

**A STUDY OF SOME CHILLING
RESPONSES OF
RECALCITRANT SEEDS OF
Avicennia marina (Forssk.) Vierh.
AND *Ekebergia capensis* Sparrm.**

BY

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I DEDICATE THIS THESIS TO MY PARENTS, JAMES AND DIETLIND LEWIS,
WHO GAVE ME THE GIFT OF AN EDUCATION.


“I can do everything through him who gives me strength”

Philippians 4: 13

PREFACE

The experimental work described in this thesis was carried out from January 2000 to January 2002 in the Plant Cell Biology Research laboratory and the Plant Physiology laboratory at the School of Life and Environmental Science, University of Natal, Durban under the supervision of Professor Patricia Berjak and Professor Norman Pammenter, the Electron Microscope Unit at the University of Natal, Durban under the supervision of Ms Priscilla Maartens and Mr. James Wesley-Smith and the Physiologie Végétale Appliquée laboratory at the Université Pierre et Marie Curie, Paris under the supervision of Professor Françoise Corbineau.

All of the work presented is original research conducted by the author and has not been submitted in any form to another University. Where use is made of the work of others it has been duly acknowledged in the text.



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ABSTRACT

Seeds remain the most convenient and successful way for storing the genetic diversity of plant species and for producing new plants routinely for agriculture and horticulture. The importance of seed storage and the ability to predict seed longevity must therefore not be underestimated. To be successful, storage conditions must maintain seed vigour and viability and ensure that normal seedlings are subsequently established under field conditions. Seed quality is best retained when deteriorative events are minimised, which is achieved by storage of low moisture-content seeds under cool to cold, or even sub-zero, temperatures. Such conditions are employed for 'orthodox' seeds, which are desiccation tolerant and able to survive at sub-zero temperatures in the dehydrated state for extended periods. It is seeds referred to as 'recalcitrant' that cannot be dehydrated and often not stored at low temperatures because they are desiccation sensitive and may not tolerate chilling. According to almost anecdotal records chilling temperatures for such seeds are those below 15°C down to 0°C, depending on the species. The limited storage lifespan of recalcitrant seeds presents a problem even for short-term storage, and as most research on chilling sensitivity has been conducted on vegetative tissue, relatively little data exist for seeds, especially recalcitrant types.

The purpose of this study was to gain an understanding of the chilling response of recalcitrant seeds, as reduced temperature could have the potential to extend, rather than curtail, storage lifespan, depending on the species. Selected physiological, biochemical and ultrastructural responses of recalcitrant seeds of *Avicennia marina* and *Ekebergia capensis* were characterised. Seeds of the two species were stored at 25, 16 and 6°C. Germination, water content (determined gravimetrically), respiration (measured as CO₂ production) and leachate conductivity (tissue electrolyte leakage over time) were assessed at regular intervals. Chilling response at the subcellular level was examined using transmission electron microscopy (TEM). Changes in sugar metabolism and activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were assessed for *A. marina* seeds, which were severely affected by the chilling temperature of 6°C, losing viability after 1 week. In contrast, the seeds of *E. capensis* retained viability after 12 weeks of storage at 6°C, indicating

the marked difference in chilling response between seeds of the two recalcitrant species, despite their common tropical provenance. However, when *E. capensis* seeds were stored at 3°C viability decreased significantly after 8 weeks, thus indicating how critically temperature must be controlled if such conditions are to be profitably employed.

Ultrastructural studies revealed that in both *E. capensis* and *A. marina* seeds vacuole formation was initiated more rapidly at lower temperatures than at higher temperatures, indicating that this was a response specific to the chilling stress imposed. Once again, 'lower temperatures' differed relative to the species concerned. In the *E. capensis* seeds, nucleolar morphology was affected and the extent of chromatin patches in the nuclei increased as the storage temperature was reduced. Other ultrastructural findings could not be linked specifically to the chilling stress imposed on the *E. capensis* and *A. marina* seeds.

Activity of the antioxidant enzymes SOD and GR was detected in the *A. marina* seeds. No measurable CAT activity was detected. Glutathione reductase activity increased in response to chilling stress, the rate of the increase depending upon the severity of the chilling stress imposed. Other than when the *A. marina* seeds were placed directly at 6°C, there were no notable increases in SOD activity. Interestingly, SOD and GR activity was not the same in the axes as in the cotyledons. Superoxide dismutase activity was found to be higher in the axes and GR activity higher in the cotyledons. It would have been beneficial to determine the extent of antioxidant enzyme activity in the *E. capensis* seeds as well if this had been possible.

Generally, chilling of recalcitrant seeds seems to evoke a response similar to that of dehydration below a critical water content. This could lead to the conclusion that recalcitrant seeds do not possess the genetic ability to cope with dehydration or chilling stress, if it were not for the existence of recalcitrant seed species that are more chilling tolerant.

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LIST OF ABBREVIATIONS

1-aminocyclopropane-1-carboxylic acid	ACC
Acyl-carrier protein	ACP
Adenosine triphosphate	ATP
Ascorbate peroxidase	APX
Carbon dioxide	CO ₂
Catalase	CAT
Centimetre	cm
Cytochrome <i>c</i>	Cyt <i>c</i>
Days	d
Degrees Celsius	°C
Dehydroascorbate	DHA
Dehydroascorbate reductase	DHAR
Deoxy ribonucleic acid	DNA
Endoplasmic reticulum	ER
Glutathione reductase	GR
Grams	g
Hours	h
Hydrogen peroxide	H ₂ O ₂
Hydroxyl radical	.OH
Infrared gas analyser	IRGA
Litre	l
Magnesium chloride	MgCl ₂
Mass by volume	m/v
Micrograms	µg
Microlitres	µl
Micrometres	µm
Micromolar	µM
Micromoles	µmol
Milligrams	mg
Millilitre	ml
Millimetre	mm
Millimolar	mM
Minutes	min
Molar (concentration)	M
Moles	mol
Monodehydroascorbate	MDHAR
Nanometres	nm
Nitroblue tetrazolium	NBT
Oxidised nicotinamide adenine diphosphate	NADP ⁺
Percentage	%
Phosphatidylglycerol	PG
Reactive oxygen species	ROS
Reduced nicotinamide adenine dinucleotide	NADH
Reduced nicotinamide adenine diphosphate	NADPH
Relative humidity	RH

Seconds (time)	s
Singlet oxygen	$^1\text{O}_2$
Sucrose fructosyl transferase	SST
Superoxide	$\cdot\text{O}_2^-$
Superoxide dismutase	SOD
Time	t
Transmission electron microscopy	TEM
Tricarboxylic acid	TCA
Volts	V

CHAPTER 1

INTRODUCTION

1.1 Seed Storage

1.1.1 The Significance of Seed Storage

Seeds remain the most convenient and successful way of storing the genetic diversity of plant species and for producing new plants routinely for agriculture and horticulture (Mayer and Poljakoff-Mayber, 1989). The importance of seed storage and the ability to predict seed longevity must therefore not be underestimated.

While genetics govern the maximum time period for which a seed can remain viable in storage, the storage conditions ultimately determine the extent to which that storage potential is realised (Mayer and Poljakoff-Mayber, 1989). Failure of a seed to germinate is the final outcome of a whole series of detrimental changes that together characterise seed storage (Simon, 1974). Due to the practical importance of maintaining seed stocks, much research has been undertaken to find the causes of deterioration in seed quality that often occurs during storage (Simon, 1974).

1.1.2 Orthodox, Intermediate and Recalcitrant Seed Types

To be successful, storage conditions must maintain seed vigour and viability and ensure that normal seedlings are subsequently established under field conditions. Seed storage conditions ideally entail sub-zero temperatures and low relative humidities (RH), requiring the seed to have a low water content to prevent freezing damage (King and Roberts, 1980). Seeds that can be stored under these conventional conditions are referred to as 'orthodox', this term referring strictly to seeds whose storage life span can be predicted from seed water content and storage temperature (Roberts, 1973). There is also a category of seeds referred to as 'recalcitrant' that are desiccation-sensitive, and so cannot be stored in the dry state, or at sub-zero temperatures. Many recalcitrant seeds (especially of tropical origin) are also chilling-sensitive, and cannot be stored at low, but above zero, temperatures (Roberts, 1973).

The terms orthodox and recalcitrant were introduced by Roberts in 1973, and have now come to refer to the different abilities of seeds to tolerate water loss or desiccation – although originally orthodox and recalcitrant were descriptors of storage behaviour. It has since been suggested that there are many seeds that cannot be exclusively categorized, and which fall somewhere between the most desiccation sensitive and the most desiccation tolerant of seeds (Berjak and Pammenter, 1994). In fact, there are three categories into which seeds can be grouped according to their desiccation tolerance: orthodox, intermediate and recalcitrant.

When orthodox seeds are mature, they have moisture contents lower than that facilitating germination (King and Roberts, 1980). This is a consequence of maturation drying, during which the loss of significant amounts of water occurs in the final stage of their development, so that when such seeds are shed they have a water content that is usually in equilibrium with the ambient relative humidity (Kermode and Bewley, 1988). Orthodox seeds can be dried to a point where all tissue water is non-freezable, existing either as a ‘glass’ or bound to the surfaces of intracellular structures and macromolecules (Vertucci, 1990). This facilitates storage at sub-zero temperatures, for example -18°C , where theoretically they should remain viable indefinitely (reviewed by Roberts and King, 1980).

Intermediate seeds can be dehydrated to relatively low levels, but not to levels as low as those typical of orthodox seeds (Ellis *et al.*, 1990). The level to which intermediate seeds can be dehydrated makes conventional storage possible, but in some cases they are sensitive to low temperatures even in the dehydrated state (Hong and Ellis, 1996). Those authors have suggested this dehydration-related chilling sensitivity to typify the seeds of some tropical species, which means that they must be stored at relatively high temperatures once they have been dehydrated. The relatively high temperatures, perhaps 15°C or more, at which they have to be stored favour the activity of fungi and other microflora, leading to deterioration and death of the seeds (Berjak, 1996).

Recalcitrant seeds are hydrated and metabolically active when they are shed, and in many cases can germinate without additional water (King and Roberts, 1980). It is

thought that the more actively metabolic a seed is when it is shed, the less water loss it will tolerate (Pammenter and Berjak, 1999). Plants producing recalcitrant seeds usually, but not exclusively, occur in humid, tropical environments where germination can be immediate, as the environment is usually suitable for seedling growth throughout the year (King and Roberts, 1980). Recalcitrant seeds are irreversibly damaged if water is lost, resulting in a loss in viability, although the degree of dehydration tolerated is a variable function of the species and the drying conditions (Pammenter *et al.*, 1998). Even at relative humidities that do not allow water loss, viability usually declines in a relatively short period of time – this is related to the onset of germinative processes that require provision of additional water (Pammenter *et al.*, 1994). Recalcitrant seeds cannot be stored at sub-zero temperatures because of ice-crystal damage, and even at low temperatures many species are chilling sensitive (King and Roberts, 1980).

Therefore, for seeds that are intermediate or recalcitrant in nature conventional seed storage cannot be used.

1.1.3 Long and Short-Term Storage of Recalcitrant Seeds

Conditions of low temperature and low relative humidity (RH) are the most useful for seed storage as metabolic rate is reduced to a minimum, thus preventing or slowing deteriorative events. Rühl (1996) states that cooling and drying form the basis for the long-term preservation of all seed types, which sums up the severity of being faced with a seed that is both desiccation and chilling sensitive. The long-term storage of orthodox seeds is relatively easy, as they should remain viable indefinitely under conventional seed storage conditions, but for recalcitrant seeds more complex techniques are necessary. Long-term storage of many recalcitrant seed species has been enabled by the development of a protocol that allows successful cryopreservation.

Cryopreservation involves the very rapid freezing, in the present case of plant material, by plunging it into a cryogen, liquid nitrogen at the temperature of -196°C often being used (Withers and Engelmann, 1998). In order for survival of the plant material, it must be dehydrated beforehand to a low enough water content, and must be frozen sufficiently rapidly, to prevent crystalline ice from forming, as this is lethal (Wesley-

Smith *et al.*, 1992; Kioko *et al.*, 1998; Berjak *et al.*, 1999). It has been found that if water is removed rapidly there is little time for any damaging reactions to occur, and so a lot more water can be removed than if this were to be done slowly (Pammenter *et al.*, 1998). Most species of recalcitrant seed are too large to be dried rapidly, and, although success with rapid drying of whole seeds has been achieved (Pammenter and Berjak, 1999), it is usual to remove the embryonic axis of a seed and to dehydrate it rapidly in a stream of air. This is called 'flash drying' (Berjak *et al.*, 1989; Berjak *et al.*, 1990). The low water contents able to be achieved by flash drying, and the rapid freezing rate achieved by plunging into a suitable cryogen, minimize ice crystal growth so that it occurs at a sub-lethal level (Wesley-Smith *et al.*, 1992). This allows good survival after cryopreservation, provided that thawing is also carried out so as to prevent ice crystal growth during re-warming. The methodology makes it possible to cryopreserve plant material that has been dehydrated to a still relatively high water content (Wesley-Smith *et al.*, 1992). If excised zygotic embryonic axes or any other plant material besides seeds are cryopreserved, it must be borne in mind that *in vitro* methods will need to be used to regenerate seedlings afterwards, as the natural food supply has been removed (Ashmore, 1997). Ashmore (1997) defines *in vitro* techniques as those that utilize tissue culture methods in the maintenance, production or modification of plant material.

The long-term storage of seeds, or any plant material, is usually in aid of genetic conservation and is aimed at maintaining seed viability and genetic stability for decades or longer (Roos, 1986). Short-term storage of seeds is required for many practical purposes, and although conventional seed storage can be used for both long and short-term storage of orthodox seeds, cryopreservation is unsuitable for the short-term storage of recalcitrant seeds. This is due mainly to the advanced techniques used, which require much expertise. A simple reduction in temperature would be ideal for short-term storage, but, as mentioned, this is not suitable for all recalcitrant seeds. For example, recalcitrant cocoa seeds cannot be cooled to below 10 to 12°C without losing their germinability, so the maximum storage life that has been achieved is eight months with an unacceptable 24% germinability being retained (Rühl, 1996). There are many similar examples of unsuccessful short-term storage of recalcitrant seeds during which

many problems are encountered, and it seems that the only way in which to improve the methods currently in use is to gain a better basic understanding of these problems.

1.2 Problems Encountered During Short-Term Storage of Recalcitrant Seeds

1.2.1 Water Loss

Water loss or dehydration of recalcitrant seeds results in irreversible metabolic disruption (Farrant *et al.*, 1988), although different degrees of dehydration are tolerated among species (Berjak *et al.*, 1989). During short-term storage it is crucial that the water content of recalcitrant seeds remains high in order to prevent a dehydration stress that would ultimately lead to loss of viability. The method most commonly used to maintain high water content is storage in an environment where the atmospheric relative humidity (RH) is in equilibrium with the seeds water content (often 100% RH), commonly referred to as wet-storage (reviewed by Berjak *et al.*, 1990). Although this prevents dehydration stress, therefore in itself enhancing storage life span, it can lead to other problems such as initiation of germinative metabolism.

1.2.2 Initiation of Germinative Metabolism

Because recalcitrant seeds are hydrated and metabolically active on shedding, and many can germinate without additional water as a result of this (King and Roberts, 1980), it is natural that wet-storage would encourage the continuation of metabolic events leading to germination. Differences in storage life span are thought to be related to differences in rates of germinative metabolism (Bilia *et al.*, 1999). Those authors, in their study on recalcitrant *Inga uruguensis*, attributed this to differences in stage of physiological maturity. Recalcitrant *A. marina* seeds have a very short storage lifespan, which may be due to the rapid initiation of germinative metabolism after shedding. Pammenter *et al.* (1984) compared the ultrastructure of embryonic root primordia of newly shed *A. marina* seeds with those that had been wet-stored for four to five days. Those authors confirmed that subcellular activity is enhanced after four to five days wet-storage and that this enhancement is similar to that occurring in root primordia of seeds set out to

germinate immediately after being shed. It was also shown that the rate of germination was higher over the first few days of storage, suggesting that initial germination processes occurred in storage. The decline in germination rate following the initial storage period of four to five days is thought to be due to the accumulation of damage that must be repaired before germination can go to completion.

1.2.3 Microbial Contamination

Short-term storage of recalcitrant seeds entailing moist conditions and ambient temperatures often allows fungal growth to proliferate (King and Roberts, 1980). It has been found that fungal attack accelerates the inherent deterioration of recalcitrant seeds (Bilia *et al.*, 1999), and depending on seed storage conditions and fungal species, recalcitrant seeds will decay completely (Berjak, 1988; Berjak, 1996). Fungistatic and fungicidal treatments have been shown to prolong the storage lifespan of wet-stored recalcitrant seeds (Calistru *et al.*, 2000), indicating that microbial contamination does play a significant role in decreasing storage life span.

1.2.4 Chilling Sensitivity

Reduction of temperature is the most convenient way of prolonging the storage lifespan of seeds, as it decreases metabolism and helps prevent water loss. It is generally accepted that storage of recalcitrant seeds in the hydrated state at as low a temperature as possible is the best way in which to retain viability in the short-term (Berjak *et al.*, 1989). Problems encountered with wet-storage of recalcitrant seeds are reduced at lower temperatures, as both germinative metabolism and fungal metabolism are significantly slowed. Each species of recalcitrant seed seems to have a specific temperature at which it incurs chilling injury, some tolerating lower temperatures than others. When mature recalcitrant *Symphonia globulifera* seeds were stored in a wet medium at 15°C viability was retained for up to two months, but when the temperature was reduced to 10 or 12°C the seeds rapidly lost their viability (Corbineau and Côme, 1986). This indicates how carefully temperature must be controlled when storing recalcitrant seeds. In view of the benefits afforded by storage at low temperatures it is

worth a thorough investigation into the responses of recalcitrant seeds to chilling temperatures and possible causes of chilling sensitivity.

1.3 Chilling Sensitivity

1.3.1 An Overview

Chilling sensitivity is a complex phenomenon characteristic of many, but not all, plants originating in tropical or subtropical climates, although plants of temperate climates can also be sensitive. A plant may be described as chilling sensitive if it suffers injury and eventually dies on exposure to chilling temperatures (Raison and Orr, 1990); i.e. temperatures cool enough to prevent normal plant growth and to produce injury, but not cool enough for ice crystal formation and therefore freezing of a plant to occur (Levitt, 1980; Taiz and Zeiger, 1998). Generally, this includes any temperature below 15°C and down to 0°C (Levitt, 1980; King and Roberts, 1980). The sensitivity of plants to chilling has been recorded for centuries (Lyons, 1973), but only more recently has this been recognised in the seeds of many tropical and subtropical plant species (King and Roberts, 1980), which is why relatively little data on chilling response in seeds, especially recalcitrant seeds, exist. Recalcitrant seeds have been compared with germinating orthodox seedlings, because they are metabolically active (Berjak et al., 1984); therefore, much of the knowledge gained on chilling response in vegetative plant tissue is expected to be relevant to recalcitrant seeds.

Chilling injury occurs as a result of exposure to a chilling stress (Saltveit and Morris, 1990), and is defined as a change in metabolism resulting in altered physiological function and appearance with adverse effects (Lyons, 1973; Hale and Orcutt, 1987). It occurs below a critical temperature (Hale and Orcutt, 1987; Raison and Orr, 1990) that is dependent on the plant species concerned and its origin or provenance (Lyons, 1973). The nature and severity of the injury is a function of the species, plant part, maturity and metabolic state, as well as being dependent on the duration and severity of the chilling stress (Saltveit and Morris, 1990; Raison and Orr, 1990). Many of the changes observed in plant tissues in response to chilling stress are the result of degenerative tissue injury, and are not the initial events that lead to alteration of metabolic processes

(Lyons, 1973; Raison and Orr, 1990). In fact, chilling stress causes a primary event that is followed by a series of secondary physiological and physical events that are damaging, and which lead to visible symptoms and cell death (Saltveit and Morris, 1990; Raison and Orr, 1990; Van Hasselt, 1990). The primary event that leads to altered metabolism and impaired functioning of the tissue is a very rapid response, even said to be instantaneous (Raison and Orr, 1990), and is reversible if the chilling stress is removed after a brief exposure (Saltveit and Morris, 1990). Each species has a different point at which the primary event is no longer reversible and gives way to degenerative secondary events that are time-dependent (Lyons, 1973; Saltveit and Morris, 1990). The 'critical temperature' referred to earlier is therefore more specifically the temperature below which the primary event of chilling injury occurs (Saltveit and Morris, 1990). This primary event has not yet been elucidated, although proposals have been put forward (Raison and Orr, 1990) and much research has been undertaken to determine the specific physiological, cellular and biochemical changes that occur in the different components of plants in response to chilling stress. Some of the main physiological symptoms include changes in membrane permeability, respiration, protoplasmic streaming and metabolic changes involving enzyme systems, sugars and other chemical substances; but presumably these are not the primary event (Lyons, 1973). Physical symptoms, which tend only to be secondary events, include necrotic lesions, increased susceptibility to decay organisms, cessation of growth and ultimately death (Lyons, 1973).

The ability of some plants to survive chilling stress seems to be related to the way in which they respond to it. The differing responses of chilling-sensitive and chilling-tolerant species have been observed and it is apparent that some plants are inherently tolerant to chilling stress, some are able to become resistant by the process of acclimation or hardening and others are not able to protect themselves at all. The terms 'acclimation' or 'hardening' are often encountered when chilling sensitivity is discussed and refer to nonheritable modifications in physiological processes caused by exposure to new environmental conditions that render plants resistant to stresses such as drought, low temperatures and freezing (Hale and Orcutt, 1987). Lyons (1973) states that in some species chilling-sensitivity can be reduced by exposure to temperatures slightly

above the chilling range. Chilling sensitive species have been divided into two categories; those unable to harden, referred to as extremely chilling-sensitive, and those able to harden, referred to as less chilling-sensitive (Graham and Patterson, 1982). Hardening involves the synthesis of proteins, enzymes, lipids, membranes and metabolites that confer tolerance or protection (Graham and Patterson, 1982), which means that a plant must have the genetic capacity to harden towards cold. In his review, Guy (1990) refers to acclimation as an inducible response, and extremely chilling sensitive plant species do not have this genetic ability to protect themselves, which is why they succumb to injury and eventually death when exposed to chilling stress. As previously mentioned, recalcitrant seeds are metabolically active and so it is appropriate to relate their inability to survive chilling stress to that of chilling-sensitive plants. Thus, the responses of plants sensitive and tolerant to chilling stress are discussed in relative detail.

1.4 Responses of Plants towards Chilling Stress

1.4.1 Membrane Alterations

Membranes consist of a lipid bilayer, but each type of cellular membrane has unique lipid-lipid and lipid-protein interactions. It is therefore likely that chloroplast, mitochondrial, endoplasmic reticulum (ER), plasma and tonoplast membranes each would respond differently towards chilling stress (Murata and Nishida, 1990). A general hypothesis is that the cellular membranes of chilling-sensitive plants undergo a physical phase transition from a flexible liquid-crystalline to a more solid gel structure when exposed to chilling temperatures (Lyons, 1973). It has since been suggested that this phase transition, which occurs in the polar lipids of cell membranes, is induced by high-melting point molecular species of phosphatidylglycerol (PG) found mainly in chloroplast membranes (Murata *et al.*, 1982). Membrane phase transitions have been suggested to be the primary response of chilling-sensitive plants to chilling stress because the temperature of the physical phase transition coincides with the temperature below which chilling injury occurs (reviewed by Wang, 1982; Raison and Orr, 1990; Nishida and Murata, 1996). Much controversy surrounds this suggestion, because

although many of the responses of plants towards chilling stress can be linked to phase changes in the membrane lipids, there are those that are totally unrelated.

Lipids control the physical state of cell membranes (Lyons, 1973) and so it is logical to assume that membranes with a high percentage of saturated fatty acid chains solidify, i.e. go into the gel state, at a temperature well above 0°C (Taiz and Zeiger, 1998). Many organisms are able to alter the ratio of saturated to unsaturated fatty acids in the polar lipids of their membranes and consequently compensate for any decrease or increase in membrane fluidity caused by changing temperature (Nishida and Murata, 1996). The unsaturation of membrane lipids, brought about by the activity of desaturase enzymes that introduce double bonds (Taiz and Zeiger, 1998), is thought to be one of the most critical parameters for the functioning of biological membranes and therefore for the survival of organisms at low temperatures (Nishida and Murata, 1996) because it provides protection against chilling injury. In plant cells, fatty acid synthesis takes place within plastids (Murata and Los, 1997). The final products are two key saturated fatty acids, palmitoyl-acyl-carrier protein (ACP) (16:0-ACP) and stearoyl-ACP (18:0-ACP); these must be converted by acyl-lipid desaturases to polyunsaturated fatty acids, because the fatty acid synthase system of plastids cannot produce unsaturated fatty acids *de novo* (Nishida and Murata, 1996). These fatty-acid desaturase enzymes, transmembrane proteins located in the endoplasmic reticulum (ER) and chloroplast membranes, are therefore central to regulating the level of unsaturated fatty acids in membrane lipids (Nishida and Murata, 1996). It has been found that the fatty acids of membrane lipids in plants are actually highly unsaturated, and so theoretically this should prevent a sharp phase transition from occurring at chilling temperatures (Markhart, 1986). Experiments have also shown that only phosphatidylglycerols (PG), and no other major lipid classes, undergo a phase transition at temperatures above 0°C (Murata and Yamaya, 1984). This lipid occurs mainly in chloroplast membranes, 70-80% of the PG from leaves (Murata and Yamaya, 1984), and accounts for approximately 6% of polar lipids in cell membranes (Raison and Orr, 1990). It seems unlikely that a phase transition localized mainly in the chloroplast membranes and involving such a small portion of membrane lipids would cause any metabolic disruption, but high proportions of saturated PG in chloroplast membrane lipids tend to

be characteristic of chilling sensitive plants (Roughan, 1985) and phase transition of PG has been found to occur at a much higher temperature in chilling-sensitive plants than it does in chilling-resistant plants. Murata and Yamaya (1984) found that 50% of the membrane area of the PG from chilling-sensitive plants was in the gel phase at 5°C, whereas only a small portion from chilling-resistant plants was in the gel phase at this temperature. Raison and Wright (1983) and Roughan (1985) have also reported similar findings.

The proposed mechanism of injury is that high-melting point molecular species of PG in the chloroplast membranes undergo a phase transition at chilling temperatures resulting in metabolic disruption, yet there is evidence that non-photosynthetic tissues of chilling-sensitive plants, which contain very little saturated PG, are also susceptible to chilling injury (Roughan, 1985; Wu and Browse, 1995). A possible explanation is that because plastids are the major site of fatty acid biosynthesis in plants, any loss of chloroplast functional integrity would rapidly affect other membranes ultimately leading to cell dysfunction (Yu *et al.*, 1996; Yu and Willemot, 1996). In tomato fruit it is not the presence of high melting point molecular species of PG that can be associated with chilling-sensitivity, but the inhibition of galactolipid biosynthesis (Yu and Willemot, 1996). Roughan (1985) investigated the apparent correlation of saturated PG with chilling sensitivity using 74 plant species. That author found that most plants of tropical origin had highly saturated PG, but that there were some chilling-sensitive plants that contained very little saturated PG. Wu and Browse (1995) worked with an *Arabidopsis* mutant (*fab 1*) that had levels of high-melting point molecular species of PG similar to those found in most chilling-sensitive plants (wild-type *Arabidopsis* is chilling-tolerant). Those authors found that when this mutant was exposed to various chilling temperatures for short periods no chilling-sensitive symptoms were visible, whereas other chilling sensitive plant species exposed to the same treatments were severely affected. The mutant plants were, however, damaged by prolonged exposure to low temperatures, which led to the conclusion that high levels of saturated PG might confer a selective advantage at non-chilling temperatures. Wu and Browse (1995) state that for tropical plants there would be no selection against traits that negatively affect growth at low temperatures, and that any particular chilling-associated trait, such as high levels of

high-melting point molecular species of PG, may not be found in all chilling-sensitive species.

As a general rule, chilling-tolerant plants do have a higher percentage of unsaturated fatty acids in their membrane lipids than do chilling-sensitive plants (Taiz and Zeiger, 1998) and in most cases, when plants acclimate to chilling temperatures the proportion of unsaturated fatty acids in their membrane lipids does increase (Markhart, 1986; Taiz and Zeiger, 1998). It has been found that the gene for the desaturases that bring about the unsaturation of membrane lipids is induced by low temperature (Sarmiento *et al.*, 1998), but it is not the absolute temperature as much as the extent of shift in temperature that brings about this induction or up-regulation (Murata and Los, 1997). Kodama *et al.* (1995) believe that the activity of desaturases is not controlled solely by the level of transcription, and Sarmiento *et al.* (1998) state that in oilseeds, the activity of a specific desaturase (oleoyl phosphatidylcholine desaturase) depended on the species of oilseed concerned. In chilling-sensitive adzuki bean (*Vigna angularis*) the desaturation of cell lipids was not marked as temperature was decreased (Kojima *et al.*, 1998), but a change in lipid unsaturation in response to low temperature has been observed in a variety of other chilling-sensitive plant species (Xu and Siegenthaler, 1997). In squash cotyledons, changes in lipid fatty acid unsaturation in response to temperature can only take place during seed development, and not once maturity has been reached (Xu and Siegenthaler, 1996). These examples indicate that it is likely that a whole host of factors influence desaturase activity, and hence the extent of unsaturation in response to chilling temperatures.

Membrane fluidity is important for the normal functioning of the cell at chilling temperatures, and much research indicates an important role for the unsaturation of membrane lipids in the growth and survival of plants at chilling temperatures. The activities of membrane proteins and enzymes that regulate metabolism are also influenced by the physical properties of membrane lipids, so when the fluidity of the membrane decreases these proteins and enzymes can no longer function normally (Taiz and Zeiger, 1998). However, there are many conflicting reports and constantly changing theories. For example, Xu and Siegenthaler (1997) have recently reported that

when plants are transferred to low temperatures both fatty acid synthesis and desaturation are inhibited, but because fatty acid synthesis is inhibited more than desaturation it appears that the level of unsaturation is increasing. In view of this, those authors believe that an apparent increase in unsaturation is unlikely to be due to a higher absolute activity of desaturases.

The alteration of membranes in response to chilling stress has been discussed in detail because much evidence indicates that many responses to chilling stress are based upon this one event, but it is not the only factor that regulates the chilling sensitivity of plants.

1.4.2 Increase in Permeability and Solute Leakage

Permeability refers to the freedom with which water and solutes can pass through a membrane; therefore the first symptom of enhanced membrane permeability would be increased solute leakage (Murata, 1990). Leakage is a passive event influenced by concentration gradient, which means that the barrier effectiveness of a membrane can be determined by placing tissue in water and measuring the quantity of solutes that have leaked out after a specific period of time (Simon, 1974). In some chilling-sensitive plant species, increased permeability, and therefore solute leakage, occurs after exposure to chilling stress (reviewed by Simon, 1974; Wang, 1982; Murata, 1990). It is generally thought that this is the result of alteration in membrane structure brought about by lipid phase transitions from the liquid-crystalline to the gel state. This transition causes the membrane to contract, opening up cracks or channels that result in increased permeability (Lyons, 1973; Levitt, 1980).

When dry seeds are imbibed, a reorganization of their membranes occurs until selective permeability is restored; this is shown by a high initial leakage rate that declines over time (Simon, 1974). Leopold (1980) observed solute leakage rates from living imbibing seed tissues to be 10-fold lower than those from dead tissues, indicating that membrane reorganization definitely occurs in dry imbibing seeds. William *et al.* (1978) showed that imposing a chilling stress on seeds during imbibition prevented proper reorganization of the dry membranes from occurring. Those authors found that the initial rapid leakage was prolonged and did not decline whilst chilling stress was

imposed, concluding that low temperature interfered with normal membrane reorganization. These studies provide evidence that increased leakage is due to impaired functioning of the cell membrane, and in chilling-sensitive plants this is probably due to phase transitions in the membrane lipids.

Although investigations show that an increased solute leakage rate usually occurs when chilling-sensitive plants are exposed to chilling stress, there are those that do not show this increase in membrane permeability (Murata, 1990). Even though high solute leakage after exposure to chilling stress might not be a general property of chilling-sensitive plant tissues (Wang, 1982) it can still be used as an indication of the extent of chilling injury in many species. For example, in young mung bean (*Vigna radiata*) seedlings, chilling treatments were observed to cause solute leakage, the degree of which was a function of the cell injury incurred (Yoshida *et al.*, 1986).

1.4.3 Increase in Free Radical Production and Antioxidant Activity

Oxygen is essential for the survival of aerobic organisms, but it can also cause lethal damage. In its ground state oxygen is relatively unreactive, but it has a tendency to react with unpaired electrons thus giving rise to reactive free radical species (Alscher, 1989; Scandalios, 1993). A free radical is any atomic species, capable of independent existence, which contains one or more unpaired electrons (Hendry, 1993). Many damaging physiological events are mediated by these free radical species and this is known as 'oxidative stress' (Scandalios, 1993). The main free radical species capable of causing oxidative damage are hydroxyl radicals ($\cdot\text{OH}$), singlet oxygen ($^1\text{O}_2$) and superoxide ($\cdot\text{O}_2^-$) (Bowler *et al.*, 1992). The production of these oxygen free radicals is a normal part of cell metabolism under aerobic conditions, and they are usually inactivated by antioxidants and free radical scavenging enzyme systems (Purvis and Shewfelt, 1993). Evidence shows that chilling increases the level of free radical species, therefore oxidative stress is thought to be a significant factor in the chilling injury of plants (Fadzillah, 1996; Prasad, 1997). The level of free radical species depends on the balance between production and the capacity for scavenging them, therefore the cold stability of scavenging enzymes and the response of enzyme-synthesis to low temperatures is of importance (Saruyama and Tanida, 1995). Chilling-

tolerant plants may avoid the production of free radicals, protect themselves by free radical scavenging or repair free radical damage, which facilitates survival or growth at chilling temperatures (Walker and Mckersie, 1993). The ability of a plant to respond appropriately towards chilling-induced oxidative stress could be one of the main factors involved in chilling sensitivity, as it is only when these defence systems have been overwhelmed that free radicals persist long enough to damage cellular processes and macromolecules (Alscher, 1989).

Plants consume oxygen during respiration and generate it during photosynthesis, so there is much scope for oxidative stress. Two major electron transport systems, located in the mitochondria and chloroplasts respectively, are the main source of free radicals during chilling stress (Purvis and Shewfelt, 1993). As previously mentioned, free radical production is a normal event occurring in plant cells, so the generation of superoxide and singlet oxygen in illuminated chloroplasts due to occasional transfer of an electron from an excited chlorophyll molecule to molecular oxygen is commonplace (Scandalios, 1993). This occurs when the ratio of NADPH to ATP formed by photosynthetic electron transport is insufficient to meet the requirements of carbon fixation. The ratio of NADPH to NADP^+ increases as ATP levels drop and reductant consumption decreases, resulting in the unavailability of NADP^+ to function as an electron acceptor (Alscher, 1989). Antioxidants would usually quench the superoxide and singlet oxygen produced, but chilling causes excess, abnormal free radical production that overwhelms the system. This excess free radical production in the chloroplast is usually the result of photo-inhibition, which is induced when chilling is accompanied by light, although increased lipid peroxidation in the thylakoid membranes may also contribute (Wise and Naylor, 1987). Photo-inhibition occurs when the light energy absorbed exceeds the capacity of the photosystems to direct it through photosynthetic electron transport (Bowler *et al.*, 1992). Because light energy is absorbed continuously, any disruption or reduction in electron transport caused by chilling immediately results in the donation of electrons to an electron acceptor other than NADP^+ , such as oxygen, because reaction-centre chlorophylls need to dispose of their excitation energy (Thompson *et al.*, 1987; Bowler *et al.*, 1992).

The reductive processes associated with the mitochondrial electron transport pathway, which consists of four closely associated electron carrier protein complexes situated in the mitochondrial inner membrane, are a source of superoxide radicals (Purvis and Shewfelt, 1993). This pathway can terminate with cytochrome oxidase or alternate oxidase, but electrons from the TCA cycle preferentially flow through the cytochrome pathway and are only diverted to the alternative pathway when the cytochrome pathway is inhibited (Purvis and Shewfelt, 1993). Disruption of electron flow through the cytochrome pathway leads to the production of superoxide and hydrogen peroxide, and this occurs during chilling when physical, disruptive changes occur in the mitochondrial membranes (Purvis and Shewfelt, 1993; Prasad *et al.*, 1994b). All of the oxygen free radical species produced are reactive and cytotoxic, so excess production as a result of metabolic disruption and failure to detoxify them has many detrimental consequences such as lipid peroxidation, protein denaturation and DNA damage (Monk *et al.*, 1989; Prasad, 1996). Free radicals are able to induce the peroxidation of membrane lipids by reacting with unsaturated fatty acids. Peroxidation damage to cell membranes can destabilize the membrane bilayer structure, which can lead to a decrease in membrane fluidity, leakage of cellular contents, loss of respiratory activity in mitochondria and loss of carbon-fixing activity in chloroplasts (Thompson *et al.*, 1987; Scandalios, 1993). Free radicals inactivate proteins by modifying amino acid residues, and enzymes are likely to lose their catalytic activity (Thompson *et al.*, 1987). In order to overcome the oxidative stress imposed by chilling, additional defences are required (Scandalios, 1993). A constitutively high antioxidant capacity, or increases in the levels of one or more antioxidants in response to chilling can prevent damage and therefore render the plant chilling-tolerant (Monk *et al.*, 1989).

Aerobic organisms possess an antioxidant defence system that consists of both enzymic and non-enzymic substances for scavenging free radicals (Monk *et al.*, 1989). They may be divided into three general classes (Walker and Mckersie, 1993):

1) Lipid soluble, membrane associated antioxidants;

- α -Tocopherol: quencher of singlet oxygen and a free radical trap for terminating lipid peroxidation chain reactions.

- Carotenoids: found in thylakoid membranes where they quench singlet oxygen and excess chlorophyll excitation energy that is not readily passed to the photosystems.
- 2) Water soluble reductants;
- Glutathione: reacts directly with free radicals or with dehydroascorbate (DHA) to regenerate ascorbate. Oxidized glutathione is in turn reduced by NADPH from photosystem I.
 - Ascorbate: reduces superoxide, hydrogen peroxide and the hydroxyl radical, or quenches singlet oxygen.
- 3) Enzymatic antioxidants;
- Superoxide dismutase (SOD): reacts with superoxide radicals and converts them to oxygen and hydrogen peroxide.
 - Catalase (CAT): detoxifies hydrogen peroxide to water and oxygen.
 - Ascorbate peroxidase (APX): main enzyme in the ascorbate-dependent hydrogen peroxide scavenging system. The enzymes monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are also required. Functional co-operation among these enzymes is important for effective scavenging.

There are many conflicting reports in which specific antioxidant enzyme activity either decreases or increases as a result of chilling stress. This is due mainly to the different plant species studied but is also because total enzyme activity is measured and not individual isozyme contribution; relative contributions of the different isozymes to the total activity might change and such a change could contribute to chilling tolerance (Prasad *et al.*, 1994a). Superoxide dismutases (SODs), which have been identified as an essential component of an organism's defence mechanism, are found in almost all aerobic organisms and subcellular compartments where oxidative stress is likely to occur (Monk *et al.*, 1989; Bowler *et al.*, 1992). SOD genes appear to be sensitive to environmental stresses responding to increased superoxide formation, therefore oxidative stress induces or enhances the activity of SOD (Bowler *et al.*, 1992; Scandalios, 1993). Plants have multiple isozymes of SOD, and there may be separate metabolic roles for each of them (Scandalios, 1993). In the context of chilling, SOD might be a primary defence, as hydroxyl radicals, formed from superoxide, easily

peroxidize unsaturated fatty acids that are thought to be directly linked to chilling tolerance (Saruyama and Tanida, 1995). SOD usually works in combination with CAT and APX because one of the dismutation products of superoxide is hydrogen peroxide, which is also highly reactive and can form hydroxyl radicals (Scandalios, 1993). CAT converts hydrogen peroxide to water and molecular oxygen, and APX reduces hydrogen peroxide to water in the presence of an appropriate oxidizable substrate (Thompson *et al.*, 1987). In research using rice cultivars, both chilling-sensitive *Oryza sativa* and chilling-tolerant Dunghan Shali (the name of the rice cultivar) were chilled at 5°C. It was noted that the total SOD activity did not change during chilling and that there was no significant difference in activity between the two cultivars, indicating that SOD itself might be cold tolerant (Saruyama and Tanida, 1995). A drastic decrease in CAT activity was noted in both cultivars, therefore it is likely to be cold labile, and an increase in APX activity was noted for the chilling-tolerant cultivar while it decreased in the chilling-sensitive cultivar (Saruyama and Tanida, 1995). Because both CAT and APX scavenge hydrogen peroxide it was concluded that a co-operation between the two was necessary for chilling-tolerance, and GR, which is required for APX activity, was not cold labile, so it is not responsible for the loss of APX activity at chilling temperatures (Saruyama and Tanida, 1995). Jahnke *et al.* (1991) found that in two maize species, chilling-sensitive *Zea mays* and chilling-tolerant *Zea diploperennis*, DHAR was the most cold-labile enzyme, and this would affect APX activity. According to MacRae and Ferguson (1985), a decrease in CAT activity is a general response to stress, and it has been found to occur in a number of chilling-sensitive and chilling-tolerant species in response to chilling stress. Those authors suggest that this observed decrease in CAT activity is due to a change in the assembly of its subunits, as the CAT precursor molecule can only become active once it has passed through the peroxisomal membrane, acquired haem groups and aggregates. Change in membrane properties occur as a result of chilling, therefore damage to the peroxisomal membrane might affect CAT assembly. In chilling-tolerant plant species hydrogen peroxide does not accumulate at chilling temperatures, this is thought to be due to the compensatory removal of hydrogen peroxide through the ascorbate/glutathione system in which APX is involved (MacRae and Ferguson, 1985). Omran (1980) found that CAT activity decreased and APX activity remained unchanged when chilling-sensitive cucumber

seeds (*Cucumis sativus* var Marketmore) were chilled. That author noted an accumulation of hydrogen peroxide in the tissue, and concluded that a decrease in CAT activity without an increase in APX activity lead to this accumulation.

Plant cells also contain relatively high levels of ascorbate, glutathione and α -tocopherol, which are efficient free radical scavengers. Thylakoid membranes in chloroplasts contain large amounts of α -tocopherol, which blocks the chain-propagating reactions of lipid peroxidation, and carotenoids, which quench singlet oxygen very rapidly (Bowler *et al.*, 1992). Besides scavenging hydrogen peroxide, GR, MDHAR, DHAR and APX catalyze reactions that maintain large pools of glutathione and ascorbate in the chloroplast (Smith *et al.*, 1989). GR has been shown to be linked with the resistance of plants to various oxidative stresses, and that an increase in GR in response to low temperatures is probably due to an induction mechanism (Alscher, 1989), which apparently is only successful in chilling-tolerant species. GR converts oxidized glutathione generated by various nonenzymic and enzymic reactions back to the reduced form. Glutathione in plants protects –SH groups in enzymes and structural proteins against oxidation either by acting as a scavenger for oxidizing substances or by repairing the –SH groups (Esterbauer and Grill, 1978), so GR is important for cold-tolerance. Walker and McKersie (1993) studied two species of tomato: chilling-tolerant *Lycopersicon hirsutum* was able to control photosynthetic electron transport reactions at low temperatures therefore avoiding the production of large quantities of free radicals, and an increase in the total ascorbate pool, carotenoids and glutathione was noted, whereas in chilling-sensitive *Lycopersicon esculentum* the carotenoid and glutathione level remained the same, total ascorbate declined and free radical production was much higher. Those authors suggested that the increase in ascorbate, carotenoids and glutathione in the chilling-tolerant cultivar was an acclimation response and that reduced free radical production was due to homeostatic control. Prasad (1997) also found that maize (*Zea mays*) seedlings exhibited an acclimation response, as they became more resistant to chilling when they were exposed to a mild oxidative stress at 14°C before being transferred to 4°C. That author suggested that the mild oxidative stress, which caused increased hydrogen peroxide levels, induced some of the antioxidant enzymes so that when the seedlings were transferred to 4°C they were more

protected, as seedlings that had not been exposed to the mild oxidative stress were severely damaged at 4°C. Fadzillah *et al.* (1996) state that even during the early stages of normal growth at 25°C there is an initial oxidative stress that induces an increase in certain enzymes such as GR. At chilling temperatures such as 4°C the initial oxidative stress is even greater, but there is no induction of GR activity in chilling-sensitive species as there is at 25°C (Fadzillah *et al.*, 1996). Alscher *et al.* (1989) consider the inducibility of enzymes in the free radical scavenging pathway and the increased synthesis of at least one of its components on exposure to oxidative stress to be crucial. In support of this, Hodges *et al.* (1996) found that chilling-sensitive inbred lines of maize had lower antioxidant enzyme capacities than chilling-tolerant lines. It has been suggested that natural antioxidant systems are disabled by chilling stress in chilling-sensitive plants, so they are unable to cope with increased free radical production, and that during chilling stress free radicals might not be generated at the same sites that they are under normal growth conditions (Hideg and Björn, 1996). Although high antioxidant activity occurs mainly in specific organelles such as the chloroplasts and mitochondria, which are the main sites of free radical production, similar scavenging pathways are operative in the cell cytoplasm (Smith *et al.*, 1989). It must be noted that there is a general decrease in all antioxidant enzymes as a plant ages or deteriorates (Pauls and Thompson, 1984; Pukacka, 1991; Bowler *et al.*, 1992), and in some instances the decrease in enzyme activity may be the result of degenerative injury induced by chilling. Reduced antioxidant enzyme activities, and therefore increased free radical accumulation, were thought to be due to reduced vigour of aged peanut seeds, thus if the vigour of chilling-sensitive seeds and plants decreases when they are exposed to chilling stress this might also affect the activity of their antioxidant enzymes (Sung and Jeng, 1994).

It is clear that the lack of a well developed free radical scavenging system in plants at chilling temperatures does contribute to chilling injury, and that all of the enzymes and antioxidants are linked in an optimized balance which reduces oxidative damage.

1.4.4 Changes in Proteins, Enzyme Activity and Respiration

The ability of a plant to survive at chilling temperatures has been attributed to a number of processes including alteration of the spectrum of proteins produced and a change in the structure or function of enzymes involved in key metabolic reactions (Graham and Patterson, 1982; Caldwell, 1990). Genetic adaptation to chilling temperatures therefore involves compensatory enzymatic reactions and continued protein synthesis at these temperatures.

Protein synthesis generally becomes ineffective in chilling-sensitive tissues during chilling and the structural and functional properties of proteins may be temperature sensitive (Wang, 1982; Graham and Patterson, 1982). Although most enzymes are more stable at low temperatures, there are those that are inactivated by cold. Cold-labile enzymes are typically complex and consist of subunits, their secondary and tertiary structure being maintained by weak chemical bonds that are easily disrupted by minor changes in cellular temperature (Caldwell, 1990). Most enzyme systems affected by chilling are associated with membranes, but there are those that are directly affected by chilling temperatures. The Arrhenius plots of membrane-associated enzymes in chilling-sensitive plants show a 'break' in activity at the same temperature at which the membrane undergoes a phase transition from the liquid-crystalline to the gel state, whereas the 'break' either does not occur or occurs at a lower temperature in chilling-tolerant plants (Wang, 1982). Membrane-associated enzyme systems demonstrated to be affected by chilling temperatures are plasma membrane ATPase, NADH Cyt *c* reductase, mitochondrial Cyt *c* oxidase and tonoplast H⁺-ATPase (Yoshida *et al.*, 1989).

Tonoplast H⁺-ATPases act as proton pumps, maintaining ionic gradients across membranes, and are thought to be used for the active transport of ions and metabolites into vacuoles (Yoshida and Matsuura-Endo, 1991; Ishikawa, 1996). They are very important in maintaining the pH and ionic concentrations of the cytoplasmic environment, so their alteration in response to chilling could lead to the disruption of many physiological functions (Yoshida and Matsuura-Endo, 1991). These membrane-associated enzymes have been the subject of much investigation as they are thought to be one of the most chilling-sensitive groups of enzymes in plant cells (Kojima *et al.*,

1998). There is a marked difference between the cold stability of tonoplast H^+ -ATPase in chilling-sensitive and chilling-tolerant cultured cells (Yoshida, 1991). It must also be noted that ATP levels decrease in response to chilling as a result of conformational change in the respiratory enzymes, thought to be induced by a phase change in the lipids of mitochondrial membranes (Wang, 1982). Thus ATP might not be available to supply the energy for the functioning of the H^+ -ATPases at chilling temperatures (Graham and Patterson, 1982).

The respiratory apparatus is not affected in chilling resistant plants even if they are held at chilling temperatures, but mitochondria show a decrease in activity when chilling-sensitive plants are held at these temperatures (Lyons, 1973; Markhart, 1986). The normal cytochrome oxidase pathway of electron transport also decreases, but in chilling-sensitive plants an initial brief decline in respiration rate usually occurs, followed by a significant increase (Graham and Patterson, 1982; Wang, 1982; Lyons and Breidenbach, 1990). This increase in respiration rate might be due to severe metabolic disruption and accumulation of oxidizable intermediates (Wang, 1982), although there is evidence of engagement of the alternative pathway of respiration, during which no ATP is generated (Graham and Patterson, 1982; Wang, 1982; Markhart, 1986). Respiration rate is often used as an index of the severity of chilling injury (Lyons, 1973) as it reflects disruption of cellular metabolism, specifically involving proteins and enzyme activity. Respiration rate can be determined by measuring the amount of carbohydrate consumed, oxygen taken up, carbon dioxide given off or heat produced, but the most common assessment is by gas analysis as it is easy and accurate (Lyons and Breidenbach, 1990).

Changes in proteins, enzymes and therefore respiration are not considered to be primary responses towards chilling, but secondary manifestations of physiological breakdown (Lyons, 1973).

1.4.5 Increase in Sugar Levels

Many reports indicate that one of the early responses of plants to chilling is the accumulation of sugars, and it is generally thought that sugar levels, or carbohydrates,

influence chilling-sensitivity (Calderon and Pontis, 1985; Purvis, 1990). Some researchers support the view that an increase in sugar levels in response to chilling is part of an acclimation process involved in rendering a plant chilling-tolerant, whereas others suggest that it is merely a consequence of the low demand for photosynthates at chilling temperatures. Sugars contribute to the osmotic potential of cells, stabilize membranes and can maintain the structural conformation of enzymes, but their role in chilling is thought to be metabolic (Purvis, 1990).

Sucrose-derived oligosaccharides, such as fructans, are often linked with chilling-tolerance (Pontis, 1989). Their accumulation in response to chilling temperatures is preceded by an increase in sucrose concentration, on which their synthesis depends (Puebla *et al.*, 1997), as sucrose is the major precursor of fructans (Pollock *et al.*, 1983). Photosynthetic processes have been shown to be less temperature sensitive than the processes controlling growth, so the increase in sucrose level often observed in response to chilling is the result of an alteration in the balance between carbon fixation and utilization (Pollock *et al.*, 1983; Calderon and Pontis, 1985; Pontis, 1989). Calderon and Pontis (1985) suggest that the increase in sucrose level as a result of decreased growth rate turns on the activity of sucrose: sucrose fructosyl transferase (SST), which initiates the synthesis of fructans and leads to their accumulation in the plant vacuole. This does not, however, explain why, for example, in wheat cultivars the rate of fructan accumulation at 4°C was three-fold higher in more chilling-tolerant cultivars than in less tolerant ones (Pontis, 1989). Tognetti *et al.* (1990) also found that in the four wheat cultivars they studied fructan levels were higher as the chilling tolerance of the cultivars increased. Those authors found that at chilling temperatures there was an increase in the activities of the enzymes that regulate sucrose concentration, and that this rise in activity was greater in the more chilling-tolerant cultivars. This points towards sucrose accumulation being part of an acclimation response in the more chilling-tolerant cultivars and therefore being under the control of low-temperature-inducible genes. Santoiani *et al.* (1993) found that the sucrose and fructan accumulated in wheat roots during exposure to chilling temperatures was reversed when the plants were returned to normal growth temperatures, which supports the suggestion of low-temperature-inducible genes. Even so, it is still unclear as to whether sugars contribute specifically

towards reduced chilling-sensitivity or if their presence is merely a consequence of chilling stress, and the primary sugars involved have also not yet been identified (Purvis, 1990).

1.4.6 Reduction of Protoplasmic Streaming

It has been observed that when chilling-sensitive plants are exposed to chilling stress, protoplasmic streaming becomes reduced or ceases, whereas this does not happen in chilling-tolerant plants (reviewed by Lyons, 1973; Wang, 1982; Raison and Orr, 1990). This streaming movement occurs around the fluid-filled vacuoles in plant cells and serves to distribute nutrients, proteins and other cellular materials (Hopson and Wessells, 1990). It requires energy in the form of ATP and is affected by the physical properties of the protoplasm, the cytoskeleton and subcellular membranes (Lyons, 1973). Temperature is thought to affect a number of the factors involved in protoplasmic streaming. These factors are thought to be cell lipid structure, energy supply from respiration, energy utilization, protoplasmic viscosity, chilling sensitivity of enzyme systems responsible for utilizing ATP for streaming and probably also thermolability of the microfilaments (Wang, 1982). This indicates that reduction or cessation of protoplasmic streaming is a secondary response occurring as the result of the effect of chilling stress on other cellular structures and metabolic processes.

1.4.7 Stimulation of Ethylene Production

Ethylene is a gaseous hormone known for its role in fruit ripening, but it also influences plant growth. Plant cells have receptors for ethylene that enable it to play a role in certain physiological responses such as leaf senescence, seed germination and cell elongation (Hopson and Wessells, 1990). It has been found that chilling temperatures stimulate the production of ethylene in a number of plant species, but the significance of this increased production is not clear as it can neither be identified as a defence mechanism nor as a cause of chilling injury (Field, 1990).

Increased ethylene production in chilled plant tissues is the result of an increased ability of the tissue to synthesize the immediate precursor of ethylene, 1-aminocyclopropane-1-

carboxylic acid (ACC) (Wang and Adams, 1982). Although some studies have shown a correlation between chilling sensitivity and high levels of ethylene production, there are those that have shown that chilling-tolerant species also produce high amounts of ethylene in response to chilling temperatures (Field, 1990). It is interesting to note that ethylene production in chilling-tolerant species can occur at low temperatures as well as once the plant has been returned to ambient temperatures, whereas in most chilling-sensitive species it can occur only on return to ambient temperatures (Field, 1990). On return to ambient temperatures after chilling there is generally an initial increase in ethylene production, after which production declines even when precursor (ACC) levels are still high. This suggests that the step converting ACC to ethylene is easily damaged by chilling temperatures (Wang, 1982). It has been suggested that this step is associated with the cell membrane, which is easily damaged by chilling, because ethylene formation capability is notably species specific and so is the extent to which the cell membrane is damaged by chilling temperatures (Wang, 1982; Field, 1990). There is not yet enough knowledge of the enzymology and endogenous factors that control ethylene biosynthesis to identify the role it plays in chilling.

1.4.8 Cytological Responses (Ultrastructure)

Of the cytological reports available, most indicate rapid disorganization of cell organelles of chilling-sensitive plants in response to chilling stress (Graham and Patterson, 1982). More specifically, some of the ultrastructural changes observed in chilling-sensitive plants immediately after exposure to chilling include structural changes of proplastids, dilation and vesiculation of endoplasmic reticulum (ER), aggregation of inner membrane particles of tonoplasts and rupture of vacuoles (Yoshida *et al.*, 1986).

Although chilling affects many organelles, undoubtedly the most drastically affected are the plastids, which show swelling, disorganization of internal lamellae and reduced levels of plastid ribosomes after exposure to chilling stress (Wang, 1982). Kimball and Salisbury (1973), who studied the effects of low temperature on three grass species, found that chloroplast ultrastructure was affected more than any other organelle. Those authors suggested that the observed swelling of chloroplasts was due to a change in

membrane permeability caused by chilling, which resulted in osmotic change and therefore an increase in volume. Disappearance of the tonoplast has also been observed in response to chilling stress. Vacuoles contain phenolic substances, and plastids, such as chloroplasts, contain polyphenol oxidase, so destruction of the tonoplast membrane and a change in plastid membrane permeability would cause accelerated oxidation of phenolics resulting in products that might adversely affect the functioning of other organelles and membranes (Abe, 1990). Partial degradation of the tonoplast membrane has been shown to occur before the appearance of physical symptoms of chilling injury in chilled eggplant fruits (Wang, 1982). Ishikawa (1996) carried out ultrastructural studies on cultured mung bean (*Vigna radiata* var. Wilczek) cells to determine what changes occurred in the cell during the early stages of chilling. That author observed swelling, disruption of the vacuolar membrane and shrinking of the plasma membrane, but swelling was the event that occurred the earliest. Swelling of the cells in response to chilling started when clear areas appeared in the cytoplasm; these two events were thought to be due to partially damaged membranes, as leaky lytic vacuole membranes would release hydrolases into the cytoplasm causing clearings. Other changes that occurred during the early stages of chilling and thought to be associated with membrane deterioration were precipitated aggregates on vacuolar membranes, whorls of ER, vesiculated membranes inside plastids, swollen mitochondria and enlarged Golgi cisternae. The other two injuries, disruption of vacuoles and shrinking of the plasma membrane, occurred later and might have been associated with the destruction of the cytoskeleton. Chilling is thought to have a direct effect on microtubules, which form part of the cytoskeleton, and in some cells these are sensitive to cold and in others they are not (Wang, 1982).

Ultrastructural observations are very useful as they can often provide structural interpretations of biochemical and physiological changes caused by chilling stress; for example, the oxidative activity of mitochondria is adversely affected during chilling and accordingly alteration in mitochondrial structure is a very obvious change that occurs (Wang, 1982). Increased exposure to a chilling stress causes increased cellular disorganization, which supports the view that the degree of injury incurred is related to the duration of exposure.

1.5 The Present Study

The purpose of this study was to gain an understanding of the chilling response of recalcitrant seeds, as reduced temperature could have the potential to extend, rather than curtail, storage life span depending on the species. Selected physiological, biochemical and ultrastructural responses of recalcitrant seeds of *Ekebergia capensis* and *Avicennia marina* towards chilling stress were characterised.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Material

2.1.1 Species Used

Experiments were carried out using the seeds of *Ekebergia capensis* Sparrm. (Meliaceae) and *Avicennia marina* (Forssk.) Vierh. (Verbenaceae), both previously identified as being recalcitrant in nature (Berjak *et al.*, 1989; Pammenter *et al.*, 1998). Two batches of *E. capensis* seeds were used. The first was collected from a population of trees in Port Elizabeth at the end of the fruiting season, May 2000, and the second from a population of trees in the suburb of Phoenix, Durban at the end of the fruiting season, February 2001. Fruiting season varied with provenance. Fruit was harvested directly from the parent trees and transferred in plastic bags or buckets to the laboratory. Two batches of *A. marina* seeds were also used. The first was a small batch collected from a population of trees in Kenya during January 2001. These were used for some preliminary experiments as the fruiting season was a few months earlier than that of the second batch, which was collected from a population of trees in the Beachwood Mangrove Nature Reserve, Durban during the April 2001 fruiting season. Mature seeds, freshly fallen from the parent trees and with intact pericarps, were collected and transferred in buckets to the laboratory.

2.1.2 Treatment on Arrival in the Laboratory

E. capensis fruits were graded according to size using a 17 mm² mesh; green fruits larger than the mesh size were used. The selected fruits were stored in open trays at 16°C until the skins had turned red in colour; at this stage the fruit pulp (mesocarp) had softened considerably and the seeds could easily be removed by applying pressure to the fruits. Once the seeds had been removed, they were rinsed briefly with tap water to remove any residual fruit pulp and spread out on paper towel until the endocarps were dry. Once dry, they were dusted with Benomyl WP® powder to minimise fungal

contamination, placed in brown paper bags within clear polythene bags and stored at 16°C until the storage experiments were initiated.

The *A. marina* seeds (fruits/propagules) were first soaked in water for 30 min to remove the pericarps. The seeds were then surface sterilised in 1% sodium hypochlorite for 20 min, rinsed three times with distilled water and sprayed with a solution of Previcur N® (2.5 ml l⁻¹) in order to minimise microbial contamination. The seeds were stored in a layer, not more than five seeds deep, on plastic mesh suspended in buckets lined with wet paper towel. The buckets were sealed using clear polythene plastic wrap and stored in the light, as *A. marina* seeds are actively photosynthetic (Steinke and Naidoo, 1991). Storage experiments were initiated immediately due to the anticipated short storage life span of the seeds.

2.2 Storage Experiments and Sampling

2.2.1 *E. capensis*

The first batch of seeds, those collected in Port Elizabeth, was kept at 16°C for approximately one month before the initiation of storage experiments; this delay was due to circumstance and not originally intended. During this time, germination capability increased from 64 to 100%, assumed to be the outcome of continued maturation during storage, so it was concluded that this delay was beneficial rather than detrimental as the seeds were more mature. To assess the chilling response of this species, seeds were stored at 6 and 16°C, with a control at 25°C, in plastic honey jars filled with dry vermiculite. The jars were opened at regular intervals to aerate the seeds and prevent anoxic conditions. To determine the effect the seed endocarp had on longevity at each temperature, the seeds were stored both with and without the endocarp. The seeds were stored for a total of 12 weeks and were sampled once a week for germination, water content, respiration rate and leachate electrical conductivity. It was found that seeds stored without the endocarp succumbed readily to fungal contamination, but that seeds stored with the endocarp retained 100% germination after 12 weeks at 25, 16 and 6°C. It was decided that a second batch of seeds was needed in

order to assess the chilling response of the seeds at temperatures lower than 6°C, and to obtain samples for ultrastructural investigation.

The second batch of seeds, those collected in Phoenix, Durban, were also kept for approximately one month before the initiation of storage experiments to allow comparison with the first batch. The seeds were stored with endocarps at 0, 3, 6 and 16°C and were sampled once for germination, water content and transmission electron microscopy (TEM) after eight weeks due to limited seed numbers and the anticipated long storage life span at low temperatures (although those seeds stored at 0°C were sampled for germination after one week as well). Enzyme assays and sugar analysis could not be undertaken due to limited seed numbers; this was considered acceptable because *E. capensis* seeds were not as chilling sensitive as *A. marina* seeds and marked fluctuations in these parameters were not expected. Nevertheless, follow-up studies to assess these parameters are planned.

2.2.2 *A. marina*

The first small batch of seeds, collected in Kenya, was used in a preliminary experiment to verify previously used storage methods and anti-fungal treatments. It was found that *A. marina* seeds retained vigour and viability best when stored in the light and sprayed twice a week with a solution of Previcur N® (2.5 ml l⁻¹) to keep fungal contamination at a minimum. It was also beneficial to remove seeds with visible fungal contamination. The second batch of seeds, those collected in Durban, were stored at 6 and 16°C, with a control at 25°C, in order to assess the chilling response of this species. Some of the seeds were also kept at 25°C for four days before removal to 6°C as *A. marina* seeds have been noted to become more metabolically active after this time period (Pammenter *et al.*, 1984). The seeds were sampled once a week for germination, water content, respiration rate, leachate electrical conductivity, TEM, enzyme assays and sugar analysis. Due to the number of analyses performed and the nature of the seeds, it was not always possible to keep sampling intervals consistent for each temperature treatment.

2.3 Experimental Procedures

2.3.1 Water Content

The water content of individual axes and cotyledons from five replicate seeds for each storage treatment of both *E. capensis* and *A. marina* was determined gravimetrically after drying in an oven at 80°C for 48 h to achieve constant weight. The water content was expressed on a dry weight basis as grams of water per gram of dry weight (g g^{-1}).

2.3.2 Germination

2.3.2.1 *E. capensis*

Endocarps were removed from ten replicate seeds for each storage treatment by careful cracking with a pair of pliers. The seeds, with the testa intact, were rinsed in a solution of Previcur N® (2.5 ml l⁻¹) and aseptically plated onto 1% water agar set in 90 mm Petri dishes, with a maximum of five seeds per plate. The plates were sprayed with the Previcur N® (2.5 ml l⁻¹) solution before being sealed with Parafilm®. The seeds were set out to germinate in a controlled environment chamber (CONVIRON) set at 25°C. Germination was assessed daily and scored positive for those seeds with a radical of 2 mm or more in length.

2.3.2.2 *A. marina*

For each storage treatment, ten replicate seeds were placed in trays that contained moist vermiculite and set out to germinate in a germination room with a temperature of approximately 25°C. The seeds were kept moist and were assessed daily for germination. The seeds were scored positive for germination when the roots had extended to 2 mm or more.

2.3.3 Respiration

Respiration was measured as CO₂ production using an ADC (Hoddesdon, Herts, U. K.) 225.MK3 infrared gas analyser (IRGA) set up as a closed gas exchange system.

2.3.3.1 *E. capensis*

Seed endocarps were removed by careful cracking with a pair of pliers and for each storage treatment five replicates consisting of whole seeds with the testa intact were used. The seeds were placed in 6 cm³ glass test tubes that were subsequently inverted, flushed with CO₂ free air and sealed with a serum stopper. Three air samples were taken from each test tube using a 1 ml insulin syringe at the start of the experiment (t = 0 h) and after 60 min (t = 1 h). The above protocol was changed for the *A. marina* seeds in order to achieve more accurate results, as it was found that 6 cm³ was too small a volume for the amount of air removed for sampling; negative pressure started to build up.

2.3.3.2 *A. marina*

For each storage treatment, three replicates each consisting of one axis tip (the part containing the root primordia), and three replicates each consisting of one cotyledon disc, obtained using a 5 mm core borer, were used. The samples were placed in 50 cm³ glass test tubes that were sealed subsequently with suba® seal lids and flushed in series with CO₂ free air via interconnecting needles and plastic tubes. The test tubes were flushed in series to save time, as it took much longer to flush the 50 cm³ test tubes individually than it did to flush the 6 cm³ ones. Two air samples were taken from each test tube using a 1 ml insulin syringe at the start of the experiment (t = 0 h) and after 30 min of incubation in a 25°C water bath (t = 0.5 h).

For both *E. capensis* and *A. marina* the air samples were injected into a gas line where CO₂ free air was streamed at 0.5 l min⁻¹ into the IRGA, which was set in absolute mode. The CO₂ concentration within the sample test tubes was determined from the height of a pulse monitored on a chart recorder for each air sample. The difference between the mean peak height after 30 or 60 min and that at the start of the experiment [(t = 0.5 h) – (t = 0 h)] or [(t = 1 h) – (t = 0 h)] was used to read the CO₂ concentration in μmol mol⁻¹ off a standard curve. The standard curve was derived by injecting 1 ml air samples that contained known CO₂ concentrations; obtained from an ADC (Hodderson, Herts, U. K.) GD-600-10248 gas diluter. In order to express the results on a dry weight basis, each seed sample used was dried in an oven at 80°C for 48 h to achieve constant weight. The

respiration rate was expressed as nmol of CO₂ produced per gram of dry weight per second [nmol CO₂ (g dry weight)⁻¹ s⁻¹]. It should be noted that this was net CO₂ produced, as the test tubes used did not exclude light, although CO₂ uptake due to photosynthesis is expected to have been minimal because of the low light intensity in the laboratory.

2.3.4 Electrolyte Leakage

Electrolyte leakage was measured using a CM 100 (Reid and Associates, Durban, S. A.) multi-cell conductivity meter at the setting of 4 V.

2.3.4.1 *E. capensis*

The seed endocarp and testa were first removed and the seeds subsequently rinsed for a few seconds in distilled water. Conductivity of the electrolyte leakage into 3 ml of distilled water was measured individually for five replicate seeds for each treatment.

2.3.4.2 *A. marina*

Five seeds for each treatment were used, from which four root primordia were excised from each axis tip (i.e. each replicate consisted of four individual root primordia and not the entire axis tip as was used in the measurement of respiration rate), and a cotyledon disc cut using a 5 mm core borer. Conductivity of the electrolyte leakage into 2 ml of distilled water was measured for each replicate of four root primordia and each replicate cotyledon disc.

For both *E. capensis* and *A. marina*, the required number of wells in a tray were filled with the specified amount of distilled water, either 2 or 3 ml, and the conductivity read over a 5 min period (5 readings at 60 s intervals) to serve as the blank reading for each well. One seed, group of four root primordia or cotyledon disc was placed per well and the conductivity of the electrolyte leakage measured over an 18 h period (36 readings at 1800 s intervals). The tray was then removed from the conductivity meter, covered with clear polythene plastic wrap and placed at -20°C overnight; to disrupt cell walls and release the maximum amount of electrolytes into the distilled water. After thawing at

25°C the polythene plastic wrap was removed and the conductivity of the electrolyte leakage measured over a 10 min period (10 readings at 60 s intervals) to serve as the maximum electrolyte leakage reading for each well. The electrolyte leakage per well was calculated as leakage after 18 h minus the blank reading and was expressed as a % of the total the seed could leak. After use, trays were washed with a 2% solution of Contrad® Concentrate followed by rinsing with distilled water.

2.3.5 Enzyme Extraction

Enzyme extraction was carried out on five *A. marina* seeds for each storage treatment. The axes and cotyledons were freeze-dried separately and stored at 16°C in plastic cryotubes in a box containing activated silica gel until further use. The freeze-dried axes and cotyledons were separately ground using a mortar and pestle and divided into three pseudo replicates each; consisting of 0.2 g of material. The enzyme extraction was done according to the method used by Bailly *et al.* (1996). Each 0.2 g of axes or cotyledons was homogenized with 0.2 g of polyvinylpyrrolidone, to remove any polyphenolics, in 6 ml of 0.1 M phosphate buffer (pH 7.8) containing 2 mM dithiothreitol, 0.1 mM EDTA and 1.25 mM PEG-4000. The homogenate was centrifuged for 15 min at 16000 g and the resulting supernatant desalted on a PD10 Sephadex column. The resulting pure solution of proteins in a phosphate buffer was used for the enzyme assays. All steps of the extraction procedure were carried out at 1-4°C.

2.3.6 Protein Determination

The protein content of each enzyme extract was determined according to the method of Bradford (1976) using the Bio-Rad® protein assay kit. The results were expressed as mg of protein per ml; read off a standard curve derived using known amounts of bovine serum albumin.

2.3.7 Enzyme Assays

Superoxide dismutase, catalase and glutathione reductase activities of each enzyme extract were measured in triplicate, and the results presented correspond to the means of the nine values obtained (Three assays on each of three extracts).

2.3.7.1 Superoxide dismutase (SOD)

SOD activity was measured according to Gianopolitis and Ries (1977). The reaction mixture contained 1.3 mM riboflavin, 13 mM methionine, 63 μ M nitroblue tetrazolium (NBT) in 0.1 M phosphate buffer (pH 7.8) and 100 μ l of enzyme extract in a final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. The reaction mixture was placed in glass test tubes in a 25°C water bath and illuminated with a fluorescent lamp. Identical test tubes that were not illuminated served as blanks. After illumination for 15 min, absorbance was measured at 560 nm. One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50%, and SOD activity of the extracts was expressed as units SOD (mg protein)⁻¹.

2.3.7.2 Catalase (CAT)

CAT activity was measured according to Clairbone (1985). The reaction mixture contained 3.125 mM H₂O₂ in 50 mM phosphate buffer (pH 7.0) and 200 μ l of enzyme extract in a total volume of 3 ml. Catalase activity was estimated by the decrease in absorbance of H₂O₂ at 240 nm at 25°C and was expressed as nmol H₂O₂ decomposed (mg protein)⁻¹ min⁻¹.

2.3.7.3 Glutathione reductase (GR)

GR activity was determined according to Esterbauer and Grill (1978), by following the rate of NADPH oxidation at 340 nm at 25°C. The reaction mixture contained 0.5 mM NADPH, 10 mM oxidized glutathione, 3 mM MgCl₂ in 0.1 M phosphate buffer (pH 7.8), and 100 μ l of enzyme extract in a total volume of 400 μ l. GR activity was expressed as nmol NADPH oxidized (mg protein)⁻¹ min⁻¹.

2.3.8 Sugar Extraction

Sugar extraction was carried out on five *A. marina* seeds for each storage treatment. The axes and cotyledons were freeze-dried separately and stored in plastic cryo-tubes in a box containing activated silica gel until further use. The freeze-dried axes and cotyledons were separately ground using a mortar and pestle and divided into three

pseudo replicates each; consisting of 50 mg of material. Each sample was homogenized in 1 ml of 80% ethanol that contained a standard sugar [1.5 mg melezitose (ml 80% ethanol)⁻¹]. The homogenate was heated for 30 min in an 80°C water bath and then centrifuged for 10 min at 12500 g. The supernatant was removed and retained, and 0.5 ml 80% ethanol added to the pellet, homogenized and centrifuged as before. The supernatant was removed and retained, and 0.35 ml 80% ethanol was added to the pellet, homogenized and re-centrifuged. The supernatant was removed and retained and the pellet discarded. For each replicate, the retained supernatant was placed in an Eppendorf tube in an RCT 60 Evaporator and evaporated at 30°C for approximately 10 h, or until all alcohol had evaporated. Once evaporated, samples were kept at -20°C until further use.

2.3.9 Sugar Analysis

The evaporated samples were removed from -20°C, dissolved in 200 µl of double-distilled water and filtered through a 0.45 µm cellulose acetate membrane. Ten µl of the filtered sample was injected into a high-pressure liquid chromatography (HPLC) column using 80% acetonitrile as a solvent. The specific types of sugars present in the samples were determined from retention time in the column, and the concentration of each was calculated using peak area monitored on a chart recorder; compared with the area of a known amount of that sugar. The standard sugar, melezitose, was used to calculate the loss coefficient, which was added to the final concentration calculated for each sugar. The concentration of each sugar in the sample was expressed as µg (mg dry mass)⁻¹, and the results presented represent the mean of three replicates.

2.3.10 Specimen Preparation for Transmission Electron Microscopy (TEM)

Five *A. marina* seeds for each storage treatment were used, from which one root primordium was excised from each, and five *E. capensis* seeds for each storage treatment were used and the axes excised. The root primordia and axes were cut on one side in order to reduce size and allow penetration of the fixative. The specimens were fixed overnight in 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2) containing 0.5% caffeine. They were then washed in a 0.1 M phosphate buffer (pH 7.2) (3 x 5

min), post-fixed in 0.5% (m/v) osmium tetroxide for 1 h, and washed again in phosphate buffer (3 x 5 min). Subsequent dehydration of the specimens was carried out in a graded series of acetone [30%, 50%, 75% and 100% (v/v)]. The 100% (v/v) acetone was then replaced with a 1: 1 mixture of epoxy resin: acetone and left for 4 h in a turntable at room temperature. The material was then immersed in full resin overnight at room temperature for infiltration. Polymerisation of individual specimens in fresh resin in silicone wells was carried out in a 70°C oven for 10 h.

2.3.11 Microtomy and Microscopy

The resin-embedded specimens were sectioned using a Reichert-Jung Ultracut E microtome, post-stained with lead and viewed using a Jeol JEM 1010 transmission electron microscope. Images were recorded photographically on Kodak TEM film.

CHAPTER 3 RESULTS

3.1 Physiological Responses

3.1.1 Water Content

3.1.1.1 *E. capensis*

The water content of *E. capensis* seeds maintained in storage at 6 and 16°C with the endocarp remained relatively stable throughout the 12 weeks of storage (Figure 1: A and B). The minor fluctuations evident may have been due to the relatively small representative seed population used, as the effect of individual seeds would have been magnified. Water content did not remain as stable when seeds were stored at 25°C with the endocarp, the axes being affected more than the cotyledons (Figure 1: C).

If the endocarp had been removed from the *E. capensis* seeds, water was lost more readily during storage. Even at the low temperatures of 6 and 16°C, water content decreased steadily with time (Figure 2: A and B). Water content decreased even further, and more rapidly, in those seeds stored at 25°C without the endocarp (Figure 2: C). In all cases the axes were affected more than the cotyledons.

3.1.1.2 *A. marina*

The water content of the *A. marina* seeds remained relatively stable throughout storage, although slight increases were evident at all temperatures (Figure 3). The wet storage conditions used were therefore successful at maintaining the initial water content of the seeds and preventing further water loss.

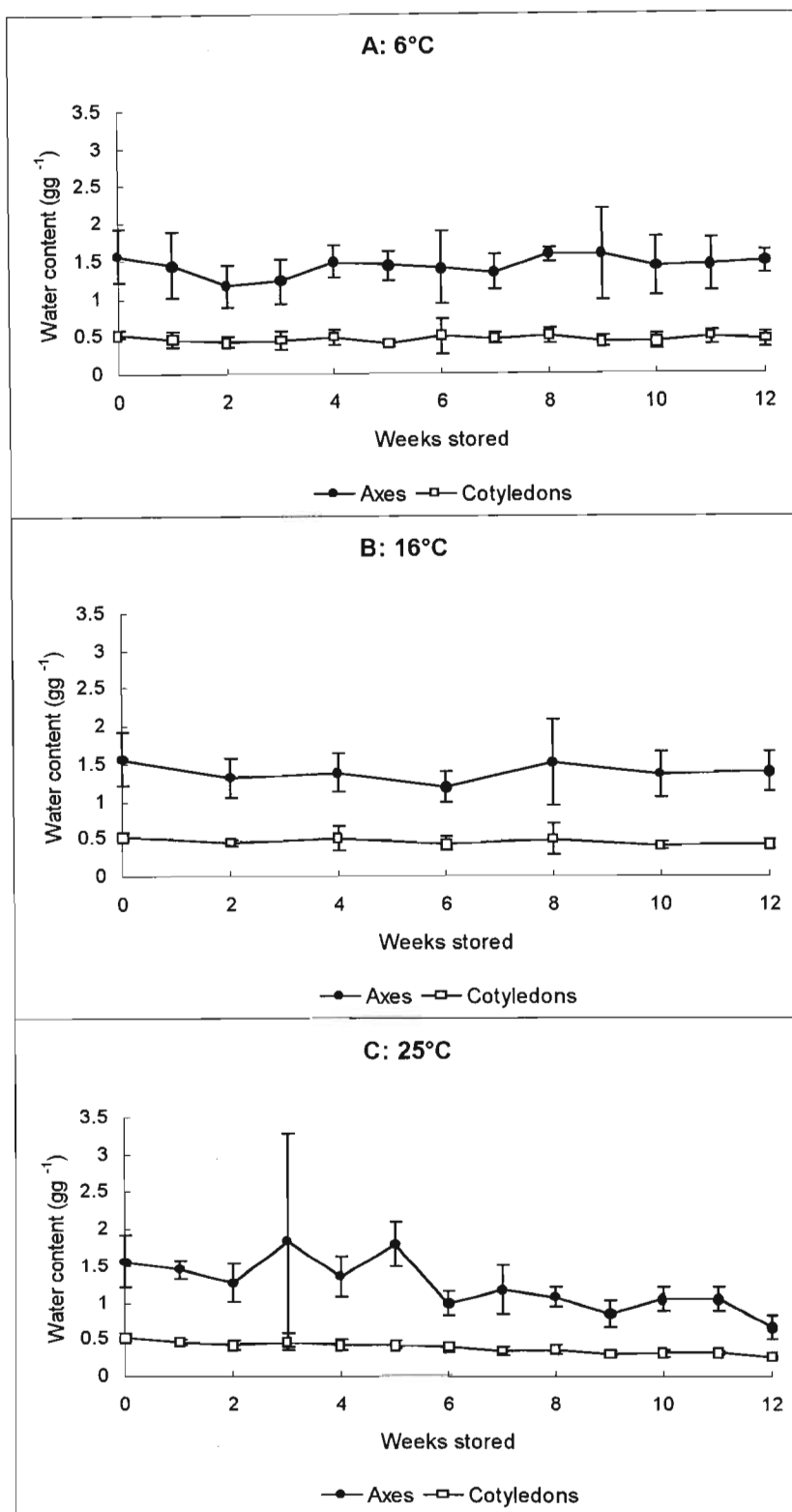


Figure 1: Water content of *E. capensis* axes and cotyledons during storage with the endocarp at different temperatures. The vertical bars correspond to standard deviation.

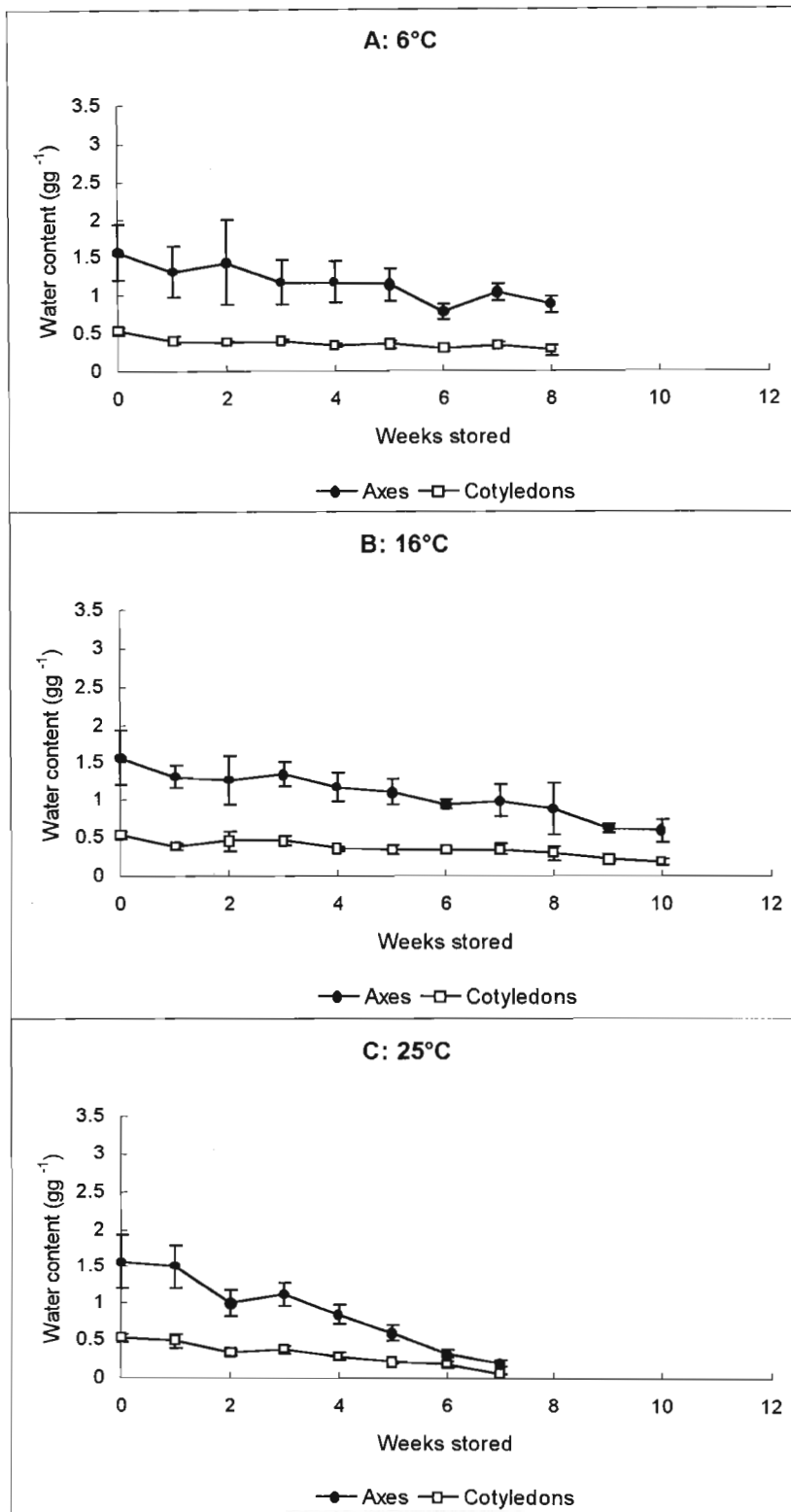


Figure 2: Water content of *E. capensis* axes and cotyledons during storage without the endocarp at different temperatures. The vertical bars correspond to standard deviation.

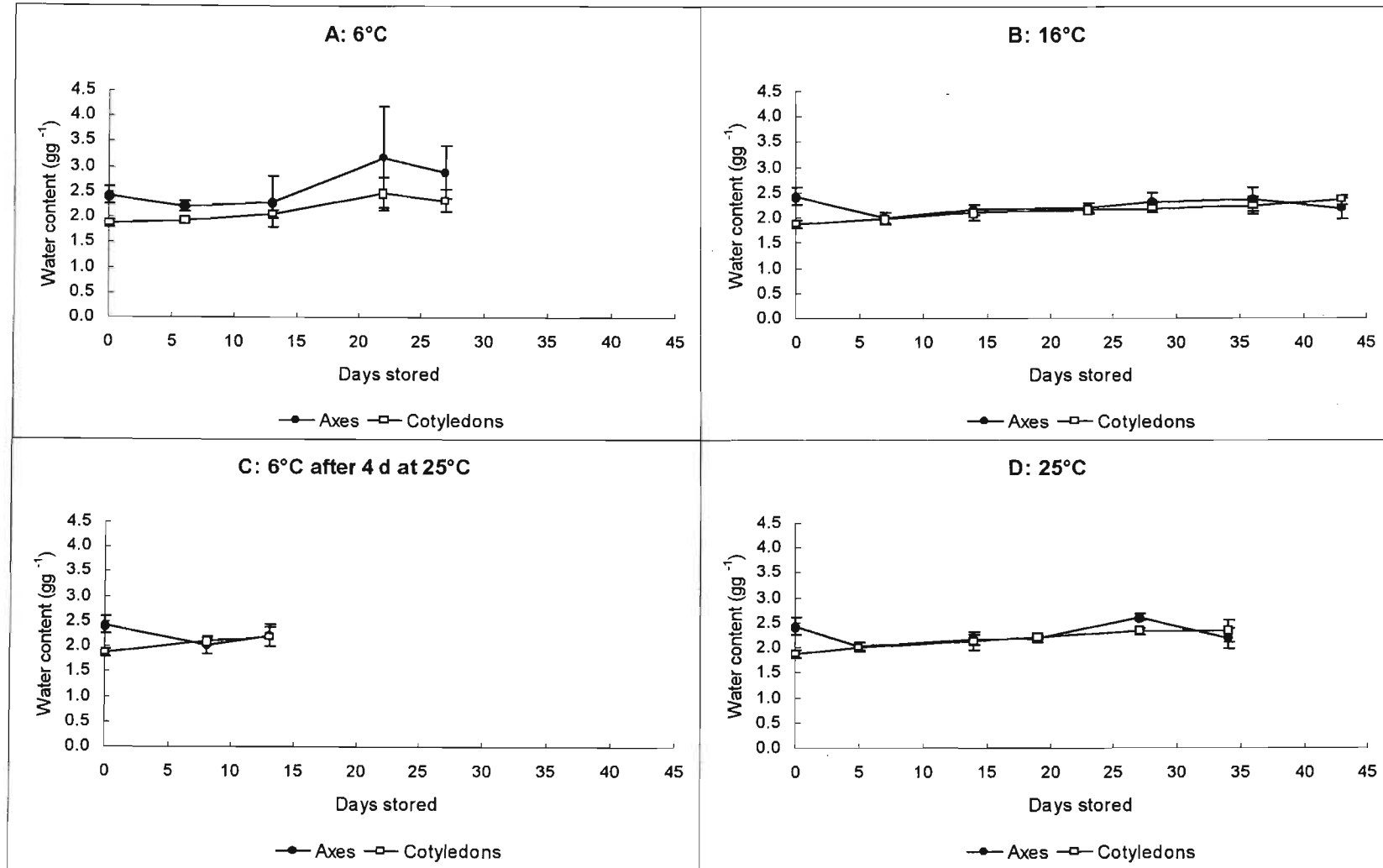


Figure 3: Water content of *A. marina* axes and cotyledons during storage at different temperatures. The vertical bars correspond to standard deviation.

3.1.2 Germination Capability

3.1.2.1 *E. capensis*

Germination capability remained 100% when *E. capensis* seeds were stored with the endocarp at 6, 16 and 25°C (Figure 4), although vigour, assessed by noting the number of days taken to germinate, did decrease slightly after storage at 25°C (compare Tables 1, 2 and 3). The retention of 100% germination capability could be linked to the initial water content being retained when the endocarp was left intact (Figure 1), but it is also likely that reduced fungal contamination played an important role. *Ekebergia capensis* seeds stored without the endocarp at 6°C succumbed to fungal contamination after 8 weeks and the experiment had to be terminated even though germination was still high (Figure 4: A). The same occurred with those seeds stored without the endocarp at 25°C, although germination had already declined considerably by 7 weeks of storage (Figure 4: C). Those seeds stored without the endocarp at 16°C retained a relatively high germination capability for a comparatively longer period, 10 weeks, than those stored without the endocarp at 6 and 25°C (Figure 4: B). Seeds stored with the endocarp at 3°C for 8 weeks had a germination capability of 40%, and those stored with the endocarp at 0°C for 1 week had a germination capability of 50%. After 8 weeks at 0°C the seeds succumbed to fungal contamination when set out to germinate. (These data are not shown graphically or in tables).

3.1.2.2 *A. marina*

When *A. marina* seeds were wet stored at 25°C, there was an initial increase in germination capability after 4 days (Figure 5). Germination then decreased gradually over 35 days, after which there was a sharp decline (Figure 5). The same initial increase in germination was seen in those seeds stored at 16°C, but after 10 days there was a steady, more rapid decline in germination than that observed at 25°C (Figure 5). At 6°C, germination capability declined rapidly (Figure 5). Only 20% germination was retained after the first week of storage, and by 27 days the seeds had lost their ability to germinate (Figure 5). There was also no initial increase in germination capability as was seen in those seeds stored at 25 and 16°C. Seeds stored at 6°C after 4 days at 25°C achieved 70% germination after the first week of storage, whereas those seeds placed

directly at 6°C achieved only 20% germination after the first week (Figure 5). This initial high germination capability did not last, and a few days later the seeds had lost their ability to germinate (Figure 5).

Days	Germination % for each week of storage												
	0	1	2	3	4	5	6	7	8	9	10	11	12
1													
2													
3	70	50	20	30		40	40	60	80	80	50	40	80
4	100	80	90	90	100	90	100	90	100	100	90	100	100
5		90	100	100		100		100			100		
6													
7													
8		100											

Table 1: The effect of storage time on the vigour of *E. capensis* seeds stored with the endocarp at 6°C.

Days	Germination % for each week of storage						
	0	2	4	6	8	10	12
1							
2							
3	70	30	30	20	40	60	30
4	100	90	90	100	70	90	80
5		100	100		100	100	100

Table 2: The effect of storage time on the vigour of *E. capensis* seeds stored with the endocarp at 16°C.

Days	Germination % for each week of storage												
	0	1	2	3	4	5	6	7	8	9	10	11	12
1													
2													
3	70	50			40	20	20	10			20		10
4	100	100	50	80	100	70	100	60	90	50	70	20	
5			80	100		90		80	100	90	80	60	70
6			90							100	100	80	90
7			100			100		90				90	100
8								100				100	

Table 3: The effect of storage time on the vigour of *E. capensis* seeds stored with the endocarp at 25°C.

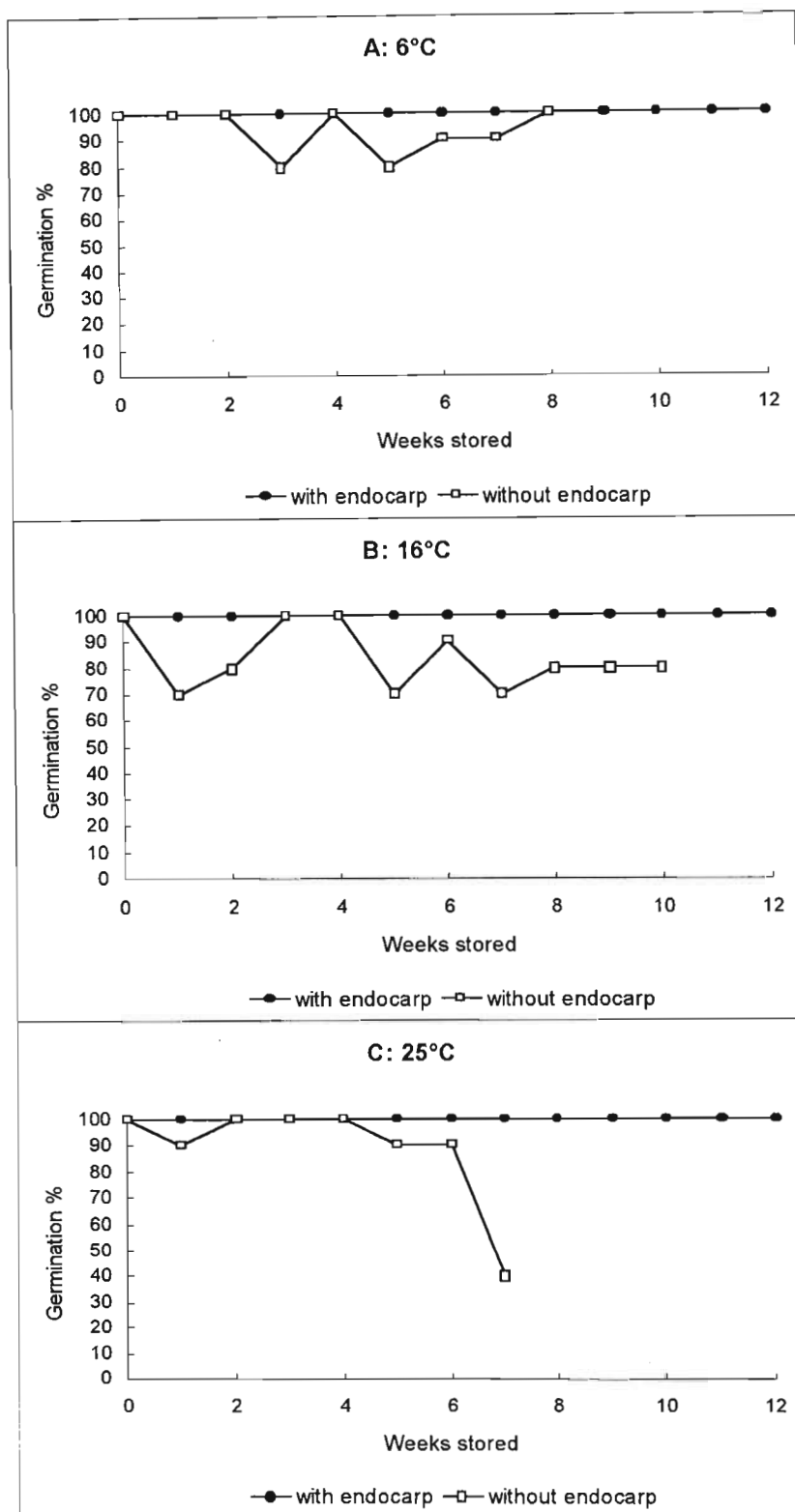


Figure 4: The effect of storage time on the germination capacity of *E. capensis* seeds stored with and without the endocarp at different temperatures.

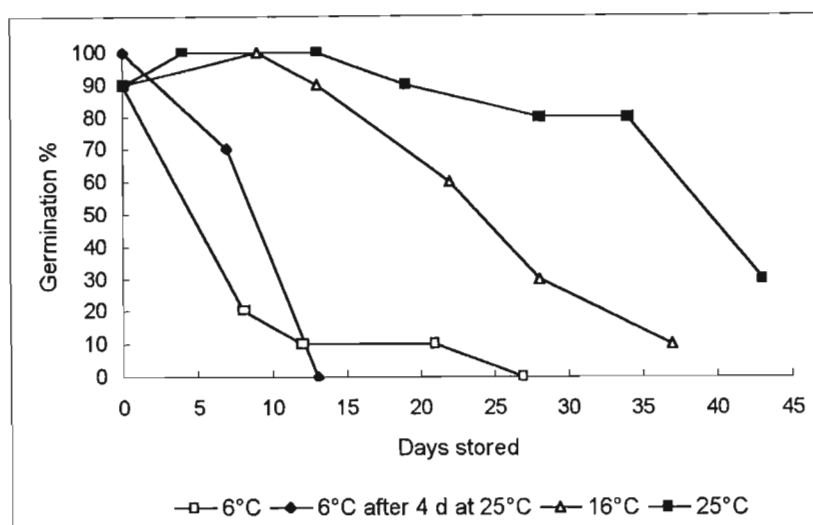


Figure 5: The effect of storage time on the germination capability of *A. marina* seeds stored at 6, 16 and 25°C and 6°C after 4 d at 25°C.

3.1.3 Respiration Rate

3.1.3.1 *E. capensis*

During storage with and without the endocarp at 25°C there was a slight initial increase in respiration rate over the first 2 weeks (Figure 6: C), but at both 6 and 16°C seeds stored without the endocarp showed a notable initial increase in respiration rate (Figure 6: A and B). It is interesting to note that seeds stored with the endocarp showed a notable initial increase only when stored at 6°C (Figure 6: A), and that in seeds stored without the endocarp the initial increase in respiration rate was $0.136 \text{ nmol of CO}_2 \text{ (g dry weight)}^{-1} \text{ s}^{-1}$ higher at 6°C than at 16°C (Figure 6: A and B). The increasing respiration rate evident after 4 weeks of storage at 25°C with the endocarp (Figure 6: C) could have been due to an acceleration of the events of germination, including a rise in respiration rate.

3.1.3.2 *A. marina*

The respiration rate of the axes and cotyledons from seeds stored at 25°C decreased up until 13 days of storage and then began to increase, but after 34 days of storage a decrease was evident again (Figure 7: D). The increase in respiration rate coincided

with the start of a gradual decline in germination capability, and the decrease with a sudden drop to 30% germination capability (Figure 5). An increased respiration rate followed by a subsequent decrease can also be seen for those seeds stored at 6 and 16°C, but a second increase in respiration rate, not observed for those seeds stored at 25°C (Figure 7: D), occurred (Figure 7: A and B). It is interesting to note that at 6°C the initial increase in respiration rate occurred when germination capability was very low, 20 to 10%, and that the second increase following the decrease in respiration rate occurred once germination capability had been lost (Figure 5). Similarly, at 16°C the initial increase in respiration rate was recorded when germination had dropped to 30%, and once again the second increase following the decrease in respiration rate occurred when germination was at its lowest; the respiration rate had remained relatively stable up until 22 days when germination capability was still a reasonable 60% (Figure 7: B and Figure 5). When seeds were stored at 6°C after 4 days at 25°C, the same trend in respiration rate was evident; that is increased respiration once germination capability had declined (Figure 7: C). There may be an inverse relationship between germination capability and respiration.

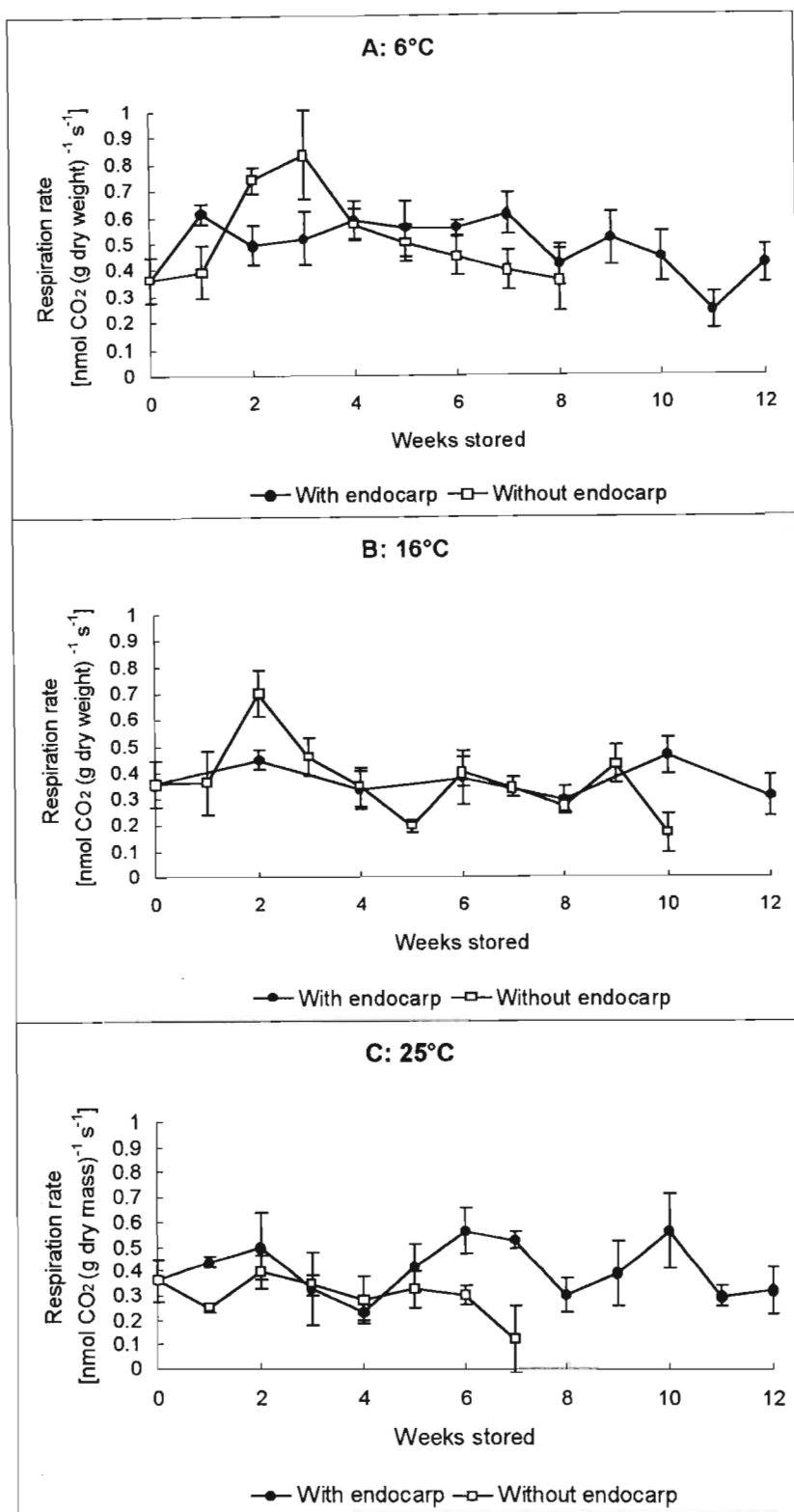


Figure 6: The effect of storage time on the respiration rate of *E. capensis* seeds stored with and without the endocarp at different temperatures. The vertical bars correspond to standard deviation.

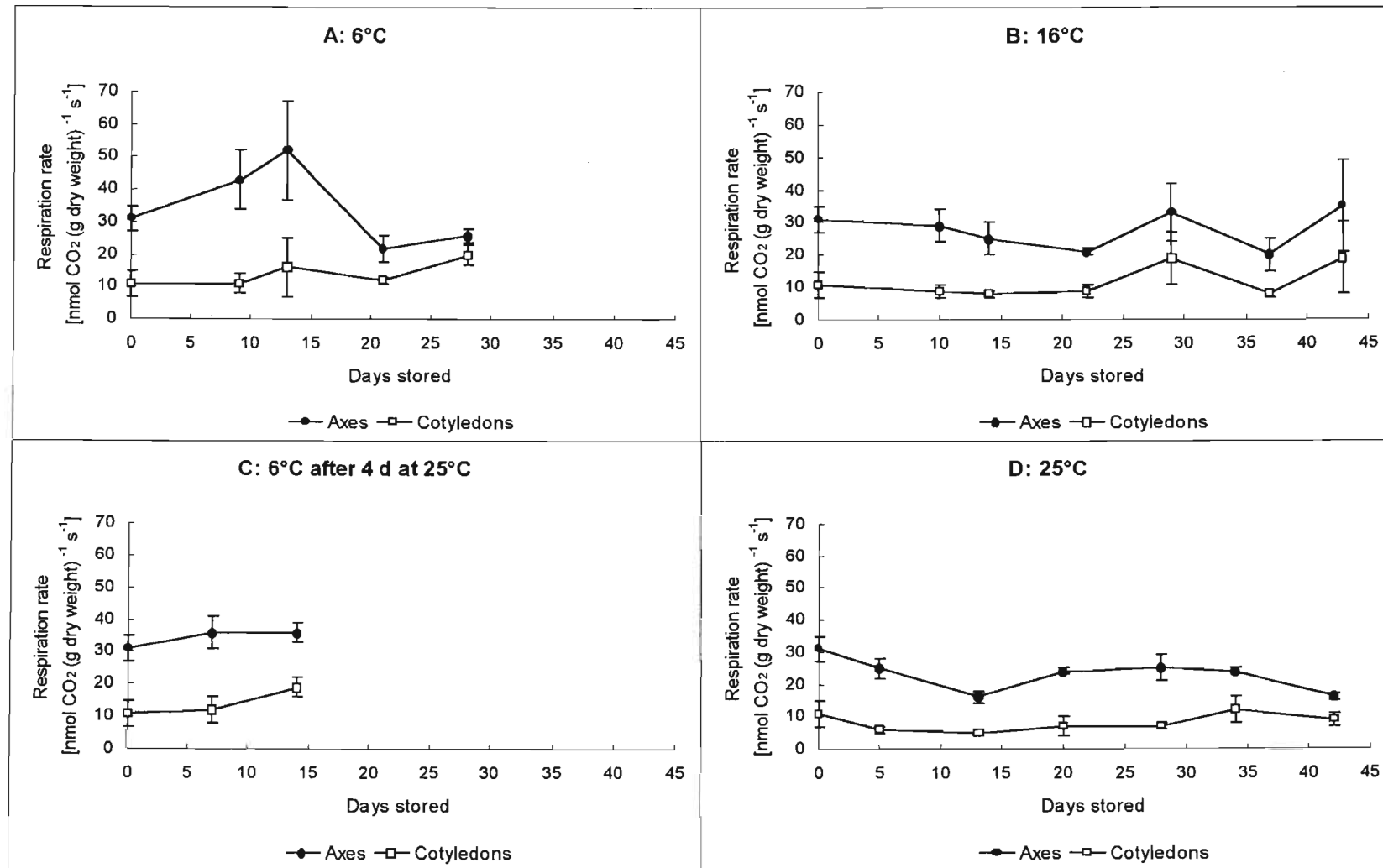


Figure 7: The effect of storage time on the respiration rate of axes and cotyledons from *A. marina* seeds stored at different temperatures. The vertical bars correspond to standard deviation.

3.1.3 Electrolyte Leakage

3.1.4.1 *E. capensis*

Seeds stored with the endocarp at 6, 16 and 25°C showed a slow, gradual increase in electrolyte leakage over the storage time of 12 weeks (Figure 8). It is interesting to note that seeds stored without the endocarp showed an obvious increase in electrolyte leakage over the first 2 weeks of storage at 6, 16 and 25°C (Figure 8: A, B and C). After 2 weeks this increase reverted back to the same level as the control, but at 25°C electrolyte leakage increased steadily again whereas it remained relatively stable at 6 and 16°C.

3.1.4.2 *A. marina*

The electrolyte leakage of the *A. marina* seed material is not shown, as there were no trends with storage time and no differences between temperature treatments. This may have been due to the cut surfaces on the samples used. A different sampling technique will have to be used if significant results are to be obtained.

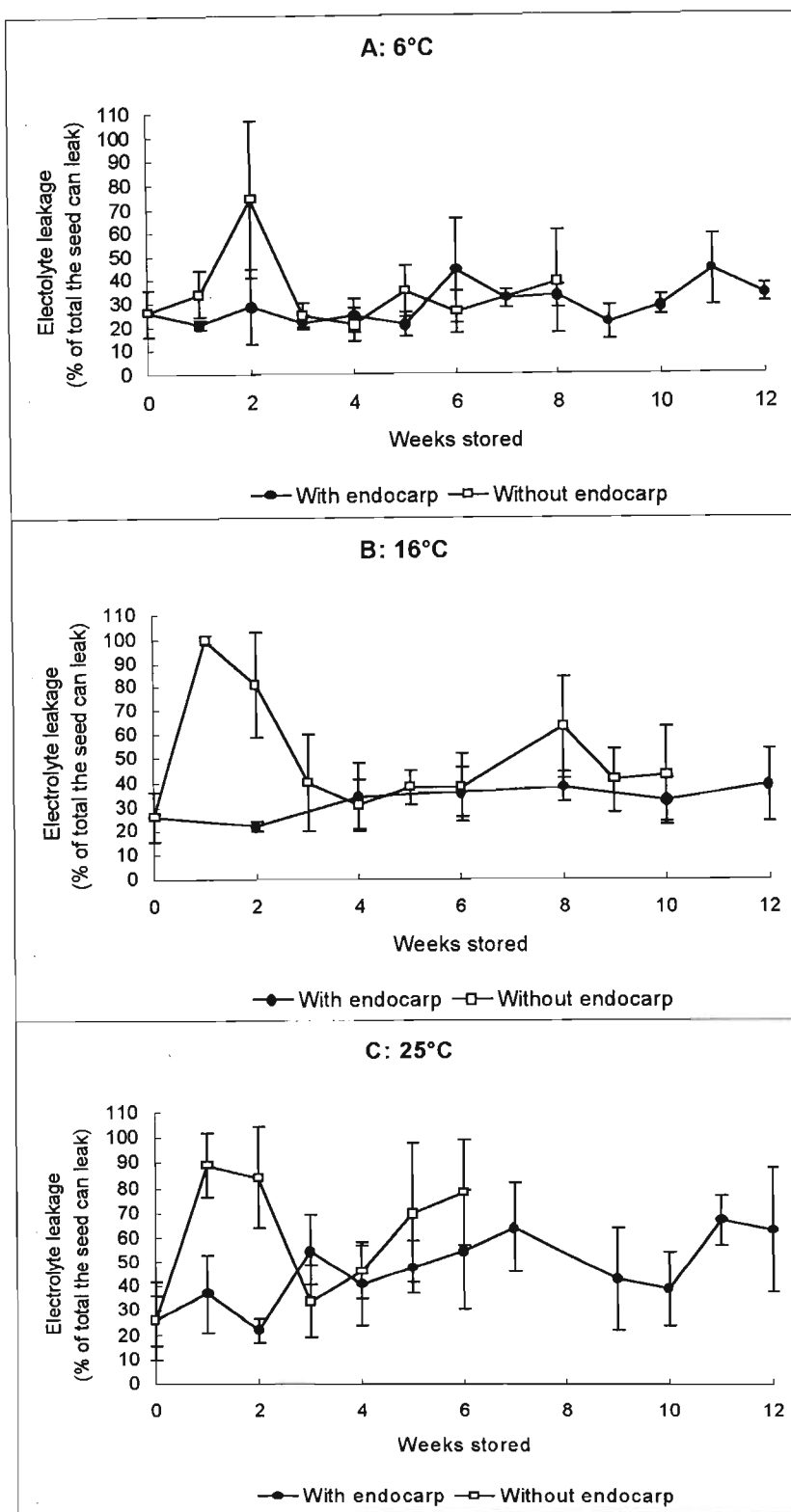


Figure 8: The effect of storage time on the electrolyte leakage of *E. capensis* seeds stored with and without the endocarp at different temperatures. The vertical bars correspond to standard deviation.

3.2 Biochemical Responses

3.2.1 Change in Enzyme Activity

3.2.1.1 Superoxide dismutase (SOD)

An initial decrease in SOD activity in both the axes and the cotyledons occurred when *A. marina* seeds were stored at 16 and 25°C (Figure 9: B and D), and similarly, a decrease was also seen in the axes of those seeds stored at 6°C after 4 days at 25°C (Figure 9: C). These decreases occurred when germination was still very high; for those seeds stored at 16 and 25°C germination was 100% and for those seeds stored at 6°C after 4 days at 25°C germination was 70% (Figure 5). In contrast to this, an initial increase in SOD activity occurred when the seeds were placed directly at 6°C (Figure 9: A) while germination was only 20% at the time (Figure 5). It is interesting to note that SOD activity in the axes of those seeds stored at 25°C for 43 days was the same in the axes of those seeds stored at 6°C for 8 days (Figure 9: A and D) coincident with 30% germination (Figure 5). Cotyledons of those seeds stored at 6°C after 4 days at 25°C showed the same increase as those seeds placed directly at 6°C (Figure 9: A and C). Interestingly, an increase in SOD activity did not occur in the axes of those seeds stored at 16°C, in fact there was quite a marked initial decrease, and a decrease also occurred in the cotyledons (Figure 9: B).

3.2.1.2 Catalase (CAT)

Catalase activity was not detected in any of the *A. marina* seed samples.

3.2.1.3 Glutathione reductase (GR)

There was an increase in GR activity in response to chilling stress. Control axes did not contain a measurable amount of this enzyme, and axes from seeds stored at 25°C started accumulating GR only after 28 days of storage (Figure 10: D). It appears that the greater the chilling stress, the more rapidly GR activity increases. This is evident when comparing GR activity in the axes and cotyledons of those seeds stored at 6°C and those stored at 16°C after 8 days (Figure 10: A and B). It is interesting to note that axes from seeds stored at 25°C for 4 days prior to storage at 6°C do not show the same rapid

increase in GR activity as those placed directly at 6°C, but that the cotyledons do (Figure 10: A and C). This could indicate a difference in the response of axes and cotyledons to chilling stress. Glutathione reductase activity was higher in the control cotyledons than in the axes, but it is apparent that the difference in GR activity between the axes and the cotyledons remains constant. Interestingly, the same extent of GR activity is reached after 8 days at 6°C after storage at 25°C for 4 days that is reached after 43 days at 25°C (Figure 10: C and D) - the only difference being the time elapsed and the germination, which is 70 and 30% respectively.

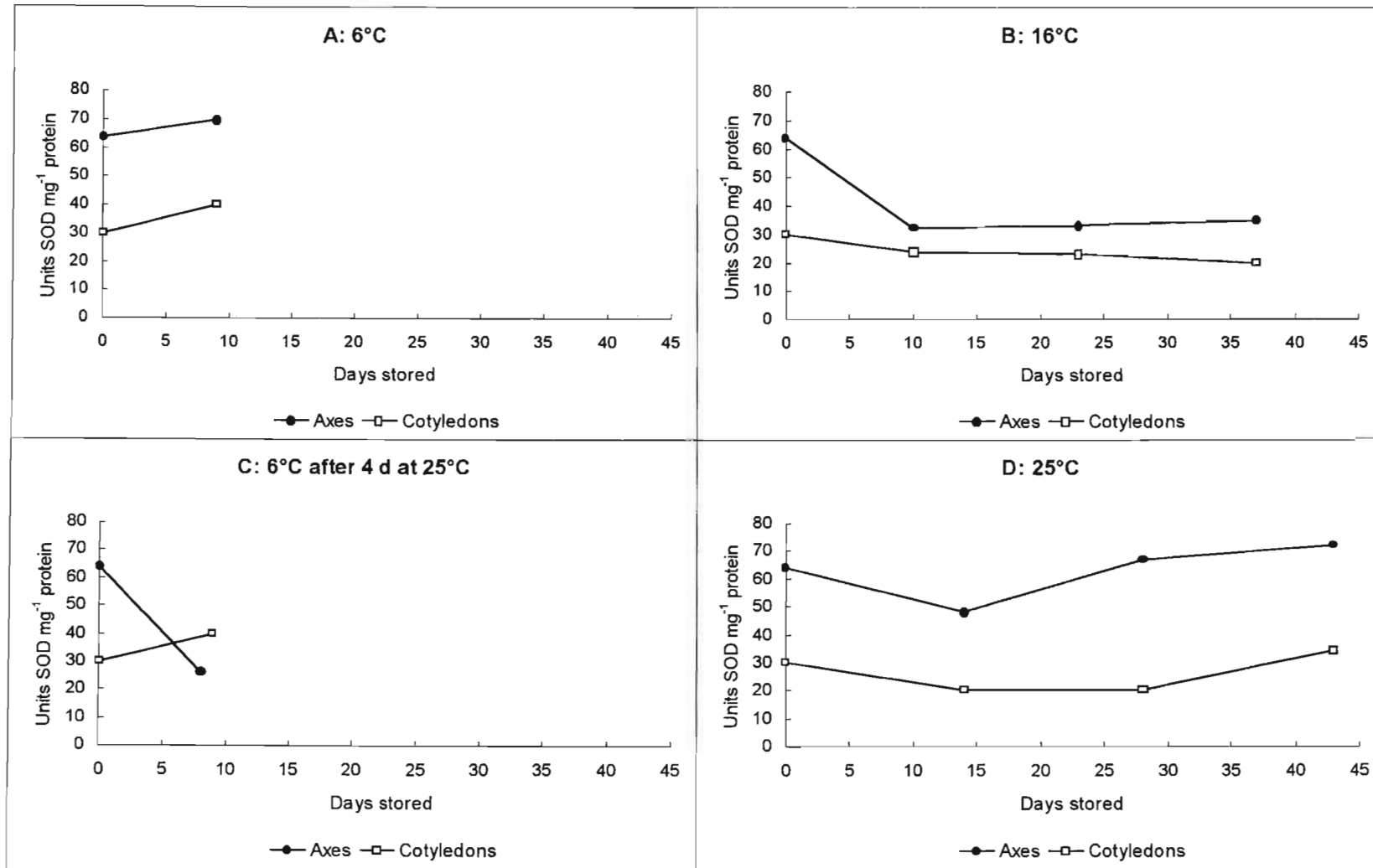


Figure 9: The effect of storage time on the activity of superoxide dismutase in the axes and cotyledons of *A. marina* seeds stored at different temperatures. The standard deviation is not shown as it was mostly below 1 unit of SOD mg⁻¹ protein.

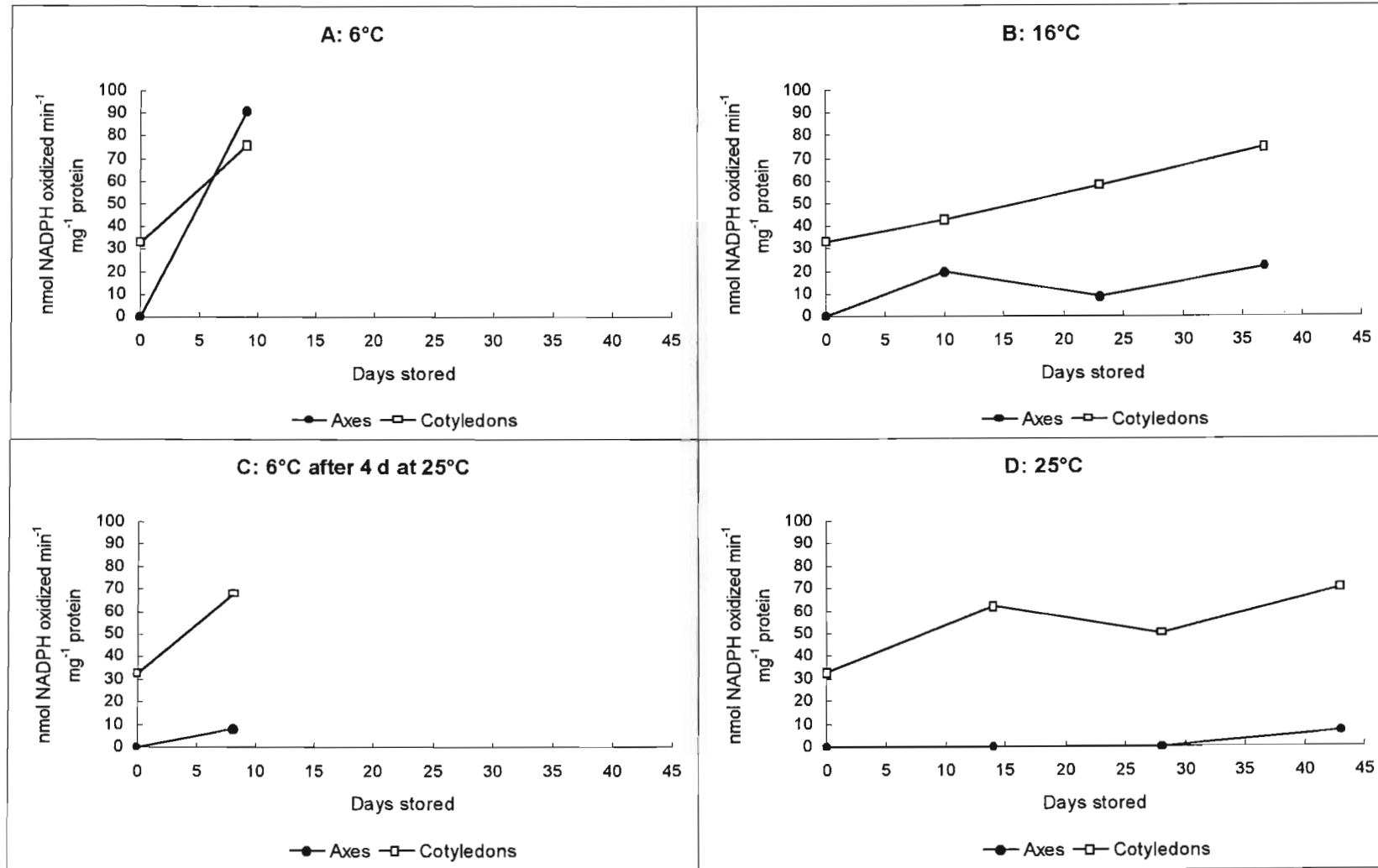


Figure 10: The effect of storage time on the activity of glutathione reductase in the axes and cotyledons of *A. marina* seeds stored at different temperatures. The standard deviation is not shown as it was below 1 nmol of NADPH oxidized min⁻¹ mg⁻¹ protein.

3.2.2 Sugar Metabolism

Fructose, glucose, sucrose and stachyose were found in the axes of *A. marina*. For the seeds stored at 6 and 16°C the same trends are evident. Sucrose and stachyose show an initial increase, while glucose and fructose show an initial decrease; although these were only marginal (Figure 11: A and B). For those seeds stored at 6°C after 4 days at 25°C the same trends can be seen for sucrose and fructose, but stachyose decreases and glucose remains more or less the same (Figure 11: C). After 23 days of storage at 16°C the level of sucrose continued to increase while stachyose decreased (Figure 11: B), whereas at 25°C the opposite occurred (Figure 11: D). Over the first 13 days at 25°C there is an increase in total sugars in the axes (Figure 11: D). A corresponding decrease in stachyose is evident in the cotyledons (Figure 12: D), indicating that sugars may have been imported into the axes from the cotyledons.

Sucrose and stachyose were found in the cotyledons of *A. marina*. Sucrose levels were consistently low throughout storage (Figure 12), this in contrast to the relatively high levels found in the axes (Figure 11). Those seeds stored at 6°C immediately, and 6°C after 4 days at 25°C show an initial increase in stachyose (Figure 12: A and C), as do those stored at 16°C but not to the same extent (Figure 12: B). Seeds stored at 25°C show a gradual decrease in stachyose (Figure 12: D).

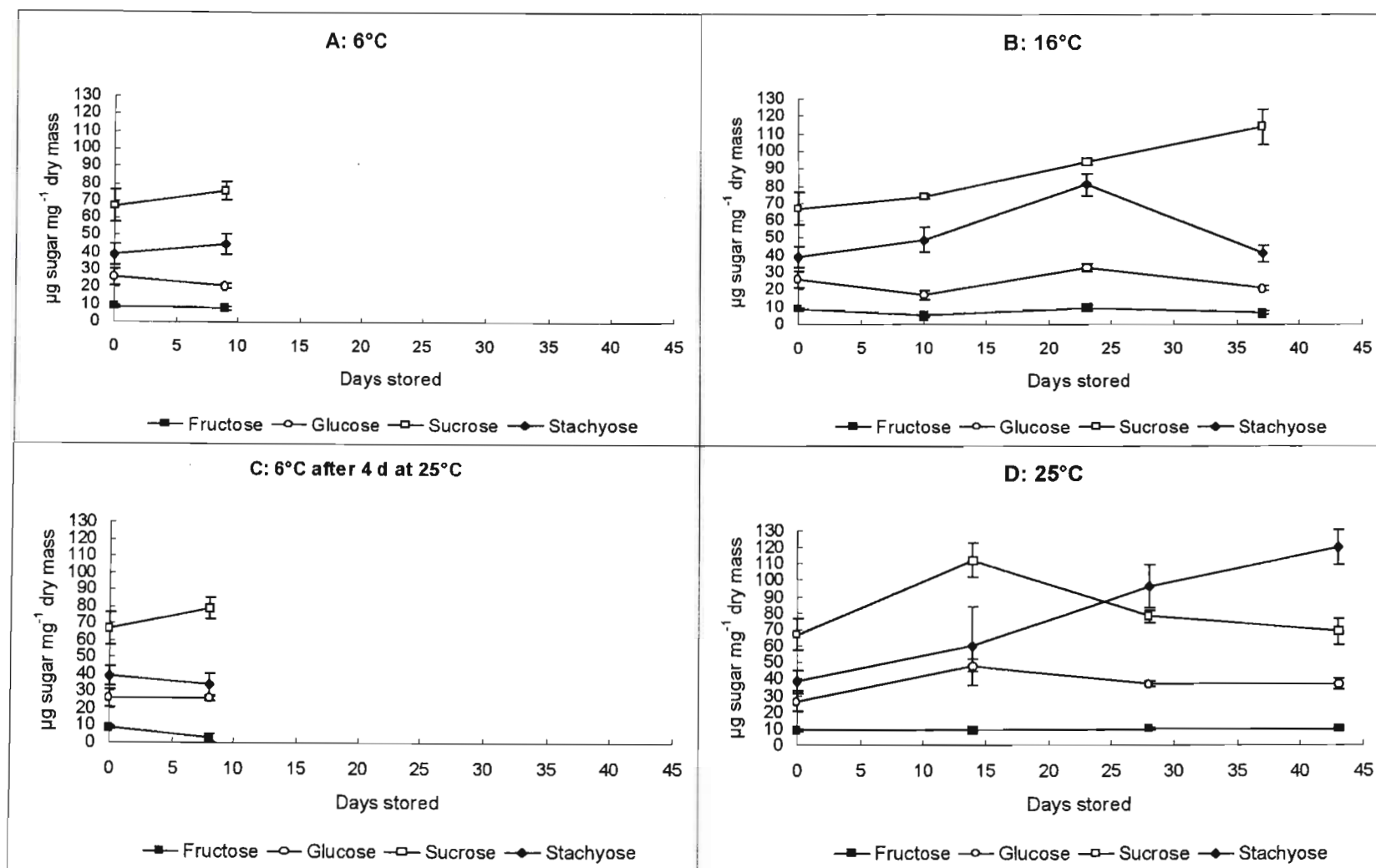


Figure 11: The effect of storage time on sugar metabolism in the axes of *A. marina* seeds stored at different temperatures. The vertical bars correspond to standard deviation.

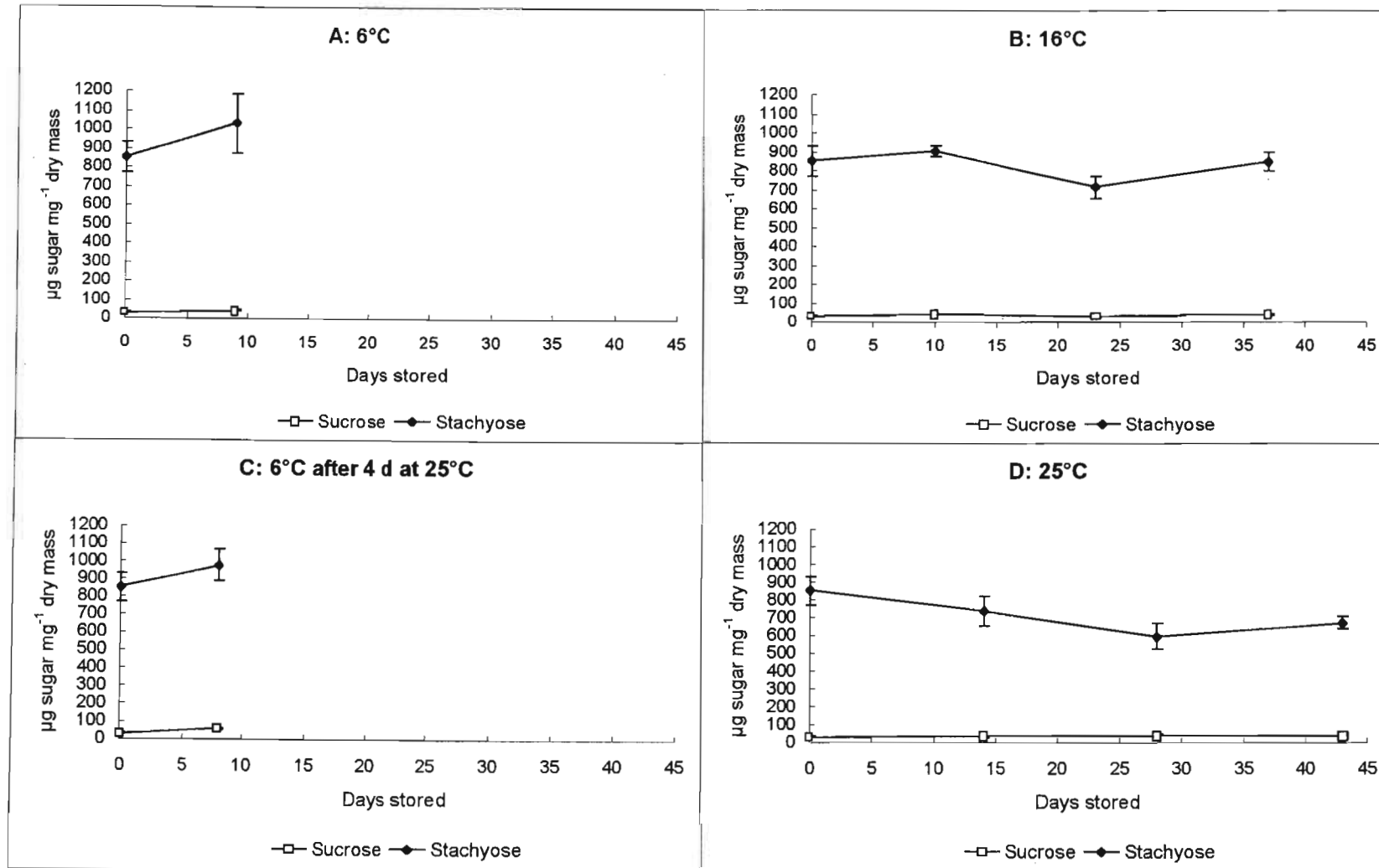


Figure 12: The effect of storage time on sugar metabolism in the cotyledons of *A. marina* seeds stored at different temperatures. The vertical bars correspond to standard deviation.

3.3 Ultrastructural Responses

The ultrastructural responses of *E. capensis* and *A. marina* seeds to chilling stress were determined by comparing the state of meristematic cells before and after chilling treatment. Transmission electron micrographs have been used to illustrate the responses observed.

Key to all transmission electron micrographs:

Cw	= Cell wall
ER	= Endoplasmic reticulum
G	= Golgi body
M	= Mitochondrion
N	= Nucleus
Ne	= Nuclear envelope
Nu	= Nucleolus
P	= Plastid
V	= Vacuole
→	= Polysomes

3.3.1 *E. capensis*

The control, which consisted of fresh seeds that had not been stored, showed metabolically active cells. This was concluded from the presence of polysomes and Golgi bodies (Figure 13.3), and many approximately spherical mitochondria with internal organisation and no electron transparent areas (Figure 13.2). Endoplasmic reticulum (ER) was also present as isolated, fairly long profiles (Figure 13.3). The cell wall appeared to be slightly 'buckled' in some of the cells (Figure 13.1), but at a higher magnification it could be seen that there was no abnormal separation of the plasmalemma from the wall and that the membrane was in good condition (Figure 13.2). Nuclear envelopes were regular, nucleoli prominent, and very few chromatin patches were visible in most of the nuclei (Figure 13.1). There were also very few vacuoles present (Figure 13.1).

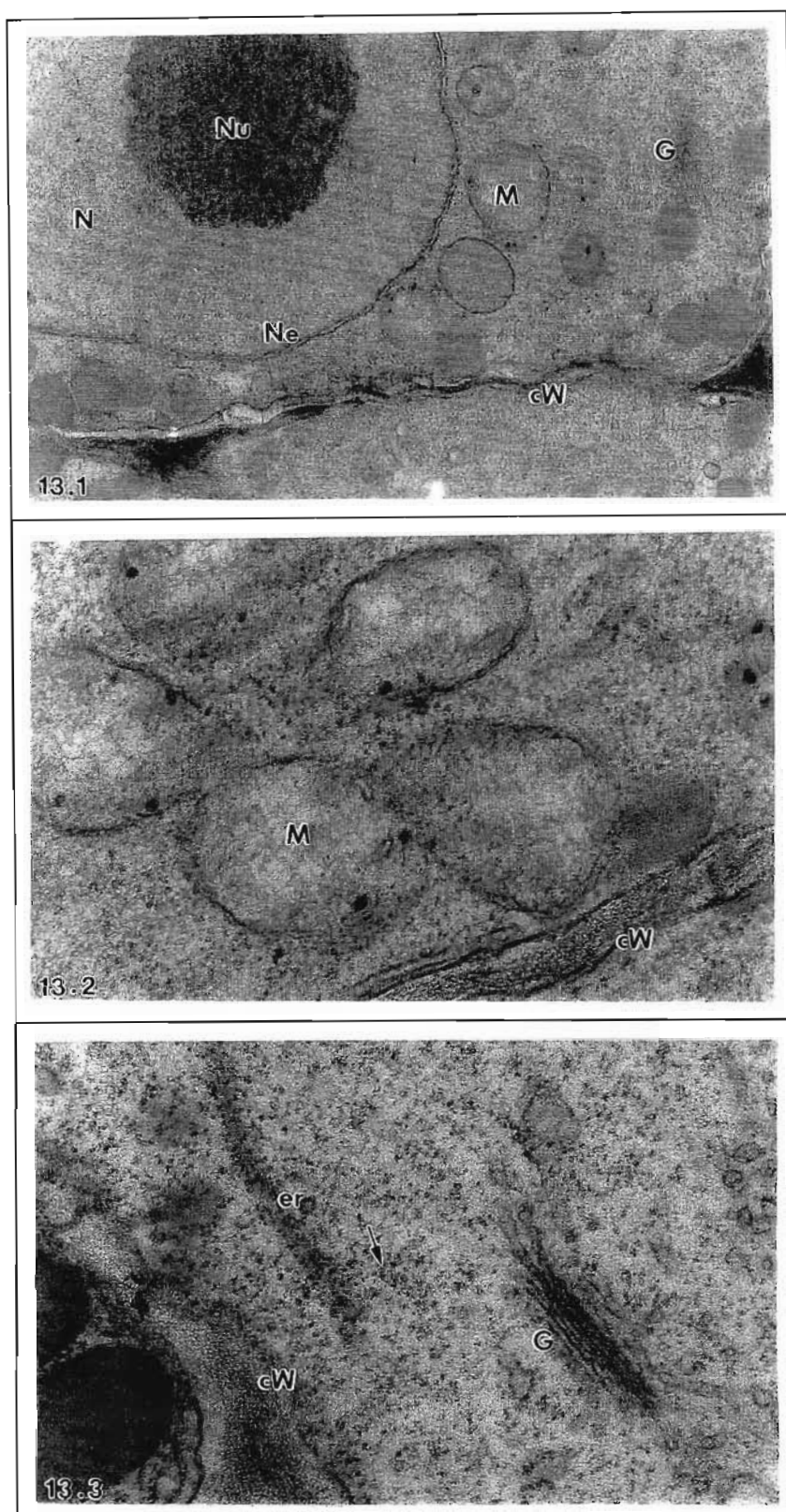


Figure 13: Transmission electron micrographs of axis meristematic cells from whole *E. capensis* seeds prior to storage (i.e. control).

Magnification: 13.1 x 12 000; 13.2 x 40 000; 13.3 x 30 000.

The cells still appeared to be relatively active after 8 weeks of storage with the endocarp at 16°C. Polysomes and Golgi bodies were visible (Figure 14.3), and there were many small mitochondria that did not show obvious deterioration, but some regression of cristae relative to the fresh condition was apparent (Figure 14.2). Some of the cell walls showed irregularities, but generally they were in good condition (Figures 14.1 and 14.3). The nuclear envelopes were regular and intact (Figures 14.1 and 14.3), and the incidence of chromatin patches had not increased. There were still very few vacuoles, but ER could be seen surrounding many of the organelles – possibly the beginning of cytolysome formation (Figure 14.1). Long profiles of ER were also apparent (Figure 14.1).

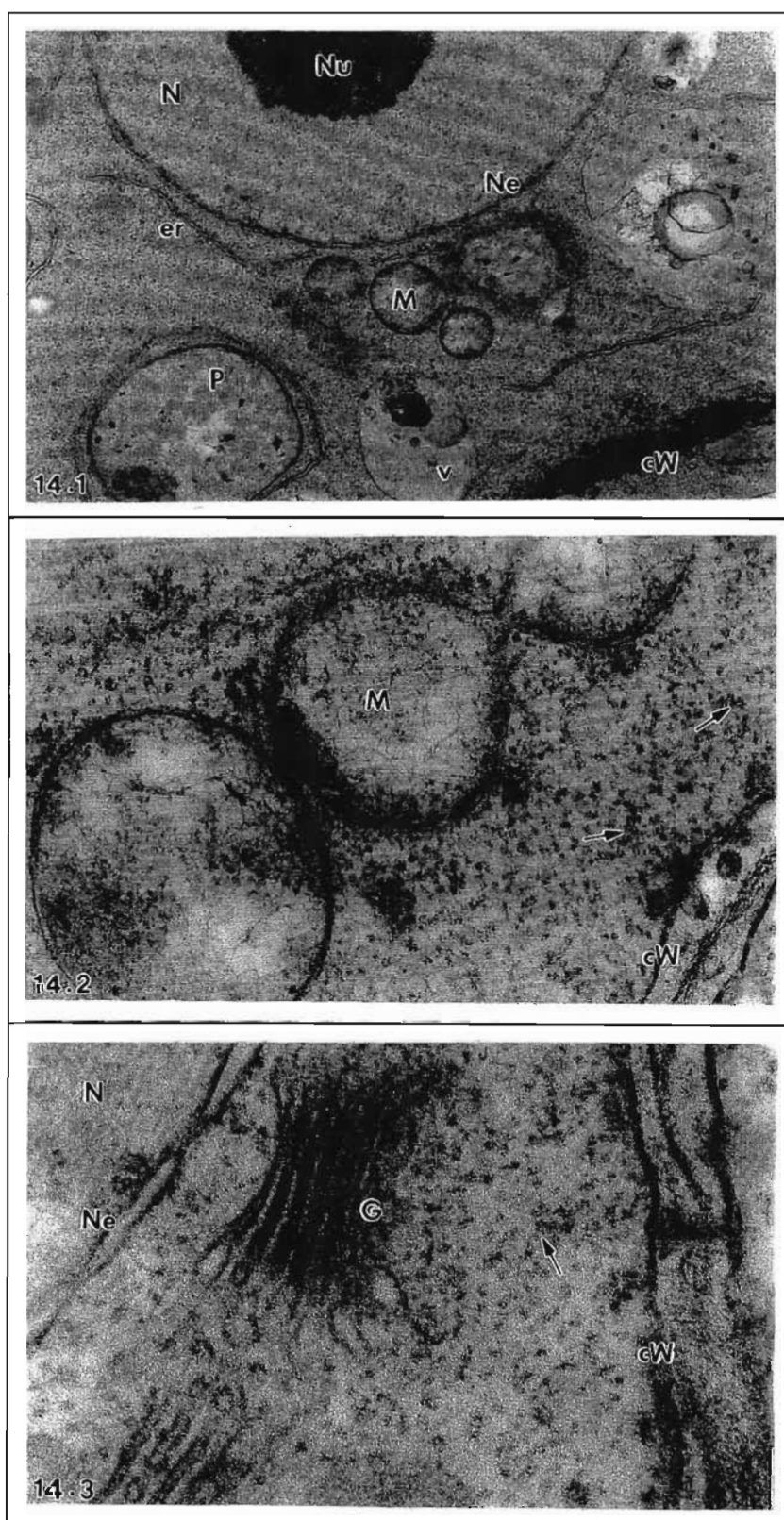


Figure 14: Transmission electron micrographs of axis meristematic cells from whole *E. capensis* seeds stored with the endocarp for 8 weeks at 16°C. Magnification: 14.1 x 10 000; 14.2 x 40 000; 14.3 x 50 000.

After 8 weeks of storage with the endocarp at 6°C there were definite signs of stress in the cells. Many organelles showed marked signs of deterioration and there was increased vacuolation, many vacuoles showing ruptured tonoplasts (Figure 15.1). The large vacuoles present are assumed to have been formed via cytolysosomes, as there were many cases of ER proliferation in whorls around deteriorated organelles and islands of cytoplasm (Figure 15.1) and residual membranes occurred inside vacuoles. Polysomes were still visible in the cytomatrix (Figure 15.2) and associated with the ER (Figure 15.1), and Golgi bodies were also still visible (Figure 15.1). Mitochondria did not show many electron transparent areas and were generally similar to those in axes from seeds stored at 16°C (compare Figure 15.2 with Figure 14.2). The cell walls had a very 'buckled' appearance (Figure 15.1) and the plasmalemma was inwardly withdrawn in some of the cells. The nuclear membranes were largely regular except for occasional localised dilations, but nucleolar vacuoles, not seen in the control and those seeds stored for 8 weeks at 16°C were present (compare Figure 15.1 with Figure 13.1 and Figure 14.1). Chromatin patches in the nuclei had become noticeable, especially at the nuclear periphery (Figure 15.1).

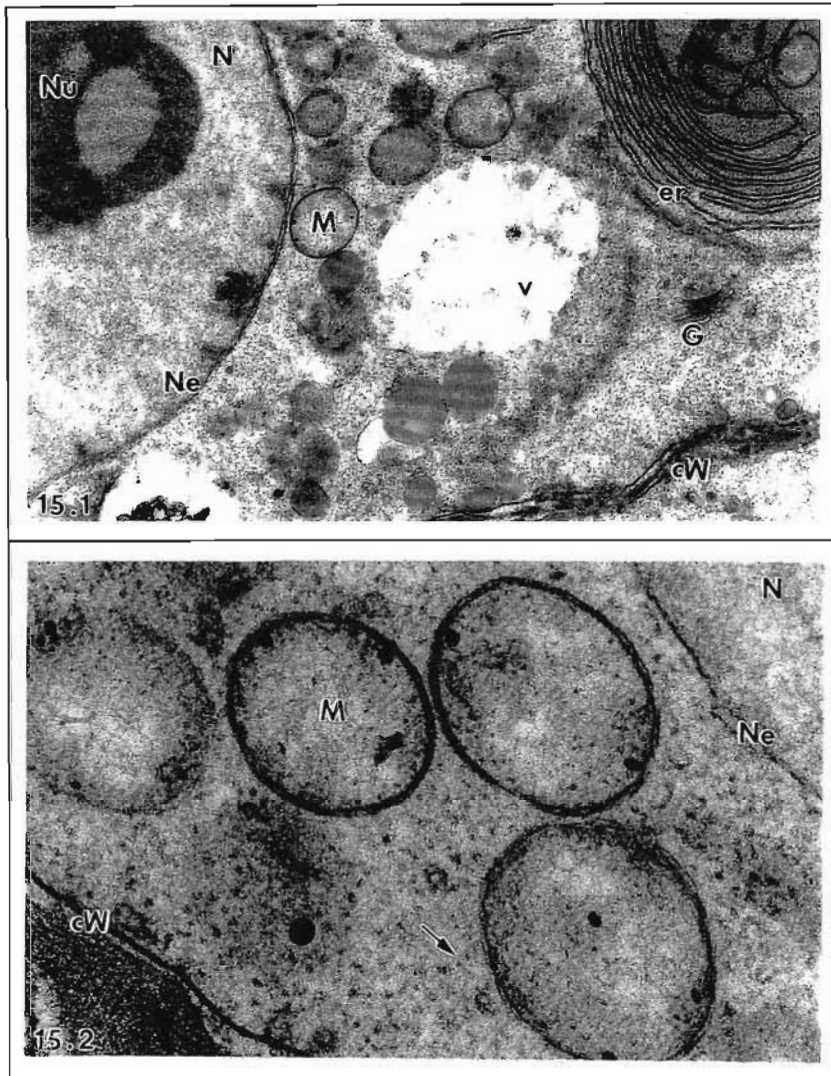


Figure 15: Transmission electron micrographs of axis meristematic cells from whole *E. capensis* seeds stored with the endocarp for 8 weeks at 6°C. Magnification: 15.1 x 10 000; 15.2 x 40 000.

In those seeds stored for 8 weeks with the endocarp at 3°C (germination did not take place and considerable fungal proliferation occurred), many of the cell walls did not have the 'buckled' appearance apparent in the cells of those seeds stored at 6°C. The mitochondria had large electron transparent areas (Figure 16.2), but some polysomes and Golgi bodies were still visible in the cytomatrix (Figure 16.3). Very large vacuoles were present in the cells (Figure 16.1) and many appeared to have engulfed other organelles. The nuclear membranes were still regular, but chromatin patches in the nuclei had increased in extent (Figure 16.1). There was no evidence of ER whorl formation, only occasional profiles were visible (Figure 16.3), although it is probable that the vacuoles present were formed via cytolysosomes as evidenced by the incidence and size of these compartments (Figure 16.1).

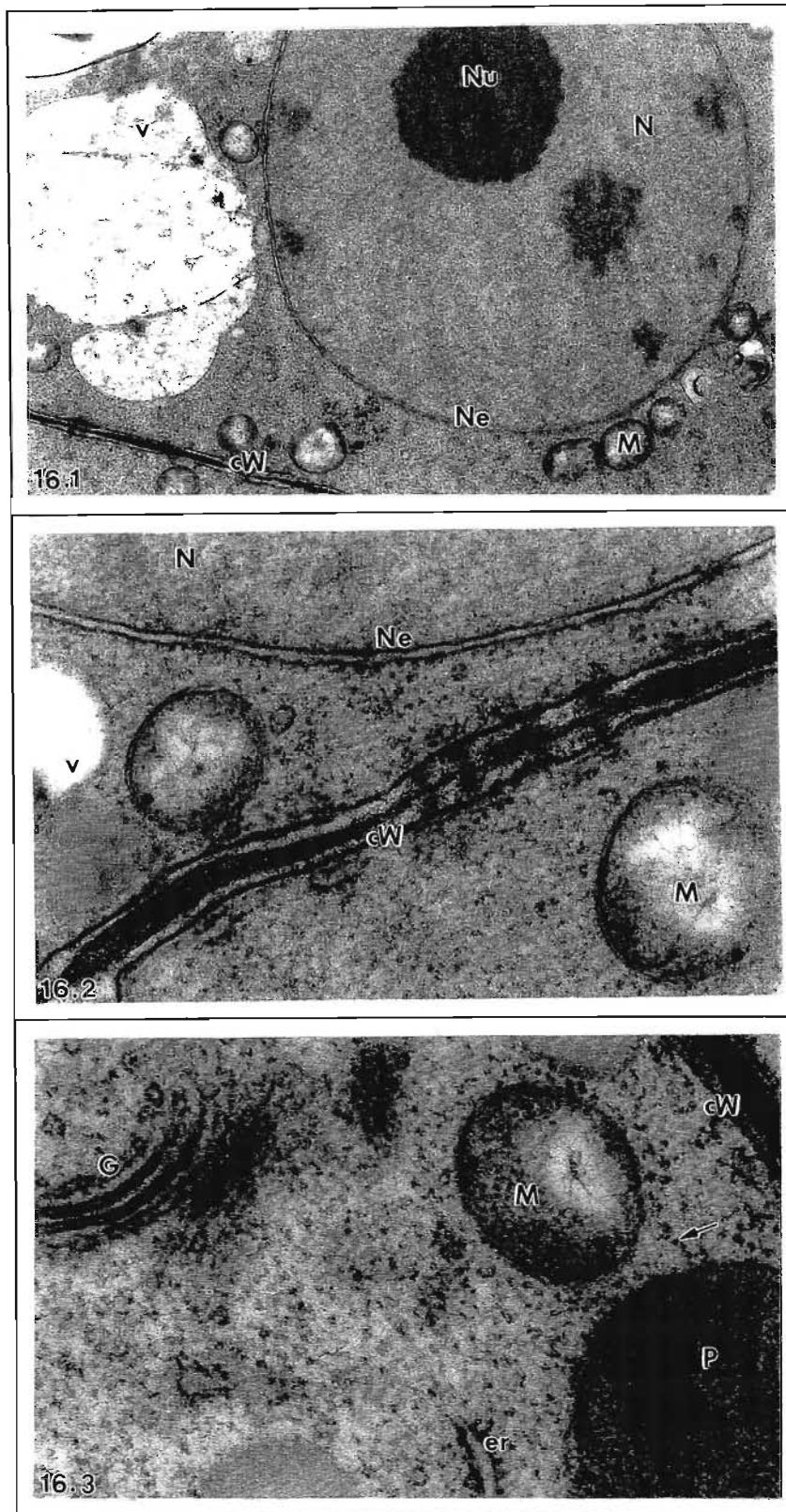


Figure 16: Transmission electron micrographs of axis meristematic cells from whole *E. capensis* seeds stored with the endocarp for 8 weeks at 3°C. Magnification: 16.1 $\times 10\,000$; 16.2 $\times 40\,000$; 16.3 $\times 40\,000$.

After 56 days at 0°C the cells were badly deteriorated. Individual organelles could not be seen, and extensive cellular disorganisation was apparent (Figure 17.1).

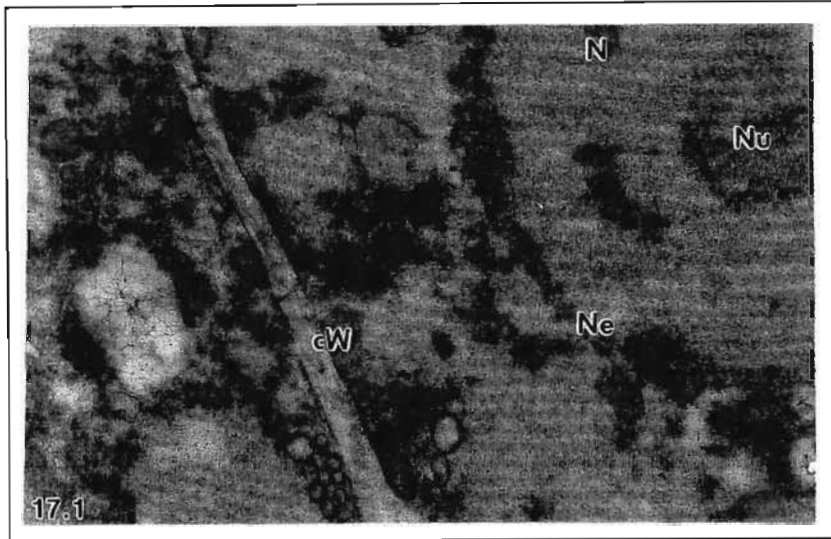


Figure 17: Transmission electron micrograph of axis meristematic Cells from whole *E. capensis* seeds stored with the endocarp for 8 weeks at 0°C. Magnification: 17.1 x 12 000.

3.3.2 *A. marina*

Cells from control seeds, i.e. fresh seeds that had not been stored, were in good condition. Although the incidence of polysomes was high (Figure 18.4), there was a general absence of Golgi bodies and mitochondria lacked the appearance of matrical density associated with a high degree of activity (Berjak *et al.*, 1995; Farrant *et al.*, 1997) (Figure 18.4). This could be indicative of relatively quiescent cells. The plasmalemma appeared to be losing close contact with the cell wall (Figures 18.3 and 18.4), but this has been observed in other ultrastructural examinations of *A. marina* (Farrant *et al.*, 1986; Calistru *et al.*, 2000) and is not indicative of deterioration. The cells contained relatively few, small vacuoles and scattered, short profiles of ER (Figure 18.1). The nuclear envelope was regular and nucleoli were prominent (Figure 18.2). The plastids in some of the cells contained dark deposits (Figure 18.1), whereas others did not (Figure 18.3). Dark deposits are characteristically laid down in the plastids a short while after the seeds have been shed (Pammenter *et al.*, 1984), indicating that some of the seeds harvested may not have been freshly fallen.

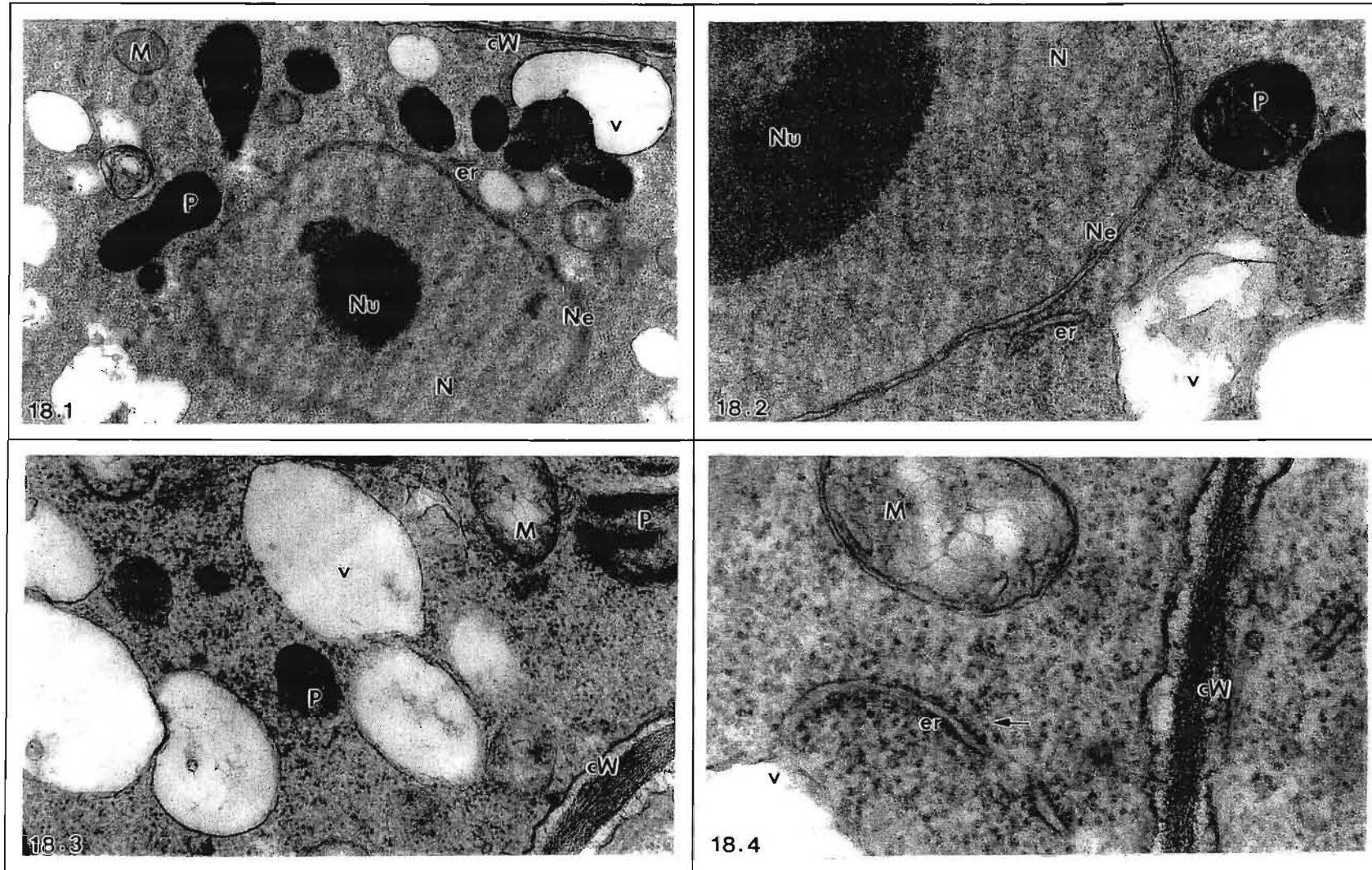


Figure 18: Transmission electron micrographs of meristematic cells from root primordia of *A. marina* seeds prior to storage, i.e. control.
Magnification: 18.1 x 12 000; 18.2 x 25 000; 18.3 x 25 000; 18.4 x 50 000.

After 4 days at 25°C the cells presented an appearance suggesting greater metabolic activity than those of the control seeds, which were freshly (or relatively freshly) fallen. More polysomes appeared to be present in the cytomatrix, mitochondria were more electron dense than those of the unstored control seeds, although the degree of mitochondrial development was essentially similar (compare Figure 19.2 with Figure 18.4), and ER appeared to be more abundant with longer profiles. No irregularities of the cell wall were evident, and plasmodesmata were well developed (Figure 19.3). Nuclear membranes were regular, vacuoles were still small and the cells generally appeared to be in good condition (Figure 19.1).

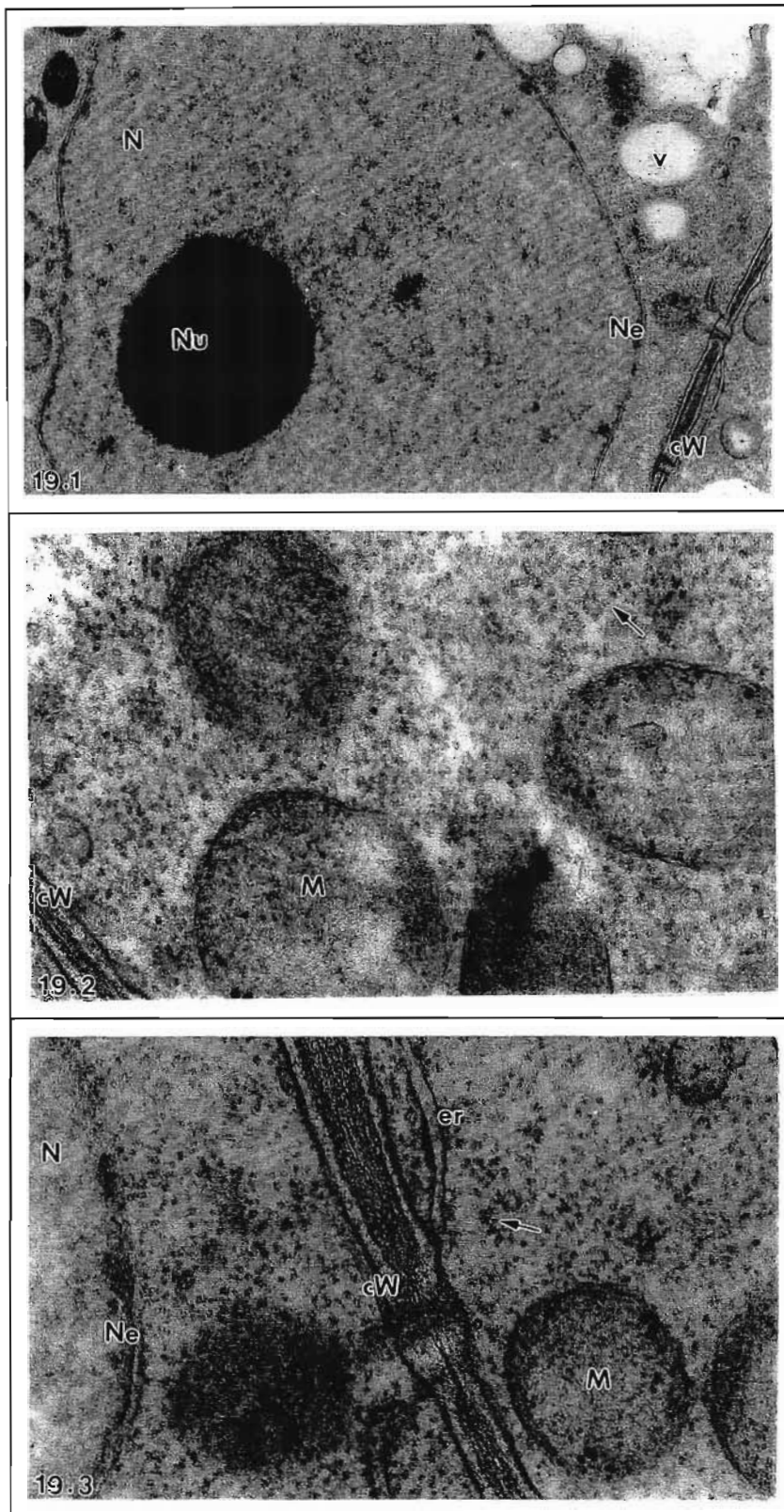


Figure 19: Transmission electron micrographs of meristematic cells from root primordia of *A. marina* seeds stored for 4 days at 25°C. Magnification: 19.1 x 10 000; 19.2 x 40 000; 19.3 x 40 000.

After 28 days of storage at 25°C there were signs of marked deterioration in the cells. The number of vacuoles had increased considerably and many of them were fusing with others in close proximity (Figure 20.1). The plasmalemma had detached from the cell walls, although in many cases adjacent cells were still connected by plasmodesmata (Figures 20.1 and 20.2). The nuclear envelopes had lost their resilience and most were very irregular; there were also signs of dissolution (Figure 20.1). The mitochondria had large, electron transparent areas and internal organisation had been lost (Figure 20.2). Membranes surrounding the mitochondria were also discontinuous in some cases (Figure 20.3). The cytomatrix had a more even granularity indicating a decrease in polysomes and an inferred decrease in protein synthesis by this stage (Figure 20.2). The only evidence of ER was the presence of occasional profiles (Figures 20.1 and 20.2), and organised Golgi bodies were not apparent.

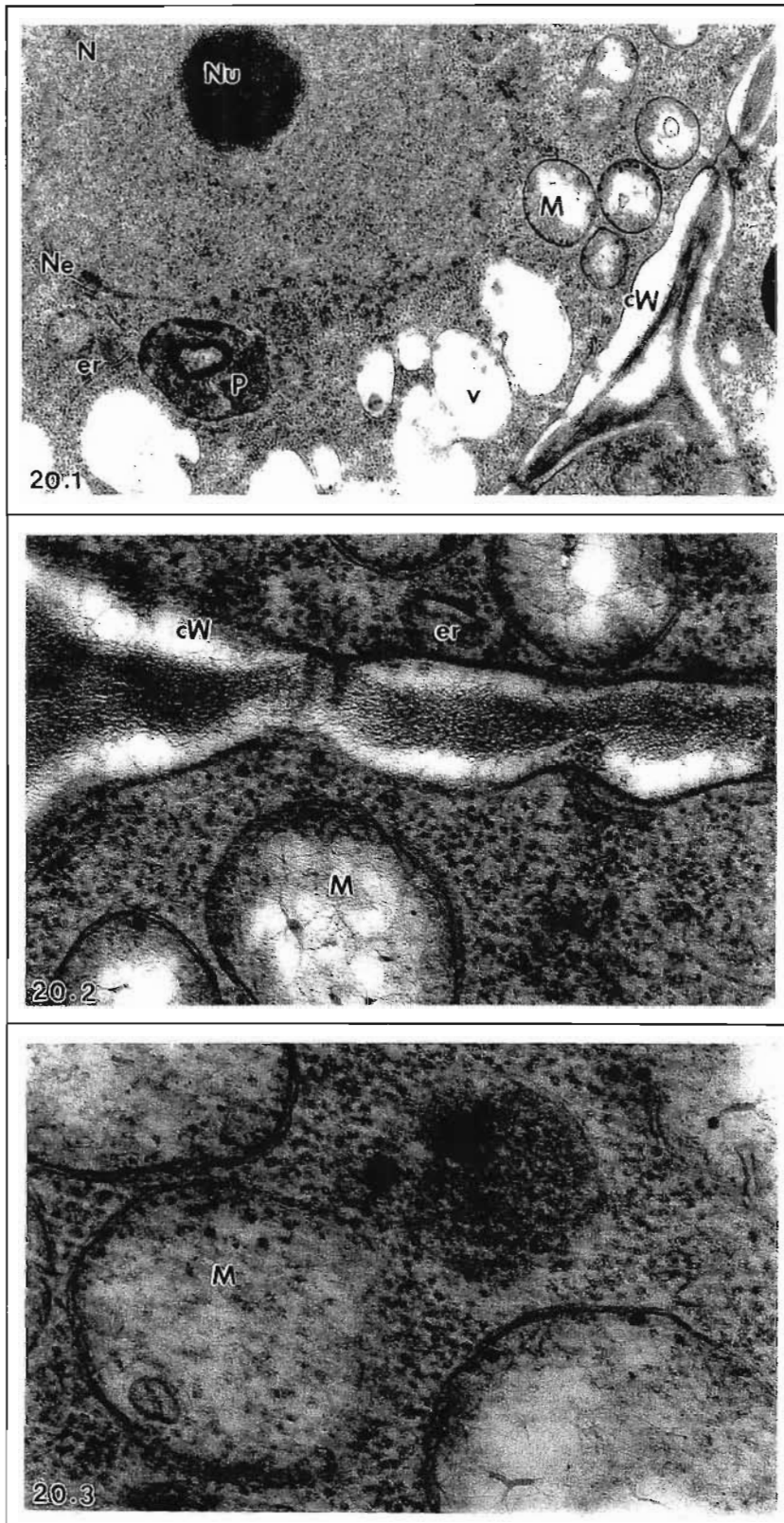


Figure 20: Transmission electron micrographs of meristematic cells from root primordia of *A. marina* seeds stored for 28 days at 25°C. Magnification: 20.1 x 12 000; 20.2 x 40 000; 20.3 x 50 000.

It is interesting to compare the cells of those seeds stored at 25°C for 28 days with the cells of those seeds stored at 16°C for the same time, as the deterioration evident was not as extensive as that noted in the former. The degree of vacuolation and vacuolar fusion was approximately the same (compare Figure 21.1 with Figure 20.1), but although both showed mitochondria with electron transparent areas these were not as extensive in the cells of seeds stored at 16°C for 28 days and cristae could still be seen in most of them (compare Figure 21.2 with Figure 20.2). Interestingly, after 28 days at 16°C there were many mitochondria that had become elongated instead of being spherical (Figure 21.2). The extent of plasmalemma withdrawal at 16°C was similar to that occurring at 25°C, with no signs of rupture and undisturbed continuity across plasmodesmata (compare Figure 21.1 with Figure 20.1). Nuclear envelopes were, however, irregular in places (Figure 21.1), but pores remained clearly defined (Figure 21.2). There was evidence of ER (Figure 21.2), but profiles were mostly distended and short. Localised, distended short aggregates of cisternae (Figure 21.3) that were most likely disassociating Golgi bodies were seen, although these may have been fragments of ER.

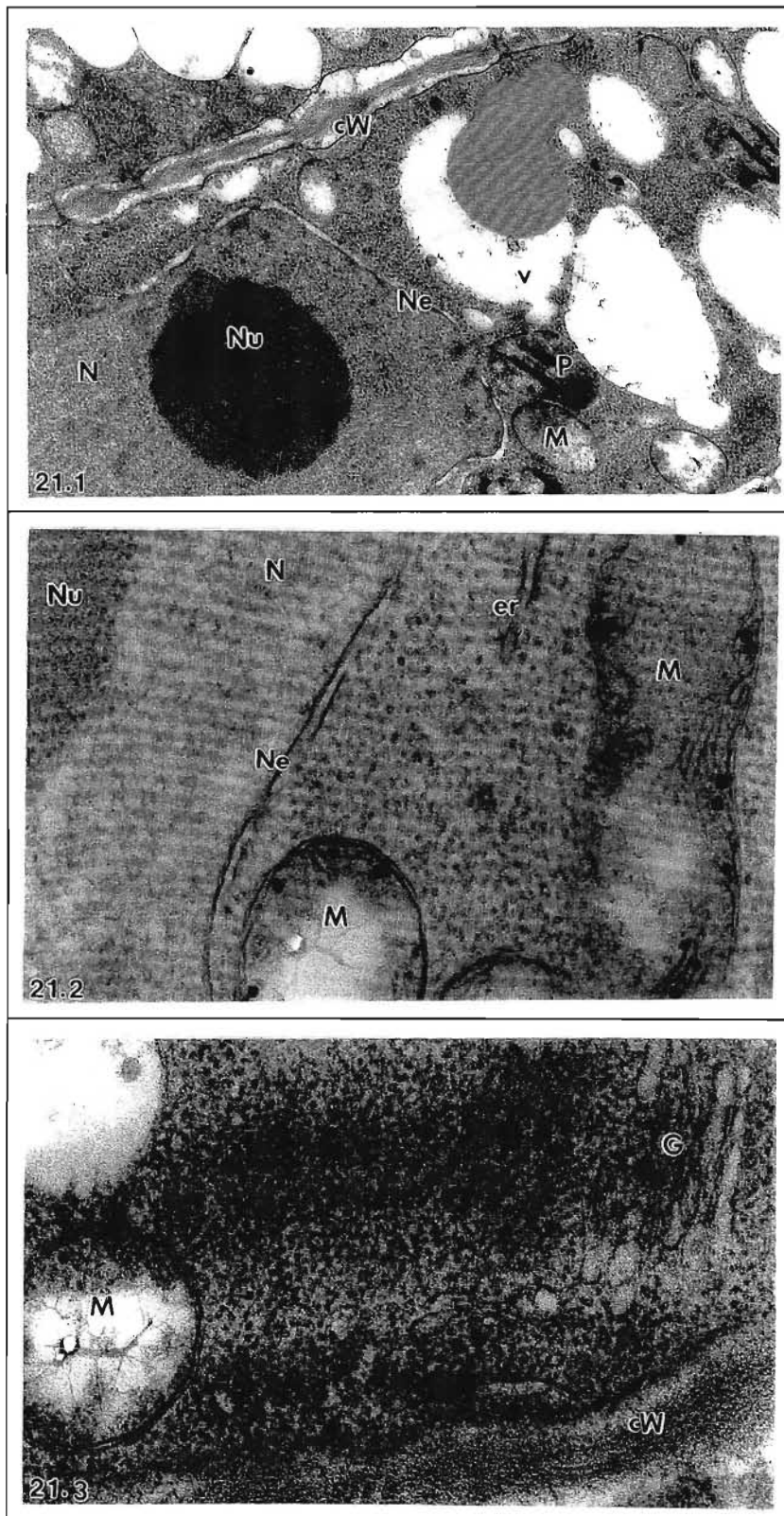


Figure 21: Transmission electron micrographs of meristematic cells from root primordia of *A. marina* seeds stored for 28 days at 16°C. Magnification: 21.1 x 12 000; 21.2 x 40 000; 21.3 x 40 000.

After 8 days at 6°C the most obvious feature was numerous, large vacuoles (Figure 22.2), which could have been formed via cytolysosomes as there were residual membranes inside many of them. The cell wall was deteriorated in many places (Figure 22.1 and 22.3) and the nuclear envelopes had lost their resilience and showed localised damage (Figure 22.1 and 22.2), there were also vacuoles in many of the nucleoli (Figure 22.4). The mitochondria had few remaining cristae and extensive electron transparent areas (Figure 22.3). Long ER profiles were present (Figure 22.1) and proliferation had occurred in some instances. It was interesting to note that polysomes were present in the cytomatrix (Figure 22.3) and Golgi bodies remained recognisable in cells, although the cisternae tended to be rather swollen (not shown).

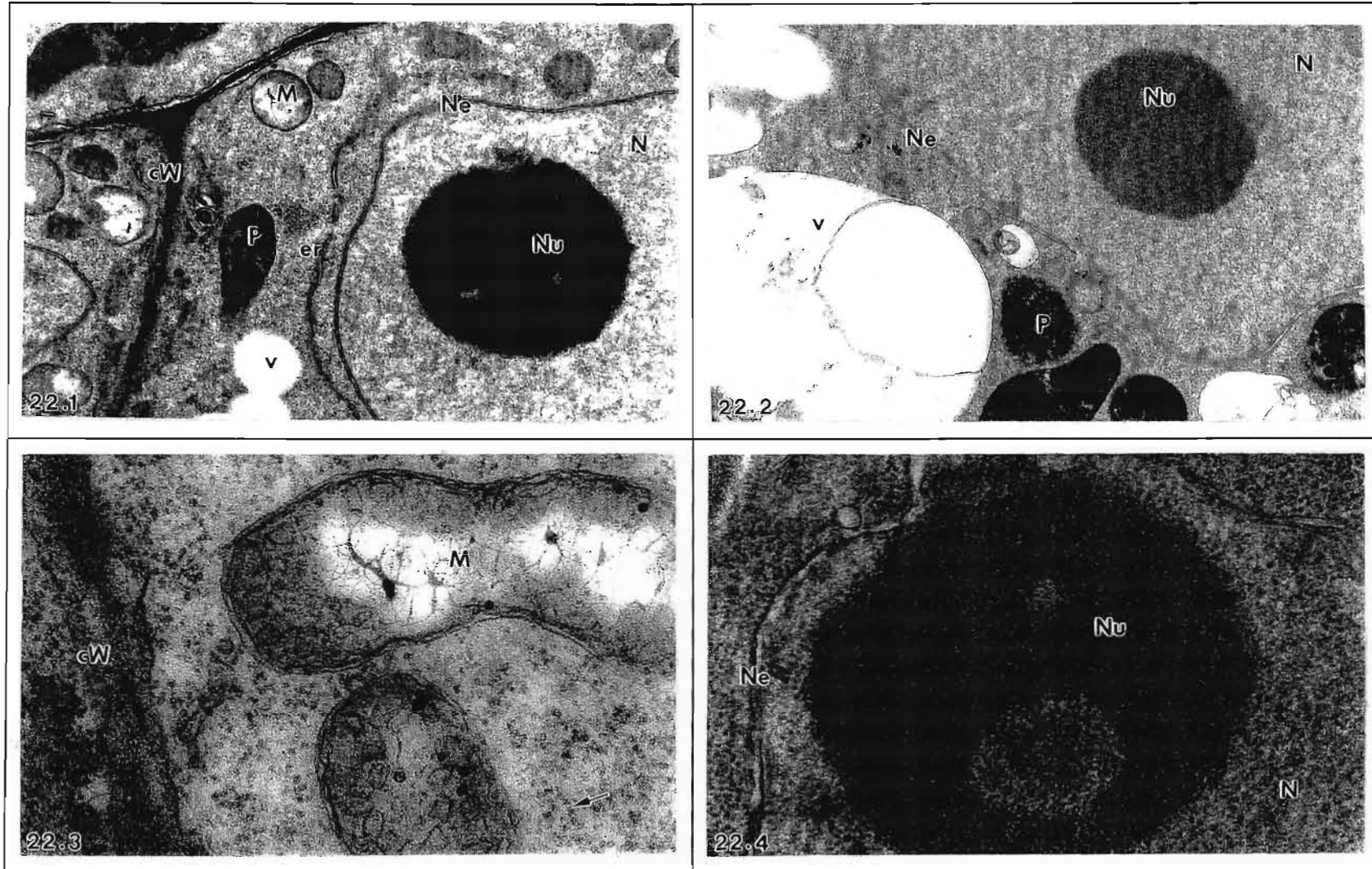


Figure 22: Transmission electron micrographs of meristematic cells from root primordia of *A. marina* seeds stored for 8 days at 6°C. Magnification: 22.1 x 10 000; 22.2 x 10 000; 22.3 x 40 000; 22.4 x 30 000.

The root primordia cells of those seeds stored for 4 days at 25°C prior to being stored for 8 days at 6°C showed similar ultrastructural organisation to those seeds exposed directly to 6°C for 8 days (compare Figure 23.1 with Figure 22.1). The many vacuoles present showed evidence of having been developed via cytolysosomes (Figure 23.3). Polysome structure had been retained, with many of these formations evident, and long profiles of ER were also present (Figures 23.2 and 23.4). Most of the mitochondria had electron transparent areas, and in some the surrounding membrane appeared discontinuous (Figure 23.4); this was also the case for many of the storage plastids (Figure 23.2). The cell wall did not appear to be as deteriorated as it was in those seeds exposed directly to 6°C, although plasmalemma withdrawal was very pronounced in places (compare Figure 23.4 and Figure 22.3). Nuclear envelopes appeared to have lost resilience to a certain extent, some nuclear profiles being more irregular than others, and there were also nucleolar vacuoles in many of the nucleoli (Figure 23.1).

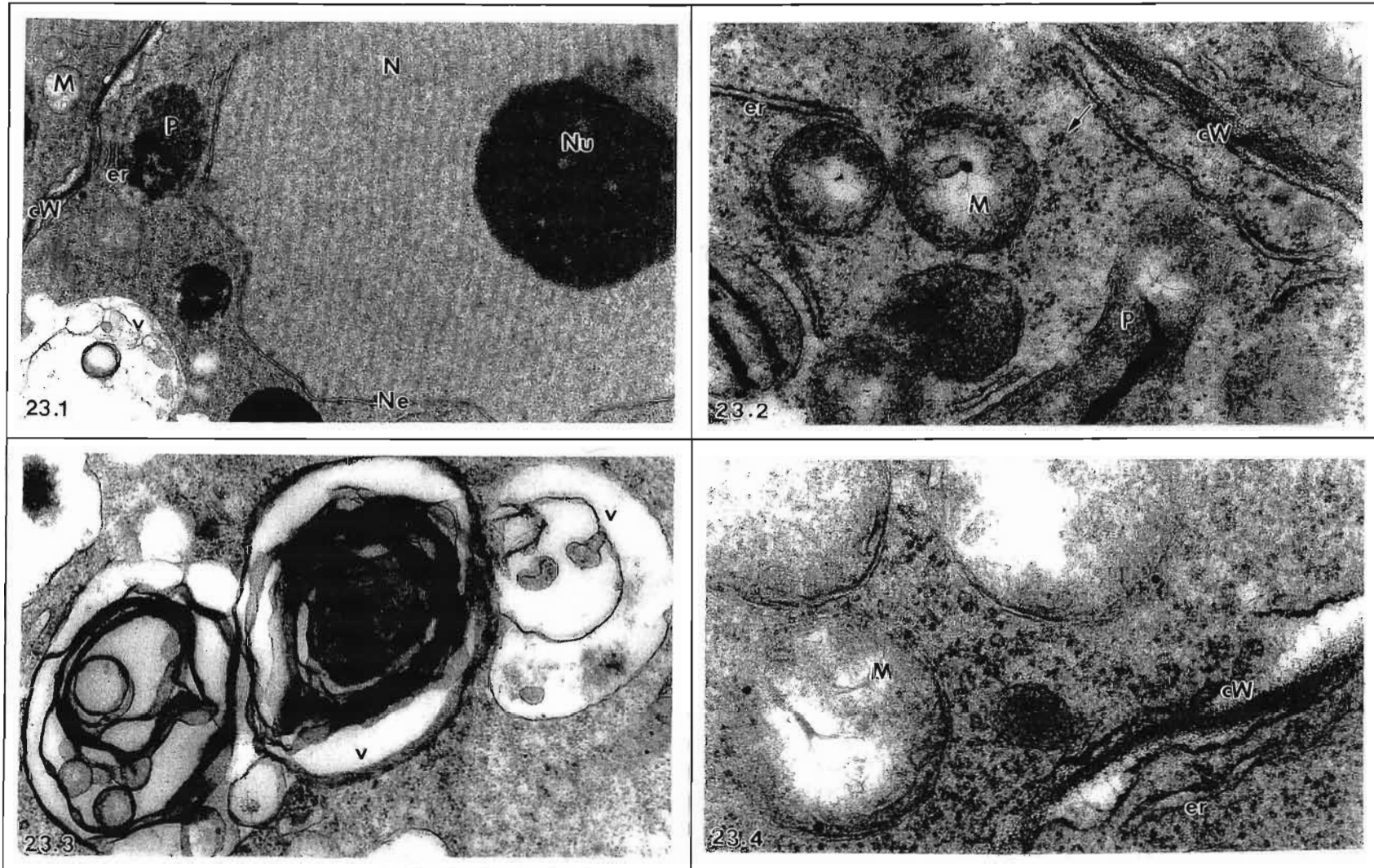


Figure 23: Transmission electron micrographs of meristematic cells from root primordia of *A. marina* seeds stored for 4 days at 25°C and 8 days at 6°C. Magnification: 23.1 x 10 000; 23.2 x 30 000; 23.3 x 20 000; 23.4 x 40 000.

CHAPTER 4

DISCUSSION

The aim of the present investigation was to characterise selected physiological, biochemical and ultrastructural responses of the recalcitrant seed species *E. capensis* and *A. marina* to chilling stress, in order to gain an understanding of the chilling response in recalcitrant seeds. The findings must be considered preliminary and the basis for further research as little is known about the nature of chilling injury and the response to chilling stress in recalcitrant seeds.

4.1 Physiological Responses

It is clear from the results obtained that the *E. capensis* seeds were able to withstand much lower temperatures for a longer period of time than those of *A. marina*, although the seeds of both species were chilling sensitive. *Ekebergia capensis* seeds stored with the endocarp were able to survive for 12 weeks at 6°C without losing any germination capability, whereas wet-stored *A. marina* seeds lasted little more than 2 weeks at 6°C, by which stage germination capability had been lost almost completely. When *E. capensis* seeds were stored with the endocarp at 3°C the germination achieved after 8 weeks was 40% and the seeds that did not germinate became over-run with fungi, suggesting that the seeds were very debilitated. This supports the view that each recalcitrant seed species has its own threshold before injury is incurred, as a result of either desiccation or chilling (Berjak and Pammenter, 1994). This indicates that it is impossible to arrive at a general optimum storage temperature for all recalcitrant seed species; each one must be individually tested. It seemed that the endocarp played an important role in maintaining the water content of the *E. capensis* seeds (compare Figures 1 and 2), which is important in maintaining seed vigour (McDonald, 1999), and protecting the seed from fungal contamination. This suggests that the nature of the seed and its components may also determine its ability to withstand chilling stress. The water content was relatively stable throughout wet-storage of the *A. marina* seeds as well (Figure 3), although it must be remembered that the methodology used measured the 'bulk tissue' water content only. The root primordia, which are essential for

germination, may have become dehydrated, as they have no protection from the external environment except that provided by the surrounding bristles (Boubriak *et al.*, 2000). Shortened storage life itself can be considered a symptom of chilling injury (Lyons, 1973). It could be assumed that more injury occurred in the *A. marina* seeds than in the *E. capensis* seeds, or that any injury incurred was more readily repaired in the *E. capensis* seeds so that germination could still take place.

It has been noted that chilling sensitivity varies with stage of development, physiological processes and biochemical reactions taking place in plant tissue at the time of chilling (Purvis, 1990), and it is evident that this is also the case for recalcitrant seeds. The enhanced metabolic activity of *A. marina* seeds after storage for 4 days at 25°C, originally documented by Pammenter *et al.* (1984), could be assumed to have initially enabled the seeds to better withstand the chilling temperature of 6°C. Germination capability of those seeds placed directly at 6°C was only 20% after 1 week opposed to 70% after 1 week for those seeds first stored at 25°C for 4 days. It must be kept in mind that this high germination capability was transient, as the seeds lost their germination capability totally, faster than those seeds placed directly at 6°C (Figure 5) despite an initially higher germination capability after 1 week. The subsequent rapid decline in germination capability could have been due to the enhanced metabolic activity giving way to germination, as it has been observed that actively growing seedlings are very susceptible to chilling stress (Purvis, 1990). Pammenter *et al.* (1984) state that the metabolic enhancement that occurs in *A. marina* seeds after short-term storage is similar to that taking place in seeds allowed to germinate immediately after shedding. Prasad (1996) found that if 2 to 5 day old maize seedlings were not acclimated, only 14 to 23% survived after 7 days at 4°C, but if they were acclimated 70 to 93% survived – this was true only for 2 to 4 day old and not 5 day old seedlings. After 5 days it is possible that the seedlings had reached a physiological age at which tolerance could no longer be induced, just as it is assumed that *A. marina* seeds became more susceptible to chilling stress once the initial stages of germination had begun. An interesting side issue is that Calistru *et al.* (2000) showed that *A. marina* seeds stored for 4 days became less susceptible to fungal infection relative to seeds infected immediately on collection.

The physical symptoms of chilling injury observed in the seeds of *A. marina* were decreased germination capability, increased decay and seed death; all typical of the type of chilling injury that takes place during early imbibition of orthodox seeds (Herner, 1990). Imbibition at low temperatures is detrimental to the seed and has been correlated with increased or sustained leakage of substances such as amino acids, sugars, organic acids, gibberellic acid, phosphates, phenolics, succinate and enzymes (Herner, 1990). It is likely that this occurred during the storage of *A. marina* seeds at 6 and 16°C, as the seeds had a slimy coating after approximately 7 days of storage and fungal proliferation increased noticeably - such leakage is associated with increased decay promoted by the loss of sugars and other substances (Herner, 1990). This might also explain the increase in water content observed after 20 days of storage at 6°C (Figure 3: A); perhaps the substances leaked were hygroscopic and/ or fungal metabolic water could have been produced.

The *E. capensis* seeds were stored for approximately 4 weeks at 16°C before any storage experiments were initiated; by this stage the seeds were mature and had a germination capability of 100%. This may have contributed to the successful storage of seeds with the endocarp at both 6 and 16°C for 12 weeks, although the presence of the endocarp might have been of greater significance. The hard endocarp kept the water content constant and fungal contamination at bay, although it cannot be said that it prevented any water uptake from occurring during storage since those seeds stored without the endocarp lost, rather than gained water. There was a significant difference in the initial water content of the *A. marina* and *E. capensis* seeds. Axes of *E. capensis* seeds had an initial water content of approximately 1.6 g g⁻¹ (Figure 1), whereas the axes of *A. marina* seeds had an initial water content of approximately 2.5 g g⁻¹ (Figure 3). The possibility of water content increase occurring at low temperatures by equilibration with high relative humidity was precluded/ minimised for the *E. capensis* seeds, as they were not wet-stored. In contrast, *A. marina* seeds, which begin germinative metabolism immediately after they have been shed regardless of whether additional water is provided (Pammenter *et al.*, 1984), were wet-stored. As the water content of these seeds did increase slightly during wet-storage, it is possible that a process equated with imbibitional chilling injury might have resulted. *Avicennia*

marina seeds may also be susceptible to light-induced damage at low temperatures, as they are actively photosynthetic (Steinke and Naidoo, 1991). In the present study the *A. marina* seeds were stored under low light conditions, but even so, chilling sensitive plants are extremely sensitive to any light at low temperatures (Van Hasselt, 1990). Reactive oxygen species (ROS) generated by a damaged photosystem may have also significantly contributed to seed deterioration.

It is said that chilling temperatures can abruptly and dramatically affect the physiological function of chilling sensitive plant species (Lyons, 1973). This was reflected in the respiration rate of *E. capensis* seeds after storage without the endocarp at 6 and 16°C for 3 and 2 weeks, respectively (Figure 6: A and B). It is interesting that this enhanced respiration rate decreased again, indicating that the upset or compensation in metabolism resulting in the enhanced respiration rate was accommodated. The initial increase in respiration rate may have been to drive the synthesis of new proteins and additional antioxidants in an attempt to adjust to the chilling temperatures, as this notable increase was not observed in those seeds stored at 25°C (Figure 6: C). In this regard, the persistence of polysomes in seeds stored at 6 and 16°C supports this contention. In the *A. marina* seeds, increased respiration rate was not an initial response to chilling stress as it was in those of *E. capensis*; it occurred at a stage when the seeds were debilitated and germination capability was low (Figure 7). It is interesting to draw a parallel between respiratory rate and the condition of mitochondria, as these organelles have a central role in respiration (Lyons, 1973) and have been found to be the cellular compartment mostly affected by chilling treatment (reviewed by De Santis *et al.*, 1999). Electron microscopy revealed that in *E. capensis* seeds electron transparent areas occurred in the mitochondria (a characteristic associated with mitochondrial inactivity [Farrant *et al.*, 1997]) only when the seeds were stored with the endocarp at 3°C (Figure 16.2), and that the mitochondria appeared to be in far better condition after storage with the endocarp at 6°C (Figure 15.2) and 16°C (Figure 14.2). The respiration rate for *E. capensis* seeds stored with the endocarp at 6°C was slightly raised throughout storage compared with the control, and seeds stored with the endocarp at 16°C maintained a respiration rate close to the control (Figure 6). Unfortunately, respiration rate during storage at 3°C could not be measured, as a consequence of the limited seed

numbers (explained in the materials and methods section, 2.2.1). In *A. marina* seeds, electron transparent areas in the mitochondria became apparent after storage for 8 days at 6°C (Figure 22.3) and also after 8 days at 6°C after 4 days at 25°C (Figure 23.4); 28 days at 16°C (Figure 21.2) and 28 days at 25°C (Figure 20.2). Respiration rate was somewhat elevated at all these sampling times (Figure 7) and germination was mostly low (Figure 5), which leads to the conclusion that this may not have been mitochondrial respiration. In support of this, a loss of respiratory activity would have occurred in the mitochondria once internal membranes had become damaged. Increases in respiratory rate in chilling-sensitive plants under cold stress have been reported previously (Graham and Patterson, 1982; Wang, 1982; Lyons and Breidenbach, 1990) and ascribed to metabolic disruption (Wang, 1982). It seems, therefore, that similar responses are inherent properties of chilling-sensitive recalcitrant seeds, although the details of the injury underlying such respiratory stimulation require elucidation. Another possibility is that the mitochondrial deterioration observed might have been associated with the short storage life span of these seeds, and not necessarily or exclusively have been the result of chilling injury. Mitochondria must be stable and in working order for a plant to survive chilling stress (Prasad, 1996), which is perhaps why *E. capensis* seeds were able to survive well for 12 weeks at 6 and 16°C but not at 3°C.

Electrolyte leakage is used as an indicator of the barrier effectiveness of cell membranes, and can therefore reflect the extent of chilling injury incurred by these. *Ekebergia capensis* seeds stored with the endocarp at 6 and 16°C showed no notable increase in electrolyte leakage throughout the 12 weeks of storage, whereas those seeds stored without the endocarp at 6 and 16°C showed a dramatic increase over the first 2 weeks of storage (Figure 8). This initial increase in electrolyte leakage reverted to the same level as the control after 2 weeks, indicating perhaps that the membranes were able to re-establish their functional integrity. It may seem plausible that the endocarp protected the seeds from any initial change in membrane structure associated with the cold, but seeds stored without the endocarp at 25°C (Figure 8: C) show the same initial increase in electrolyte leakage as that observed when the seeds were stored without the endocarp at 6 and 16°C (Figure 8: A and B). It is also interesting that a gradual, but relatively steady, increase in electrolyte leakage occurred when *E. capensis* seeds were

stored with the endocarp at 25°C (Figure 8: C). This increase in electrolyte leakage with storage time occurred to a much lesser extent in those seeds stored with the endocarp at 6 and 16°C (Figure 8: A and B), so it can be concluded that it was not the chilling temperatures of 6 and 16°C that affected the membrane permeability of the stored *E. capensis* seeds. The cryptic effects of fungi below the endocarp cannot be precluded. Their deleterious effects on the seed (cotyledon) surface cells would have been most rapid at 25°C. Steady water loss with storage time did occur in the *E. capensis* seeds stored without the endocarp at 6, 16 and 25°C (Figure 2), those seeds stored at 25°C being affected the most (Figure 2: C). This indicates the possibility of desiccation damage resulting in increased membrane permeability. This may also account for the increased membrane permeability with storage time observed for *E. capensis* seeds stored with the endocarp at 25°C (Figure 8: C), as the seeds lost water at this temperature even when stored with the endocarp (Figure 1: C). This still leaves the dramatic increase in leakage observed within the first 2 weeks of storage for seeds without the endocarp at all three temperatures conjectural.

4.2 Biochemical Responses

It is unreliable to make far-reaching assumptions to explain responses, based on the presence or absence of a particular substance or class of substances, in a seed. This caution must be exercised when reviewing the consequences of the presence or absence of various antioxidant enzymes. For example, dehydrin or late embryogenesis abundant (LEA) proteins have been implicated in the acquisition of desiccation tolerance of seeds because they accumulate in orthodox seeds as desiccation tolerance increases prior to dehydration or maturation drying on the plant (Kermode, 1990). They are thought to prevent cellular damage during desiccation by binding to macromolecules, so it has been suggested that the desiccation sensitivity of recalcitrant seeds is due to a lack of dehydrins. Finch-Savage *et al.* (1994) and Han *et al.* (1995) tested for the presence of dehydrins in recalcitrant seeds and found that they were not universally absent. Those authors state that the presence of dehydrins alone is insufficient to confer full desiccation tolerance in seeds, so an insufficient quantity of any particular dehydrin may result in desiccation sensitivity. A similar situation might exist when it comes to

antioxidants or antioxidant enzymes; they may need to be present in sufficient quantity, or in combination with some other substance or process, rather than just be present.

The antioxidant enzyme glutathione reductase (GR) was found to be present in the axes and cotyledons of the *A. marina* seeds, but, as mentioned in the introduction, GR is implicated in the cold tolerance of many plants. *Avicennia marina* seeds are extremely chilling sensitive, which confirms that it is not merely the presence of a single substance or enzyme that confers chilling tolerance. It would be interesting to compare the activity of antioxidant enzymes in a chilling tolerant and a chilling sensitive recalcitrant seed species to determine whether their contribution towards chilling sensitivity or tolerance is quantitative. Glutathione reductase is not cold labile (Saruyama and Tanida, 1995), and in the present study its activity increased in response to chilling stress. It appeared that the rate of the increase depended upon the severity of the chilling stress. After 1 week at 6°C the activity of GR in the *A. marina* axes and cotyledons was over 90 and almost 80 nmol NADPH oxidized (mg protein)⁻¹ min⁻¹, respectively (Figure 10: A). In contrast, after 1 week at 16°C the activity of GR in the axes and cotyledons were only 20 and just over 40 nmol NADPH oxidized (mg protein)⁻¹ min⁻¹, respectively (Figure 10: B). There was no measurable GR activity in the control axes of *A. marina* until after 28 days of storage (Figure 10: D) and activity was much lower after 8 days in those seeds stored for 4 days prior to storage at 6°C than in those placed immediately at 6°C (Figure 10: C), which suggests that increased GR activity in the seed is a response to increased oxidative stress in general. It must be borne in mind that GR forms a part of the ascorbate-dependent H₂O₂ scavenging system, and that even though GR is not cold-labile other enzymes in this system are. This would prevent GR from fulfilling all of its functions.

Superoxide dismutase (SOD) activity was also found to be present in the seeds of *A. marina*. A notable increase in SOD activity occurred only when the seeds were placed directly at 6°C (Figure 9: A). The immediate decrease in SOD activity observed when the seeds were placed at 25, 16 and 6°C, after 4 days of storage at 25°C, occurred when germination capability was still high (Figure 9: B, C and D), whereas the germination capability of those seeds placed directly at 6°C was only 20% when SOD activity

increased. This could indicate that increased SOD activity in the seed is a response to increased oxidative stress in general, which would have been the greatest in those seeds placed directly at 6°C. Even so, it is difficult to explain why SOD activity in the *A. marina* seeds, especially in the axes, decreased after harvest. Monk *et al.* (1989) found that chilling injury could be related to the extent of SOD activity in strains of *Chlorella ellipsoidea*, the higher the activity the greater was the resistance. This suggests that generally a high antioxidant status in seeds on harvest would contribute positively to storage quality. The increase in SOD activity when the seeds were placed directly at 6°C was apparently not sufficient to prevent chilling injury, which occurs as a result of chilling stress. It is interesting to note that SOD and GR activity was not the same in the axes as in the cotyledons; SOD activity tended to be higher in the axes than in the cotyledons (Figure 9) and GR activity tended to be higher in the cotyledons than in the axes (Figure 10). Hendry *et al.* (1992) found that the defences against oxidative attack in recalcitrant *Quercus robur* L. seeds were different in the axes and in the cotyledons; the cotyledons contained mostly enzymatic defences with SOD and GR activity 6 times higher than in the axes, and the axes contained mostly anti-oxidant compounds such as ascorbic acid and alpha-tocopherol. Those authors concluded that the axes were a 'weak link' when it came to desiccation tolerance, as they did not possess adequate oxidative defences. In *A. marina* seeds stored at 25°C, GR and SOD activity gradually increased over the storage period (Figure: 9D and 10D). This indicates that rather than the antioxidant enzymes being constitutive, they are induced as oxidative stress increases in the seed. It has been suggested that the increased resistance of *A. marina* seeds to stress after 4 days of storage compared with newly shed seeds might be due to the seed attaining a greater ability to induce defence mechanisms when metabolic activity is enhanced (Calistru *et al.*, 2000), but since both GR and SOD activity was not unusually high in the seeds placed at 6°C after storage for 4 days at 25°C this may pertain particularly to defences against pathogens. Anguelova-Merhar *et al.* (2002) found that once *A. marina* seeds had been wet-stored for 4 days after harvesting, the activity of β -1,3-glucanase and chitinase, both antimicrobial enzymes, increased more than two-fold compared with newly shed seeds. Another consideration is that the results obtained for antioxidant enzyme activity are not entirely representative, by virtue of the fact that seed samples probably included viable and nonviable seeds from which

the enzymes were extracted. The result reported, in all likelihood, represents a non-uniform population that consisted of some seeds that were more deteriorated than others. This makes it difficult to distinguish the cause and effect of deterioration (McDonald, 1999).

It was also interesting that no measurable catalase (CAT) activity was found in the axes or cotyledons of the *A. marina* seeds. According to MacRae and Ferguson (1985) reduction in CAT activity is a general response to stress, and is characteristic of leaves and cotyledons exposed to low temperatures. Initially this seems highly detrimental, as CAT mediates the detoxification of H_2O_2 , which is generated mostly by mitochondrial respiration. Cakmak *et al.* (1993) state that CAT has a fairly low affinity for H_2O_2 and that in the seed its activities are usually very low or undetectable in the cytoplasm, mitochondria and chloroplasts; it is the ascorbate-dependent H_2O_2 scavenging system that is more effective. The enzyme ascorbate peroxidase (APX) increases the earliest and the most during imbibition and germination, especially in the embryo, as H_2O_2 generation increases with the increased respiratory activity following seed imbibition (Cakmak *et al.*, 1993). Cakmak *et al.* (1993) mention that the stimulation of this pathway during imbibition and germination may be crucial for the detoxification of H_2O_2 . One of the enzymes essential for the operation of this pathway, dehydroascorbate reductase (DHAR), is cold labile (Jahnke *et al.*, 1991), therefore preventing the operation of this pathway at low temperatures. This would be detrimental to *A. marina* seeds stored at chilling temperatures because some water uptake, and germination, continues during storage. It might be part of the reason why *A. marina* seeds succumb so readily to chilling injury; free radicals produced during water uptake and germination cannot be quenched at chilling temperatures because the main detoxification pathway is inoperable.

Prasad (1996) suggested that the death of non-acclimated and 5 day old acclimated maize seedlings was due to insufficient of antioxidant activity to scavenge the damaging levels of reactive oxygen species (ROS) generated during chilling. Evidence for this suggestion lay in the fact that a number of antioxidant enzymes were rapidly induced in the early stages of acclimation in seedlings less than 5 days old. It would be interesting

to compare the level of antioxidant enzyme activity in chilling sensitive seeds with that of chilling tolerant seeds, as it seems clear that antioxidants contribute largely towards chilling tolerance. It would also have been beneficial to determine the extent of antioxidant enzyme activity in the *E. capensis* seeds as well, as this species was not as sensitive to the higher chilling temperatures as *A. marina*.

Of the sugars found in the *A. marina* seeds, sucrose and stachyose were the most prominent. In the axes of *A. marina* seeds stored at 16°C sucrose accumulated while stachyose declined (Figure 11: B). This could indicate possible inhibition of one or more enzymes in the sugar synthesising pathway, because stachyose requires sucrose for synthesis and should therefore increase while sucrose decreases (Krebbbers *et al.*, 1997). The increase in sucrose at 16°C may have occurred because it was no longer being used in the synthesis of stachyose, and the decrease in stachyose because it was being utilised and not replenished. The opposite occurred in the axes of *A. marina* seeds stored at 25°C; sucrose initially increased and then decreased while stachyose increased, indicating that at ambient temperatures stachyose is synthesised from the available sucrose (Figure 11: D). Pollock (1984) also found that sucrose accumulation during light was higher at low temperatures (5°C) in the species *Lolium temulentum* L. According to Levitt (1980), some enzymes are inhibited more than others by low temperatures, which can result in the relative enhancement of a particular enzyme and therefore an increase in a specific metabolic pathway. The high levels of stachyose found in the cotyledons of *A. marina* are normal, as it has been reported to be the most abundant oligosaccharide present in the axes and cotyledons of these seeds (Farrant *et al.*, 1992). Stachyose aids in maintaining the low water potentials necessary in the seed, as they are shed into a saline environment – osmotic potential of *A. marina* seeds is said to be approximately -3 Mpa and that of sea water approximately -2.5 Mpa (Pammenter *et al.*, 1997), and sucrose would consequently be made available for immediate translocation to the axes for germination initiation immediately on shedding (Farrant *et al.*, 1993).

4.3 Ultrastructural Responses

Ultrastructural changes can provide a structural interpretation of biochemical and physiological changes caused by chilling stress (Wang, 1982), and are therefore very useful. The root primordia of the *A. marina* seeds and the axes of the *E. capensis* seeds contain meristematic regions that are critical for germination but also, being relatively superficially located, are particularly susceptible to deterioration, so any ultrastructural changes in response to chilling stress should have been clearly evident. When the ultrastructural responses of the *E. capensis* and *A. marina* seeds to chilling stress were assessed it was sometimes difficult to distinguish changes, and damage was not always evident even though germination capability was almost zero. The reason for this may have been because chilling injury is often not immediately evident, sometimes becoming evident only 2 or 3 days later, once the seed has been removed to ambient temperatures or set out to germinate (Lyons, 1973). In the present study, samples for electron microscopy were taken immediately after removal from the low temperature. It may have been beneficial to set the seeds out for germination 1 or 2 days before sampling was done.

One of the most interesting ultrastructural responses observed was cytolysosome formation. This is a type of vacuolation that involves the enclosure of a volume of cytoplasm, which usually contains various organelles, by specialised ER elements (Lamb and Berjak, 1981). Those authors mention that the organelles and membranes isolated in the vacuole eventually degrade so that a normal, single-membrane vacuole results. ER proliferation seemed to precede cytolysosome formation, but it appeared that this occurred only at certain temperatures. It may be possible that cytolysosome formation occurred more rapidly as the temperature was reduced and that the stage of ER proliferation had already occurred, as there was evidence of this type of vacuolar ontogeny at the lower temperatures. Residual membranes could be seen inside a number of vacuoles from the cells of *A. marina* seeds stored at 6°C after 4 days at 25°C, presumably still needing time to degrade completely (Figure 23.3). In *E. capensis* seeds stored for 8 weeks at 16°C there were not many vacuoles present, but ER proliferation and ER surrounding various organelles was evident (Figure 14.1). This is suggested to be indicative of the beginning stages of cytolysosome formation. After 8 weeks at 6°C

there were considerably more vacuoles, many of which still contained membranes, and ER was proliferating in whorls around organelles (Figure 15.1), indicating that vacuolation was ongoing. After 8 weeks at 3°C there was no evidence of ER proliferation or whorl formation, but many vacuoles were present – some of which still contained membranes (Figure 16.1). The conclusion is therefore that vacuole formation was initiated more quickly at lower temperatures, in both *A. marina* and *E. capensis*, than at higher temperatures, and was therefore a definite response to the chilling stress. It is interesting to note that extensive vacuolation also occurs in response to desiccation stress in recalcitrant seeds. Pammenter *et al.* (1998), studying the effects of drying rate on viability retention in *E. capensis* seeds, found that seeds dried rapidly for 6 hours to a water content of 1.3 g g⁻¹ appeared normal and had enhanced germination rates. The ultrastructural studies of those authors showed that ER proliferation had occurred in the axis cells of these seeds, which indicates that this response is not limited to chilling stress.

Irregularity of the nuclear profile, i.e. the change from an essentially spherical to a lobed profile, was a common feature of *A. marina* seeds stored for any length of time at any temperature. This could, at least partly, be a consequence of deterioration of nucleoskeletal structure. Compare the control prior to storage (Figure 18.2) with seeds stored for 8 days at 6°C (Figure 22.2), 8 days at 6°C after 4 days at 25°C (Figure 23.1), 28 days at 16°C (Figure 21.1) and 28 days at 25°C (Figure 20.1). Merhar *et al.* (2002) found that after rehydration of desiccated recalcitrant *Trichilia dregeana* seeds, nucleoskeletal elements became abnormally aggregated. Those authors suggested that dehydration lead to progressive loss of the ability for nucleoskeleton reassembly; it is possible that chilling has a similar effect. Nucleolar vacuoles were seen in those seeds stored for 8 days at 6°C (Figure 22.4) and 8 days at 6°C after 4 days at 25°C (Figure 23.1). These are considered to be indicative of high rRNA transcription, which is not necessarily a pathology but could be a compensatory response – especially in view of the other stress related occurrences such as ER proliferation and cytolysosome formation (Smith, pers. comm.¹). In the *E. capensis* seeds nucleolar morphology was affected and the extent of chromatin patches in the nucleus increased as the storage temperature was

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reduced. This first became evident after storage for 8 weeks at 6°C (Figure 15.1) and could also be seen after storage for 8 weeks at 3°C (Figure 16.1).

In a comparison of *A. marina* seeds that had been stored for 28 days at 25°C with those that had been stored for 28 days at 16°C, the signs of cellular deterioration evident were: increased vacuolation and vacuolar fusion, plasmalemma detached from the cell walls, ruptured and irregular nuclear membranes, mitochondria with electron transparent areas and ruptured external membranes and distended ER. Although seeds stored at both temperatures showed these deteriorative trends, those stored at 25°C seemed to be in a worse condition or in a more advanced stage of deterioration (compare Figure 20.1 with Figure 21.1) even though viability was higher at 25°C than at 16°C (Figure 5). The most noticeable differences could be seen in the mitochondria and the cell wall. The plasmalemma had not become detached to the same extent, the cell wall was not as 'buckled' and the mitochondria had much smaller electron transparent areas in the seeds stored at 16°C. It is natural to anticipate more damage and deterioration in the seeds stored at 16°C because *A. marina* seeds are chilling sensitive, but this did not appear to be the case. Although the present work shows that a storage temperature of 16°C is detrimental to *A. marina* seeds, such temperatures may be expected, though occurring sporadically, in the natural habitat. *Avicennia marina* is a tropical species, but its southern most limits in the Eastern Cape province are not even sub-tropical (Coates Palgrave, 1983). Thus 16°C may represent the upper threshold of a temperature range that is damaging, if sustained. However, the most likely explanation is that at the higher temperature, the damage was a consequence of a water stress imposed as the seeds progressed towards germination (Farrant *et al.*, 1986; Farrant *et al.*, 1988). Also, the deteriorative effects of any seed-associated fungi would have been enhanced at the higher temperature.

4.4 Further Investigations

Due to the preliminary nature of the present study and limited seed numbers, it would be beneficial to further investigate a number of areas.

Anabolic enzymes can often repair damage that occurs during seed storage. If they are effective the seed should be capable of normal germination, if not, the damage that occurred during storage will lead to detrimental physiological consequences and the seed will be nongerminable (McDonald, 1999). It would be interesting to determine at what point chilling damage becomes irreversible in recalcitrant seeds of particular species. Subsequent transfer to room temperature after specific periods of chilling could facilitate this.

The antioxidant activity in chilling sensitive recalcitrant seeds needs to be compared with that of chilling tolerant recalcitrant seeds before, during and after chilling for a range of species. It is said that the ability of maize seedlings to withstand chilling stress depends on their ability to increase the synthesis and activity of antioxidant enzymes during acclimation (reviewed by De Santis *et al.*, 1999). Li and Sun (1999) found that the rapid decrease in the activity of free radical scavenging enzymes contributed to the desiccation sensitivity and loss of viability of *Theobroma cacao* (cocoa) seeds during desiccation. It is implicit that antioxidant activity is of great importance in the seed when it is subjected to stress conditions. The effect of chemicals that limit ROS or trigger activation of antioxidant systems would also be of interest.

Lipid peroxidation begins with the generation of a free radical, so it can be taken as an indication of how efficiently the antioxidant scavenging systems are operating (McDonald, 1999). Lipid-soluble antioxidants are thought to contribute to the free radical tolerance of soybean seed membranes during early germination and therefore to the dehydration tolerance of this tissue (Senaratna *et al.*, 1985). This is possibly also important for chilling tolerance if damage is mediated via free radicals. It would therefore be interesting to compare lipid peroxidation occurring at chilling temperatures in chilling sensitive and chilling tolerant recalcitrant seeds.

It would be interesting to investigate the presence and up-regulation of certain genes involved with chilling tolerance and to compare this in chilling sensitive and chilling tolerant recalcitrant seeds. Stewart *et al.* (1990) found that a number of genetic determinants influenced the chilling tolerance of maize seedlings.

4.5 Conclusion

Chilling seems to have a similar effect on recalcitrant seeds as dehydration below a critical water content, and it is also species dependent. When recalcitrant seeds are severely dehydrated there is a loss of membrane integrity (Berjak *et al.*, 1984) and a disruption in metabolism that together result in loss of viability because there is an inability to repair such damage (Nautiyal and Purohit, 1985). Membranes and antioxidant systems in recalcitrant seeds are generally not adapted for severe dehydration or chilling stress, and most, but not all, are genetically suited to moist, warm, tropical climates that do not experience such conditions. It is assumed that the genetic mechanisms that control the process of maturation drying in orthodox seeds do not operate in recalcitrant seeds (Berjak *et al.*, 1984) or some of the suite of controlling mechanisms are absent (Pammenter and Berjak, 1999). The same could be assumed for genetic mechanisms and consequent processes controlling chilling sensitivity. There is evidence that drought stress can increase low temperature tolerance in some plant species, thus it is possible that the response of plants to drought and low temperature that lead to tolerance, share related genetic mechanisms and gene products (Liu *et al.*, 1998). The existence of recalcitrant seed species that are more chilling tolerant is a complicating factor in the attempt to elucidate the cause of chilling sensitivity in chilling sensitive recalcitrant seed species. Rather than simple comparisons with orthodox seeds, comparisons with temperate recalcitrant seeds, such as *Quercus robur* and *Aesculus hippocastanum*, which are more chilling tolerant, would be very useful.

REFERENCES

- Abe, K. (1990) Ultrastructural changes during chilling stress. *In*: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 71-84.
- Alscher, R. G. (1989) Biosynthesis and antioxidant function of glutathione in plants. *Physiologia Plantarum* 77, 457-464.
- Anguelova-Merhar, V. S., Calistru, C. and Berjak, P. (2002) Biochemical and histopathological studies of wet-stored recalcitrant seeds of *Avicennia marina* infected by *Fusarium moniliforme*. In press.
- Ashmore, S. E. (1997) *Status report on the development and application of in vitro techniques for the conservation and use of plant genetic resources*. Rome, Italy: International Plant Genetic Resources Institute.
- Bailly, C., Benamar, A., Corbineau, F. and Côme, D. (1996) Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated aging. *Physiologia Plantarum* 97, 104-110.
- Berjak, P. (1988) Stored seeds: the problems caused by micro-organisms (with particular reference to the fungi). *Proceedings of the Seed Pathology International Advanced Course*. Brasilia, ABRATES. pp. 38-50.
- Berjak, P. (1996) The role of micro-organisms in deterioration during storage of recalcitrant and intermediate seeds. *In*: Quédrago, A. S., Poulsen, K. and Stubsgaard (eds.) *Proceedings of a workshop on Improved Methods for handling and Storage of Intermediate/Recalcitrant Tropical Forest tree seeds*. Rome, International Plant Genetic Resources Institute. pp. 121-126.

- Berjak, P., Campbell, G. K., Farrant, J. M., Omondi-Oloo, W. and Pammenter, N. W. (1995) Responses of seeds of *Azadirachta indica* (neem) to short-term storage under ambient or chilled conditions. *Seed Science and Technology* **23**, 779-792.
- Berjak, P., Dini, M. and Pammenter, N. W. (1984) Possible mechanisms underlying the dehydration responses in recalcitrant and orthodox seeds: desiccation-associated subcellular changes in propagules of *Avicennia marina*. *Seed Science and Technology* **12**, 365-384.
- Berjak, P., Farrant, J. M., Mycock, D. J. and Pammenter, N. W. (1990) Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation-sensitivity. *Seed Science and Technology* **18**, 297-310.
- Berjak, P., Farrant, J. M. and Pammenter, N. W. (1989) The basis of recalcitrant seed behaviour: cell biology of the homoiohydrous seed condition. *In*: Taylorson, R. B. (ed.) *Recent advances in the development and germination of seeds*. Plenum Press, New York. pp. 89-108.
- Berjak, P., Mycock, D., Wesley-Smith, J., Dumet, D. and Watt, P. (1996) Strategies for in vitro conservation of hydrated germplasm. *In*: Normah, M. N., Narimah, M. K. and Clyde, M. M. (eds.) *In vitro conservation of plant genetic resources*. Kuala Lumpur, Malaysia: Percetakan Watan Sdn.Bhd.
- Berjak, P. and Pammenter, N. W. (1994) Recalcitrance is not an all-or-nothing situation. *Seed Science Research* **4**, 263-264.
- Berjak, P., Walker, M., Watt, M. P. and Mycock, D. J. 1999. Experimental parameters underlying failure or success in plant germplasm cryopreservation: a case study on zygotic axes of *Quercus robur* L. In press *Cryo-Letters*.

- Bilia, D. A., Marcos-Filho, J. and Novembre, A. D. C. L. (1999) Desiccation tolerance and seed storability of *Inga uruguensis* (Hook. et Arn). *Seed Science and Technology* **27**, 77-89.
- Boubriak, I., Dini, M., Berjak, P. and Osborne, D. J. (2000) Desiccation and survival in the recalcitrant seeds of *Avicennia marina*: DNA replication, DNA repair and protein synthesis. *Seed Science Research* **10**, 307-315.
- Bowler, C., Van Montagu, M. and Inzé, D. (1992) Superoxide dismutase and stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**, 83-116.
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry* **72**, 248-254.
- Cakmak, I., Strbac, D. and Marschner, H. (1993) Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. *Journal of Experimental Botany* **44** (258), 127-132.
- Calderon, P. and Pontis, H. G. (1985) Increase of sucrose synthase activity in wheat plants after a chilling shock. *Plant Science* **42**, 173-176.
- Caldwell, C. R. (1990) Plant enzymes in relation to chilling sensitivity. In: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 165-180.
- Calistru, C., McLean, M., Pammenter, N. W. and Berjak, P. (2000) The effects of mycofloral infection on the viability and ultrastructure of wet-stored recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh. *Seed Science Research* **10**, 341-353.

- Clairbone, A. (1985) Catalase activity. *In*: Greenwald, R. A. (ed.) *Handbook of methods for oxygen radical research*. CRC Press: Boca Raton, Florida. pp. 283-284.
- Coates Palgrave, K. (1983) *Trees of Southern Africa*. Struik Publishers: Cape Town. (2)
- Corbineau, F. and Côme, D. (1986) Experiments on the storage of seeds and seedlings of *Symphonia globulifera* L. f. (Guttiferae). *Seed Science and Technology* **14**, 585-591.
- De Santis, A., Landi, P. and Genchi, G. (1999) Changes of mitochondrial properties in maize seedlings associated with selection for germination at low temperature. Fatty acid composition, cytochrome *c* oxidase, and adenine nucleotide translocase activities. *Plant Physiology* **119**, 743-754.
- Ellis, R. H., Hong, T. D. and Roberts, E. H. (1990) An intermediate category of seed storage behaviour? I. Coffee. *Journal of Experimental Botany* **41**, 1167-1174.
- Esterbauer, H. and Grill, D. (1978) Seasonal variation of glutathione and glutathione reductase in needles of *Picea abies*. *Plant Physiology* **61**, 119-121.
- Fadzillah, N. M., Gill, V., Finch, R. P. and Burdon, R. H. (1996) Chilling, oxidative stress and antioxidant responses in shoot cultures of rice. *Planta* **199**, 552-556.
- Farrant, J. M., Pammenter, N. W. and Berjak, P. (1986) The increasing desiccation sensitivity of recalcitrant *Avicennia marina* seeds with storage time. *Physiologia Plantarum* **67**, 291-298.
- Farrant, J. M., Pammenter, N. W. and Berjak, P. (1988) Recalcitrance – a current assessment. *Seed Science and Technology* **16**, 155-166.

- Farrant, J. M., Pammenter, N. W. and Berjak, P. (1992) Development of the recalcitrant (homoiohydrous) seeds of *Avicennia marina*: anatomical ultrastructural and biochemical events associated with development from histodifferentiation to maturation. *Annals of Botany* **70**, 75-86.
- Farrant, J. M., Pammenter, N. W. and Berjak, P. (1993) Seed development in relation to desiccation tolerance: a comparison between desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation-tolerant types. *Seed Science Research* **3**, 1-13.
- Farrant, J. M., Pammenter, N. W., Berjak, P. and Walters, C. (1997) Subcellular organization and metabolic activity during the development of seeds that attain different levels of desiccation tolerance. *Seed Science Research* **7**, 135-144.
- Field, R. J. (1990) Influence of chilling stress on ethylene production. In: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 235-253.
- Finch-Savage, W. E., Pramanik, S. K. and Bewley, J. D. (1994) The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. *Planta* **193**, 478-485.
- Giannopolitis, C. N. and Ries, S. K. (1977) Superoxide dismutases. I. Occurrence in higher plants. *Plant physiology* **59**, 309-314.
- Graham, D. and Patterson, B. D. (1982) Responses of plants to low, nonfreezing temperatures: proteins, metabolism, and acclimation. *Annual Review of Plant Physiology* **33**, 347-372.
- Guy, C. L. (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 187-223.

- Hale, M. G. and Orcutt, D. M. (1987) *The physiology of plants under stress*. Canada: John Wiley & Sons, Inc.
- Han, B., Pammenter, N., Farrant, J., Berjak, P. and Kermode, A. R. (1995) A comparative analysis of dehydrin synthesis in seeds and seedlings of desiccation tolerant and recalcitrant species. *Plant Growth Regulator Society of America* **23(3)**, 244-248.
- Hendry, G. A. F. (1993) Oxygen, free radical processes and seed longevity. *Seed Science Research* **3**, 141-153.
- Hendry, G. A. F., Finch-Savage, W. E., Thorpe, P. C., Atherton, N. M., Buckland, S. M., Nilsson, K. A. and Seel, W. E. (1992) Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. *New Phytologist* **122**, 273-279.
- Herner, R. C. (1990) The effects of chilling temperatures during seeds germination and early seedling growth. In: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 51-69.
- Hideg, E. and Björn, L. O. (1996) Ultraweak light emission, free radicals, chilling and light sensitivity. *Physiologia Plantarum* **98**, 223-228.
- Hodges, D. M., Andrews, C. J., Johnson, D. A. and Hamilton, R. I. (1996) Antioxidant compound responses to chilling stress in differentially sensitive inbred maize lines. *Physiologia Plantarum* **98**, 685-692.
- Hong, T. D. and Ellis, R. H. (1996) *A protocol to determine seed storage behaviour*. Rome, IPGRI.
- Hopson, J. L. and Wessells, N. K. (1990) *Essentials of biology*. McGraw-Hill publishing company: U.S.A.

- Ishikawa, H. A. (1996) Ultrastructural features of chilling injury: injured cells and the early events during chilling of suspension-cultured Mung bean cells. *American Journal of Botany* **83**(7), 825-835.
- Jahnke, L. S., Hull, M. R. and Long, S. P. (1991) Chilling stress and oxygen metabolizing enzymes in *Zea mays* and *Zea diploperennis*. *Plant, Cell and Environment* **14**, 97-104.
- Kermode, A. R. (1990) Regulatory mechanisms involved in the transition from seed development to germination. *Critical Review Plant Science* **9**, 155-195.
- Kermode, A. R. and Bewley, J. D. (1988) The role of maturation drying in the transition from seed development to germination V. Responses of the immature castor bean embryo to isolation from the whole seed: a comparison with premature desiccation. *Journal of Experimental Botany* **39**, 487-497.
- Kimball, S. L. and Salisbury, F. B. (1973) Ultrastructural changes of plants exposed to low temperatures. *American Journal of Botany* **60**(10), 1028-1033.
- King, M. W. and Roberts, E. H. (1980) Maintenance of recalcitrant seeds in storage. In: Chin, H. F. and Roberts, E. H. (eds.) *Recalcitrant crop seeds*. Kuala Lumpur, Malaysia: Tropical Press SDN.BHD pp. 53-89.
- Kioko, J., Berjak, P., Pammenter, N. W., Watt, M. P. and Wesley-Smith, J. (1998) Desiccation and cryopreservation of embryonic axes of *Trichilia dregeana* Sond. *Cryo-Letters*, **19**: 5-14.
- Kodama, H., Horiguchi, G., Nishiuchi, T., Nishimura, M. and Iba, K. (1995) Fatty acid desaturation during chilling acclimation is one of the factors involved in conferring low-temperature tolerance to young Tobacco leaves. *Plant Physiology* **107**, 1177-1185.

- Kojima, M., Suzuki, H., Ohnishi, M. and Ito, S. (1998) Effects of growth temperature on lipids of Adzuki bean cells. *Phytochemistry* **47(8)**, 1483-1487.
- Krebbers, E., Broglie, R., Hitz, B., Jones, T. and Hubbard, N. (1997) Biotechnological approaches to altering seed composition. *In: Larkins, B. A. and Vasil, I. K. (eds.) Cellular and molecular biology of plant seed development.* Dordrecht: Kluwer academic publishers. pp. 595-633.
- Lamb, J. M. and Berjak, P. (1981) A unifying view of vacuolar ontogeny from studies on the root cap of *Zea mays* L. *South African Journal of Science* **77**,
- Leopold, A. C. (1980) Temperature effects on Soybean imbibition and leakage. *Plant Physiology* **65**, 1096-1098.
- Levitt, J. (1980) *Responses of plants to environmental stresses.* Volume 1: *Chilling, freezing and high temperature stresses.* New York: Academic Press, Inc. (2)
- Li, C. and Sun, W. Q. (1999) Desiccation sensitivity and activities of free radical-scavenging enzymes in recalcitrant *Theobroma cacao* seeds. *Seed Science Research* **9**, 209-217.
- Liu, J. J., Krenz, D. C., Galvez, A. F. and De Lumen, B. O. (1998) Galactinol synthase (GS): increased enzyme activity and levels of mRNA due to cold and desiccation. *Plant Science* **134**, 11-20.
- Lyons, J. M. (1973) Chilling injury in plants. *Annual Review of Plant Physiology* **24**, 445-466.
- Lyons, J. M. and Breidenbach, R. W. (1990) Relation of chilling stress to respiration. *In: Wang, C. Y. (ed.) Chilling injury of Horticultural crops.* CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 223-233.

- MacRae, E. A. and Ferguson, I. B. (1985) Changes in catalase activity and hydrogen peroxide concentration in plants in response to low temperature. *Physiologia Plantarum* **65**, 51-56.
- Markhart, A. H. (1986) Chilling injury: a review of possible causes. *Hort Science* **21(6)**, 1329-1333.
- Mayer, A. M. and Poljakoff-Mayber, A. (1989) *The germination of seeds*. UK, Oxford: Pergamon Press plc. (4)
- McDonald, M. B. (1999) Seed deterioration: physiology, repair and assessment. *Seeds Science and Technology* **27**, 177-237.
- Merhar, V. S., Gumede, Z. P. and Berjak, P. (2002) Sensitivity of the nucleoskeleton to dehydration in recalcitrant seeds of *Trichilia dregeana* Sond. *Proceedings of the 15th International Congress on Electron Microscopy*. Durban, South Africa. pp. 745-746.
- Monk, L. S., Fagerstedt, K. V. and Crawford, R. M. M. (1989) Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress. *Physiologia Plantarum* **76**, 456-459.
- Murata, T. (1990) Relation of chilling stress to membrane permeability. *In*: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 201-209.
- Murata, N. and Los, D. A. (1997) Membrane fluidity and temperature perception. *Plant Physiology* **115**, 875-879.
- Murata, N. and Nishida, I. (1990) Lipids in relation to chilling sensitivity in plants. *In*: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 181-199.

- Murata, N. and Yamaya, J. (1984) Temperature-dependent phase behavior of phosphatidylglycerols from chilling-sensitive and chilling-resistant plants. *Plant Physiology* **74**, 1016-1024.
- Murata, N., Sato, N., Takahashi, N. and Hamazaki, Y. (1982) Compositions and positional distributions from leaves of chilling-sensitive and chilling-resistant plants. *Plant and Cell Physiology* **23**(6), 1071-1079.
- Nautiyal, A. R. and Purohit, A. N. (1985) Seed viability in sal II: Physiological and biochemical aspects of ageing in seeds of *Shorea robusta*. *Seed Science and Technology* **13**, 69-76.
- Nishida, I. and Murata, N. (1996) Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 541-568.
- Omran, R. G. (1980) Peroxide levels and the activities of catalase, peroxidase, and indolacetic acid oxidase during and after chilling cucumber seedlings. *Plant Physiology* **65**, 407-408.
- Pammenter, N. W. and Berjak, P. (1999) A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Science Research* **9**, 13-37.
- Pammenter, N. W., Berjak, P., Farrant, J. M., Smith, M. T. and Ross, G. (1994) Why do stored hydrated recalcitrant seeds die? *Seed Science Research* **4**, 187-191.
- Pammenter, N. W., Farrant, J. M. and Berjak, P. (1984) Recalcitrant seeds: Short-term storage effects in *Avicennia marina* (Forssk.) Vierh. may be germination-associated. *Annals of Botany* **54**, 843-846.

- Pammenter, N. W., Greggains, V., Kioko, J. I., Wesley-Smith, J., Berjak, P. and Finch-Savage, W. E. (1998) Effects of differential drying rates on viability retention of *Ekebergia capensis*. *Seed Science Research* **8**, 463-471.
- Pammenter, N. W., Motete, N. and Berjak, P. (1997) The response of hydrated recalcitrant seeds to long-term storage. *In*: Ellis, R. H., Black, M., Murdoch, A. J and Hong, T. D. (eds.) *Basic and applied aspects of seed biology: proceedings of the 5th international workshop on seeds*. Dordrecht: Kluwer academic publishers. pp. 689-703.
- Pauls, K. P. and Thompson, J. E. (1984) Evidence for the accumulation of peroxidized lipids in membranes of senescing cotyledons. *Plant Physiology* **75**, 1152-1157.
- Pollock, C. J. (1984) Sucrose accumulation and the initiation of fructan biosynthesis in *Lolium temulentum* L. *New Phytologist* **96**, 527-534.
- Pollock, C. J., Lloyd, E. J., Stoddart, J. L. and Thomas, H. (1983) Growth, photosynthesis and assimilate partitioning in *Lolium temulentum* exposed to chilling temperatures. *Physiologia Plantarum* **59**, 257-262.
- Pontis, H. G. (1989) Fructans and cold stress. *Journal of Plant Physiology* **134**, 148-150.
- Prasad, T. K. (1996) Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: changes in antioxidant system, oxidation of proteins and lipids, and protease activities. *The Plant Journal* **10** (6), 1017-1026.
- Prasad, T. K. (1997) Role of catalase in inducing chilling tolerance in pre-emergent maize seedlings. *Plant Physiology* **114**, 1369-1376.

- Prasad, T. K., Anderson, M. D., Martin, B. A. and Stewart, C. R. (1994a) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *The Plant Cell* **6**, 65-74.
- Prasad, T. K., Anderson, M. D. and Stewart, C. R. (1994b) Acclimation, hydrogen peroxide, and abscisic acid protect mitochondria against irreversible chilling injury in maize seedlings. *Plant Physiology* **105**, 619-627.
- Puebla, A. F., Salerno, G. L. and Pontis, H. G. (1997) Fructan metabolism in two species of *Bromus* subjected to chilling and water stress. *New Phytologist* **136**, 123-129.
- Pukacka, S. (1991) Changes in membrane lipid components and antioxidant levels during natural ageing of seeds of *Acer platanoides*. *Physiologia Plantarum* **82**, 306-310.
- Purvis, A. C. (1990) Relation of chilling stress to carbohydrate composition. In: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 211-221.
- Purvis, A. C. and Shewfelt, R. L. (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiologia Plantarum* **88**, 712-718.
- Raison, J. K. and Orr, G. R. (1990) Proposals for a better understanding of the molecular basis of chilling injury. In: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 145-164.
- Raison, J. K. and Wright, L. C. (1983) Thermal phase transitions in the polar lipids of plant membranes. Their induction by disaturated phospholipids and their possible relation to chilling injury. *Biochim Biophys Acta* **731**, 69-78.

- Roberts, E. H. (1973) Predicting the storage life of seeds. *Seed Science and Technology* **1**, 499-514.
- Roberts, E. H. and King, M. W. (1980) The characteristics of recalcitrant seeds. *In*: Chin, H. F. and Roberts, E. H. (eds.) *Recalcitrant crop seeds*. Kuala Lumpur, Malaysia: Tropical Press SDN.BHD. pp. 1-5.
- Roos, E. E. (1986) Precepts of successful seed storage. *In*: McDonald, M. B. and Nelson, C. J. (eds.) *Physiology of seed deterioration*. CSSA special publication no. 11. USA: Crop science society of America, Inc.
- Roughan, P. G. (1985) Phosphatidylglycerol and chilling sensitivity in plants. *Plant Physiology* **77**, 740-746.
- Rühl, G. (1996) Microscopic methods for subcellular examination of desiccation and chilling sensitivity. *In*: Ouedraogo, A. S., Poulsen, K. and Stubsgaard, F. (eds.) *Intermediate/recalcitrant tropical forest tree seeds: proceedings of a workshop on improved methods for handling and storage of intermediate/recalcitrant tropical forest tree seeds 8-10 June 1995*. Humlebaek, Denmark: IPGRI. pp. 44-73.
- Saltveit, M. E. and Morris, L. L. (1990) Overview on chilling injury of horticultural crops. *In*: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 3-15.
- Santoiani, C. S., Tognetti, J. A., Pontis, H. G. and Salerno, G. L. (1993) Sucrose and fructan metabolism in wheat roots at chilling temperatures. *Physiologia Plantarum* **87**, 84-88.
- Sarmiento, C., Garcés, R. and Mancha, M. (1998) Oleate desaturation and acyl turnover in sunflower (*Helianthus annuus* L.) seed lipids during rapid temperature adaptation. *Planta* **205**, 595-600.

- Saruyama, H. and Tanida, M. (1995) Effect of chilling on activated oxygen-scavenging enzymes in low temperature-sensitive and -tolerant cultivars of rice (*Oryza sativa* L.). *Plant Science* **109**, 105-113.
- Scandalios, J. G. (1993) Oxygen stress and superoxide dismutases. *Plant Physiology* **101**, 7-12.
- Senaratna, T., Mckersie, B. D. and Stinson, R. H. (1985) Antioxidant levels in germinating Soybean seed axes in relation to free radical and dehydration tolerance. *Plant Physiology* **78**, 168-171.
- Simon, E. W. (1974) Phospholipids and plant membrane permeability. *New Phytologist* **73**, 377-420.
- Smith, I. K., Vierheller, T. L. and Thorne, C. A. (1989) Properties and functions of glutathione reductase in plants. *Physiologia Plantarum* **77**, 449-456.
- Steinke, T. D. and Naidoo, Y. (1991) Respiration and net photosynthesis of cotyledons during establishment and early growth of propagules of *Avicennia marina* at three temperatures. *South African Journal of Botany* **57**, 171-174.
- Stewart, C. F., Martin, B. A., Reding, L. and Cerwick, S. (1990) Seedling growth, mitochondrial characteristics, and alternative respiratory capacity of corn genotypes differing in cold tolerance. *Plant Physiology* **92**, 761-766.
- Sung, J. M. and Jeng, T. L. (1994) Lipid peroxidation and peroxide-scavenging enzymes associated with accelerated aging of peanut seed. *Physiologia Plantarum* **91**, 51-55.
- Taiz, L. and Zeiger, E. (1988) *Plant Physiology*. Sunderland, Massachusetts: Sinauer Associates, Inc. (2)

- Thompson, J. E., Legge, R. L. and Barber, R. F. (1987) The role of free radicals in senescence and wounding. *New Phytologist*, **105**, 317-344.
- Tognetti, J. A., Salerno, G. L., Crespi, M. D. and Pontis, H. G. (1990) Sucrose and fructan metabolism of different wheat cultivars at chilling temperatures. *Physiologia Plantarum* **78**, 554-559.
- Van Hasselt, P. R. (1990) Light-induced damage during chilling. *In*: Wang, C. Y. (ed.) *Chilling injury of Horticultural crops*. CRC Press Inc.: Boca Raton, Florida, U.S.A. pp. 113-128.
- Vertucci, C. W. (1990) Calorimetric studies of the state of water in seed tissues. *Biophysical Journal* **58**, 1463-1471.
- Walker, M. A. and McKersie, B. D. (1993) Role of the ascorbate-glutathione antioxidant system in chilling resistance of tomato. *Journal of Plant Physiology* **141**, 234-239.
- Wang, C. Y. (1982) Physiological and biochemical responses of plants to chilling stress. *HortScience* **17**(2), 173-186.
- Wang, C. Y. and Adams, D. O. (1982) Chilling-induced ethylene production in cucumbers (*Cucumis sativus* L.). *Plant Physiology* **69**, 424-427.
- Wesley-Smith, J., Vertucci, C. W., Berjak, P., Pammenter, N. W. and Crane, J. (1992) Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. *Journal of Plant Physiology* **140**, 596-604.
- William, J., Bramlage, A., Leopold, C. and Parrish, D. J. (1978) Chilling stress to soybeans during imbibition. *Plant Physiology* **61**, 525-529.

- Wise, R. J. and Naylor, A. W. (1987) Chilling-enhanced photooxidation: the peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure. *Plant Physiology* **83**, 272-277.
- Withers, L. A. and Engelmann, F. (1998) In vitro conservation of plant genetic resources. In: Altman, A. (ed.) *Agricultural Biotechnology*. New York: Marcel Dekker, Inc. pp. 57-88.
- Wu, J. and Browse, J. (1995) Elevated levels of high-melting-point phosphatidylglycerols do not induce chilling sensitivity in an Arabidopsis mutant. *The Plant Cell* **7**, 17-27.
- Xu, Y. and Siegenthaler, P. (1996) Effect of non-chilling temperature and light intensity during growth of squash cotyledons on the composition of thylakoid membrane lipids and fatty acids. *Plant Cell Physiology* **37(4)**, 471-479.
- Xu, Y. and Siegenthaler, P. (1997) Low temperature treatments induce an increase in the relative content of both linolenic and Δ^3 -*trans*-hexadecenoic acids in thylakoid membrane phosphatidylglycerol of squash cotyledons. *Plant Cell Physiology* **38(5)**, 611-618.
- Yoshida, S. (1991) Chilling-induced inactivation and its recovery of tonoplast H⁺-ATPase in Mung Bean cell suspension cultures. *Plant Physiology* **95**, 456-460.
- Yoshida, S., Kawata, T., Uemura, M. and Niki, T. (1986) properties of plasma membrane isolated from chilling-sensitive etiolated seedlings of *Vigna radiata* L. *Plant Physiology* **80**, 152-160.
- Yoshida, S. and Matsuura-Endo, C. (1991) Comparison of temperature dependency of tonoplast proton translocation between plants sensitive and insensitive to chilling. *Plant Physiology* **95**, 504-508.

- Yoshida, S., Matsuura, C. and Etani, S. (1989) Impairment of tonoplast H⁺-ATPase as an initial physiological response of cells to chilling in Mung Bean (*Vigna radiata* [L.] Wilczek). *Plant Physiology* **89**, 634-642.
- Yu, H. L. and Willemot, C. (1996) Inhibition of eukaryotic galactolipid biosynthesis in mature-green tomato fruits at chilling temperature. *Plant Science* **113**, 33-41.
- Yu, H., Thompson, J. E., Yelle, S. and Willemot, C. (1996) Impairment of galactolipid biosynthesis in tomato pericarp at chilling temperature. *Journal of Plant Physiology* **149**, 171-178.