis* (E) (Experiment 1). The first arrow in each graph represents the change in temperature from 20°C to 4°C, while the second represents the change from 4°C to the 20°C recovery period.

marked affect of chilling on GRA in *E. smithii* (Figure 3.2 B). The level of GRA in this species was the lowest of all the plants examined. During the chilling period a slight increase in GRA was observed. This continued once the plant was returned to 20°C conditions. As a result, the during the overall recovery period GRA levels were slightly higher than the mean initial GRA levels. There was a steady, relatively high level of GRA in *E. macarthurii* during the initial 20°C period (Figure 3.2 C). On chilling (day 6) a marked decrease in GRA was observed, which was followed

by an increase and another decrease. On days 6, 7, 9 and 10 similar proline levels were observed, perhaps indicating that the increase in GRA seen on day 8 was transitory. Return to 20°C resulted in slightly higher levels of GRA, which were similar to those levels observed during the initial period. The GRA levels observed in the hybrid, GN1026, were higher than in any of the other species (5 times higher than that of *E. smithii*) (Figure 3.2 D). The levels were also very variable. There was a steady enhancement during the first 20°C period, but as the standard error bars were large, this increase is questionable. When the plants were placed at 4°C, there was a marked decline in GRA. On days 8 and 9 there was a rise in GRA, but it was inhibited on day 10. The standard error bars for the results on days 6, 7 and 8 were large compared to those obtained for other species, possibly indicating that the decrease observed on these days was not as marked. When the plants were returned to 20°C, GRA declined slightly before increasing. The final level of GRA was similar to the level of GRA before chilling. GRA levels in *E. grandis* were fairly constant over the fifteen day experiment, although there was a decrease in day 4 (Figure 3.2 E). Chilling appeared to enhance GRA a little and this level was maintained for the duration of the stress. When the plants were returned to 20°C, a slight inhibition of GRA was observed, but the overall levels were higher than the overall initial GRA levels. GRA in *E. grandis* was higher than in *E. nitens* or *E. smithii*, but lower than in *E. macarthurii* or GN1026.

Statistical analysis showed that there was interaction at the species level, that is, all the species were statistically different from each other, with GN1026 and *E. macarthurii* having the greatest difference (Appendix I). There were no significant interactions at the temperature levels or when the species and temperature levels were combined.

As for the proline data, no trend was found, although some of the species showed considerably higher GRA levels than others, it was not possible to objectively rank them. Consequently, this parameter was not included in the ranking system.

3.2 Biophysical Parameters

3.2.1 **Fluorescence** Characteristics

Photochemical reactions are complemented by nonphotochemical reactions. These reactions are able to make use of excess light, so preventing damage to the photosynthetic apparatus. Fluorescence is a well known nonphotochemical reaction which absorbs and then reflects excess light at a longer wavelength. Should photosynthetic processes be adversely affected, nonphotochemical reactions, including fluorescence, would increase. It was hypothesised that the photosynthetic apparatus of chilling-tolerant plants would be able to withstand or tolerate high light

under chilling stress conditions and would, therefore, show less fluorescence or show less of a decrease in photosynthetic efficiency over longer periods of stress.

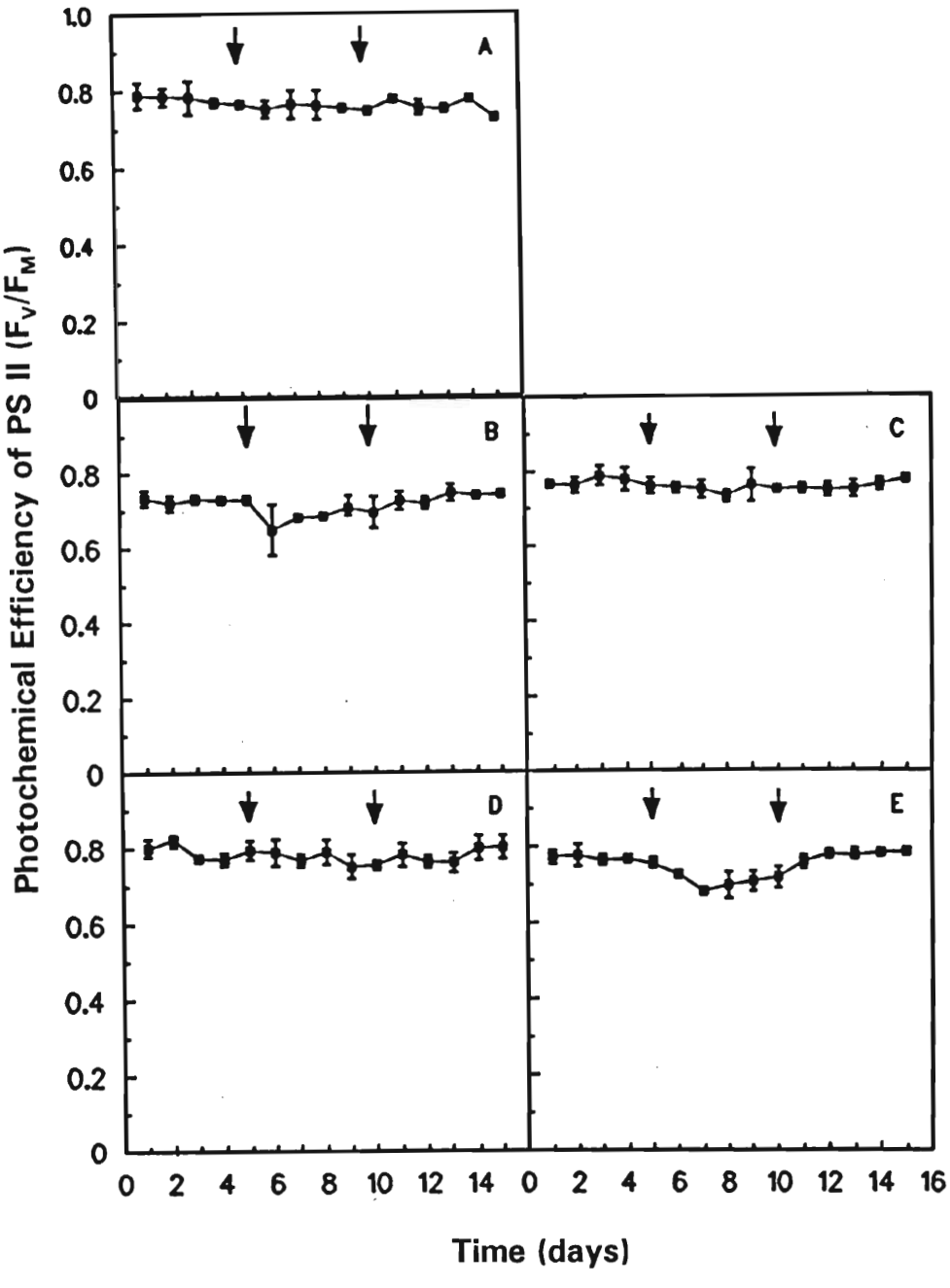

Figure 3.3 The effect of temperature on photsynthetic efficiency (F_V/F_M) in *Eucalyptus nitens* (A), *E. smithii* (B), *E. macarthurii* (C), GN1026 (D) and *E. grandis* (E) (Experiment 1). The first arrow in each graph represents the change in temperature from 20°C to 4°C, while the second represents the change from 4°C to the 20°C recovery period.

On chilling, photosynthetic efficiency was generally found to decrease (Figure 3.3). A decrease in photosynthetic efficiency (F_V/F_M) is seen as an indication of chilling sensitivity. Those species that showed the most noticeable decreases were *E. smithii* and *E. grandis* (Figure 3.3 B and E,

respectively). In both of these cases, the ratio returned to initial levels once the plants were returned to 20°C. In the other three species, the pattern was less clear, and there appeared to be more fluctuation in the data, especially for *E. nitens* and GN1026. *E. nitens* (Figure 3.3 A) showed a decrease in photosynthetic efficiency when placed at 4°C, but the decrease was more gradual than in either *E. smithii* or *E. grandis*. Once the plants were returned to 20°C, photosynthetic efficiency levels returned to those of the initial period, although there was more fluctuation. In GN1026 no constant rate of photosynthetic efficiency was observed (Figure 3.3 D), making it difficult to determine whether there was a real change when the plants were subjected to chilling. The photosynthetic ratios observed in *E. macarthurii* followed the general trend (Figure 3.3 C), with a gentle decrease on chilling and a return to prechilling levels when the plants were replaced at 20°C. There was a slight increase in the overall efficiency during the 20°C recovery period, compared to that during the initial 20°C period.

Statistical analysis showed that the chilling period gave statistically different F_V/F_M ratios to the initial and recovery 20°C periods. No differences were found between the two 20°C periods. There was a little interaction between *E. nitens* and *E. smithii* and between the 4°C and 20°C periods, but this was not significant (Appendix I).

In order to quantify the decrease in photosynthetic efficiency, a line was drawn from day 5 (onset of chilling) to day 10 (onset of recovery temperature) along the average F_V/F_M ratio. The area under this line was calculated and the results plotted (Figure 3.4). This graph clearly indicates the differences between the five species, with *E. smithii* showing the least decrease in photosynthetic efficiency, followed by *E. nitens, E macarthurii,* GN1026 and *E. grandis*. These differences were used to rank the species in terms of their chilling tolerance, with *E. smithii* being the most tolerant and ranked as **5**, followed by *E. nitens* (**4**), *E. macarthurii* (**3**), GN1026 (**2**) and *E. grandis* (**1**), hypothetically the least tolerant.

During the chilling period, the mean maximum non-photosynthetic quenching (Q_{NP}) values were also recorded. This is quenching of the nonphotosynthetic or protective reactions. A decrease in Q_{NP} represents uncoupling of the thylakoids and an increase indicates their energisation. It was hypothesised that those plants able to cope with stress conditions would show high Q_{NP} values as their photosynthetic apparatus would be minimally affected, while those plants unable to cope with the stress, would show uncoupling of thylakoids, or low Q_{NP} values. *E. grandis* had the lowest Q_{NP} value, followed by *E. smithii* and *E. macarthurii*, GN1026 and *E. nitens* (Figure 3.5). These data were used to rank the species in terms of chilling tolerance. *E. grandis* was ranked as the least chilling-tolerant and given the value **1**, while *E. nitens* was the most tolerant and given the value **5**. *E. smithii, E. macarthurii* and GN1026 were intermediate and placed as **2, 3** and **4,**

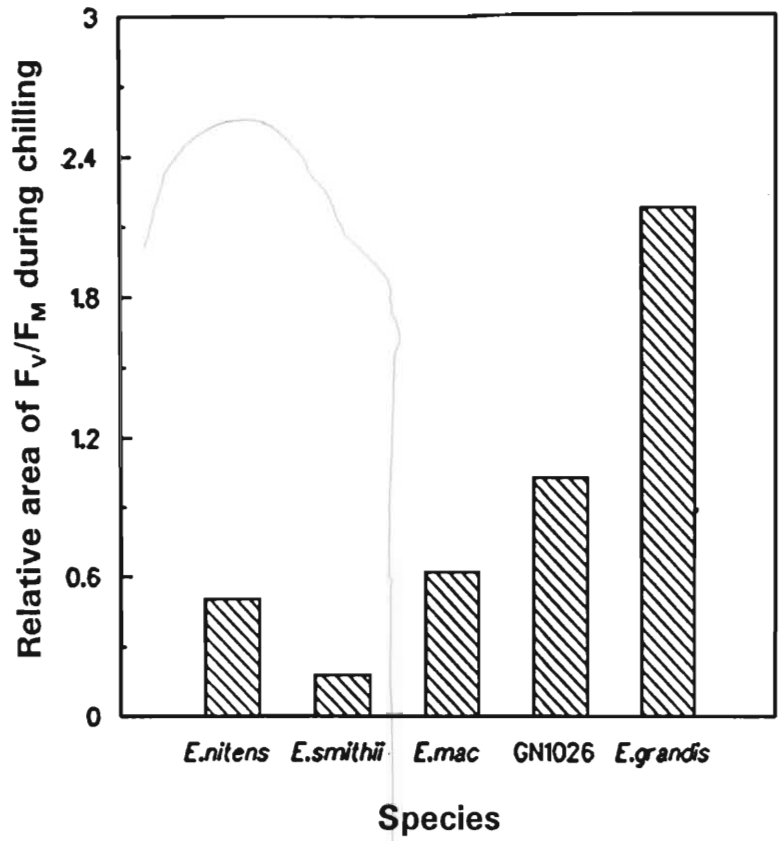

Figure 3.4 Area of F_V/F_M during the chilling period (Experiment 1 - see text for details). *E. mac* represents *E. macarthurii*.

respectively.

The slope of Q_{NP} was correlated with nonphotochemical quenching reaction rate. Hence a decrease in the slope of Q_{NP} would indicate a decrease in the rate of nonphotochemical reactions, implying a decreased level of protection against stress conditions. Those plants more tolerant of chilling would show little change or an increase in the slope of QNP. The effects of chilling on the slope of Q_{NP} are given in Figure 3.6. Because *E. smithii* (Figure 3.6 A) showed the greatest decrease of slope on chilling, it was given the ranking of 1 to indicate that for this parameter, this species was the least tolerant of chilling. *E. nitens* (Figure 3.6 B) also exhibited a decreased slope when placed in chilling conditions, but this was not as large as for the previous species. *E. nitens* was given the ranking of 2. Chilling did not appear to affect the slope of Q_{NP} of *E. grandis* which showed little variation over the whole experimental period (Figure 3.6 E). As such, this species was given a ranking of 3. *E. macarthurii* showed an increase in Q_{NP} slope on chilling, but this chilling-induced increase was temporary as the slope of Q_{NP} returned to a similar pre-chilling level and remained there for the remainder of the experiment (Figure 3.6 C). This species was given a rank value of 4. GN1026 also showed an increase in Q_{NP} slope on exposure to chilling. In this

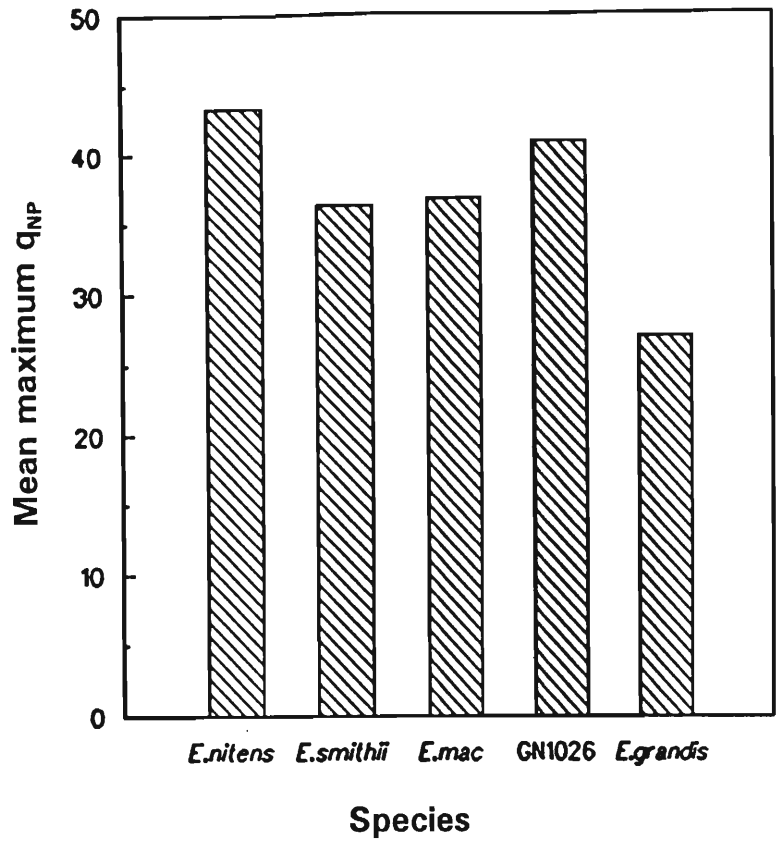

Figure 3.5 Comparison of mean maximum q_{NP} values during the 4°C chilling period.

case, the increase was constant and appeared to be maintained until the end of the experimental period (Figure 3.6 D). The hybrid was given a ranking value of 5.

Because the fluorescence data, particularly the data for the F_V/F_M ratios, appeared to be potentially useful, this experiment was repeated. The results can be seen in Figure 3.7. There are many similarities with the previous experiment, but some of the trends were emphasised. There was a marked decrease in photosynthetic efficiency when the plants were subjected to chilling temperatures, but in some of the species there appeared to be two phases in the chilling curve. This is most apparent in *E. nitens* (Figure 3.7 A) and GN1026 (Figure 3.7 D), although it can be seen in *E. grandis* (Figure 3.7 E). This may be related to the efficiency with which these plants are able to cope with stress conditions. The areas under the curve during the chilling period for each of the species was measured, as before, and the results were compared to those of the previous experiment (Figure 3.8). The smallest area was observed for *E. smithii*, followed by *E. macarthurii*, *E. grandis*, GN1026 and *E. nitens*. Using this data, GN1026 would have been given a ranking value of 1, *E. nitens* one of 2, *E. grandis* one of 3, *E. macarthurii* one of 4 and *E. smithii* one of 5. While the order of ranking is different to the previous experiment, the areas were much larger overall (data not shown). Unfortunately, during this experiment the temperature in

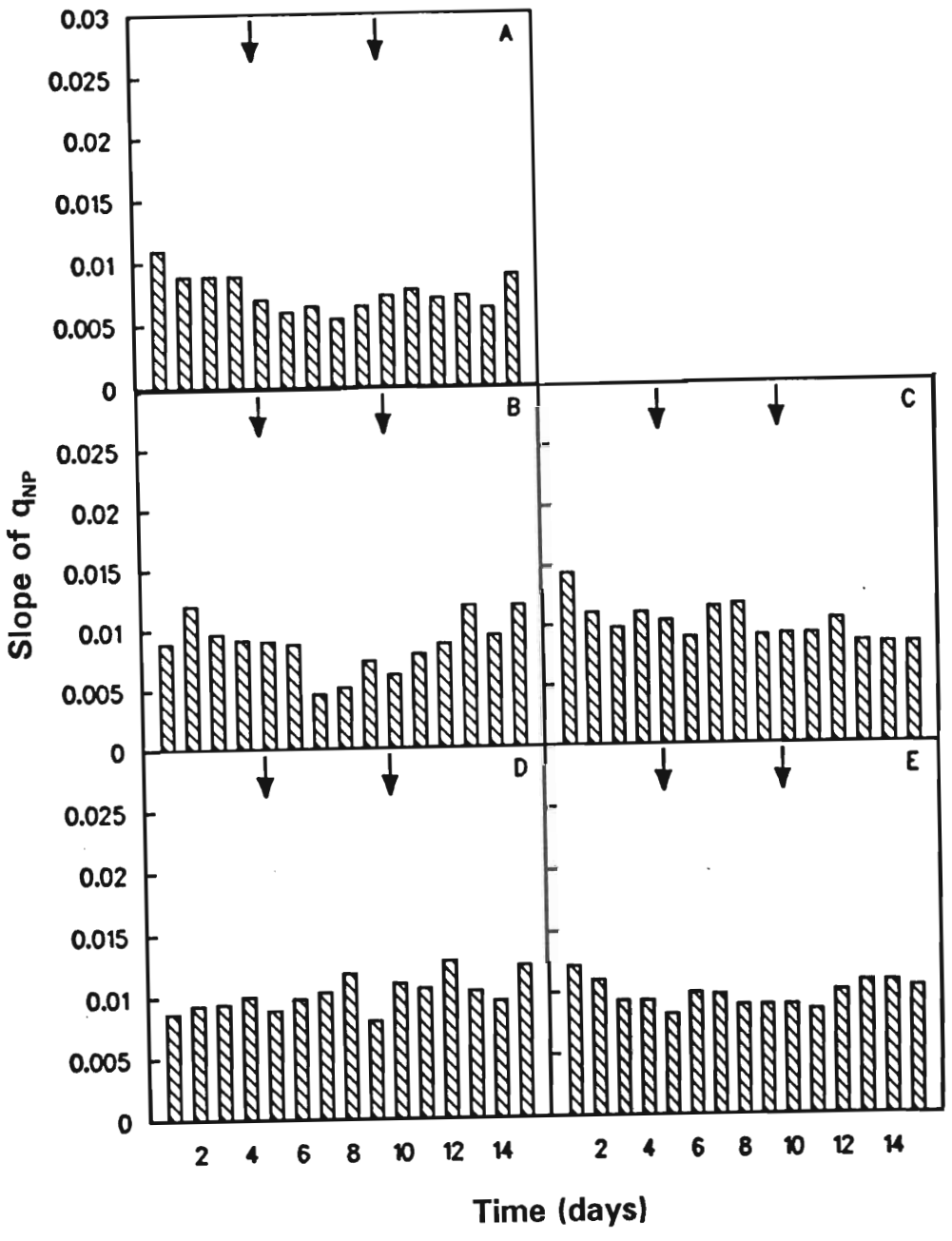

Figure 3.6 The effect of temperature on the slope of q_{NP} in *Eucalyptus nitens* (A), *E. smithii* (B), *E. macarthurii* (C), GN1026 (D) and *E. grandis* (E) (Experiment 1). The first arrow in each graph represents the change in temperature from 20°C to 4°C, while the second represents the change from 4°C to the 20°C recovery period.

the CONVIRON was unstable, particularly when set to the chilling temperature (4°C), and it was decided not to include these data in the final ranking to prevent confounding of the results .

3.2.2 Onset of Temperature of Melt

The amount of water in a cell affects the temperature at which the tissue will freeze. Both the "bound" and "free" water affects the temperature at which tissue freezes. "Bound" water, in other words water attached to membranes and organelle surfaces, does not freeze (Berjak *et al.*, 1990)

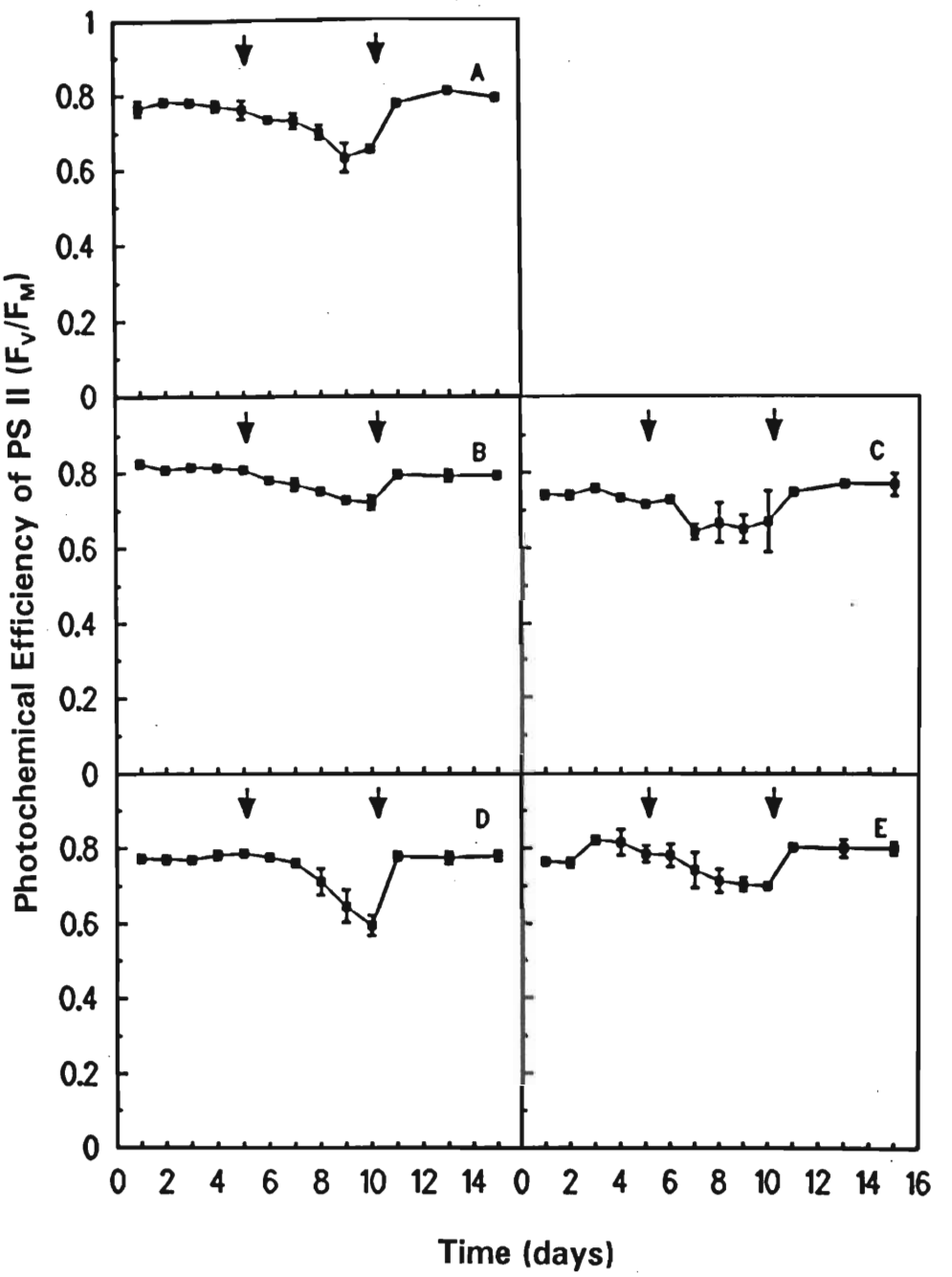

Figure 3.7 The effect of temperature on photsynthetic efficiency (F_V/F_M) in *Eucalyptus nitens* (A), *E. smithii* (B), *E. macarthurii* (C), GN1026 (D) and *E. grandis* (E) (Experiment 2). The first arrow in each graph represents the change in temperature from 20°C to 4°C, while the second represents the change from 4°C to the 20°C recovery period.

and, as such, it was hypothesised that chilling-tolerant plants would have more of this type of water in their cells. "Free" water is not bound to surfaces or organelles and may freeze. It was assumed that plants exhibiting lower onset of melt temperatures would freeze at lower temperatures. It was, therefore, hypothesised that those plants with low onset of melt temperatures would be more chilling and/or freezing tolerant.

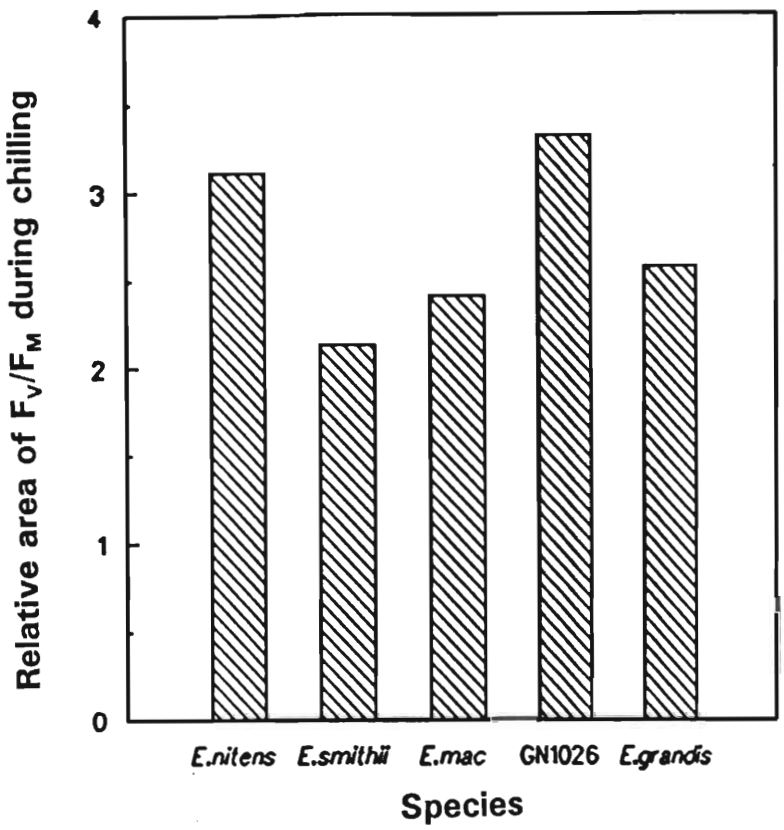

Figure 3.8 Area of F_V/F_M during the chilling period (Experiment 2 - see text for details). *E. mac* represents *E. macarthurii*.

External temperature appears to affect the onset of temperature of melt, generally causing a decrease towards the end of the chilling period (Figure 3.9). A decline in the temperature of melt during the initial 20°C period was seen in *E. nitens* (Figure 3.9 A). This may be artificial because the error bars for this period are large. On the third day of chilling (day 8) there was a marked decline in the onset of temperature of melt. On return to 20°C, the onset of temperature of melt in *E. nitens* increased, but gradually decreased over the five day period. The overall temperature of melt was lower than that of the initial period, but it is difficult to determine the extent of the difference because of fluctuation during the initial 20°C period. A similar trend was seen in *E. smithii* (Figure 3.9 B). There was a decrease in onset of temperature of melt during the initial 20°C period, followed by a decline on day 8 of the chilling treatment. When the plants were returned to 20°C, there was a slight increase in onset of temperature of melt. Once again, the overall level during this period was lower than in the initial 20°C period. *E. macarthurii* was little affected by chilling and in this way was different to the other species (Figure 3.9 C). There was a certain amount of fluctuation, and a slight decrease over the five day chilling period. The level to which the onset of temperature of melt returned after the chilling was similar to that during the initial 20°C period. GN1026 showed a similar trend over the fifteen day period to that in *E. nitens* and *E. smithii* (Figure 3.9 D). The decrease during the initial 20°C period may be artificial due to the large error bars for some of the data, but there did appear to be a slight increase in the onset

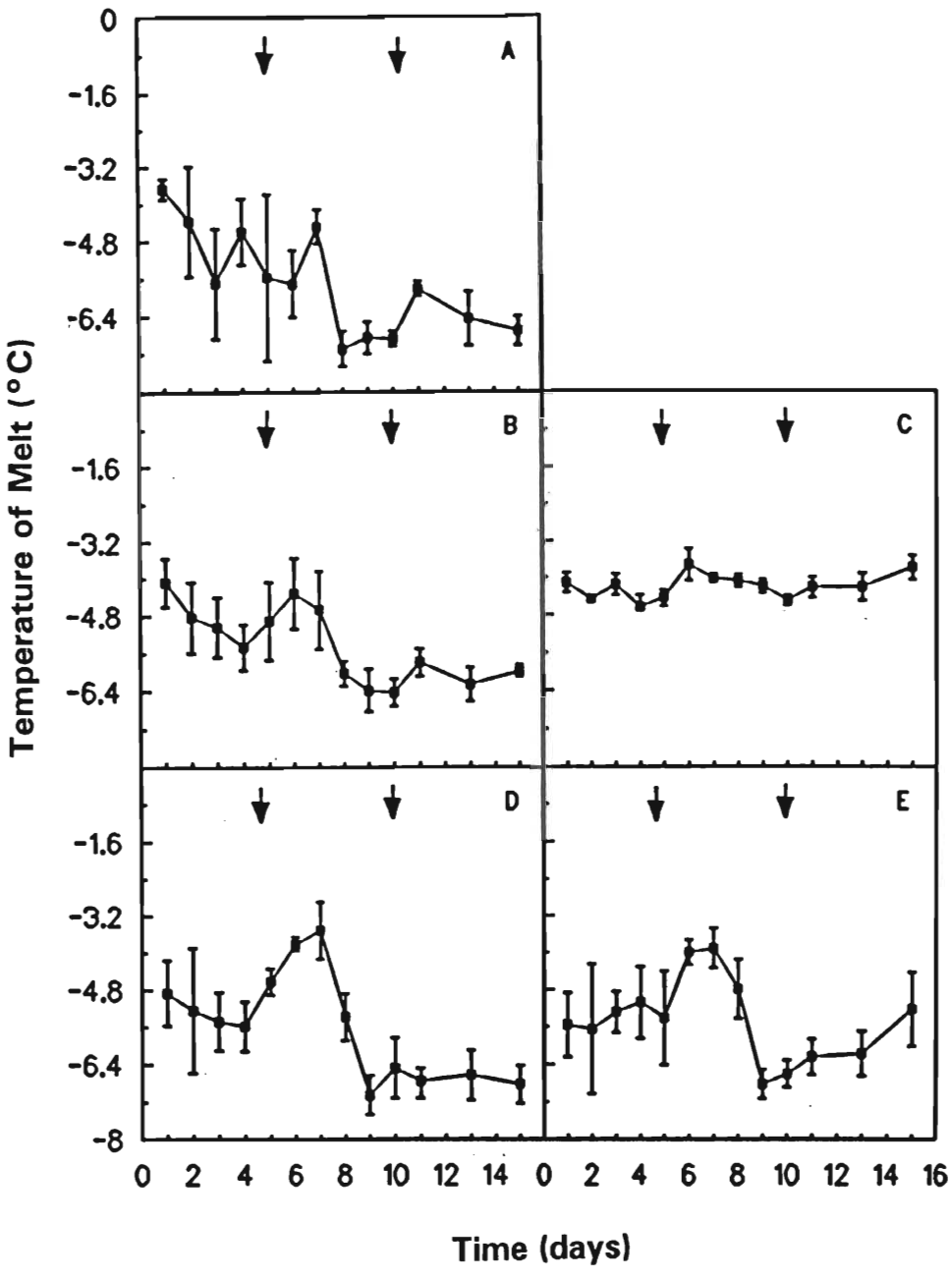

Figure 3.9 The effect of temperature on the onset temperature of melt in *Eucalyptus nitens* (A), *E. smithii* (B), *E. macarthurii* (C), GN1026 (D) and *E. grandis* (E) (Experiment 2). The first arrow in each graph represents the change in temperature from 20°C to 4°C, while the second represents the change from 4°C to the 20°C recovery period.

of temperature of melt until day 7. After this there was a marked decrease until day 10, when the temperature was returned to 20°C. The overall onset of temperature of melt was constant during the recovery period, and was lower than the overall value during the initial 20°C period. A similar trend was observed in *E. grandis* (Figure 3.9 E). Although there did not appear to be the initial decrease observed in the other species, there was a slight increase in onset of temperature of melt up until the seventh day, as seen in GN1026. After day 7, the onset temperature declined rapidly until the temperature was increased. The onset of temperature of melt appeared to increase

during the recovery period, but this may be artificial as the data points are not significantly different.

Analysis showed that both the initial 20°C and the 4°C chilling periods differed from the 20°C recovery period. There was a little interaction when the species and temperature levels were combined, showing that *E. macarthurii* and *E. nitens* interacted at the initial 20°C and the 4°C periods.

The species with the lowest onset of temperature of melt was *E. nitens* (-7.46°C), followed by GN1026 (-7.06°C), *E. grandis* (-6.82°C) and *E. smithii* (-6.44°C). *E. macarthurii* was found to be significantly different from all of the other species (Figure 3.32 and Appendix I) as it did not show much variation in the onset of temperature of melt over the whole experimental period. Using these results it was possible to rank the data in order of chilling tolerance. The most tolerant species was taken to be *E. macarthurii*, which showed very little variation over the entire experiment, including the chilling period, and was given a value of **5**. The other species were ranked on the basis of onset of temperature of melt, the species with the lowest temperature being regarded as the more tolerant. As a result, the ranking values assigned were *E. nitens* (4), GN1026 (3), *E. grandis* (2) and *E. smithii* (1).

3.3 Ranking of *Eucalyptus* species

The ranking data taken from the biophysical parameters was placed in a parameter ranking co-efficient table (Table 3.1). *E. nitens* was found to be the most chilling-tolerant plant on the basis of having a low onset of temperature of melt, the smallest area under the F_V/F_M curve and the highest maximum Q_{NP} during the chilling period. This was followed by *E. macarthurii*, GN1026 and *E. smithii*. *E. grandis* ranked as the most chilling sensitive of the five species.

Table 3.1. Ranking co-efficient table for some *Eucalyptus* species.

	E. nitens	*E. smithii*	*E.macarthurii*	GN1026	*E.grandis*
i	4	1	5	3	2
ii	5	4	3	2	1
iii	5	2	3	4	1
iv	2	1	4	5	3
Total	16	8	15	14	7

i = Onset of temperature of melt, ii = Area under the F_V/F_M curve (during chilling), iii = Maximum q_{NP} (during chilling) and iv = the slope of q_{NP}. GRA and proline concentration data were not included in this table as attempting to include them would have been too subjective.

CHAPTER 4
DISCUSSION

Plants are subjected to many and varied forms of stress, one of the most common being temperature. Many attempts have been made to determine the causes of and to find a means of measuring the damage caused by temperature stress. Low temperature stress has been well researched in many species (Blum, 1988; Guy 1990) and progress has been made towards discovering the reasons for damage under such conditions. Many techniques have been used to assess cold tolerance (Blum, 1988; Burr *et al.*, 1990; Anderson *et al.*, 1992), but few have been positively correlated with chilling tolerance. In this study, four of the most commonly tested parameters proline concentration, glutathione reductase activity, fluorescence characteristics and temperature of melt, were chosen in the present attempt to rank five *Euclayptus* species in terms of their chilling tolerance.

4.1 Biochemical Parameters

4.1.1 Proline Content

Proline has been found to accumulate under a variety of stress conditions, including water stress (Bates *et al.*, 1973; Argandona and Pahlich, 1984; Treichel *et al.*, 1984; Voetberg and Sharp, 1991, Naidu *et al.*, 1992a), salt stress (Maslenkova *et al.*, 1992), metal ion stress (Amundson *et al.*, 1993) and chilling stress (Chu *et al.*, 1978; Kushad and Yelenosky, 1987; Charest and Phan, 1991; Dörffling *et al.*, 1993). In this study an attempt was made to correlate chilling stress with increased proline concentrations in a variety of *Eucalyptus* species.

The results of this study showed great variation in proline concentration, both within and between *Eucalyptus* species. This variation was observed when the plants were placed at 20°C as well as when they were chilled to and maintained at 4°C. The data from all the species did appear to show a decline in proline concentration on transferal to 4°C, although this was not always clearly visible (Figure 3.1). Because this is contrary to most other data - most authors have found that proline levels increase on exposure to stress in both tolerant and sensitive species - this data should be viewed with caution. Other authors have also shown interspecific (Chu *et al.*, 1978; Ilhai and Dörffling, 1982) and intraspecific (Palfi-Deim *et al.*, 1989; Ilhai and Dörffling, 1982; Naidu *et al.*, 1992b) variation in proline concentrations, even under similar stress conditions. The variation observed in the *Eucalyptus* species examined may not be due to inherent, natural variations, but to interference of other free amino acids with the proline assay (Chinard, 1952). This factor may not influence proline accumulation data significantly as the levels of other free amino acids are reportedly low under stress conditions (Barnett and Naylor, 1966; Bates *et al.*, 1973). Sugars have

also been found to interfere with free proline determination (Magné and Larher, 1992). Growth and experimental conditions such as light intensity and quality and vapour pressure deficit may also influenced proline accumulation (Naidu *et al.*, 1992a).

Light appears to affect proline accumulation but not the rate or manner in which it is utilised (Chu *et al.*, 1978). During chilling stress, light caused increased proline accumulation in *Lolium perenne* (Draper, 1972), wheat and barley (Chu *et al.*, 1978; Joyce *et al.*, 1992), potato (van Swaaij *et al.*, 1985), *Myrtus communis, Pinus halepensis, Ceratonia siliqua* and *Laurus nobilis* (Diamantolou and Rhizopoulou, 1992). It appears that light induced proline accumulation is related to photosynthesis as it may be affected by the carbohydrate supply in the plant (Stewart *et al.*, 1966). Trapped energy during the light reaction of photosynthesis may supply the reductant and ATP demands of proline synthesis (Joyce *et al.*, 1992). Proline accumulation could result from higher proteolysis as indicated by lower protein content in stressed plants (Mukherjee and Choudjuri, 1983). Stress-tolerant plants may have increased incorporation of proline into newly synthesised proteins which would make the plant tolerant of stress as proline has a number of protective roles in the plant (Mukherjee and Choudhuri, 1983; Alia *et al.*, 1991; Joyce *et al.*, 1993).

Other authors have found that tolerant plants accumulate more proline than sensitive plants (Tal *et al.*, 1979; Ilhai and Dörffling, 1982; Dörffling *et al.*, 1993), especially under salt and water stress. The contradiction of results has led to divided opinion as to the suitability of using proline accumulation as an indicator of stress tolerance. Some authors have used accumulation of this compatible solute as an evaluating parameter in, for example, apple shoots (Benko, 1968), barley (Singh *et al.*, 1972), soybean and sorghum (Bates *et al.*, 1973), tomato (Aloni and Rosenshtein, 1984) and wheat (Palfi-Deim *et al.*, 1989). The work on apple shoot, barley and wheat went as far as using proline as a measure of comparitive frost or drought tolerance. Lalk and Dörffling (1985) have shown that in wheat there was a significant correlation between the ability of test plants to survive freezing damage and proline accumulation. This has been repeated by Kushad and Yelenosky (1987) with citrus plants. In other work, proline appears to indicate the deleterious response or senescence response of the plant to stress conditions, for example a fall in leaf water potential in barley leaves (Chu *et al.*, 1978), with little adaptive value being present (Hanson *et al.*, 1979; Hitz and Hanson, 1980; Aspinall and Paleg, 1981).

A factor that appears to separate the species is the behavior of the plants during the recovery period. In both *E. nitens* and *E. smithii* there was a return of proline concentrations to prechilling levels. *E. macarthurii* and *E. grandis* showed recovery levels of approximately half those of proline accumulation during the initial 20°C period. Proline levels in GN1026 did not return to

the concentrations observed before chilling once the plants were returned to 20°C. These variations could be ascribed to interspecific variation and may or may not be relevant to chilling tolerance. The considerable intraspecific variation does little to clarify the situation.

Koster and Lynch (1992) found that the proline content in Puma rye only increased after three weeks of exposure to stress (chilling) conditions, but that after six weeks it had still not reached a plateau. Kushad and Yelenosky (1987) found that proline levels had increased in chilling sensitive citrus trees after three weeks, and concluded that the overall amount of proline accumulated was important rather than its rate of accumulation. Results of a similar nature were observed by Lalk and Dörffling (1985) in winter wheat and in citrus (Yelenosky, 1979). A comparable response was recorded for drought stressed plants by Aspinall and Paleg (1981). On the other hand, authors working on a variety of plants have noted increased proline concentrations from one to five day stress periods (Tal *et al.*, 1979; Voetberg and Stewart, 1984; Palfi-Deim *et al.*, 1989; Naidu *et al.*, 1992a,b). This would imply that it is theoretically possible to assess proline accumulation after short stress periods.

In this study plants were grown and acclimated under the same experimental conditions and it is not likely that proline accumulation would have been affected by variations in environmental conditions. As there did not appear to be any significant trends, either from observation or statistical analysis, it was decided not to incorporate these data in the final ranking for cold tolerance. Another factor that points towards the questionnability of including this data in a ranking system is the length of time many authors have found necessary for proline accumulation. Because of the short time scale of this work, it is possible that the variation seen in the results presented is caused by intraspecific variation, rather than variation caused by differences in the ability to accumulate proline.

4.1.2 Glutathione Reductase Activity

Chilling may influence the amount of photoinhibition a plant is subjected to by preventing excess energy dissipation through photosynthesis (see Jahnke *et al.*, 1991). The rate at which thylakoid membranes produce superoxide radicals may be affected by chilling. If the rate of electron transfer to NADP is impaired, then more radicals may be produced. This would lead to oxidative damage which could affect photosynthesis to such an extent that it is inhibited (Hodgson and Raison, 1991). To improve the photosynthetic rate, toxic oxygen species must be removed by the antioxidant pathway, of which glutathione reductase appears to be a rate-limiting enzyme (Jablonski and Anderson, 1981). It is thought that individual plants unable to respond to the increased demand for glutathione reductase during chilling or freezing stress periods are susceptible to chilling injury (Doulis *et al.*, 1993).

Chilling did not appear to affect GRA to any marked extent in any of the species examined in this study. The hybrid, GN1026, showed the most variation, but these results had high standard error bars, especially during the initial 20°C period. The species in this study can be divided into those that showed a decrease and those that showed an increase in GRA when placed at 4°C. *Eucalyptus nitens, E. macarthurii* and GN1026 fall into the first and *E. smithii* and *E. grandis* the second group. This division is very tenuous as there was much intrinsic variation in the data. Other authors have found these two types of response of GRA to chilling, usually in different species and under different conditions.

When wheat was placed at 10°C (a cold, but non-chilling temperature), the glutathione pool increased (Badiani *et al.*, 1993). This was due to an increase in oxidised glutathione (GSSG) rather than to an increase in reduced gluthathione (GSH) and resulted in a decrease in the GSH:GSSG ratio. In pine trees, stressed over a two year period, there was a decrease in GRA levels especially when exposed to low winter temperatures (Winglse and Hällgren, 1993). Hariyadi and Parkin (1993) found that GSH levels decreased in cold-stressed cucumber fruit peel decreased. On the other hand, those authors found little difference in GSH and GRA levels in chilled and unchilled cucumber seedlings. Wingsle *et al.* (1992) found that there was little difference in GRA levels between stressed and non-stressed Scots pine seedlings. Similar results were obtained for soybean (Smith *et al.*, 1989). In chilling-sensitive maize (*Zea mays*) GRA levels decreased when plants were exposed to chilling conditions, but increased initially in chilling tolerant *Z. diploperennis*. After this initial increase, GRA levels stabilised at a level 50% higher than prechilling levels (Jahnke *et al.*, 1991). The results of those authors were similar to those obtained in the present study, although changes in GRA levels in chill-treated *Eucalyptus* were inconsistent. As inconsistency hindered analysis of GRA during chilling, these data were not included when ranking the *Eucalyptus* species in terms of chilling tolerance.

In apple callus, GRA levels gradually increased, after an initial decrease, during cold treatment (Kuroda *et al.*, 1991). Anderson *et al.* (1992) found that the highest GRA levels in Eastern white pine needles were in winter, especially when the temperature was below -5°C. Increases in GRA were also observed in conifers (Esterbauer and Grill, 1978), cotton (Foster and Hess, 1980), wheat (Gamble and Burke, 1984), spinach (de Kok and Oosterhuis, 1983; Tanaka *et al.*, 1988) and bean (Schmidt and Kunert, 1986; Cakmak and Marschner, 1992). In the latter case, there was a general increase in the activities of scavenging enzymes when increased light intensity was combined with another stress. GRA levels were found to increase gradually as the length of the stress period increased (Tanaka *et al.*, 1988; Cakmak and Marschner, 1992).

Glutathione reductase activity may increase as part of the response to stress conditions and as protection against toxic oxygen species (Foster and Hess, 1980; Gamble and Burke, 1984; Tanaka *et al.*, 1988). As such, it may also be involved in protection against photooxidative stress (Rennenberg, 1982; Smith *et al.*, 1989; Anderson *et al.*, 1992; Aono *et al.*, 1993). The role GR plays in the hydrogen peroxide/superoxide scavenging pathway in chloroplasts (and possibly also in the cytoplasm) has been discussed in detail in the Introduction (section 1.3.5.2). The scavenging systems in the cytosol and chloroplasts may cooperate in transporting their substrates and products across the chloroplastic membranes resulting in one system affecting that of the other (Comai *et al.*, 1985; Aono *et al.*, 1993). Although GR may be involved in the scavenging system, authors have found that there was no clear correlation between increased GRA and stress, particularly ozone stress, tolerance (see Wingsle *et al.*, 1992).

An increase in glutathione reductase may be caused by stimulation of either GR biosynthesis or the depression of GR activation (Tanaka *et al.*, 1988). Further work is needed to clarify what triggers GR biosynthesis and at which step the enzyme's levels are controlled (Tanaka *et al.*, 1988). GR could increase as a result of increased protein levels. Multiple forms of GR have been found in spinach, with isoforms appearing in chill-hardened spinach that were not observed in non-hardened plants (Guy and Carter, 1984). There was also an increase in the relative abundance of isoforms common to both types of spinach (Smith *et al.*, 1989). It was thought by this author that changes in the total pool of GR may be less significant than changes in the levels of individual isozymes. Anderson *et al.*, (1992) did not observe new or different isoforms when comparing summer and winter data of white pine and implied that there was doubt as to whether these isoforms existed.

GR of cold-hardened plants may have a higher affinity for oxidised than reduced glutathione at low temperatures than non-hardened plants as the oxidised form of GR is more stable than the reduced form (Guy and Carter, 1984; Smith *et al.*, 1989; Jahnke *et al.*, 1991). This may account for the differences Jahnke *et al.* (1991) obtained in chill-hardened and non-hardened maize, mentioned above. The level of GRA may also be affected by light quantity and quality (Gamble and Burke, 1984).

These differences in GRA response to stress conditions may be due to seasonal variation as this has been observed in *Pinus* abies (Esterbauer and Grill, 1978) and poplar (Sagisaka, 1985). There is also evidence for differences in the intrinsic temperature responses of different species and of different genotypes within the same species (Ellis *et al.*, 1992). The diversity observed may also be accounted for by different levels of stress tolerance within one species (Polle *et al.*, 1993).

Because of conflicting results in the literature, more work is necessary to determine the possibility of a correlation between chilling and increased of decreased GRA levels.

4.2 Biophysical Parameters

4.2.1 Fluoresence Characteristics

The fluorescence characteristics, primarily the F_V/F_M ratio, of all the *Eucalyptus* species studied appear to have been affected by cold conditions. This is particularly noticeable in Figure 3.32, although it is evident in Figure 3.31 to a lesser extent. The F_V/F_M ratio is thought to indicate the photosynthetic efficiency of the plant and as such can be used to assess photoinhibitory damage to the photosynthetic apparatus (Krause and Weis, 1991). Interspecific variation in photosynthetic efficiency was apparent (Figure 3.4) and was also seen in other *Eucalyptus* species (Ferrar *et al.*, 1989). This variation was correlated with variation in chilling stress tolerance.

Photoinhibition is often enhanced when light is combined with another stress, for example chilling stress (Greer, 1988; Adams III *et al.*, 1990; Ögren and Evans, 1992). This was also the case in chilling-tolerant plants (Boese and Huner, 1992). Chilling induces a decrease in the F_V/F_M ratio (indicating photoinhibition) in all the *Eucalyptus* species examined, with the greatest decrease being evident in *Eucalyptus grandis* and the smallest in *E. nitens* (Figure 3.3). Other authors have reported similar results in *Nerium oleander* (Demmig *et al.*, 1988), Actinidia deliciosa (Greer, 1988), grapevine leaves (Düring *et al.*, 1990), soybean (Adams III *et al.*, 1990; Neuner and Larcher, 1991), cucumber, sunflower, cotton, *Monstera deliciosa* and *Schefflera arboricola* (Adams III *et al.*, 1990), spinach (Somersalo and Krause, 1990a,b; Boese and Huner, 1992), tomato (Walker *et al.*, 1991), *Picea rubens* (Adams and Perkins, 1993) and *Picea abies*, *Pinus sylvestris* and *Thuja plicata* (Weger *et al.*, 1993).

Inhibition of photosynthesis by chilling temperatures is light-dependant (Powles *et al.*, 1983; Hetherington *et al.*, 1989), although there does not appear to be synergism between light stress and preceding frost stress (Ögren and Evans, 1992). Chilling at high light intensity results in an irreversibly changed capacity of undergo photosynthetic energisation in grapevine leaves (Báló *et al.*, 1991). This type of inhibition does not appear to involve damage to PSII, but could be related to a disproportionate decrease in the photosynthetic activity of the plants at chilling temperatures (Hodgson and Raison, 1989).

A decreased F_V/F_M ratio may occur together with a decrease in the photon yield of PSII (Adams III *et al.*, 1990). This has been attributed to a decrease in variable fluorescence (F_V) in some cases

(Adams III *et al.*, 1990; Boese and Huner, 1992) and to a decrease in maximum fluorescence (F_M) in others (Düring *et al.*, 1990).

F_V is related to increased thermal dissipation of excitation energy in the photosynthetic apparatus and may decrease during low temperatures as a result of the deactivation of excited chlorophyll molecules (Ögren and Evans, 1992). This may indicate that chilling stress, as such, does not affect the excitation energy transfer from PSII to PSI (Somersalo and Krause, 1990a), but that there are changes in PSII that correspond to low frost hardiness during chilling or freezing stress (Brennan and Jeffries, 1990). As injury increases, F_V decreases (Greer and Laing, 1988; Krause, 1988), indicating that the state of photosynthetic electron transport primarily affects the water-splitting side of PSII (Sundbom *et al.*, 1982). The decrease in F_V may be attributed to fluorescence quenching by the oxidised reaction centres of PSII (Butler, 1978). It could also be caused by the primary electron acceptor of PSII (reduced Q_A) being unable to accumulate. This would be because electrons are not supplied to the photoreaction of PSII (Barnes and Wilson, 1984).

Although F_V may decrease when plants are exposed to chilling temperatures, some authors have found that baseline fluorescence (F_0) does not alter (Boese and Huner, 1992). Somersalo and Krause (1990a) have reported that F_0 remains constant when the plants tested were cold-hardened, or when high light was combined with chilling stress (1990b), but increased in unhardened plants (see also Hurry and Huner, 1991; Neuner and Larcher, 1991). An increase in F_0 may indicate impaired energy trapping efficiency in the PSII reaction centre, or a partial disconnection of antennae from the centres (Somersalo and Krause, 1990a; Neuner and Larcher, 1991). In contrast to this, F_0 has been reported to decrease in stressed, including chill-stressed, plants (Adams and Perkins, 1993; Osmond *et al.*, 1993). This has been supported by a decrease in the quantum yield of PSII in chill-treated tissue as a result of a chill-induced decrease in the rate constant of PSII photochemistry and/or an increase in the rate constant for transfer of energy out of PSII chlorophyll matrices (Baker *et al.*, 1983; Demmig *et al.*, 1987, 1988). As a result, PSI is favoured in chill-treated plants and cyclic electron transport is enhanced with modifications in the ATP:NADPH levels (Baker *et al.*, 1983; Krause, 1988). F_0 may decrease as a result of larger antennae systems as chlorophyll content is often found to increase under stress conditions (Badiani *et al.*, 1993).

Numerous explanations for the decrease in the F_V/F_M ratio have been advanced. One of these is concerned with the direct effect of chilling on thylakoid membranes. Fluorescence quenching is brought about by the development of a high energy status of the thylakoid membranes which is a result of photoinduced protonation of the interior of the thylakoid (see Barnes and Wilson, 1984). As such, fluorescence is an indication of the integrity of the thylakoid system (Terashima *et al.*,

1991b). Thylakoid membrane fluidity influences the temperature at which PSII denatures, which is correlated with the temperature at which photosynthesis becomes unstable (Raison *et al.*, 1982). As chilling or freezing injury increases, the thylakoid membranes are less able to quench fluorescence as they may be restricted in their ability to maintain the proton electrochemical gradient because membranes become "leaky" to protons and cations (Palta and Li, 1978; Murata and Tatsuma, 1979; Baker *et al.*, 1983; Düring *et al.*, 1990). Leakage of metal ions would promote spill-over of excitation energy from PSII to PSI, reducing fluorescence emission and decreasing F_V. Photophosphorylation would be uncoupled and photosynthetic flux inactivated (Düring *et al.*, 1990). Partial dissociation of CF_1 complexes in the thylakoid membranes can contribute to the uncoupling of thylakoids (Terashima *et al.*, 1991a). In opposition to the above reasoning, Brüggeman *et al.*, (1992) have found no evidence for direct membrane damage resulting in impaired electron transport. Lapointe *et al.*, (1991) found that resistance to low temperature induced photoinhibition is associated with higher levels of cell organisation of thylakoid membranes.

One of the major causes of decreased photosynthetic efficiency in plants at low temperatures is decreased water oxidation (Sundbom *et al.*, 1982; Barnes and Wilson, 1984). Water oxidation is a temperature sensitive process in the chloroplasts in many species and often results in impaired electron transport. Any condition that impairs this process beyond PSII would result in a greater decrease in photon yield than in the F_V/F_M ratio and in increased thermal dissipation within PSII (Adams III *et al.*, 1990).

Low temperature may affect the enzymatic steps (Calvin cycle and/or the site of photophosphorylation) of photosynthesis (Strand and Öquist, 1985). For example, low activity of Rubisco could play a role in decreasing photosynthetic capacity during chilling (Boese and Huner, 1992; Brüggeman *et al.*, 1992). This enzyme appears to play a large role in chilling acclimation in various *Eucalyptus* species and *Nerium oleander* (Ferrar *et al.*, 1989). Photosynthetic efficiency may also be affected by a possible ortho-phosphate mediated feedback inhibition by internal sugar accumulation (Pollock *et al.*, 1983, Bagnall *et al.*, 1988). In this case, free inorganic phosphate may be sequestered from the cytoplasmic pool (Foyer, 1990).

An increase in the radiationless dissipation of excitation energy (non-photochemical quenching) may result in a decrease in F_V/F_M (Demmig and Björkman, 1987; van Wijk and van Hasselt, 1993). This increase does not appear to be temperature dependent (Greer, 1988). Such an increase may be mediated by the carotenoid zeaxanthin (Demmig *et al.*, 1987; 1988; Giersch and Krause, 1991; Eickmeier *et al.*, 1993). In the light, both the xanthophyll cycle and fluorescence quenching are governed by the transthylakoid pH gradient and a redox component (Sieferman and

Yamamoto, 1975). The way in which zeaxanthin interacts with chlorophyll is unknown (Eickmeier *et al.*, 1993), but the *in vitro* quenching of chlorophyll *a* fluorescence by carotenoids has been reported (Beddard *et al.*, 1977). Zeaxanthin may operate as a fluorescence quencher itself by operating as an overflow valve under conditions of excess excitation. It could be absent or ineffective under control conditions to allow for efficient excitation energy transfer (Demmig *et al.*, 1987; 1988). Zeaxanthin may help to stabilise chlorphyll *a/b* complexes that may be transformed to a unique state when thylakoid membranes are affected by cold stress (Eickmeier *et al.*, 1993). Another carotenoid, rhodoxanthin, increases in western red cedar during high light and chilling (Weger *et al.*, 1993). This carotenoid changes the leaf colour from green to red-brown and may decrease the light intensity reaching the photosynthetic apparatus, so acting as a protective mechanism. In *Eucalyptus grandis* new, apical, leaves changed colour to a pink-brown after two days at 4°C. This colouration disappeared after three days at the 20°C recovery temperature. The formation of a pink-brown colour may be due to the formation of a protective carotenoid, possibly rhodoxanthin or xanthophyll (D. McKellar, *pers. comm.*) and may function to lessen the effect of stress on the plant. This colouration has been observed in *E. grandis* leaves when changed from a low to higher light intensity under ambient temperatures (personal observation) and therefore may be a response to high light and not chilling stress. Chilling may reduce the ability of this species to withstand light stress. As this phenomenon only appeared in *E. grandis*, it appears that chilling may be affecting the ability to withstand light stress in this species to a greater degree than that of the other species examined. This substantiates the finding that *E. grandis* is less tolerant of chilling than *E. nitens, E. macarthurii, E. smithii* or GN1026 ().

A recent hypothesis as to the decrease of photosynthetic efficiency or reasons for fluorescence quenching involves the transformation of fractions of PSII reaction centres into two populations (van Wijk and van Hasselt, 1993). The first population comprises non-inhibited centres while the second inhibited centres, called fluorescence quenchers, with high rate constants for thermal deactivation (Giersch and Krause, 1991). The latter would trap excess excitation energy which would be dissipated as heat. This system is thought to operate when repair of PSII (synthesis of the reaction centre protein D1) is not rapid enough, so conferring protection on the remaining active centres (Greer *et al.*, 1991; Osmond *et al.*, 1993).

Non-photochemical quenching (q_{NP}) reflects the energisation and/or coupling status of the thylakoid membranes *in vivo*. Under chilling conditions, Terashima *et al.*, (1991b) found that chilling-sensitive cucumber leaves showed marked declines in q_{NP}. Non-photochemical quenching appears to be a protective mechanism (Krause *et al.*, 1988). As such, it was assessed in each of the five study species in an attempt to distinguish them on the basis of cold tolerance. q_{NP} values were taken during the 4°C chilling period (Figure 3.5) and compared. The results indicated differences

among the species, making it possible to use this fluorescence characteristic as a means of ranking the given species in terms of chilling tolerance. *Eucalyptus grandis* had the lowest q_{NP} value indicating a low level of energisation or a larger amount of uncoupling of the thylakoid membranes. Because of this, it was assumed that this species was least able to tolerate chilling conditions as it was most adversely affected by them. *E. nitens* appeared to be the least affected by chilling as it had the highest q_{NP} value of the species compared. This was followed by GN1026 and then *E. smithii* and *E. macarthurii*. On each day of the experiment, the slope of q_{NP} over a twenty minute period was assessed (Figure 3.6). The results obtained from this assessment were different to those obtained for other paramters. The slope of q_{NP} would indicate the rate at which non-photochemical quenching occurred. Presumably the more tolerant plants would have a higher rate of non-photochemical quenching because they are more able to withstand adverse conditions and would therefore be protected by such a mechanism. This rate would be expected to remain constant or to increase under stress conditions in tolerant plants to maintain efficient working photosynthetic systems. The order of ranking will be discussed later (section 4.4).

All the *Eucalyptus* species the F_V/F_M ratio appeared to recover rapidly once the plants were returned to 20°C. This was similar to recover in kiwifruit leaves (Greer, 1988) and western red cedar (Weger *et al.*, 1993), which occurred only when the plants were returned to at least 20°C. Recovery in this case was dependent on the extent of photoinhibition (Krause *et al.*, 1985; Demmig and Björkman, 1987, Greer, 1988; Greer and Laing, 1988). Recovery in the *Eucalyptus* species studied appeared complete, possibly because they were not exposed to high light conditions during the stress period. In other *Eucalyptus* species (Ögren and Evans, 1992) frost-induced changes in photosynthesis were not permanent, requiring only several frostless days and nights in the field for total recovery. This may indicate that in *Eucalyptus* species photoinhibition is common and that the plants are adapted to overcome it (Ögren and Evans, 1992).

Fluorescence has been used by many authors to assess and detect stress damage, particularly chilling stress damage (see Smillie and Hetherington, 1983; Düring *et al.*, 1990; Adams and Perkins, 1993). Stress affects intensity of fluorescence (Kaczmarek *et al.*, 1991). Temperature-induced fluorescence changes have been used to monitor progressive damage to potato chloroplast membranes. Using these changes, it was possible to rank the *Eucalyptus* species in order of tolerance to low temperature (Smillie, 1979: Sundbom *et al.*, 1982; Brennan and Jeffries, 1990). Fluorescence has been found a useful tool in studying the effects of photosynthetic light reactions in a number of species as thylakoid membrane organisation and function are influenced by temperature (Fork, 1979; Havaux and Lannoye, 1983).

The F_V/F_M ratio has been found to be a good diagnostic probe for photoinhibition of photosynthesis in many plants (Hurry and Huner, 1991; Ögren, 1991; Osmond *et al.*, 1993), including *Eucalyptus* species (Ögren and Evans, 1992). In the latter species the authors concerned were able to distinguish between species more and less tolerant of photoinhibition using fluorescence. Fluorescence quenching and maximum rates of induction and quenching appear to be reliable indicators for *in vivo* screening of stress tolerant (particularly salt-tolerant) wheat (KrishnaRaj *et al.*, 1993). Photoinhibition has been induced in both cold tolerant and sensitive species at moderate light intensities and has been monitored by assessing changes in the F_V/F_M ratio (Hetherington *et al.*, 1989). These changes have been seen as a quantitative measure of photosynthesis when determined at ambient temperature (Barnes and Wilson, 1984; Adams III *et al.*, 1990; Brennan and Jeffries, 1990; Düring *et al.*, 1990). The F_V/F_M ratio is useful as a measure of photoinhibition because a linear relationship between it and the optimal quantum yield of photosynthetic oxygen yield is often observed (Giersch and Krause, 1991). The ratio is also an assessment of the potential photochemical yield of PSII (Butler, 1978) and a reliable indicator of photosynthetic oxygen evolution (Adams III *et al.*, 1990).

Fluorescence appears to be a sensitive, informative and rapid method to characterise the disintegration of membranes caused by chilling temperatures (Adams and Perkins, 1993). It has also been used to analyse plant temperature optima (Burke and Oliver, 1993). There has been correlation between visual assessments of chilling or freezing stress on plants and chlorophyll fluorescence, for example between the yield of F_V in clover and visual effects of chilling (Düring *et al.*, 1990). The latter is useful as a screening technique in this species. Fluorescence has also been used in red spruce to assess chilling tolerance as it is rapid (Adams and Perkins, 1993), and in cereals (Öquist *et al.*, 1993) because strong positive correlation was found between cold-hardening and a capacity to maintain a high oxidised to reduced Q_A ration under high light at 5°C.

Many authors have looked at chlorophyll fluorescence induction which is affected by many factors such as photosynthetic electron transport, excitation energy transfer between photosystems and the Calvin cycle and the energy state of thylakoid membranes (Krause and Weis, 1984). Marked differences have been found between chill-treated and non-chilled *Zea mays* (Baker *et al.*, 1983) and *Lycopersicon esculentum* (Janssen *et al.*, 1992) during the induction kinetic of chlorophyll fluorescence. Although not examined in this work, to study the effects of chilling on the induction kinetics of *Eucalyptus* species could be of interest and be of possible use in ranking these plants in terms of their chilling tolerance.

4.2.2 Onset of Temperature of Melt

Different types or states of water have been observed in seed and *Artemia* cyst material (Clegg, 1986; Vertucci, 1990; Pammenter *et al.*, 1991; Ramlov and Hvidt, 1992). These states exhibit different responsed when frozen, particularly when melted (Vertucci, 1990). This information was obtained using differential scanning analysis (Vertucci, 1990) or differential thermal analysis (Burr *et al.*, 1990). Thermal analysis is the measurment of changes in physical properties of a substance as a function of temperature whilst the substance is subjected to a controlled temperature programme (Brown, 1988).

Work on *Eucalyptus* species has shown hardened and unhardened plants are able to withstand low temperature to different extents (Tibbits and Reid, 1987). Hardened plants were able to withstand temperatures of -7.5°C while unhardened were only able to withstand -3.4°C (Tibbits and Reid, 1987). In this study it was hypothesised that the onset of temperature of melt would be able to elucidate intrinsic differences between species before they underwent cold-hardening. Accordingly, the onset of temperature of melt of five *Eucalyptus* species was examined. The temperature at which melt is initiated is thought to provide information about the nature of water that is present and therefore undergoing change as a result of freezing (Berjak *et al.*, 1992). In the *Eucalyptus* species studied, the onset of temperature of melt appeared to decline after a few days at 4°C (Figure 3.9). The extent to which it declined (became more negative) varied among species, with the lowest temperature of melt being recorded in *E. nitens*. This was thought to imply that this species was able to withstanding lower environmental temperatures and was possibly more tolerant of these. Interspecific comparisons may have confounded, though, by clinal variations in frost resistance within one species (Davidson and Reid, 1987).

As a consequence of the effect on onset of temperature of melt three moisture regions or states were hypothesised for pea and soybean (Vertucci, 1990). At least four states of hydration were found for *Landolphia kirkii* cotyledon tissue (Pammenter *et al.*, 1991; Berjak *et al.*, 1992) and for *Artemia* cysts (Ramlov and Hvidt, 1992). The first and second states of hydration refers to water contents of 0.05 to 0.10 $g.gdwt^{-1}$ and 0.10 to 0.24 $g.gdwt^{-1}$ respectively. In the first state water is unable to freeze, unable to exist as a glass at ambient temperature and has limited mobility (Williams and Leopold, 1989). The second state is similar except it is able to form glasses and there is molecular movement evident. The third state is of water contents of 0.24 to 0.33 $g.gdwt^{-1}$. In this state water melts at lower temperatures than pure water (Simatos *et al.*, 1975; Vertucci, 1989a, 1989b) and has low enthalpies of melt which may reflect the dissolution of hydrophilic substances (Vertucci, 1990). The fourth water state is of water contents between 0.33 and 0.55 $g.gdwt^{-1}$. This water has thermal properties similar to pure water. There is no devitrification and dilution of hydrophilic solutes may result in the energy of melt being less than pure water (Vertucci, 1990). The fifth water state contains more water than 0.55 $g.gdwt^{-1}$. This state has

properties similar to water in dilute solutions. Onset of temperature of melt has been observed to increase (become less negative) when the water content of pea and soybean seed tissues increased (Vertucci, 1990). Similar results have been recorded in *Landolphia kirkii* (Pammenter *et al.*, 1991) and *Artemia* cysts (Ramlov and Hvidt, 1992) and may be due to varying permeability of cell components to liquid water (Ramlov and Hvidt, 1992). Very low temperatures of melt were observed in these cysts when hydration was low. These increased when hydration increased until a plateau was reached of around -10°C when hydration was 1.25 $g.gdwt^{-1}$ (Ramlov and Hvidt, 1992). Fully hydrated leaves (on average 2.1 $g.gdwt^{-1}$) were used to measure the onset of temperature of melt in *Eucalyptus* species. At this hydration level, it could be assumed that there would be little difference in the state of water in the different species (Ramlov and Hvidt, 1992). Any differences in temperature of melt observed could then be accounted for by differences in the species. This was then correlated to differences in chilling or freezing tolerance, with those plants most tolerant of chilling conditions showing lower temperatures of melt. Although the onset of temperature of melt was a useful tool for assessing chilling tolerance, more useful information could have been obtained as to the status of each plant if, for example, water content had been varied. As this procedure would have taken some time, it was not attempted during this study as one of the aims was to find a rapid technique for assessing cold tolerance.

4.3 Comparison of Tests Used to Rank Species

Many of the tests used to rank species have already been discussed. But there are a few other tests used by authors to rank species, particularly *Eucalyptus* species. These fall into three main categories: electrolyte leakage, differential thermal analysis and whole plant freeze tests. Electrolyte tests are precise, sensitive and do not destroy whole plants (Tibbits, 1986; Tibbits and Reid, 1986,). They are thought to be possible because stress, particularly hydration after dehydration, may induce phase transitions in the lipid bilayers of membranes (Crowe and Crowe, 1986). These tests have been those most favoured to assess membrane damage and to rank species in terms of stress tolerance. Differential thermal analysis has also been used to rank *Eucalyptus* species. This is also a sensitive, objective, rapid technique which is non-destructive to the whole plant (Burr *et al.*, 1990). On the other hand it is difficult to calibrate the equipment used and estimates were more conservative that those from field trials. The last type of general test used for assessment of frost or chilling tolerance is whole plant freezing tests. These are accurate, but require destructive sampling and show poor precision with small samples (Burr *et al.*, 1990). Other tests already mentioned include enzyme and soluble protein assays which are destructive, time consuming and require precision from the tester. Various fluorometric tests have been mentioned, particularly measurement of the F_V/F_M ratio. These are rapid, non-destructive, sensitive and easy to perform.

4.4 Ranking order of *Eucalyptus* species

The parameters used to rank *E. nitens, E. macarthurii, E. smithii, E. grandis* and GN1026 were the onset of temperature of melt, the area under the F_V/F_M curve, maximum q_{NP}, and the slope of q_{NP} over 20 minutes during the chilling treatment. The first three of these parameters gave comparable results, placing the species in similar orders of chilling tolerance. The latter gave almost the opposite ranking to these. In general, the species given as the most chilling-tolerant was *E. nitens*. This is in agreement with opinion in both Australia and South Africa as this species is regarded as an extremely chilling-tolerant one in both countries (Tibbits and Reid, 1986; Hallam *et al.*, 1989; B. Herman, *pers. comm.*). The ranking of *E. grandis* also complied with general consensus. This is regarded as a chilling-sensitive species and is only found in subtropical to temperate climates in its natural habitat (Hallam *et al.*, 1989). In this study, *E. macarthurii, E. smithii* and GN1026 were ranked as 4, 2 and 3, respectively (4 representing more tolerant and 2 more sensitive). Foresters at Mondi have ranked *E. smithii* above GN1026 on the basis of field trials (B. Herman, *pers. comm.*). As a result, the ranking parameters used in this study, with the exception of slope of q_{NP}, gave results that correlated well with those results from field trials (Figure 4.1). If the measurements of q_{NP} slope are excluded from the ranking, little difference is

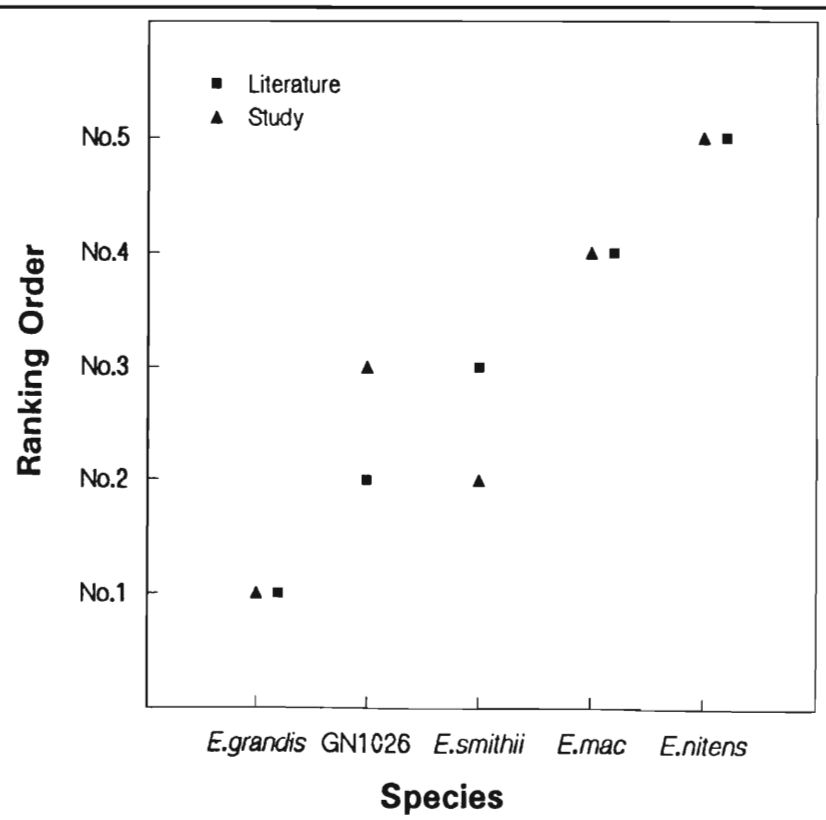

Figure 4.1 Comparison between the rank order from the literature and that obtained in this study of *Eucalyptus nitens, E. macarthurii, E. smithii, E. grandis* and GN1026. The parameters used to rank the species were onset temperature of melt, area under the F_V/F_M curve, maximum q_{NP} and the slope of q_{NP} over the chilling treatment. *E. mac* represents *E. macarthurii*.

noted. It is suggested that measurement of the slope of q_{NP} be excluded as a means of ranking *Eucalyptus* plants in future. It should be possible to use the onset temperature of melt, the area under the F_V/F_M curve and maximum q_{NP} as a fast and efficient means of assessing cold tolerance of *Eucalyptus* seedlings.

4.5 Concluding Remarks

A clear understanding of frost hardening in *Eucalyptus* species is necessary to make full use of these species in commerce and industry. As a consequence, it is necessary to understand biochemical and biophysical factors that could contribute to low temperature tolerance or to the hardening process. Of the parameters tested in this study, the biochemical ones appeared to be of little use in assessing cold tolerance in *Eucalyptus* species. This may have been caused by the nature of *Eucalyptus* tissue. The biophysical parameters measured, fluorescence and onset of temperature of melt, showed most promise in assessing cold tolerance in this species. In particular, fluorescence characteristics appeared to be the most accurate and repeatable and were not time consuming or destructive to any part of the plant. Furthermore, this technique can be performed in the field and on any size of tree. Further study of *Eucalyptus* species using fluorescence (for example, fast-induction kinetics) is recommended and is potentially useful as an added means of ranking *Eucalyptus* species in terms of chilling tolerance.

REFERENCES

Adams, E. & Frank, L. 1980. Metabolism of proline and hydroxyproline. **Annual Review of Biochemistry** *49*: 1005-1062

Adams, G.T. & Perkins, T.D. 1993. Assessing cold tolerance in *Picea* using chlorophyll fluorescence. **Environmental and Experimental Botany** *33*: 377-382

Adams, W.W. III, Winter, K. & Lanzi, A. 1989. Light and the maintenance of photosynthetic competence in leaves of *Populus balsamifera* L. during short-term exposures to high concentrations of sulfer dioxide. **Planta** *177*: 91-97

Adams, W.W. III, Demmig-Adams, B., Winter, K. & Schreiber, U. 1990. The ratio of variable to maximum chlorophyll fluorescence from photosystem II, measured in leaves at ambient temperature and at 77K, as an indicator of the photon yield of photosynthesis. **Planta** *180*: 166-174

Alia, Saradhi, P.P. & Mohanty, P. 1991. Proline enhances primary photochemical activities in isolated thylakoid membranes of *Brassica juncea* by arresting photoinhibitory damage. **Biochemical and Biophysical Communications** *181*: 1238-1244

Allen, J.F., Bennett, J., Steinback, K.E. & Arntzen, C.J. 1981. Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of energy between photosystems. **Nature** *291*: 21-25

Aloni, B. & Rosenshtein, G. 1984. Proline accumulation: A parameter for evaluation of sensitivity of tomato varieties to drought stress? **Physiologia Plantarum** *61*: 231-235

Alscher, R.G. 1989. Biosynthesis and antioxidant function of glutathione in plants. **Physiologia Plantarum** *77*: 475-464

Amundson, R.G., Kohut, R.J., Laurence, J.A., Fellows, S. & Colavito, L.J. 1993. Moderate water stress atlers carbohydrate content and cold tolerance of Red Spruce foliage. **Environmental and Experimental Botany** *33*: 383-390

Anchoroguyit, T.J., Rudolph, A.S., Carpenter, J.F. & Crowe, J.H. 1987. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. **Cryobiology** *24*: 324-331

Anderson, J.W., Foyer, C.H. & Walker, D.A. 1983. Light-dependent reduction of dehydroascorbate and uptake of exogenous ascorbate by spinach leaves. **Planta** *158*: 442-450

Anderson, J. V., Chevone, B.I. & Hess, H.L. 1992. Seasonal variation in the antioxidant system of Eastern White pine needles. **Plant Physiology** *98*: 501-508

Aono, M., Kubo, A., Saji, H., Tanaka, K. & Kondo, N. 1993. Enhanced tolerance to photooxidative stress of transgenic *Nicotiana tobacum* with high chloroplastic glutathione reductase activity. **Plant Cell Physiology** *34*: 129-135

Argandona, V. & Pahlich, E. 1991. Water stress on proline content and enzyme activities in barley seedlings. **Phytochemistry** *30*: 1093-1094

Arntz, B., & Trebst, A. 1986. On the role of the Q_B protein of PS II in photoinhibition. **FEBS Lett.** *194*: 43-49

Asada, K. 1984. Chloroplasts: formation of active oxygen and its scavenging. **Methods in Enzymology** *105*: 422-429

Asada, K. & Badger, M.R. 1984. Photoreduction of O_2 and H_2O_2 with concomitant evolution of O_2 in intact spinach chloroplasts: Evidence for scavenging of hydrogen peroxide by peroxidase. **Plant and Cell Physiology** *25*: 1169-1179

Asada, K., Kiso, K. & Yoshikawa, K. 1974. Univalent reduction of molecular oxygen by spinach chloroplasts on illumination. **Journal of Biological Chemistry** *249*: 2175-2181

Asada, K., Kanematsu, S. & Uchida, K. 1977. Superoxide dismutases in photosynthetic organisms: Absence of the cuprozinc enzyme in eukaryotic algae. **Archives of Biochemistry and Biophysics** *179*: 243-256

Asada, K. & Takahashi, M. 1987. Production and scavenging of active oxygen in photosynthesis. In: Photoinhibion. D.J. Kyle, C.B. Osmond, and C.J. Arntzen (eds). Elsevier: Amsterdam.

Aspinall, D. & Paleg, L.G. 1981. Proline accumulation: physiological aspects. In: L.G. Paleg and D. Aspinall (eds). Physiology and Biochemistry of Drought Resistance in Plants. Academic Press: Sydney, Australia.

Badger, M.R. 1985. Photosynthetic oxygen exchange. **Annual Review of Plant Physiology** *36*: 27-53

Badiani, M., Paolacci, A. R., D'Annibale, A. & Sermanni, G.G. 1993. Antioxidants and photosynthesis in the leaves of *Triticum durum* L. seedlings acclimated to low, non-chilling temperature. **Journal of Plant Physiology** *142*: 18-24

Bagnall, D.J., King, R.W. & Farquhar, G.D. 1988. Temperature-dependent feedback inhibition of photosynthesis in peanut. **Planta** *175*: 348-354

Baker, N.R., East, T.M. & Long, S.P. 1983. Chilling damage to photosynthesis in young *Zea mays*. II. Photochemical function of thylakoids *in vivo*. **Journal of Experimental Botany** *34*: 189-197

Baker, N.R. 1991. A possible role for photosystem II in environmental perturbations of photosynthesis. **Physiologia Plantarum** *81*: 563-570

Báló, B., Lajkó, F. & Garab, G. 1991. Effects of chilling on photosynthesis of grapevine leaves. **Photosynthetica** *25*: 227-230

Barber, J. 1982. Influence of surface charge on thylakoid structure and function. **Annual Review of Plant Physiology** *33*: 261-295

Barclay, L.C.R. 1988. The cooperative antioxidant role of glutathione with a lipid-soluble and a water-soluble antioxidant during peroxidation of liposomes initiated in the aqueous phase and in the lipid phase. **Journal of Biological Chemistry** *263*: 16138-16142

Barényi, B. & Krause, G.H. 1985. Inhibition of photosynthetic reactions by light. A study with isolated spinach chloroplasts. **Planta** *163*: 218-226

Barnett, N.M. & Naylor, A.W. 1966. Amino acid and protein metabolism in Bermuda grass during water stress. **Plant Physiology** *41*: 1222-1230

Barnes, J.D. & Wilson, J.M. 1984. Assessment of the frost sensitivity of *Trifolium* species by chlorophyll fluorescence analysis. **Annuls of Applied Biology** *105*: 107-116

Bassi, R. & Sharma, S.S. 1993. Proline accumulation in wheat seedlings exposed to zinc and copper. **Phytochemistry** *33*: 1339-1342

Bates, L.S., Waldren, R.P & Teare, I.D. 1973. Rapid determination of free proline for water-stress studies. **Plant and Soil** *39*: 205-207

Beddard, G.S., Davidson, R.S. & Tretheway, K.R. 1977. Quenching of chlorophyll fluorescence by ß-carotene. **Nature** *267*: 373-374

Benko, B. 1968. The content of some amino acids in young apple shoots in relation to frost resistance. **Biologia Plant (Prague)** *11*: 334-337

Berjak, P., Farrant, J.M., Mycock, D.J. & Pammenter, N.W. 1990. Recalcitrant (homoihydrous) seeds: The enigma of their desiccation-sensitivity. **Seed Science Technology** *18*: 297-310

Berjak, P., Pammenter, N.W. & Vertucci, C. 1992. Homoiohydrous (recalcitrant) seeds: Developmental status, desiccation sensitivity and the state of water in axes of *Landolphia kirkii* Dyer. **Planta** *186*: 249-261

Berry, J. & Raison, J.K. 1982. Responses of macrophytes to temperature. In: Encyclopedia of Plant Physiology. Lange, O.B., Osmond, C.B.Nobel, P.G. (eds.). Springer Verlag: Berlin. *12a*: 277-338

Bielawski, W. & Joy, K.W. 1986. Reduced and oxidized glutathione and glutathione reductase activity in tissues of *Pisum sativum*. **Planta** *169*: 267-272

Björkman, O. 1968. Further studies on differentiation of photosynthetic properties in sun and shade ecotypes of *Solidago virgaurea*. **Physiologia Plantarum** *21*: 84-99

Björkman, O. 1987. Low temperature chlorophyll fluorescence in leaves and its relationship to photosynthetic yield of photosynthesis in photoinhibition. In: Topics in Photosynthesis. Volume 9: Photoinhibition. D.J. Kyle & C.B. Osmond (eds). Elsevier: Amsterdam. Pp 123-144

Björkman, O. 1989. Some viewpoints on photosyntheitc response and adaptation to environmental stress. In: Photosynthesis: Proc. of the C.S. French Symposium. W.R. Briggs (ed). Alan R. Liss Inc.: New York. Pp 45-58

Black, M.T., Brearley, T.H. & Horton, P. 1986. Heterogeneity in chloroplast photosystem II. **Photosynthetic Research** *8*: 193-207

Blum, A. 1988. Plant Breeding for Stress Environments. CRC Press Inc.: Florida.

Burke, J.J. & Oliver, M.J. 1993. Optimal thermal enviroments for plant metabolic processes (*Cucumis sativus* L.). Light-harvesting chlorophyll *a/b* pigment protein complex of photosystem II and seedling establishment in cucumber. **Plant Physiology** *103*: 295-302

Boese, S.R. & Huner, N.P.A. 1992. Developmental history affects the susceptibility of spinach leaves to *in vivo* low temperature photoinhibition. **Plant Physiology** *99*: 1141-1145

Boggess, S.F. & Stewart, C.R. 1980. The relationship between water stress induces proline accumulation and inhibition of protein synthesis in tobacco leaves. **Plant Science Letters** *17*: 245-252

Boggess, S.F., Stewart, C.R., Aspinall, D. & Paleg, L.G. 1976. Effect of water stress on proline synthesis from radioactive precursors. **Plant Physiology** *58*: 398-401

Boveris, A. 1984. Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. **Methods in Enzymology** *105*: 65-82

Bradbury, M. & Baker, N.R. 1986. The kinetics of photoinhibition of the photosynthetic apparatus in pea chloroplasts. **Plant, Cell and Environment** *9*: 289-297

Breton, J. 1982. Hypothesis - The F695 fluorescence of chloroplasts at low temperature is emitted from the primary acceptor of photosystem II. **FEBS Letters** *147*: 16-20

Briantais, J.-M., Cornic, G. & Hodges, M. 1988. The modification of chlorophyll fluorescence of *Chlamydomonas reinhardtii* by photoinhibition and chloramphenicol addition suggest a form of PS II less susceptible to degradation. **FEBS Letters** *236*: 226-230

Briantais, J.-M., Ducruet, J.-M., Hodges, M. & Krause, G.H. 1992. The effects of low temperature acclimation and photoinhibitory treatments on Photosystem 2 were studied by thernoluminescence and fluorescence decay kinetics. **Photosynthesis Research** *31*: 1-10

Briantais, J.-M., Vernotte, C., Picaud, M. & Krause, G.H. 1979. A quantitative study of the slow decline of chlorophyll *a* fluorescence in isolated chloroplasts. **Biochimica et Biophysica Acta** *548*: 128-138

Bridges, S.M. & Salin, M.L. 1981. Distribution of iron containing superoxide dismutase in vascular plants. **Plant Physiology** *68*: 275-278

Brüggemann, W.,van der Kooij, T.A.W. & van 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 rebulose-1,5-bisphosphate carboxylase/oxygenase. **Planta** *186*: 179-187

Butler, W.L. 1978. Energy distribution in the photochemical apparatus of Photosynthesis. **Annual Review of Plant Physiology** *29*: 345-378

Brown, M.E. 1988. Introduction to thermal analysis techniques and applications. Chapman and Hall University Press: Cambridge.

Burke, J.J. 1990. High temperature stress and adaptations in crops. In: Stress Responses in Plants: Adaptations and Acclimation Mechanisms, Plant Biology Series. Vol. 12. R.G. Alscher & J.R. Cumming (eds). Wiley-Liss: New York.

Burke, M.J., Gusta, L.V., Quamme, H.A., Weisser, C.J. & Li, P.H. 1976. Freezing and injury in plants. **Annual Review of Plant Physiology** *27*: 507-528

Burr, K.E., Tinus, R.W., Wallner, S.J. & King, R.M. 1990. Comparison of three cold hardiness tests for conifer seedlings. **Tree Physiology** *6*: 351-369

Cabané, M., Calvet, P, Vincens, P. & Boudet, A.M. 1993. Characterization of chilling-acclimation-related proteins in soybean and identification of one as a member of the heat shock protein (HSP 70) family. **Planta** *190*: 346-353

Cakmak, I. & Marschner, H. 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione peroxidase in bean leaves. **Plant Physiology** *98*: 1222-1227

Caldwell, M.M., Robberecht, F.V. & Flint, S.D. 1983. Internal filters: Prospects for UV-acclimation in higher plants. **Physiologia Plantarum** *58*: 445-450

Callahan, F.E., Becker, D.W. & Cheniae, G.M. 1986. Studies on the photoactivation of the water oxidizing enzyme. II. Characterization of weak light photoinhibition of PS II and its light-induced recovery. **Plant Physiology** *82*: 261-269

Cao, J. & Govindjee. 1990. Chlorophyll *a* fluorescence transient as an indicator of active and inactive Photosystem II in thylakoid membranes. **Biochimica et Biophysica Acta** *1015*: 180-188

Carlberg, I. & Mannervik, B. 1985. Glutathione reductase. In: **Methods of Enzymology** *113*: 484-490. A. Meister (ed).

Carri, M.T., Galiazzo, F., Ciriolo, M.R. & Rotilio, G. 1991. Evidence for co-regulation of Cu, Zn superoxide dismutase and metallothionien gene expression in yeast through transcripional control by copper via the ACEI factor. **FEBS Letters** *2278*: 263-266

Casano, L.M. & Trippi, V.S. 1992. The effect of oxygen radicals on proteolysis in isolated oat chloroplasts. **Plant and Cell Physiology** *33*: 329-332

Castonguay, Y., Nadeau, P. & Laberge, S. 1993. Freezing tolerance and alteration of translatable mRNAs in alfalfa (*Medicago sativa* L.) hardened at subzero temperatures. **Plant Cell Physiology** *34*: 31-38

Catavelli, L. & Bartels, D. 1989. Cold induced mRNAs accumulate with different kinetics in barley coleoptiles. **Planta** *178*: 184-188

Chapin, F.S. III, Bloom, A.J., Field, C.B. & Waring, R.H. 1987. Plant responses to multiple environmental factors. **BioScience** *37*: 49-57

Charest, C. & Phan, C.T. 1991. Kinetic properties of four enzymes involved in proline metabolism in cold-acclimated wheat. **Journal of Experimental Botany** *42*: 672-678

Charles, S.A. & Halliwell, B. 1980. Effect of hydrogen peroxide on spinach (*Spinacea oleracea*) chloroplast fructose bisphosphatase. **Biochemical Journal** *189*: 373-376.

Chen, P.M. & Li, P.H. 1977. Induction of frost hardiness in stem cortical tissues of *Cornus stolonifera* Michx. by water stress. II. Biochemical changes. **Plant Physiology** *69*: 256-258

Chinard, F.P. 1952. Photometric estimation of proline and ornithine. **Journal of Biological Chemistry** *199*: 91-95

Chongpraditnun, P., Mori, S. & Chino, M. 1992. Excess copper induces a cytosolic Cu, Zn-superoxide dismutase in soybean root. **Plant and Cell Physiology** *33*: 239-244

Chow, W.S., Osmond, C.B. & Huang, L.K. 1989. Photosystem II function and hervicide binding sites during photoinhibition of spinach chloroplasts in-vivo and in-vitro. **Photosynthetic Research** *21*: 17-26

Christophersen, B.O. 1969. Induction and prevention of chilling injury to radicle tips of imbibing cotton seed. **Plant Physiology** *43*: 743-746

Chu, T.M., Jusaitis, M. Aspinall, D. & Paleg, L.G. 1978. Accumulation of free proline at low temperatures. **Physiologia Plantarum** *43*: 254-260

Chylla, R.A. & Whitmarsh, J. 1989. Inactive photsystem II complexes in leaves. **Plant Physiology** *90*: 765-772

Clarkson, D.T., Hall, K.C. & Roberts, J.K.M. 1980. Phospholipid composition and fatty acid desaturation in the roots of rye, *Secale cereale* cv Lovatz-patoni, during acclimation of low temperature: positional analysis of fatty acids. **Planta** *149*: 464-471

Clegg, J.S. 1986. The physical properties and metabolic status of *Artemia* cysts at low water contents: The "Water Replacement Hypothesis". In: Membranes, Metabolism and Dry Organisms. A.C. Leopold (ed). Cornell University Press: New York. Pp 169-187.

Cleland, R.E. & Melis, A. 1987. Probing the events of photoinhibition by altering electron-transport activity and light-harvesting capacity in thylakoids. **Plant, Cell and Environment** *10*: 747-752

Cleland, R.E., Melis, A. & Neale, P.J. 1986. Mechanism of photoinhibition: photochemical reaction center inactivation in system II of chloroplasts. **Photosynthetic Research** *9*: 79-88

Cloutier, Y. 1983. Changes in the electrophoretic patterns of the soluble proteins of winter wheat and rye following cold acclimation and desiccation stress. **Plant Physiology** *71*: 400-403

Comai, L., Facciotti, D., Hiatt, W.R., Thompson, G., Rose, R.E. & Stalker, D.M. 1985. Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. **Nature** *317*: 741-744

Conn, E.E. & Vennesland, B. 1951. Glutathione reductase of wheat germ. **Journal of Biological Chemistry** *192*: 17-28

Connell, J.P. & Mullet, J.E. 1986. Pea chloroplast glutathione reductase: purification and characterisation. **Plant Physiology** *82*: 351-356

Cossins, E.A., Scott, R.A. & Jaleel, N. 1988. Formate synthesis in bundle sheath cells of corn and sorghum. **Journal of Plant Physiology** *132*: 266-272

Coulson, M., Huckett, B.I. & Watt, M.P. 1993. An investigation into the development of chloroplast isolation and "in-organelle" digestion procedures for the routine production of chloroplast DNA fingerprints of *Eucalyptus* species. **South African Forestry Journal** *164*: 1-7

Cramer, W.A., Whitmarsh, J. & Low, P.S. 1981. Differential scanning calorimetry of chloroplast membranes: Identification of an endothermic transition associated with the water splitting complex of photosystem II. **Biochemistry** *20*: 157-162

Crawford, R.M.M. & Huxter T.J. 1977. Root growth and carbohydrate metabolism at low temperatures. **Journal of Experimental Botany** *28*: 917-925

Creissen, G., Edwards, E.A., Enard, C., Wellburn, A. & Mullineaux, P. 1991. Molecular characterization of glutathione reductase cDNAs from pea (*Pisum sativum* L.) **The Plant Journal** *2*: 129-131

Crowe, L.M. & Crowe, J.H. 1986. Hydration-dependant phase transitions and permeability properties of biological membranes. In: Membranes, Metabolism and Dry Organisms. A.C. Leopold (ed). Cornell University Press: New York. Pp 210-229.

Dallmier, K.A. & Stewart, C.R. 1992. Effect of exogenous abscisic acid on proline dehydrogenase activity in maize (*Zea mays* L.). **Plant Physiology** *99*: 762-764

Dalton, D., Hanus, F.J., Pascoe, G.A. & Evans, H.J. 1987. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. **Plant Physiology** *83*: 789-794

Davidson, N.J. & Reid, J.B. 1987. The influence of hardening and waterlogging on the frost resistance of subalpine eucalypts. **Australian Journal of Botany** *35*: 91-101

de Kok, L.J. & Oosterhuis, F.A. 1983. Effects of frost-hardening and salinity on glutathione reductase activity in spinach leaves. **Physiologia Plantarum** *58*: 47-51

Delauney, A.J. & Verma, D.P.S. 1990. A soybean gene encoding δ^1-pyrroline-5-carboxylate reductase was isolated by functional complementation in *Eschericia coli* and is found to be osmoregulated. **Molecular and General Genetics** *221*: 299-305

Demmig, B. & Björkman, O. 1987. Comparison of the effect of excessive light on chlorophyll fluorescence (77K) and photon yield of O_2 evolution in leaves of higher plants. **Planta** *171*: 171-184

Demmig, B., Winter, K., Krüger, A. & Czygan, F-C. 1987. Photoinhibition and zeaxanthin formation in intact leaves. A possible role of the xanthophyll cycle in the dissipation of excess light energy. **Plant Physiology** *84*: 218-224

Demmig, B., Winter, K., Krüger, A. & Czygan, F-C. 1988. Zeaxanthin and the heat dissipation of excess light energy in *Nerium oleander* exposed to a combination of high light and water stress. **Plant Physiology** *87*: 17-24

Demmig-Adams, B & Adams, W.W. III. 1992. Photoprotection and other responses of plants to high light stress. **Annual Review of Plant Physiology and Plant Molecular Biology** *43*: 599-626

Demmig-Adams, B., Adams, W.W. III, S., Winter, K. Meyer, A., Schreiber, U, Pereira, J.S., Krüger, A., Czygan, F.-C. & Lange, O.L. 1990. Photochemical efficiency of photosystem II, photon yield of O_2 evolution photosynthetic capacity, and carotenoid composition during the midday depression of net CO_2 uptake in *Arbutus unedo* growing in Portugal. **Planta** *177*: 377-387

Dhindsa, R.S. 1991. Drought stress, enzymes of glutathione metabolism, oxidation injury, and protein synthesis in *Tortila ruralis*. **Plant Physiology** *95*: 648-651

Diamantoglou, S. & Rhizopoulou, S. 1992. Free proline accumulation in sapwood, bark and leaves of three evergreen sclerophylls and a comparison with an evergreen conifer. **Journal of Plant Physiology** *140*: 361-365.

Dörffling, K., Dörffling, H. & Lesselich, G. 1993. *In vitro*-selection and regeneration of hydroxyproline-resistant lines of winter wheat with increased proline content and increased frost tolerance. **Journal of Plant Physiology** *142*: 222-225

Doulis, A.G., Hausladen, A., Mondy, B., Alscher, R.G., Chevone, B.I. & Weiser, R.L. 1993. Antioxidant response and winter hardiness in red spruce *Picea rubens* Sarg.). **New Phytologist** *123*: 365-374

Dowgert, M.F. & Steponkus, P.L. 1984. Behaviour of the plasma membrane of isolated protoplasts during a freeze-thaw cycle. **Plant Physiology** *75*: 1139-1151

Duncan, D.R. & Widholm, J.M. 1987. Proline accumulation and its implication in cold tolerance of regenerable maize callus. **Plant Physiology** *83*: 703-708

Draper, S.R. 1972. Amino acid changes associated with low temperature treatment of *Lolium perenne*. **Phytochemistry** *11*: 639-641

Düring, H., Ortoidze, T.V. & Bushnell, B. 1990. Effects of subzero temperature on chlorophyll fluorescence of grapevine buds. **Journal of Plant Physiology** *136*: 758-760

Edwards, E.A., Rawsthorne, S. & Mullineaux, P.M. 1990. Subcellular distrubution of multiple forms of glutathione reductase in leaves of pea (*Pisum sativum* L.). **Planta** *180*: 276-284

Edwards, R., Blount, J.W. & Dixon, R.A. 1991. Glutathione and elicitation of the phytoalexin response in legume cell cultures. **Planta** *184*: 403-409

Egneus, H., Heber, U. & Kirk, M. 1975. reduction of oxygen by the electron transport chain of choroplasts during assimilation of carbon dioxide. **Biochemica et Biophysica Acta** *408*: 252-268

Eickmeier, W.G., Caspar, C. & Osmond, C.B. 1993. Chlorophyll fluorescence in the resurrection plant *Selaginella lepidophylla* (Hook & Grev.) spring during high-light and desiccation stress, and evidence for zeaxanthin-associated photoprotection. **Planta** *189*: 30-38

Ellis, R.H., Summerfield, R.J., Edmeades, G.O. & Roberts, E.H. 1992. Photoperiod, temperature, and the interval from sowing to tassel initiation in diverse cultivars of maize. **Crop Science** *32*: 1225-1232

Elstner, E.F. 1982. Oxygen activation and oxygen toxicity. **Annual Review of Plant Physiology** *33*: 73-96

Esterbauer, H. & Grill, D. 1978. Seasonal variation of glutathione and glutathione reductase in needles of *Picea abies*. **Plant Physiology** *61*: 119-121

Faye, L., Monatassim, B. & Ghorbel, A. 1986. Cell wall and cytoplasmic isozymes of ß-fructosidase have different N-linked oligosaccharides. **Plant Physiology** *80*: 27-33

Fee, J.A. & Valentine, J.S. 1977. Chemical and Physical properties of superoxide. In: Superoxide and Superoxide Dismutases. A.M. Michelson, J.M. McCordI. Fridovich (eds). Academic Press: New York. Pp 19-60.

Ferrar, P.J., Slayter, R.O. & Vranjic, J.A. 1989. Photosynthetic temperature acclimation in *Eucalyptus* species from diverse habitats, and a comparison with *Nerium oleander*. **Australian Journal of Plant Physiology** *16*: 199-217

Foote, C.S. 1979. Quenching of singlet oxygen. In: Singlet Oxygen. H.H.Wasserman and R.W. Murray (eds). Academic Press: New York. Pp 77-105

Fork, D.C. 1979. The influence of changes in the physical phase of thylakoid membrane lipids on photosynthetic activity. In: Low Temperature Stress in Crop Plants. J.M. Lyons, D. Graham & J.K. Raison (eds). Academic Press: New York. Pp 215-229

Foster, J.G. & Hess, J.L. 1980. Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere enriched in oxygen. **Plant Physiology** *66*: 482-487

Foyer, C.H. 1990. The effect of sucrose and mannose on cytoplasmic protein phosphorylation, sucrose phosphate synthetase activity and photosynthesis in leaf protoplasts from spinach. **Plant Physiology and Biochemistry** *28*: 151-160

Foyer, C.F. & Halliwell, B. 1976. The presence of gluthathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. **Planta** *133*: 21-25

Foyer, C.F. & Halliwell, B. 1977. Purification and properties of dehydroascorbate reductase from spinach leaves. **Phytochemistry** *16*: 1347-1350

Fridovich, I. 1970. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. **Journal of Biological Chemistry** *245*: 4053-4057

Fridovich, I. 1974. Superoxide dismutases. **Advances in Enzymology** *41*: 35-97

Fridovich, I. 1975. Superoxide dismutases. **Annual Review of Biochemistry** *44*: 147-159

Fridovich, I. 1976. Oxygen radicals, hydrogen peroxide and oxygen toxicity. In: Free Radicals in Biology. W.A. Pryor (ed). Academic Press: New York. Pp 239-277

Fridovich, I. 1986. Biological effects of superoxide radical. **Archives of Biochemistry and Biophysics** *247*: 1-11

Gamble, P.E. & Burke, J.J. 1984. Effect of water stress on the chloroplast antioxidant system. I. Alteration in glutathione reductase activity. **Plant Physiology** *76*: 615-621

Garber, M.P. 1977. Effect of light and chilling temperatures on chilling-sensitive and chilling-resistant plants. Pretreatment of cucumber and spinach thylakoids *in vivo* and *in vitro*. **Plant Physiology** *59*: 981-985

Genty, B., Briantais, J.-M. & Baker, N.R. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of cholorphyll fluorescence. **Biochimica et Biophysica Acta** *990*: 87-92

Genty, B., Harbinson, J., Briantias, J.-M. & Baker, N.R. 1990. The relationship between non-photochemical quenching of chlorophyll fluorescence and the rate ofphotosystem 2 photochemistry in leaves. **Photosynthetic Research** *25*: 249-257

Giersch, C. & Krause, H. 1991. A simple model relating photoinhibitory fluorescence quenching in chloroplasts to a population of altered photosystem II reaction centres. **Photosynthetic Research** *30*: 115-121

Giersch, C. & Robinson, S.P. 1987. Effects of photoinhibition on photosynthetic carbon metabolism in intact isolated spinach chloroplasts. **Australian Journal of Plant Physiology** *14*: 439-449

Gillham, D.J. & Dodge, A.D. 1985. Chloroplast protection in greening leaves. **Physiologia Plantarum** *65*: 393-396

Gillham, D.J. & Dodge, A.D. 1986. Hydrogen peroxide-scavenging systems within pea chloroplasts. **Planta** *167*: 246-251

Gilmour, S.J., Artus, N.N & Thomashow, M.F. 1992. cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. **Plant Molecular Biology** *18*: 13-21

Gong, H. & Nilsen, S. 1989. Effect of temperature on photoinhibition of photosynthesis, recovery and turnover of the 32 kD chloroplast protein in *Lemna gibba*. **Journal of Plant Physiology** *135*: 9-14

Graham, D. & Patterson, B.D. 1982. Responses of plants to low, nonfreezing temperatures: Proteins, metabolism and acclimation. **Annual Review of Plant Physiology** *33*: 347-372

Green, M.J. & Hill, H.O.A. 1984. Chemistry of dioxygen. **Methods in Enzymology** *105*: 3-22

Greer, D.H. 1988. Effect of temperature on photoinhibition and recovery in *Actinidia deliciosa*. **Australian Journal of Plant Physiology** *15*: 195-205

Greer, D.H., Berry, J. & Björkman, O. 1986. Photoinhibition of photosynthesis in intact bean leaves: role of light and temperature, and requirement for chloroplast-protein synthesis during recovery. **Planta** *186*: 253-260

Greer, D.H. & Laing, W.A. 1988. Photoinhibition of photosynthesis in intact kiwifruit (*Actinidia deliciosa*) leaves: recovery and its dependence on temperature. **Planta** *174*: 159-165

Greer, D.H., Laing, W.A. & Kipnis, T. 1988. Photoinhibition of photosynthesis in intact kiwifruit (*Actinidia deliciosa*) leaves: effect of temperature. **Planta** *174*: 152-158

Greer, D.H., Ottander, C. & Öquist, G. 1991. Photoinhibition and recovery of photosynthesis in intact barley leaves at 5° and 20°C. **Physiologia Plantarum** *81*: 203-210

Groden, D. & Beck, E. 1979. H_2O_2 destruction by ascorbate dependant systems from chloroplasts. **Biochemica et Biophysica Acta** *546*: 426-435

Grodzinsky, B. & Butt, V.S. 1976. Hydrogen peroxide production and the release of carbon dioxide during glycollate oxidation in leaf peroxisomes. **Planta** *128*: 225-231

Guenther, J.E. & Melis, A. 1990. The physiological significance of photosystem II heterogeneity in chloroplasts. **Photosynthetic Research** *23*: 105-109

Guy, C.L. 1990. Cold acclimation and freezing stress tolerance: Role of membrane proteins. **Annual Review of Plant Physiology and Plant Molecular Biology** *41*: 187-223

Guy, C.L. & Carter, J.V. 1984. Characterization of partially purified gluthathione reductase from cold-hardened and non-hardened spinach leaf tissue. **Cryobiology** *21*: 454-464

Guy, C.L., Carter, J.V., Yelenosky, G. & Guy, C.T. 1984. Changes in glutathione content during cold acclimation in *Cornus sericea* and *Citrus sinensis*. **Cryobiology** *21*: 433-454

Hallam, P.M. & Reid, J.B. 1989. Seasonal and genetic variation in frost hardiness of *Eucalyptus delegatensis*. **Canadian Journal of Forestry Research** *19*: 480-488

Hallam, P.M., Reid, J.B. & Beadle, C.L. 1989. Frost hardiness of commercial *Eucalytus* species at different elevations. **Canadian Journal of Forestry Research** *19*: 1235-1239

Halliwell, B. 1982. The toxic effects of oxygen on plant tissues. In: Superoxide Dismutase. L.L. Oberley (ed). CRC Press: Boca Raton, Florida. Pp 213-259

Halliwell, B. & Foyer, C.H. 1978. Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. **Planta** *139*: 9-17

Halliwell, B. & Gutteridge J.M.C. 1985. Free radicals in Biology and Medicine. Oxford University Press: London. Pp 54-83

Hanson, A.D., Nelsen, C.E., Pedersen, A.R. & Everson, E.H. 1979. Capacity for proline accumulation during water stress in barley and its implications for breeding for drought stress. **Crop Science** *19*: 489-493

Hariyadi, P. & Parkin, K.L. 1993. Chilling-induced oxidative stress in cucumber (*Cucumis sativus* L. cv. Calypso) seedlings. **Journal of Plant Physiology** *141*: 733-738

Harris, P. & James, A.T. 1969. Effect of low temperature on fatty acid biosynthesis in seeds. **Biochimica et Biophysica Acta** *187*: 13-18

Havaux, M. 1993. Rapid photosynthetic adaptations to heat stress triggered in potato leaves by moderately elevated temperatures. **Plant, Cell and Environment** *16*: 461-467

Havaux, M. & Lannoye, R. 1983. Temperature dependence of delayed chlorophyll fluorescence in intact leaves of higher plants. A rapid method for detecting the phase transition of thylakoid membrane lipids. **Photosynthetic Research** *4*: 257-263

Heber, U.L., Tyankova, K.A. & Santarius. 1971. Stabilization and inactivation ofbiological membranes during freezing in the presence of amino acids. **Biochimica et Biophysica Acta** 241: 587-592

Hellergren, J. & Li, P.H. 1981. Survival of *Solanum tuberosum* suspension cultures to -14°C: The mode of action of proline. **Physiologia Plantarum** 52: 449-453

Herman, B. Personal communication. Mondi Forests, Research and Development, Natal Office, P.O. Box 39, Pietermaritzburg, 3200

Hetherington, S.E., He, J. & Smillie, R.M. 1989. Photoinhibition at low temperature in chilling-sensitive and -resistant plants. **Plant Physiology** *90*: 1609-1615

Hipkins, M.F. & Baker, N.R. 1986. Photosynthesis: energy transduction: A practical approach. IRL Press Limited, Oxford. Pp 91

Hitz, W.D. & Hanson, A.D. 1980. Determination of glycine-betaine by pyrolysis-gas-chromatography in cereals and grasses. **Phytochemistry** *19*: 2371-2374

Hodgson, R.A.J., Orr, G.R. & Raison, J.K. 1987. Inhibition of photosynthesis by chilling in the light. **Plant Science** *49*: 75-79

Hodgson, R.A.J. & Raison, J.K. 1989. Inhibition of photosynthesis by chilling in moderate light: A comparison of plants sensitive and insensitive to chilling. **Planta** *178*: 545-552

Hodgson, R.A.J. & Raison, J.K. 1991. Superoxide production by thylakoids during chilling and its implication in the susceptiblility of plants to chilling-induced photo-inhibition. **Planta** *183*: 222-228

Horton, P. 1985. Interactions between electron transfer and carbon assimilation. In: Photosynthetic mechanisms and the environment. Topics in photosynthesis. Vol. 6. Barber, J. & Baker, N.R. (eds). Elsevier Scientific Publications, B.V.: Amsterdam. Pp 135-187

Horton, P. & Hague, A. 1988. Studies of the induction of chlorophyll fluorescence in isolated barley protoplasts. IV. Resolution of non-photochemical quenching. **Biochimica et Biophysica Acta** *932*: 107-115

Hossain, M.A., Nakano, Y. & Asada, K. 1984. Monodehydroascorbate reductase in spinachi chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. **Plant and Cell Physiology** *25*: 385-395

Houde, M., Danyluk, J., Laliberte, J.-F., Rassart, E., Dhindsa, R.S. & Sarhan, F. 1992. Cloning, characterization and expression of a cDNA encoding a 50 kD protein specifically induced by cold acclimation in wheat. **Plant Physiology** *99*: 1381-1387

Huner, N.P.A. & Macdowell, F.D.H. 1979. The effects of low temperature acclimation of winter rye on catalytic properties of its ribulose bisphosphate carboxylase-oxygenase. **Canadian Journal of Biochemistry** *57*: 1036-1041

Huner, N.P.A., Palta, J.P., Li, P.H. & Carter, J.V. 1981. Comparison of the structure and function of ribulose bisphosphate carboxylase-oxygenase from a cold-hardy and nonhardy potato species. **Canadian Journal of Biochemistry** *39*: 280-289

Hurry, V.M., Gardeström, P. & Öquist, G. 1993. Reduced sensitivity to photoinhibition following frost-hardening of winter rye is due to increased phosphate availability. **Planta** *190*: 484-490

Hurry, V.M. & Huner, N.P.A. 1991. Low growth temperature effect a differential inhibition of photosynthesis in spring and winter wheat. **Plant Physiology** *96*: 491-497

Ilhai I. & Dörffling,K. 1982. Changes in abscisic acid and proline levels in maize varieties of different drought resistance. **Physiologia Plantarum** *55*: 129-135

Jablonski, P.P. & Anderson, J.W. 1978. Light-dependent reduction of oxidised glutathione by ruptured chloroplasts. **Plant Physiology** *61*: 221-225

Jablonski, P.P. & Anderson, J.W. 1981. Light-dependent reduction of dehydrascorbate by ruptured pea chloroplasts. **Plant Physiology** *67*: 1239-1244

Jablonski, P.P. & Anderson, J.W. 1982. Light-dependent reduction of hydrogen peroxide by ruptured pea chloroplasts. **Plant Physiology** *69*: 1407-1413

Jackson, W.M., Kostyla, J., Nordin, J.H. & Brandts, J.F. 1973. Calorimetric study of protein transitions in human erythrocyte ghosts. **Biochemistry** *12*: 3662-3667

Jankhe, L.S., Hull, M.R. & Long, S.P. 1991. Chilling stress and oxygen metabolizing enzymes in *Zea mays* and *Zea diploperennis*. **Plant, Cell and Environment** *14*: 97-104

Janssen, L.H.J., Wams, H.E. & van Hasselt, P.R. 1992. Temperature-dependence of chlorophyll fluorescence induction and photosynthesis in tomato as affected by temperature and light conditions during growth. **Journal of Plant Physiology** *139*: 549-554

Jarillo, J.A., Leyva, A., Salinas, J. & Martínez-Zapter, J.M. 1993. Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Aribidopsis thaliana*, a chill-tolerant plant. **Plant Physiology** *101*: 833-837

Jeffree, C.E., Read, N.D., Smith, J.A.C. & Dale, J.E. 1987. Water droplets and ice deposits in leaf intracellular spaces: Redistribution of water during cryofixation for scanning electron microscopy. **Planta** *172*: 20-37

Jegerschöld, C., Virgin, I. & Strying, S. 1990. Light-dependent degradation of the D_1 protein in Photosystem II is accelerated after inhibition of the water splitting reaction. **Biochemistry** *29*: 6179-6186

Jocelyn, P.C. 1972. The Biochemistry of the SH Group. Academic Press: New York.

Johnson-Flanagan, A.M. & Singh, J. 1987. Alteration of gene expression during the induction if freezing tolerance in *Brassica napus* suspension cultures. **Plant Physiology** *85*: 699-705

Jolivet, Y., Hamelin, J. & Larher, F. 1983. Osmoregulation in halophytic higher plants: The protective rffects of glycine betaine and other related solutes against the oxalate destabilisation of membranes in beet root cells. **Zeitschrift für Pflanzenphysiologie** *109*: 171-180

Joyce, P.S., Paleg, L.G. & Aspinall, D. 1984. The requirement for low-intensity light in the accumulation of proline as a response to water deficit. **Journal of Experimental Botany** *151*: 209-215

Joyce, P.A., Aspinall, D. & Paleg, L.G. 1992. Photosynthesis and the accumulation of proline in response to water deficit. **Australian Journal of Plant Physiology** *19*: 249-261

Kaczmarek, J., Prokowski, Z. & Brzóstowicz, A. 1991. Low temperature affects on fluorescence in leaves of unhardened and hardened winter wheat. **Photosynthetica** *25*: 215-217

Kaiser, W.M. 1979. Reversible inhibition of the calvin cycle and activation of oxidative pentose phosphate cycle in isolated intact chloroplast by hydrogen peroxide. **Planta** *145*: 377-382

Kalk-Torres, W., Burke, J.J. & Anderson, J.M. 1984. Chloroplast glutathione reductase: Purification and properties. **Physiologia Plantarum** *61*: 271-278

Katsaras, J., Stinson, R.H., Kendall, E.J. & McKersie, B.D. 1986. Structural simulation of free radical damage in a model membrane system: a small angle X-ray study. **Biochemica et Biophysica Acta** *861*: 243-250

Kaufmann, M.R. 1975. Leaf water stress in Engelmann spruce: Influence of the root and shoot environments. **Plant Physiology** *56*: 841-844

Kendall, E.J. & McKersie, B.D. 1989. Free radical and freezing injury to cell membranes of wheat. **Physiologia Plantarum** *76*: 86-94

Keuh, J.S., Hill, J.M., Smith, S.J. & Bright, S.W.J. 1984. Proline biosynthesis in a proline-accumulating barley mutant. **Photosynthesis** *23*: 2207-2210

Kidambi, S.P., Mahan, J.R. & Matches, A.G. 1990. Purification and thermal dependence of glutathione reductase from two forage legume species. **Plant Physiology** *92*: 363-367

Klimov, V.V. & Krasnovskii, A.A. 1981. Pheophytin as a primary electron acceptor in photsystem II reaction centres. **Photosynthetica** *15*: 592-609

Kohl, D.H., Schubert, K.R., Carter, M.B., Hagehorn, C.H. & Shearer, G. 1988. Proline metabolism in N_2 fixing root nodules: energy transfer and regulation of purine synthesis. **Proceedings of the National Academy of Science, USA** *85*: 2036-2040

Kohl, D.H., Kennelly, E.J., Zhu, Y., Schubert, K.R. & Shearer, G. 1991. Proline accumulation, nitrogenase (C_2H_2 reducing) activity and activities of enzymes related to proline metabolism in drought stressed soybean nodules. **Journal of Experimental Botany** *42*: 831-837

Koster, K.L. & Lynch, D.V. 1992. Solute accumulation and compartmentation during the cold acclimation of puma rye. **Plant Physiology** *98*: 108-113

Krause, G.H. 1988. Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. **Physiologia Plantarum** *74*: 566-574

Krause, G.H. & Cornic, G. 1987. CO_2 and O_2 interactions in photoinhibition. In: Photoinhibition. D.J. Kyle, C.B. Osmond & C.J. Arntzen (eds). Elsevier: Amsterdam. Pp 169-196

Krause, G.H., Briantias, J.-M. & Vernotte, C. 1983. Characterization of chlorophyll fluorescence quenching in chloroplasts by fluorescence specrtoscopy at 77 K. I. $\triangle$pH-Dependent quenching. **Biochimica et Biophysica Acta** *723*: 169-175

Krause, G.H., Köster, S. & Wong, S.C. 1985. Photoinhibition of photosynthesis under anaerobic conditions studied with leaves and chloroplasts of *Spinacia oleracea* L. **Planta** *165*: 430-438

Krause, G.H., Laasch, H. & Weis, E. 1988. Regulation of thermal dissipation of absorbed light energy in chloroplasts indicated by energy-dependent fluorescence quenching. **Plant Physiology and Biochemistry** *26*: 445-452

Krause, G.H., Somersalo, S., Zumbusch, E., Weyers, B. & Laasch, H. 1990. On the mechanism of photoinhibition in chloroplasts. Relationship between changes in fluorescence and activatity of Photosystem II. **Journal of Plant Physiology** *136*: 472-479

Krause, G.H. & Weis, E. 1991. Chlorophyll fluorescence and photosynthesis: The basics. **Annual Review of Plant Physiology and Plant Molecular Biology** *42*: 313-349

Krasnuk, M. Witham, F.H. & Jung, G.A. 1976. Electrophoretic studies of several hydrolytic enzymes relation to cold tolerance of alfalfa. **Cryobiology** *13*: 225-242

KrishnaRaj, S., Mawson, B.T., Yeung, E.C. & Thorpe, T.A. 1993. Utilization of induction and quenching kinetics of chlorophyll *a* fluorescence for *in vivo* salinity screening studies in wheat (*Triticum aestivum* vars. Kjarchia-65 and Fielder). **Canadian Journal of Botany** *71*: 87-92

Kruger, R., Jager, H.J., Hintz, M. & Pahlich, E. 1986. Purification to homogeneity of pyrroline-5-carboxylate reductase of barley. **Plant Physiology** *80*: 142-144

Krinsky, N. 1979. Biological roles of singlet oxygen. In: Singlet Oxygen. H.H. Wasserman and R.W. Murray (eds). Academic Press: New York. Pp 73-89

Kuroda, H., Sagisaka, S., Asada, M. & Chiba, K. 1991. Peroxide-scavenging systems during cold acclimation of apple callus in culture. **Plant and Cell Physiology** *32*: 635-641

Kushad, M.M. & Yelenosky, G. 1987. Evaluations of polyamine and proline levels during low temperature acclimation of citrus. **Plant Physiology** *84*: 692-695

Kuthan, H. & Ullrich, V. 1982. Oxidase and oxygenase function of the microsomal cytochrome P_{450}. **European Journal of Biochemistry** *126*: 583-588

Kyle, D.J. 1987. The biochemical basis for photoinhibition of photosystem II. In: Photoinhibition. D.J. Kyle, C.B. Osmond & C.J. Arntzen (eds). Elsevier: Amsterdam. Pp 167-226

Kyle, D.J., Osmond, C.B. & Arntez, C.J. (eds). 1987. Photoinhibition. Elsevier: Amsterdam. Pp 155-183

Laasch, H. 1987. Non-photochemical quenching of chlorophyll *a* fluorescence in isolated chloroplasts under condition of stressed photsynthesis. **Planta** *171*: 220-226

Laliberté, G. & Hallibust, J.A. 1989. Pyrroline-5-carboxylate reductase in *Chlorella autotrophica* and *Chlorella saccarophila* in relation to osmoregulation. **Plant Physiology** *91*: 917-923

Lalk, I. & Dörffling, J. 1985. Hardening, abscisic acid, proline and freezing resistance in 2 winter wheat varieties. **Plant Physiology** *63*: 287-292

Lapointe, L., Huner, N.P.A., Carpentier, R. & Ottander, C. 1991. Resistance to low temperature photoinhibition is not associated with isolated thylakoid membranes of winter rye. **Plant Physiology** *97*: 804-810

Larson, R.A. 1988. The antioxidants of higher plants. **Phytochemistry** *27*: 969-978

Lawton, M.A., Clouse, S.D. & Lamb, C.J. 1990. Glutathione-elicited changes in chromatin structure within the promoter of the defense gene chalcone synthase. **Plant Cell Reports** *8*: 561-564

Levitt, J. 1962. A sulfhydryl-disulphide hypothesis of frost injury and resistance in plants. **Journal of Theoretical Biology** *3*: 355-391

Levitt, J. 1972. Responses of plants to environmental stresses. 1st edition. Academic Press: New York.

Levitt, J. 1980. Responses of plants to environmental stresses. 2nd edition. Academic Press: New York

Li, P.H. 1984. Subzero temperature stress physiology of herbaceous plants. **Horticultural Review** *6*: 373-416

Li, P.H. & Sakai, A. 1982. (eds) Plant cold hardiness and freezing stress mechanisms and crop implications. Academic Press: New York. Pp 416

Lieberman, M., Craft, C.C., Audia, W.V. & Wilcox, M.S. 1958. Biochemical studies of chilling injury in sweet potatoes. **Plant Physiology** *33*: 307-311

Loomis, W.D. & Battaile, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. **Phytochemistry** *5*: 423-438

Lumsden, J., Henry, L. & Hall, D.O. 1977. Superoxide dismutase in photosynthetic organisms. In: Superoxide and Superoxide Dismutases. A.M. Michelson, J.M. McCord, I. Fridovich (eds). Academic Press: New York. Pp 218

Lynch, D.V. & Thompson, G.A. 1984. Microsomal phospholipid molecular species alterations during low temperatures acclimation in *Dunaliella*. **Plant Physiology** *74*: 193-197

Lyons, J.M. 1973. Chilling injury in plants. **Annual Review of Plant Physiology** *24*: 445-466

Madamanchi, N.R., Anderson, J.V., Alscher, R.G., Cramer, C.L. & Hess, J.L. 1992. Purification of multiple forms of glutathione reductase from pea (*Pisum sativum* L.) seedlings and enzyme levels in ozone-fumigated pea. **Plant Physiology** *100*: 138-145

Mäenpää, P., Aro, E.M., Somersalo, S. & Tyystjärvi, E. 1988. Rearrangement of the chloroplast thylakoid at chilling temperature in the light. **Plant Physiology** *87*: 762-766

Magné, C. & Larher, F. 1992. High sugar content of extracts interferes with calorimetric determination of amino acids and free proline. **Analytical Biochemistry** *200*: 115-118

Mahan, J.R. & Burke, J.J. 1987. Purification and characterisation of glutathione reductase from corn mesophyll chloroplasts. **Physiologia Plantarum** *71*: 352-358

Mahan, J.R., Burke, J.J. & Orzech, K.A. 1987. The "thermal kinetic window" as an indicator of optimum plant temperature. **Plant Physiology** *83*: S87

Mahan, J.R., Burke, J.J. & Orzech, K.A. 1990. Thermal dependance of the apparent K_M of glutathione reductases from three plant species. **Plant Physiology** *93*: 822-824

Maniatis, T., Fritsch, E.F., & Sambrook, J. 1982. Molecular cloning. A laboratory manual. Cold Springer Verlag: New York

Mapson, L.W. & Goddard, D.R. 1951. Reduction of glutathione by co-enzyme II. **Nature** *167*: 975-977

Marmiroli, N., Terzi, V., Odoardi Stanca, M., Lorenzoonj, C. & Stanca, A.M.1986. Protein synthesis during cold shock in barley tissues. **Theoretical and Applied Genetics** *73*: 190-196

Maslenkova, L.T., Miteva, T.S. & Popova, L.P. 1992. Changes in the polypeptide patterns of barley seedlings exposed to jasmonic acid and salinity. **Plant Physiology** *98*: 700-707

Mazur, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. **Journal of General Physiology** *47*: 347-369

Mazur, P. 1969. Freezing injury in plants. **Annual Review of Plant Physiology** *20*: 419-448

McKellar, D. Personal communication. Mondi Forests, Research and Development, Natal Office, P.O.Box 39, Pietermaritzburg, 3200

McNamer, A.D. & Stewart, C.R. 1974. Nicotinanide adenine dinucleotide-dependent proline dehydrogenase in *Chlorella*. **Plant Physiology** *53*: 440-444

Mead, J.F. 1976. Free radical mechanisms of lipid damage and consequences for cellular membranes. In: Free Radicals in Biology. W.A. Pryor (ed). Academic Press: New York. Pp 159

Mehler, A.H. 1951. Studies on reactions of illuminated chloroplasts. I. Mechanisms of the reduction of oxygen and other Hill reactions. **Archives of Biochemistry and Biophysics** *33*: 65-77

Melcarek, P.K. & Brown, G.N. 1977. Effects of chill stress on prompt and delayed chlorophyll fluorescence from leaves. **Plant Physiology** *60*: 822-825

Miler, P.M. & Stewart, C.R. 1976. Pyrroline-5-carboxylic acid reductase from soybean leaves. **Phytochemistry** *15*: 1855-1857

Meils, A. 1985. Functional properties of photosystem IIß in spinach chloroplasts. **Biochimica et Biophysica Acta** *808*: 334-342

Mishra, N.P., Mishra, R.K. & Singhal, G.S. 1993. Changes in the activities of anti-oxidant enzymes during exposure of intact wheat leaves to strong visible light at different temperatures in the presence of protein synthesis. **Plant Physiology** *102*: 903-910

Moll, B.A. & Steinback, K.E. 1986. Chilling sensitivity in *Oryza sativa*: The role of protein phosphorylation in protection against photoinhibition. **Plant Physiology** *80*: 420-423

Mukherjee, S.P. & Choudhuri, M.A. 1983. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vignia* seedlings. **Physiologia Plantarum** *58*: 166-170

Murata, N. 1969. Magnesium ion dependent distribution of excitation energy between the two pigment systems in spinach chloroplasts. **Biochimica et Biophysica Acta** *189*: 171-181

Murata, T. & Tatsumi, Y. 1979. Ion leakage in chilled plant tissues. In: Low Temperature Stress in Crop Plants. J.M. Lyons, D. Graham & J.K. Raison (eds). Academic Press: New York. Pp 141-151

Murray, R.W. 1979. Chemical sources of singlet oxygen. In: Singlet Oxygen. H.H. Wasserman and R.W. Murray (eds). Academic Press: New York.

Naidu, B.P., Aspinall, D. & Paleg, L.G. 1992a. Variability in proline accumulating ability of barley (*Hordeum vulgaris* L.) cultivars induced by vapour pressure deficit. **Plant Physiology** *98*: 719-722

Naidu, B.P., Paleg, L.G., Aspinall, D., Jennings, A.C. & Jones, G.P. 1991. Amino acid and glycine betaine accumulation in cold-stressed wheat seedlings. **Phytochemistry** *30*: 407-409

Naidu, B.P., Paleg, L.G. & Jones, G.P. 1992b. Nitrogenous compatible solutes in drought stressed *Medicago* spp. **Phytochemistry** *31*: 1195-1197

Nakagawara, S. & Sagisaka, S. 1984. Increases in enzyme activities related to ascorbate metabolism during cold acclimation in poplar twigs. **Plant and Cell Physiology** *25*: 899-906

Nakano, Y. & Asada, K. 1981. Hydrogen peroxide is scavenged by ascorbate peroxidase in spinach chloroplasts. **Plant, Cell and Environment** *22*: 867-880

Nakano, Y. & Asada, K. 1987. Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. **Plant and Cell Physiology** *28*: 131-140

Nikolopoulos, D. & Manetas, Y. 1991. Compatible solutes and *in vivo* stability of *Salsola soda* enzymes: Proline incompatibility. **Phytochemistry** *30*: 411-413

Neuner, G. & Larcher, W. 1991. The effect of light, during and subsequent to chilling, on the photosynthetic activity of two soybean cultivars, measured by *in vivo* chlorophyll fluorescence. **Photosynthetica** *25*: 257-266

Noguchi, M., Koiwai, A., Yokoyama, M. & Tamaki, E. 1968. Studies on nitrogen metabolism in tobacco plants. IX. Effect of various compounds on proline biosynthesis in the green leaves. **Plant Cell Physiology** *9*: 35-47

Nordin, A. 1977. Effects of low root temperature on ion uptake and ion translocation in wheat. **Physiologia Plantarum** *39*: 305-310

Ögren, E. 1991. Prediction of photoinhibition of photosynthesis from measurements of fluorescence quenching components. **Planta** *184*: 538-544

Ögren, E. & Evans, J.R. 1992. Photoinhibition of photosynthesis *in situ* in six species of *Eucalyptus*. **Australian Journal of Plant Physiology** *19*: 223-232

Ohad, I., Koike, H., Shochat, S. & Inoue, Y. 1988. Changes in the properties of reaction centers during the initial stages of photoinhibition as revealed by thermoluminescence measurements. **Biochimica et Biophysica Acta** *993*: 288-298

Ohad, I., Kyle, D.J. & Arntzen, C.J. 1984. Membrane protein damage and repair: removal and replacement of inactivate 32-kilodalton polypeptides in chloroplast membranes. **Journal of Cell Biology** *99*: 481-485

Ohad, I., Kyle, D.J. & Hirschenberg, J. 1985. Light-dependent degradation of the Q_B-protein in isolated pea thylakoids. **EMBO Journal** *4*: 1655-1659

Olien, C.R. 1967. Freezing stresses and survival. **Annual Review of Plant Physiology** *18*: 387-408

Oliver, D.J. 1979. Mechanism of decarboxylation of glycine and glycolate by isolated soybean cells. **Plant Physiology** *64*: 1048-1052

Ono, T-A. & Murata, N. 1981. Chilling susceptibility of the blue-green alga *Anacystis nidulans*. II. Stimulation of the passive permeability of cytoplasmic membrane at chilling temperatures. **Plant Physiology** *67*: 182-187

Öquist, G., Chow, W.S. & Anderson, J.M. 1992. Photoinhibition of photosynthesis represents a mechanism for the long-term regulation of photosystem II. **Planta** *186*: 450-460

Öquist, G., Greer, D.H. & Ögren, E. 1987. Light stress at low temperature. In: Topics in photosynthesis. Vol. 9. D.J. Kyle, C.B. Osmond & C.J.Arntzen (eds). Elsevier: Amsterdam. Pp 67-88

Öquist, G. & Huner N.P.A. 1991. Effects of cold acclimation on the susceptibility of photosynthesis to photoinhibition in Scots pine and in winter and spring cereals: a fluorescence analysis. **Functional Ecology** *5*: 91-100

Öquist, G. & Huner N.P.A. 1993. Cold-hardening-induced resistance to photoinhibition of photosynthesis in winter rye is dependent upon an increased capacity for photosynthesis. **Planta** *189*: 150-156

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

Öquist, G. & Martin, B. 1986. Cold climates. In: Photosynthesis in Contrasting Environments. Baker, N.R.Long, S.P. (eds.). Elsevier Science Publishers B.V. Biomedical Division. Pp 341-582

Orrenius, S. 1985. Oxidative stress studied in intact mammalian cells. **Philosophical Transactions of the Royal Society of London** *B 311: 673-677*

Osmond, C.B. 1981. Photosrespiration and photoinhibition. Some implication for the energetics of photosynthesis. **Biochimica et Biophysical Acta** *639*: 77-98

Osmond, C.B., Ramus, J., Levavasseur, G., Franklin, L.A. & Henley, W.J. 1993. Fluorescence quenching during photosynthesis and photoinhibition of *Ulva rotundata* Blid. **Plant** *190*: 97-106

Ottander, C. & Öquist, G. 1991. Recovery of photosynthesis in winter-stressed Scots pine. **Plant, Cell and Environment** *14*: 345-349

Paleg, L.G., Douglas, T.J., van Dall, A. & Keech, D.B. 1981. Proline, betaine and other organic solutes protect enzymes against heat inactivation. **Australian Journal of Plant Physiology** *8*: 107-114

Paleg, L.G., Stewart, G.R. & Bradbeer, J.W. 1984. Proline and glycine betaine influence protein solvation. **Plant Physiology** *75*: 974-978

Pálfi-Deim, G., Gulyás, S. & Cseuz, L. 1989. Demonstration of the extient of drought resistance in winter wheat varieties, and study of the proline accumulation ability of 25 cultivated species. **Acta Biologica Szeged** *35*: 81-89

Palta, J.P. & Li, P.H. 1978. Cell membrane properties in relation to freezing injury. In: Plant Cold Hardiness and Freezing Stress. Mechanisms and Crop Implications. P.H. Li & A. Sakai (eds). Academic Press: New York. Pp 135-182

Pammenter, N.W., Vertucci, C.W. & Berjak, P. 1991. Homeohydrous (recalcitrant) seeds: Dehydration, the state of water and viability characteristics in *Landolphia kirkii*. **Plant Physiology** *96*: 1093-1098

Patterson, B.D., Payne, L.A., Chen, Y-Z. & Graham, D. 1974. An inhibitor of catalase induced by cold in chilling-sensitive plants. **Plant Physiology** *76*: 1014-1018

Patton, S.E., Rosen, G.M. & Rauckman, E.J. 1980. Superoxide production by purified hamster hepatic nuclei. **Molecular Pharmacology** *18*: 588-593

Penfold, A.R. & Willis, J.L. 1961. The Eucalypts. Botany, Cultivation, Chemistry and Utilization. Interscience Publishers, Inc.: New York.

Penning de Vreis, F.W.T. 1975. The cost of maintenence processess in plant cells. **Annuls of Botany** *39*: 77-92

Pinto, M.C., Mata, A.M. & Lopez-Barea, J. 1985. The redox interconversion mechanisms of *Saccharomycae cerevisiae* glutathione reductase. **European Journal of Biochemistry** *151*: 275-281

Polle, A., Chakrabarte, K., Chakrabarti, S., Seufert, F., Schramel, P. & Rennenberg, H. 1992. Antioxidants and mangenese deficiency in needles of Norway spruce (*Picea abies* L.) trees. **Plant Physiology** *99*: 1084-1089

Polle, A., Pfirrmann, T., Chakrabarti, S. & Rennenberg, H. 1993. The effects of enhanced ozone and enhanced carbon dioxide concentrations on biomass, pigments and antioxidative enzymes in spruce needles (*Picea abies* L.). **Plant, Cell and Environment** *16*: 311-316

Pollock, C.J., Lloyd, E.J., Stoddart, J.L. & Thomas, H. 1983. Growth, photosynthesis and assimilate partitioning in *Lolium temulentum* exposed to chilling temperatures. **Physiologia Plantarum** *59*: 257-262

Pomeroy, M.K., Pihakaski, S.J. & Andrews, C.J. 1983. Membrane properties of isolated winter wheat cells in relation to icing stress. **Plant Physiology** *72*: 535-539

Powles, S.B. 1984. Photoinhibition of photosynthesis induced by visible light. **Annual Review of Plant Physiology** *35*: 15-44

Powles, S.B., Berry, J.A. & Björkman, O. 1983. Interaction between light and chilling temperature on the inhibition of photosynthesis in chilling sensitive plants. **Plant, Cell and Evironment** *6*: 117-123

Poynton, R.J. 1979. Tree planting in Southern Africa. Vol. II. The Eucalypts. South African Forestry Research Institute, VR Printers: Pretoria, S.A. Pp 350-370; 433-437; 532-536; 741-744

Radmer, R.J. & Kok, B. 1976. Photoreduction of O_2 primes replaces CO_2 assimilation. **Plant Physiology** *58*: 336-340

Raison, J.K. 1980. in The Biochemistry of Plants: Metabolism and Rich, Respiration. Volume 2. D. Davies (ed). Academic Press: New York.

Raison, J.K. & Orr, G.R. 1986. Phase transitions in thylakoid polar lipids of chilling sensitive plants. **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

Ramlov, H. & Hvidt, A. 1992. *Artemia* cysts at subzero temperatures studied by differential scanning calorimetry. **Cryobiology** *29*: 131-137

Rao, M.V. 1992. Cellular detoxifying mechanisms determine the age-dependent injury in tropical trees exposed to SO_2. **Journal of Plant Physiology** *140*: 733-740

Rawn, J.D. 1983. Biochemistry. Harper & Row Publishers, Inc., New York. Pp 874

Rayapati, P.J., Stewart, C.R. & Hack, E. 1989. Pyrroline-5-carboxylate reductase is in pea (*Pisum sativum* L.) leaf chloroplasts. **Plant Physiology** *91*: 581-586

Rees, D., Young, A., Noctor, G., Britton, G. & Horton, P. 1989. Enhancement of the ΔpH-dependant dissipation of excitation energy in spinach chloroplasts by light activation: correlation with the synthesis of zeaxanthin. **FEBS Letters** *256*: 85-90

Reismann, S., Michaels, A. & Ohad, I. 1986. Lack of recovery from photoinhibition in a temperature-sensitive *Chlamydomonas reinhardtii* mutant T44 unable to synthesize and/or integrate the Q_B protein of photosystem II at 37°C. **Biochimica et Biophysica Acta** *849*: 41-50

Reismann, S. & Ohad, I. 1986. Light dependent degradation of the thylakoid 32 kDa Q_B protein in isolated chloroplast membranes of *Chlamydomonas reinhardtii*. **Biochimica et Biophysica Acta** *849*: 51-69

Rennenberg, H. 1982. Glutathione metabolism and possible biological roles in higher plants. **Phytochemistry** *21*: 2771-2781

Rich, P.R. & Bonner, W.D. 1978. The sites of superoxide anion generation in higher plant mitochondria. **Archives of Biochemistry and Biophysics** *188*: 206-213

Roberts, D.W.A. 1978. The invertase complement of cold-hardy and cold-sensitive wheat leaves. **Canadian Journal of Botany** *53*: 1333-1337

Robinson, J.M. 1988. Does O_2 photoreduction occur within chloroplasts in vivo? **Physiologia Plantarum** *72*: 666-680

Robinson, J.M. & Gibbs, M. 1982. Hydrogen peroxide synthesis in isolated spinach chloroplast lamellae. **Plant Physiology** *70*: 1249-1254

Robinson, J.M., Smith, M.G. & Gibbs, M. 1980. Influence of hydrogen peroxide upon carbon dioxide photoassimilation in the spinach chloroplast. I. Hydrogen peroxide generated by broken chloroplasts in an "intact" chloroplast preparation in causal agent of the Warburg effect. **Plant Physiology** *65*: 755-759

Sagisaka, S. 1982. Comparitive studies on the metabolic function of differentiated xylem and living bark of wintering perennials. **Plant and Cell Physiology** *23*: 1337-1346

Sagisaka, S. 1985. Injuries of cold acclimated poplar twigs resulting from enzyme inactivation and substrate depression during frozen storage at ambient temperatures for a long period. **Plant and Cell Physiology** *26*: 1135-1145

Sagisaka, S. & Asada, M. 1981. Coordinate and noncoordinate changes in enzyme activities in pentose phosphate cycle in poplar: a control of enzyme activities in differentiated xylem. **Plant and Cell Physiology** *22*: 1459-1468

Salerno, G.L. & Pontis, H.G. 1982. Raffinose synthesis in *Chlorella vulgaris* cultures after a cold shock. **Plant Physiology** *89*: 648-651

Salin, M.L. 1987. Toxic oxygen species and protective systems of the chloroplast. **Physiologia Plantarum** *72*: 681-68

Salin, M.L. & Bridges, S.M. 1981. Absence of the iron-containing superoxide dismutase in mitochondria from mustard (*Brassica capestris*). **Biochemical Journal** *195*: 229-233

Sandalio, L.M., Fernández, V.M., Rupérez, F.L. & Del Río, L.A. 1988. Superoxide free radicals are produced in glyoxisomes. **Plant Physiology** *87*: 1-4

Sarhan, F. & Perras, M. 1987. Accumulation of a high molecular weight protein during cold hardening of wheat (*Triticum aestivum* L.). **Plant and Cell Physiology** *75*: 818-826

Sawada, S-I, Matsushima, H. & Miyachi, S. 1974. Effects of growth temperature on photosynthetic carbon metabolism in green plants.III. Differences in structure, photosynthetic activities and activities of ribulose diphosphate carboxylase and glycolate oxidase in leaves of wheat grown under varied temperatures. **Plant Cell Physiology** *15*: 239-248

Scandalios, J.G. 1993. Oxygen stress and superoxide dismutase. **Plant Physiology** *101*: 7-12

Schmidt, G. & Kunert, K.J. 1986. Lipid peroxidation in higher plants: The role of glutathione reductase. **Plant Physiology** *82*: 700-703

Schöner, S. & Krause, G.H. 1990. Protective systems against active oxygen species in spinach: response to cold acclimation in excess light. **Planta** *180*: 383-389

Serenata, T., McKersie, B.D. & Stinson, R.H. 1985. Simulation of dehydration injury to membranes from soybean axes by free radicals. **Plant Physiology** *77*: 474-477

Sevilla, F., Del Rio, L.A. & Hellin, E. 1984. Superoxide dismutases from a citrus plant: Presence of two iron-containing isoenzymes in leaves of lemon trees *(Citrus limonum* R.). **Journal of Plant Physiology** *116*: 381-387

Sieferman, D. & Yamamoto, H.Y. 1985. Light-induced de-epoxidation of violaxanthin in lettuce chloroplasts. IV. The effect of electron transport conditions on violaxanthin availability. **Biochimica et Biophysica Acta** *387*: 149-158

Simatos, D., Faure, M., Bonjour, E. & Couach, M. 1975. The physical state of water at low temperatures in plasma with different water contents as studied by differential thermal analysis and differential scanning calorimetry. **Cryobiology** *12*: 202-208

Siminovitch, D. & Briggs, D.R. 1953. Studies on the chemistry of the living bark of the black locust tree in relation to frost hardiness. IV. Effects of ringing on translocation, protein synthesis, and the development of hardiness. **Plant Physiology** *28*: 177-200

Siminovitch, D. & Cloutier, Y. 1982. Twenty-four-hour induction of freezing and drought tolerance in plumules of winter rye seedlings by desiccation stress at room temperature in the dark. **Plant Physiology** *69*: 250-255

Simon, J-P. 1979. Differences in thermal properties of NADmalate dehydrogenase in genotypes of *Lathyrus japonicus* Willd (*Leguminosae*) from maritime and continental sites. **Plant Cell and Environment** *2*: 23-33

Singh, T.N., Aspinall, D. & Paleg, L.G. 1972. Proline accumulation and varietal adaptability to drought in barley: a potential metabolic measure of drought resistance. **Nature** *236*: 188-190

Singh, T.N., Paleg, L.G. & Aspinall, D. 1973. Stress metabolism. I. Nitrogen metabolism and growth in barley plants during water stress. **Australian Journal of Biological Science** *26*: 5-46

Skogen, D., Chaturvedi, R., Weidemann, F. & Nilsen, S. 1986. Photoinhibition of photosynthesis: Effect of light quality and quantity on recovery from photoinhibition in *Lemna gibba*. **Journal of Plant Physiology** *126*: 195-205

Smillie, R.M. & Hetherington, S.E. 1983. Stress tolerance and stress-induced injury in crop plants measured by chlorophyll fluorescence *in vivo*. **Plant Physiology** *72*: 1043-1050

Smirnoff, N. & Colombé, S.V. 1988. Drought influences the activity of enzymes of the chloroplast hydrogen peroxide scavenging system. **Journal of Experimental Botany** *39*: 1097-1108

Smirnoff, N. & Cumbes, Q.J. 1989. Hydroxyl radical scavenging activity of compatible solutes. **Phytochemistry** *28*: 1057-1060

Smith, I.K., Polle, A. & Rennenberg, H. 1990. Glutathione. In: Stress Responses in Plants: Adaptation and Acclimation Mechanisms, Plant Biology Series. Vol. 12. R.G. Alscher & J.R. Cumming (eds). Wiley-Liss: New York. Pp 128-194

Smith, I.K., Vierheller, T.L. & Thorne, C.A. 1989. Properties and functions of glutathione reductase in plants. **Physiologia Plantarum** *77*: 449-456

Somersalo, S. & Krause, G.H. 1988. Changes in chlorophyll fluorescence related to photoinhibition of photosynthesis and cold acclimation of green plants. In: Applications of chlorophyll fluorescence. H.K. Lichtenthaler (ed). Kluwer: Dordrecht. Pp 91-146

Somersalo, S. & Krause, G.H. 1989. Photoinhibition at chilling temperature. Fluorescence characteristics of unhardened and cold-acclimated spinach leaves. **Planta** *177*: 409-416

Somersalo, S. & Krause, G.H. 1990a. Reversible photoinhibition of unhardened and cold-acclimated spinach leaves at chilling temperatures. **Planta** *180*: 181-187

Somersalo, S. & Krause, G.H. 1990b. Photoinhibition at chilling temperatures and effects of freezing stress on cold acclimated spinach leaves in the field. A fluorescence study. **Physiologia Plantarum** *79*: 617-622

Stewart, C.R. 1972. The effect of wilting on proline metabolism in excised bean leaves in the dark. **Plant Physiology** *51*: 508-511

Stewart, C.R. 1978. Role of carbohydrates in proline accumulation in wilted barley leaves. **Plant Physiology** *61*: 775-778

Stewart, C.R., Morris, C. & Thompson, J.F. 1966. Changes in amino acid content of excised leaves during incubation. II. Role of sugar in the accumulation of proline in wilted leaves. **Plant Physiology** *41*: 1585

Stewart, G.R. & Lee, J.A. 1974. The role of proline accumulation in halophytes. **Planta (Berl)** *120*: 279-289

St John, J.B., Christiansen, M.N., Ashworth, E.N. & Genter, W.A. 1979. Effect of BASF-13-338, a substituted pyridazinone on linoleic acid levels and winter hardiness of crops. **Crop Science** *19*: 65-69

Steponkus, P.L. 1984. Role of the plasma membrane in freeze injury and cold acclimation. **Annual Review of Plant Physiology** *35*: 543-584

Stewart, G.R. & Lee, J.A. 1974. The role of proline accumulation in halophytes. **Planta** *120*: 279-289

Strand, M. & Öquist, G. 1985. Inhibition of photosynthesis by freezing temperatures and high light levels in cold-acclimated seelings of Scots pine (*Pinus sylvestris*). I. Effect on the light limited and light saturated rates of CO_2 assimilation. **Physiologia Plantarum** *64*: 425-430

Sundbom, E., Strand, M. & Hällgren, J.E. 1982. Temperature-induced fluorescence changes. A screening method for frost-tolerance of potato (*Solanum* sp.). **Plant Physiology** *70*: 1299-1302

Takahama, U. 1983. Suppression of carotenoid photobleaching by kaempferol in isolated chloroplasts. **Plant and Cell Physiology** *23*: 859-864

Tal, M., Katz, A., Heikin, H. & Dehan, K. 1979. Salt tolerance in the wild relatives of the cultivated tomato: Proline accumulation in *Lycopersicon esculentum* Mill., *L. peruvianum* Mill. and *Solanum pennelli* Cor. and polyethylene glycole. **New Phytologist** *82*: 349-355

Tanaka, K., Saji, H. & Kondo, N. 1988. Immunological properties of spinach glutathione reductase and inductive biosynthesis of the enzyme with ozone. **Plant and Cell Physiology** *29*: 637-642

Tanaka, K., Suda, Y., Kondo, N. & Sugahara, K. 1985. O_3 tolerance and the ascorbate-dependant H_2O_2 decompostion system in chloroplasts. **Plant and Cell Physiology** *26*: 1425-1431

Taylor, A.O. & Rowley, J.A. 1971. Platns under climatic stress. I. Low temperature, high-light effects on photosynthesis. **Plant Physiology** *47*: 713-718

Terashima, I., Kashino, Y. & Katoh, S. 1991a. Exposure of leaves of *Cucumis sativus* L. to low temperatures in the light causes uncoupling of thylakoids. I. Studies with isolated thylakoids. **Plant Cell Physiology** *32*: 1267-1274

Terashima, I., Sonioke, K, Kawazu, T. & Katoh, S. 1991b. Exposure of leaves of *Cucumis sativus* L. to low temperatures in the light causes uncoupling of thylakoids. II. Non-destructive measurements with intact leaves. **Plant Cell Physiology** *32*: 1275-1283

Tibbits, W.N. 1986. Frost resistance in *Eucalyptus nitens* (Deane and Maiden) Maiden. PhD Thesis. University of Tasmania.

Tibbits, W.N. & Reid, J.B. 1986. Frost resistance in *Eucalyptus nitens* (Deane & Maiden) Maiden: Genetic and seasonal aspects of variation. **Australian Forestry Research** *17*: 29-47

Tibbits, W.N. & Reid, J.B. 1987. Frost resistance in *Eucalyptus nitens* (Deane & Maiden) Maiden: Physiological aspects of hardiness. **Australian Journal of Botany** *35*: 235-250

Tolbert, N.E. 1971. Peroxisomes and glyoxisomes. **Annual Review of Plant Physiology** *22*: 45-74

Torel, J., Cillard, J.& Cillard, P. 1986. Antioxidants activity of flavenoids and ractivity with peroxy radicals. **Phytochemistry** *25*: 383-385

Triechel, S., Brinkmann, E., Scheitler, B. & von Willert, D.J. 1984. Occurence and changes of proline content in plants in the Southern Namib Desert in relation to increasing and decreasing drought. **Planta** *162*: 236-242

Tsang, E.W.T., Bowler, C., Hérouart, D., van Camp, W., Villarroel, R., Genetello, C., van Montagu, M & Inzé, D. 1991. Differential regulation of superoxide disumases in plants exposed to environmental stress. **The Plant Cell** *3*: 783-792

Tschiersch, H. & Ohmann, E. 1993. Photoinhibition in *Euglena gracilis*: Involvement of reactive oxygen species. **Planta** *191*: 319-323

Tseng, M.I. & Li, P.H. 1987. Changes in nucleic acid and protein synthesis during induction of cold hardiness. In: Plant Cold Hardiness. Li, P.H.Sakai, A. (eds.) Academic Press. Wiley Liss: New York. Pp 1-27

Turner, L.B. & Pollock, C.J. 1993. The effects of temperature and pH on the apparent Michaelis constant of glutathione reductase from maize (*Zea mays* L.). **Plant, Cell and Environment** *16*: 289-295

Uemura, M. & Yoshida S. 1984. Involvement of plasma membrane alterationsn cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). **Plant Physiology** *75*: 818-826

van Dorssen, R.J., Breton, J., Plijter, J., Satoh, K., van Gorkom, H.J., & Amez, J. 1987. Spectroscopic properties of the reaction center and of the 47 kDa chlorophyll protein of photosystem II. **Biochimica et Biophysica Acta** *893*: 267-274

van Ginkel, G. & Brown, J.S. 1978. Endogenous catalase and superoxide dismutase activities in photosynthetic membranes. **FEBS Letters** *94*: 284-286

van Hassalt, P.R. & van Berlo, H.A.C. 1980. Photooxidative damage to the photosynthetic apparatus during chilling. **Physiologia Plantarum** *50*: 52-56

van Swaaij, A.C., Jacobsen, E. & Feenstra, W.J. 1985. Effect of cold hardening, wilting and exogenously applied proline on leaf proline content and frost tolerance of several genoty0pes of *Solanum*. **Physiology Plantarum** *64*: 230-236

van Wijk, K.J. & Krause, G.H. 1991. Oxygen dependence of photoinhibition at low temperature in intact protoplasts of *Valerianella locusta* L. **Planta** *186*: 135-142

van Wijk, K.J. & van Hasselt, P.R. 1993. Photoinhibition of photosystem II in vivo is preceded by down-regulation through light-induced acidification of the lumen: Consequences for the mechanisim of photoinhibition in vivo. **Planta** *189*: 359-368

Venekamp, J.H. 1989. Regulation of cytosol acidity in plants under conditions of drought. **Physiologia Plantarum** *76*: 112-117

Venkateswarlu, B. & Ramesh, K. 1993. Cell membrane stability and biochemical response of cultured cells of groundnut under polyethylene glycol-induced water stress. **Plant Science** *90*: 179-185

Vertucci, C.W. 1989a. The relationship between thermal transitions and freezing injury in pea and soybean seeds. **Plant Physiology** *90*: 1121-1128

Vertucci, C.W. 1989b. Effects of cooling rate on seeds exposed to liquid nitrogen temperatures. **Plant Physiology** *90*: 1478-1485

Vertucci, C.W. 1990. Calorimetric studies of the state of water in seed tissues. **Biophysical Journal** *58*: 1463-1471

Voetberg, G. & Sharp, R.E. 1991. Growth of the maize primary root at low water potentials. III. Role of increased proline deposition in osmotic adjustment. **Plant Physiology** *96*: 1125-1130

von Gadow, K., van der Zyl, D.W., van Laar, A., Schöner, A.P.G., Kassier, H.W., Warkotsch, P.W., Vermaas, H.F., Owen, D.L. & Jordaan, J.V. 1987. South African Forestry Handbook. Southern African Institute of Forestry, VR Printers: Pretoria, S.A.

Voetberg, S. & Stewart, C.R. 1984. Steady state proline levels in salt-shocked barley leaves. **Plant Physiology** *76*: 567-570

Walker, M.A., McKersie, B.D. & Pauls, K.P. 1991. Effects of chilling on the biochemical and functional properties of thylakoid membranes. **Plant Physiology** *97*: 663-669

Walker, M.A. & McKersie, B.D. 1993. Role of the ascorbate-glutathione antioxidant system in chilling resistance of tomato. **Journal of Plant Physiology** *141*: 234-239

Weger, H.G., Silim, S.N. & Guy, R.D. 1993. Photosynthetic acclimation to low temperature by western red cedar seedlings. **Plant, Cell and Environment** *16*: 711-717

Weis, E. & Berry, J.A. 1987. Quantum efficiency of photosystem II in relation to 'energy'-dependent quenching of chlorophyll fluorescence. **Biochimica et Biophysica Acta** *894*: 198-208

Weiser, C.J. 1970. Cold resistance and injury in woody plants. **Science** *169*: 1269-1278

Wenkert, W. 1980. Measurement of tissue osmotic pressure. **Plant Physiology** *65*: 614-617

Wetter, L.R. 1984. Protein extraction and analysis. In: Cell Cultures and Somatic Cell Genetics of Plants. Laboratory Procedures and their Application. Volume 1. I.K. Vasil (ed). Academic Press: Orlando. Pp 54

Williams, R.J. 1981. Frost desiccation: an osmotic model. In: Analysis and Improvement of Plant Cold Hardiness. Olien, C.R. & Smith, M.N. (eds.). CRC Press: Boca Raton, Florida. Pp 89

Williams, R.J. & Leopold, A.C. 1989. The glassy state in corn embryos. **Plant Physiology** *89*: 977-981

Williams, W.P. & Allen, J.F. 1987. State 1 / State 2 changes in higher plants and algae. **Photosynthesis Research** *13*: 19-45

Williams, W.P. & Gounaris, K. 1992. Stabilization of PSII - mediated electron transport in oxygen-evolving PSII core preparations by addition of compatible co-solutes. **Biochimica et Biophysica Acta** *1100*: 92-97

Wingate, V.P.M., Lawton, M.A. & Lamb, C.J. 1988. Glutathione elicits a massive and selective induction of plant defense genes. **Plant Physiology** *87*: 206-210

Wingsle, G. 1989. Purification and characterization of glutathione reductase from Scots pine needles. **Physiologia Plantarum** *76*: 24-30

Wingsle, G. & Hällgren, J.-E. 1993. Influence of SO_2 and NO_2 exposure on glutathione, superoxide dismutase and glutathione reductase activities in Scots pine needles. **Journal of Experimental Botany** *44*: 463-470

Wingsle, G., Mattson, A., Ekblad, A., Hällgren, J-E. & Selstam, E. 1992. Activities of glutathione reductase and superoxide dismutase in relation to changes of lipids and pigments due to ozone in seedlings of *Pinus sylvestris* (L.). **Plant Science** *82*: 167-178

Whittaker, A. 1990. The use of transgenic tobacco plants containing the *E. coli* glutathione reductase gene to investigate the role of glutahione reductase duing oxygen toxicity. MSc Thesis. University of Natal, Durban, South Africa.

Wolfe, J. 1978. Chilling injury in plants - the role of membrane lipid fluidity. **Plant, Cell and Environment** *1*: 241-247

Yamazaki, I. & Pietts, L.H. 1968. The mechanisms of aerobic oxidase reaction catalysed by peroxidase. **Biochemica et Biophysica Acta** *77*: 47-64

Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. & Somero, G.N.1982. Living with water stress: evolution of osmolyte systems. **Science** *217*: 1214-1222

Yelenosky, G. 1979. Accumulation of free proline in citrus leaves during cold hardening of young leaves in controlled temperature regimes. **Plant Physiol.** *64*: 425-427

Yoshida, S. & Sakai, A. 1973. Phospholipid changes associated with the cold hardiness of cortical cells from poplar stem. **Plant and Cell Physiology** *14*: 353-359

Young, L.C.T. & Conn, E.E. 1956. The reduction and oxidation of glutathione by plant mitochondria. **Plant Physiology** *31*: 205-211

Ziegler, D.M. 1985. Role of reversible oxidation-reduction of enzyme thiols-disulphides in metabolic regulation. **Annual Review of Biochemistry** *54*: 305-329

Zopes, H., Klapheck, S. & Bergmann, L. 1993. The function of homoglutahione and hydroxymethylglutathione for the scavenging of hydrogen peroxide. **Plant Cell Physiology** *34*: 515-521

APPENDIX I.

The analysis of data from this study is included for the reader's information. The following symbols are used: P, S and L. "P" represents parameter. These are onset of temperature of melt (1), proline concentration (2), photosynthetic efficiency (3) and glutathione reductase activity (4). "S" represents the *Eucalyptus* species used in the study: *E. nitens* (1), *E. macarthurii* (2), *E. smithii* (3), GN1026 (4) and *E. grandis* (5). "L" represents the level of the experiment: (1) is the initial 20°C period, (2) is the 4°C chilling period and (3) is the 20°C recovery period. Analysis was performed to determine whether there was interaction between any or all of the above mentioned factors. The program written by Professor Trofsky of the Mathematical Statistics Department, UND, to determine interaction is also included.

A. Program for Statistical Analysis

```
data gms1;
infile 'd:\atros\gms\all.dat';
input p s l t y;
proc sort ;
by p;
proc print;run;
proc glm data=gms1 ;
by  p;
class s l ;
model y=s|l;
means s|l;
lsmeans s|l/tdiff;
contrast'S*L S12 vs L12 ' S*L 1 -1 0 -1 1 0 0 0 0 0 0 0 0 0 0;
contrast'S*L S12 vs L13 ' S*L 1 0 -1 -1 0 0 0 0 0 0 0 0 0 0 0;
contrast'S*L S12 vs L23 ' S*L 0 1 -1 0 -1 0 0 0 0 0 0 0 0 0 0;
contrast'S*L S13 vs L12 ' S*L 1 0 -1 0 0 -1 1 0 0 0 0 0 0 0 0;
contrast'S*L S13 vs L13 ' S*L 1 0 -1 0 0 -1 0 1 0 0 0 0 0 0 0;
contrast'S*L S13 vs L23 ' S*L 0 1 -1 0 0 0 -1 1 0 0 0 0 0 0 0;
contrast'S*L S14 vs L12 ' S*L 0 -1 0 0 0 0 0 0 0 -1 0 0 0 0 0;
contrast'S*L S14 vs L13 ' S*L 0 0 -1 0 0 0 0 0 0 -1 0 1 0 0 0;
contrast'S*L S14 vs L23 ' S*L 0 1 -1 0 0 0 0 0 0 0 -1 1 0 0 0;
contrast'S*L S15 vs L12 ' S*L 1 -1 0 0 0 0 0 0 0 0 0 0 -1 1 0;
contrast'S*L S15 vs L13 ' S*L 1 1 -1 0 0 0 0 0 0 0 0 0 -1 0 1;
contrast'S*L S15 vs L23 ' S*L 0 1 -1 0 0 0 0 0 0 0 0 0 0 -1 1;
contrast'S*L S23 vs L12 ' S*L 0 0 0 1 -1 0 -1 1 0 0 0 0 0 0 0;
```

```
contrast'S*L S23 vs L13 ' S*L 0 0 0 1 0 -1 -1 0 1 0 0 0 0 0 0;
contrast'S*L S23 vs L23 ' S*L 0 0 0 0 1 -1 0 -1 1 0 0 0 0 0 0;
contrast'S*L S24 vs L12 ' S*L 0 0 0 1 -1 0 0 0 0 -1 1 0 0 0 0;
contrast'S*L S24 vs L13 ' S*L 0 0 0 1 0 -1 0 0 0 -1 0 1 0 0 0;
contrast'S*L S24 vs L23 ' S*L 0 0 0 0 1 -1 0 0 0 0 -1 1 0 0 0;
contrast'S*L S25 vs L12 ' S*L 0 0 0 1 -1 0 0 0 0 0 0 0 -1 1 0;
contrast'S*L S25 vs L13 ' S*L 0 0 0 1 0 -1 0 0 0 0 0 0 -1 0 1;
contrast'S*L S25 vs L23 ' S*L 0 0 0 0 1 -1 0 0 0 0 0 0 0 -1 1;
contrast'S*L S34 vs L12 ' S*L 0 0 0 0 0 0 1 -1 0 -1 1 0 0 0 0;
contrast'S*L S34 vs L13 ' S*L 0 0 0 0 0 0 1 0 -1 -1 0 1 0 0 0;
contrast'S*L S34 vs L23 ' S*L 0 0 0 0 0 0 0 1 -1 0 -1 1 0 0 0;
contrast'S*L S35 vs L12 ' S*L 0 0 0 0 0 0 1 -1 0 0 0 0 -1 1 0;
contrast'S*L S35 vs L13 ' S*L 0 0 0 0 0 0 1 0 -1 0 0 0 -1 0 1;
contrast'S*L S35 vs L23 ' S*L 0 0 0 0 0 0 0 1 -1 0 0 0 0 -1 1;
contrast'S*L S45 vs L12 ' S*L 0 0 0 0 0 0 0 0 0 1 -1 0 -1 1 0;
contrast'S*L S45 vs L13 ' S*L 0 0 0 0 0 0 0 0 0 1 0 -1 -1 0 1;
contrast'S*L S45 vs L23 ' S*L 0 0 0 0 0 0 0 0 0 0 1 -1 0 -1 1;
run;
```

B. Statistical Analysis Results

The SAS System 72

P = 1

General Linear Models Procedure

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	102.545236	7.324660	8.42	0.0001
Error	180	156.588502	0.869936		
Corrected Total	194	259.133738			

R-Square	C.V.	Root MSE	Y Mean
0.395723	-17.79446	0.93270	-5.24154

Source	DF	Type I SS	Mean Square	F Value	Pr > F
S	4	56.1241641	14.0310410	16.13	0.0001
L	2	22.0746834	11.0373417	12.69	0.0001
S*L	8	24.3463888	3.0432986	3.50	0.0009

Source	DF	Type III SS	Mean Square	F Value	Pr > F

S	4	62.3027056	15.5756764	17.90	0.0001
L	2	22.0746834	11.0373417	12.69	0.0001
S*L	8	24.3463888	3.0432986	3.50	0.0009

The SAS System 10

P = 1

General Linear Models Procedure

Level of ----------Y----------

S	N	Mean	SD
1	39	-4.18794872	0.33153693
2	39	-5.68384615	1.33863170
3	39	-5.52205128	1.26230403
4	39	-5.40794872	0.95240466
5	39	-5.40589744	0.96954606

Level of ----------Y----------

L	N	Mean	SD
1	75	-4.91120000	0.96472508
2	75	-5.23853333	1.28885209
3	45	-5.79711111	1.01425437

Level of Level of ----------Y----------

S	L	N	Mean	SD
1	1	15	-4.35600000	0.26925293
1	2	15	-4.09133333	0.32741120
1	3	9	-4.06888889	0.34818976
2	1	15	-4.78000000	1.41622738
2	2	15	-6.20133333	1.11496872
2	3	9	-6.32777778	0.57082786
3	1	15	-5.16600000	0.89226678
3	2	15	-5.25533333	1.53987878
3	3	9	-6.56000000	0.66537583
4	1	15	-5.38466667	0.83482134
4	2	15	-5.07333333	1.13976606
4	3	9	-6.00444444	0.45208161
5	1	15	-4.86933333	0.82933249
5	2	15	-5.57133333	1.09917415
5	3	9	-6.02444444	0.35436955

The SAS System 11

P=1

General Linear Models Procedure

Least Squares Means

S Y T for HO: LSMEAN(i)=LSMEAN(j) / Pr > |T|

	LSMEAN	i/j	1	2	3	4	5
1	-4.17207407	1	.	7.349344	6.846735	6.051078	6.055167
			0.0001	0.0001	0.0001	0.0001	
2	-5.76970370	2	-7.34934	.	-0.50261	-1.29827	-1.29418
			0.0001		0.6159	0.1959	0.1973
3	-5.66044444	3	-6.84673	0.50261	.	-0.79566	-0.79157
			0.0001	0.6159		0.4273	0.4297
4	-5.48748148	4	-6.05108	1.298266	0.795656	.	0.004089
			0.0001	0.1959	0.4273		0.9967
5	-5.48837037	5	-6.05517	1.294177	0.791567	-0.00409	.
			0.0001	0.1973	0.4297	0.9967	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

L Y T for HO: LSMEAN(i)=LSMEAN(j) / Pr > |T|

	LSMEAN	i/j	1	2	3
1	-4.91120000	1	.	2.149128	5.037241
			0.0330	0.0001	
2	-5.23853333	2	-2.14913	.	3.176042
			0.0330		0.0018
3	-5.79711111	3	-5.03724	-3.17604	.
			0.0001	0.0018	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

S	L	Y LSMEAN	
		LSMEAN	Number
1	1	-4.35600000	1
1	2	-4.09133333	2
1	3	-4.06888889	3
2	1	-4.78000000	4
2	2	-6.20133333	5

2	3	-6.32777778	6
3	1	-5.16600000	7
3	2	-5.25533333	8
3	3	-6.56000000	9
4	1	-5.38466667	10
4	2	-5.07333333	11
4	3	-6.00444444	12
5	1	-4.86933333	13
5	2	-5.57133333	14
5	3	-6.02444444	15

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

The SAS System 12

P=1

General Linear Models Procedure

Dependent Variable: Y

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
S*L S12 vs L12	1	10.6597350	10.6597350	12.25	0.0006
S*L S14 vs L23	1	2.5573168	2.5573168	2.94	0.0882
S*L S15 vs L12	1	3.5041667	3.5041667	4.03	0.0462
S*L S15 vs L23	1	0.6360556	0.6360556	0.73	0.3936
S*L S23 vs L12	1	6.6533400	6.6533400	7.65	0.0063
S*L S23 vs L13	1	0.0665089	0.0665089	0.08	0.7825
S*L S23 vs L23	1	3.9043339	3.9043339	4.49	0.0355
S*L S24 vs L12	1	11.2580017	11.2580017	12.94	0.0004
S*L S24 vs L13	1	2.4220800	2.4220800	2.78	0.0969
S*L S24 vs L23	1	1.8210613	1.8210613	2.09	0.1497
S*L S25 vs L12	1	1.9404017	1.9404017	2.23	0.1371
S*L S25 vs L13	1	0.4336513	0.4336513	0.50	0.4811
S*L S25 vs L23	1	0.3001250	0.3001250	0.34	0.5577
S*L S34 vs L12	1	0.6020017	0.6020017	0.69	0.4066
S*L S34 vs L13	1	1.6858689	1.6858689	1.94	0.1656
S*L S34 vs L23	1	0.3924668	0.3924668	0.45	0.5027
S*L S35 vs L12	1	1.4076017	1.4076017	1.62	0.2050
S*L S35 vs L13	1	0.1605035	0.1605035	0.18	0.6680
S*L S35 vs L23	1	2.0394756	2.0394756	2.34	0.1275
S*L S45 vs L12	1	3.8506667	3.8506667	4.43	0.0368

S*L S45 vs L13	1	0.8060112	0.8060112	0.93	0.3371
S*L S45 vs L23	1	0.6426113	0.6426113	0.74	0.3912

The SAS System 76

P=2

General Linear Models Procedure

Class Level Information

Class	Levels	Values
S	5	1 2 3 4 5
L	3	1 2 3

Number of observations in by group = 191

The SAS System 77

P=2

General Linear Models Procedure

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	416.09359435	29.72097102	84.27	0.0001
Error	176	62.07427058	0.35269472		
Corrected Total	190	478.16786492			

R-Square	C.V.	Root MSE	Y Mean
0.870183	47.29259	0.5938811	1.2557592

Source	DF	Type I SS	Mean Square	F Value	Pr > F
S	4	406.63408406	101.65852101	288.23	0.0001
L	2	2.15597225	1.07798613	3.06	0.0496
S*L	8	7.30353803	0.91294225	2.59	0.0107

Source	DF	Type III SS	Mean Square	F Value	Pr > F
S	4	385.33249379	96.33312345	273.13	0.0001
L	2	2.38249721	1.19124861	3.38	0.0364
S*L	8	7.30353803	0.91294225	2.59	0.0107

The SAS System 78

P=2

General Linear Models Procedure

Level of ----------Y----------

S	N	Mean	SD
1	37	0.96378378	0.38236364
2	38	0.50894737	0.34743687
3	38	0.57342105	0.20795369
4	39	4.08487179	1.25731517
5	39	0.09615385	0.05896544

Level of ----------Y----------

L	N	Mean	SD
1	74	1.35932432	1.72534637
2	75	1.09293333	1.39524944
3	42	1.36404762	1.66402401

Level of S	Level of L	N	----------Y---------- Mean	SD
1	1	15	1.20333333	0.20030928
1	2	13	0.55307692	0.26889851
1	3	9	1.15777778	0.23493498
2	1	14	0.70928571	0.48889930
2	2	15	0.46333333	0.07907200
2	3	9	0.27333333	0.14949916
3	1	15	0.40400000	0.18753285
3	2	15	0.72266667	0.10010471
3	3	8	0.61125000	0.16530815
4	1	15	4.37400000	1.50305308
4	2	15	3.67333333	1.02931785
4	3	9	4.28888889	1.08637522
5	1	15	0.06266667	0.01667619
5	2	17	0.11117647	0.06863694
5	3	7	0.13142857	0.06256425

The SAS System 79

P = 2

General Linear Models Procedure

Least Squares Means

S Y T for H0: LSMEAN(i) = LSMEAN(j) / Pr > |T|

LSMEAN i/j 1 2 3 4 5

1	0.97139601	1	.	3.48274	2.765253	-22.456	6.07421
			0.0006	0.0063	0.0001	0.0001	
2	0.48198413	2	-3.48274	.	-0.69007	-26.0993	2.66985
		0.0006		0.4911	0.0001	0.0083	
3	0.57930556	3	-2.76525	0.690073	.	-25.168	3.324034
		0.0063	0.4911		0.0001	0.0011	
4	4.11207407	4	22.45596	26.09932	25.16801	.	28.2898
		0.0001	0.0001	0.0001		0.0001	
5	0.10175724	5	-6.07421	-2.66985	-3.32403	-28.2898	.
		0.0001	0.0083	0.0011	0.0001		

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

L	Y	T for H0: LSMEAN(i) = LSMEAN(j) / Pr > |T|		
	LSMEAN	i/j	1	2	3
1	1.35065714	1	.	2.522437	0.504903
			0.0125	0.6143	
2	1.10471735	2	-2.52244	.	-1.63363
		0.0125		0.1041	
3	1.29253571	3	-0.5049	1.633629	.
		0.6143	0.1041		

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

The SAS System 80

P = 2

General Linear Models Procedure

Dependent Variable: Y

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
S*L S12 vs L12	1	0.58029983	0.58029983	1.65	0.2013
S*L S14 vs L23	1	0.00032209	0.00032209	0.00	0.9759
S*L S15 vs L12	1	1.81460655	1.81460655	5.14	0.0245
S*L S15 vs L23	1	0.87648787	0.87648787	2.49	0.1167
S*L S23 vs L12	1	1.17450668	1.17450668	3.33	0.0697

S*L S23 vs L13	1	1.10556463	1.10556463	3.13	0.0784
S*L S23 vs L23	1	0.01671521	0.01671521	0.05	0.8279
S*L S24 vs L12	1	0.76176609	0.76176609	2.16	0.1434
S*L S24 vs L13	1	0.34161430	0.34161430	0.97	0.3264
S*L S24 vs L23	1	1.82508681	1.82508681	5.17	0.0241
S*L S25 vs L12	1	0.32895588	0.32895588	0.93	0.3355
S*L S25 vs L13	1	0.64973280	0.64973280	1.84	0.1764
S*L S25 vs L23	1	0.11649746	0.11649746	0.33	0.5662
S*L S34 vs L12	1	3.89640167	3.89640167	11.05	0.0011
S*L S34 vs L13	1	0.23136095	0.23136095	0.66	0.4191
S*L S34 vs L23	1	1.43049549	1.43049549	4.06	0.0455
S*L S35 vs L12	1	0.28198646	0.28198646	0.80	0.3725
S*L S35 vs L13	1	0.04780510	0.04780510	0.14	0.7132
S*L S35 vs L23	1	0.04407470	0.04407470	0.12	0.7241
S*L S45 vs L12	1	2.16852535	2.16852535	6.15	0.0141
S*L S45 vs L13	1	0.06113299	0.06113299	0.17	0.6777
S*L S45 vs L23	1	0.93392624	0.93392624	2.65	0.1055

The SAS System 81

P=3

General Linear Models Procedure

Class Level Information

Class	Levels	Values
S	5	1 2 3 4 5
L	3	1 2 3

Number of observations in by group = 195

The SAS System 82

P=3

General Linear Models Procedure

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	0.36880097	0.02634293	20.23	0.0001
Error	180	0.23439378	0.00130219		
Corrected Total	194	0.60319475			

R-Square	C.V.	Root MSE	Y Mean
0.611413	4.792047	0.0360858	0.7530359

Source	DF	Type I SS	Mean Square	F Value	Pr > F
S	4	0.10940952	0.02735238	21.00	0.0001
L	2	0.24602017	0.12301009	94.46	0.0001
S*L	8	0.01337128	0.00167141	1.28	0.2546

Source	DF	Type III SS	Mean Square	F Value	Pr > F
S	4	0.09155612	0.02288903	17.58	0.0001
L	2	0.24602017	0.12301009	94.46	0.0001
S*L	8	0.01337128	0.00167141	1.28	0.2546

The SAS System 83

P = 3

General Linear Models Procedure

Level of ----------Y----------

S	N	Mean	SD
1	39	0.71641026	0.05348326
2	39	0.74564103	0.05552595
3	39	0.77051282	0.04656341
4	39	0.74692308	0.06070437
5	39	0.78569231	0.03460617

Level of ----------Y----------

L	N	Mean	SD
1	75	0.77880000	0.03183403
2	75	0.70820000	0.05936261
3	45	0.78482222	0.02206013

Level of Level of ----------Y----------

S	L	N	Mean	SD
1	1	15	0.73666667	0.01877181
1	2	15	0.67066667	0.05775152
1	3	9	0.75888889	0.02027588
2	1	15	0.77466667	0.01457330
2	2	15	0.68933333	0.04861902
2	3	9	0.79111111	0.01691482

3	1	15	0.79066667	0.03305119
3	2	15	0.73133333	0.04356713
3	3	9	0.80222222	0.01715938
4	1	15	0.77666667	0.01046536
4	2	15	0.69866667	0.07548573
4	3	9	0.77777778	0.01563472
5	1	15	0.81533333	0.00915475
5	2	15	0.75100000	0.02840775
5	3	9	0.79411111	0.01207730

The SAS System 84

P = 3

General Linear Models Procedure

Least Squares Means

S Y T for H0: LSMEAN(i) = LSMEAN(j) / Pr > |T|

	LSMEAN	i/j	1	2	3	4	5
1	0.72207407	1	.	-3.52294	-6.26203	-3.44368	-7.69763
				0.0005	0.0001	0.0007	0.0001
2	0.75170370	2	3.522943	.	-2.73909	0.079266	-4.17469
			0.0005		0.0068	0.9369	0.0001
3	0.77474074	3	6.262031	2.739088	.	2.818354	-1.4356
			0.0001	0.0068		0.0054	0.1529
4	0.75103704	4	3.443677	-0.07927	-2.81835	.	-4.25395
			0.0007	0.9369	0.0054		0.0001
5	0.78681481	5	7.69763	4.174687	1.435599	4.253954	.
			0.0001	0.0001	0.1529	0.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

L Y T for H0: LSMEAN(i) = LSMEAN(j) / Pr > |T|

	LSMEAN	i/j	1	2	3
1	0.77880000	1	.	11.98074	-0.88505
				0.0001	0.3773
2	0.70820000	2	-11.9807	.	-11.2607
			0.0001		0.0001
3	0.78482222	3	0.885047	11.26067	.

0.3773 0.0001

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

The SAS System 85

P = 3

General Linear Models Procedure

Dependent Variable: Y

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
S*L S12 vs L12	1	0.00140167	0.00140167	1.08	0.3009
S*L S14 vs L23	1	0.00023347	0.00023347	0.18	0.6725
S*L S15 vs L12	1	0.00001042	0.00001042	0.01	0.9288
S*L S15 vs L23	1	0.00572347	0.00572347	4.40	0.0374
S*L S23 vs L12	1	0.00253500	0.00253500	1.95	0.1647
S*L S23 vs L13	1	0.00006722	0.00006722	0.05	0.8205
S*L S23 vs L23	1	0.00268347	0.00268347	2.06	0.1529
S*L S24 vs L12	1	0.00020167	0.00020167	0.15	0.6944
S*L S24 vs L13	1	0.00066125	0.00066125	0.51	0.4770
S*L S24 vs L23	1	0.00144500	0.00144500	1.11	0.2936
S*L S25 vs L12	1	0.00165375	0.00165375	1.27	0.2613
S*L S25 vs L13	1	0.00399031	0.00399031	3.06	0.0817
S*L S25 vs L23	1	0.00968000	0.00968000	7.43	0.0070
S*L S34 vs L12	1	0.00130667	0.00130667	1.00	0.3178
S*L S34 vs L13	1	0.00030681	0.00030681	0.24	0.6280
S*L S34 vs L23	1	0.00019014	0.00019014	0.15	0.7028
S*L S35 vs L12	1	0.00009375	0.00009375	0.07	0.7888
S*L S35 vs L13	1	0.00302170	0.00302170	2.32	0.1294
S*L S35 vs L23	1	0.00217014	0.00217014	1.67	0.1984
S*L S45 vs L12	1	0.00070042	0.00070042	0.54	0.4643
S*L S45 vs L13	1	0.00140281	0.00140281	1.08	0.3007
S*L S45 vs L23	1	0.00364500	0.00364500	2.80	0.0961

The SAS System 86

P = 4

General Linear Models Procedure

Class Level Information

Class	Levels	Values
S	5	1 2 3 4 5

L 3 1 2 3

Number of observations in by group = 195

The SAS System 87

P=4

General Linear Models Procedure

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	818.30664973	58.45047498	39.62	0.0001
Error	180	265.54954029	1.47527522		
Corrected Total	194	1083.85619002			

R-Square	C.V.	Root MSE	Y Mean
0.754996	22.31474	1.2146091	5.4430795

Source	DF	Type I SS	Mean Square	F Value	Pr > F
S	4	630.23519667	157.55879917	106.80	0.0001
L	2	39.68646325	19.84323163	13.45	0.0001
S*L	8	148.38498980	18.54812373	12.57	0.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
S	4	529.60779000	132.40194750	89.75	0.0001
L	2	39.68646325	19.84323163	13.45	0.0001
S*L	8	148.38498980	18.54812373	12.57	0.0001

The SAS System 88

P=4

General Linear Models Procedure

Level of ----------Y----------

S	N	Mean	SD
1	39	4.62319231	1.43100125
2	39	8.97848718	2.35911833
3	39	4.31758974	0.85821577
4	39	5.12646154	1.60708137
5	39	4.16966667	1.00246988

Level of ----------Y----------

L	N	Mean	SD
1	75	6.01274667	3.02166731
2	75	5.11306667	1.90445034
3	45	5.04365556	1.50847453

Level of	Level of	----------Y----------	
S	L	N	Mean	SD
1	1	15	5.2096000	1.23217414
1	2	15	3.7318667	1.30504290
1	3	9	5.1313889	1.29016413
2	1	15	11.3966000	1.60634740
2	2	15	7.7856667	1.36684181
2	3	9	6.9363333	0.57241681
3	1	15	4.2883333	0.95193320
3	2	15	3.9603333	0.70675927
3	3	9	4.9617778	0.57517188
4	1	15	4.9735333	1.72255275
4	2	15	5.3111333	1.71477387
4	3	9	5.0735556	1.34854200
5	1	15	4.1956667	0.81299487
5	2	15	4.7763333	0.93561314
5	3	9	3.1152222	0.37778988

The SAS System 89

P = 4

General Linear Models Procedure

Least Squares Means

S	Y	T for H0: LSMEAN(i) = LSMEAN(j) / Pr > |T|				
	LSMEAN	i/j	1	2	3	4	5
1	4.69095185	1	.	-14.1838	1.015483	-1.51351	2.33807
			0.0001	0.3112	0.1319	0.0205	
2	8.70620000	2	14.18378	.	15.19926	12.67027	16.52185
			0.0001		0.0001	0.0001	0.0001
3	4.40348148	3	-1.01548	-15.1993	.	-2.52899	1.322586
			0.3112	0.0001		0.0123	0.1877
4	5.11940741	4	1.51351	-12.6703	2.528994	.	3.85158
			0.1319	0.0001	0.0123		0.0002
5	4.02907407	5	-2.33807	-16.5219	-1.32259	-3.85158	.
			0.0205	0.0001	0.1877	0.0002	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

L	Y	T for H0: LSMEAN(i) = LSMEAN(j) / Pr > |T|		
	LSMEAN	i/j	1	2	3
1	6.01274667	1	.	4.535939	4.231305
			0.0001	0.0001	
2	5.11306667	2	-4.53594	.	0.303067
			0.0001		0.7622
3	5.04365556	3	-4.23131	-0.30307	.
			0.0001	0.7622	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

The SAS System 90

P = 4

General Linear Models Procedure

Dependent Variable: Y

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
S*L S12 vs L12	1	17.06453340	17.06453340	11.57	0.0008
S*L S14 vs L23	1	7.53777115	7.53777115	5.11	0.0250
S*L S15 vs L12	1	15.88878960	15.88878960	10.77	0.0012
S*L S15 vs L23	1	26.34602738	26.34602738	17.86	0.0001
S*L S23 vs L12	1	40.41619227	40.41619227	27.40	0.0001
S*L S23 vs L13	1	74.12340873	74.12340873	50.24	0.0001
S*L S23 vs L23	1	9.63387670	9.63387670	6.53	0.0114
S*L S24 vs L12	1	58.46593307	58.46593307	39.63	0.0001
S*L S24 vs L13	1	58.48941023	58.48941023	39.65	0.0001
S*L S24 vs L23	1	1.05256367	1.05256367	0.71	0.3994
S*L S25 vs L12	1	65.88566460	65.88566460	44.66	0.0001
S*L S25 vs L13	1	32.12774509	32.12774509	21.78	0.0001
S*L S25 vs L23	1	1.85339014	1.85339014	1.26	0.2638
S*L S34 vs L12	1	1.66133760	1.66133760	1.13	0.2900

S*L S34 vs L13	1	0.92478669	0.92478669	0.63	0.4296
S*L S34 vs L23	1	4.31768269	4.31768269	2.93	0.0888
S*L S35 vs L12	1	3.09628167	3.09628167	2.10	0.1492
S*L S35 vs L13	1	8.65160503	8.65160503	5.86	0.0164
S*L S35 vs L23	1	19.93838087	19.93838087	13.52	0.0003
S*L S45 vs L12	1	0.22155527	0.22155527	0.15	0.6988
S*L S45 vs L13	1	3.91922311	3.91922311	2.66	0.1049
S*L S45 vs L23	1	5.69938261	5.69938261	3.86	0.0509