

Responses to chilling and cryopreservation of
recalcitrant seeds of *Ekebergia capensis*
from different provenances

By

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Durban Westville
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As the candidate's supervisor I have/have not approved this thesis/dissertation for
submission.

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ABSTRACT

Recalcitrant seeds are shed at relatively high water contents and are metabolically active. The effect of chilling the recalcitrant seeds of purportedly a single species, *Ekebergia capensis*, from Port Elizabeth (PE; Eastern Cape), St Lucia (KwaZulu-Natal [KZN]) and Tanzania (tropical southern Africa) was tested. Viability and axis ultrastructure, solute leakage and protein synthesis were investigated. Additionally, cryopreservation of embryonic axes (explants) was studied. In particular, the ability of cathodic water to improve explant survival was tested, and related to its effect on the production of potential harmful reactive oxygen species such as superoxide ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2), and its ability to maintain levels of total aqueous antioxidants (TAA). Shoot and root ultrastructure were examined after each cryopreparative step in explants treated with and without cathodic water, and ultrastructure correlated with survival.

The seeds from PE retained 80% viability after 12 weeks storage at 1° and 3°C and 100% when stored at 6°C. Those from St Lucia were dead after 38 d storage at 3°C. All the seeds from Tanzania were dead after 9 d when stored at 3°C.

The rate of protein synthesis decreased gradually over the storage period, irrespective of the provenance. Electrolyte leakage from axes showed that those from St Lucia and Tanzania ‘leaked’ solutes irrespective of the duration or temperature or storage, but those from PE showed an initial increase, which then decreased. A 30% mass loss was achieved after 48 d, 230 h and 21 h for seeds from PE, St Lucia and Tanzania, respectively. Nuclear ribosomal ITS1 sequences revealed the presence of three well-to-strongly-supported monophyletic clades corresponding to the geographical areas from which the seeds were sampled (PE, KZN and Tanzania). Axes from the seeds from St Lucia and Tanzania lost ultrastructural integrity during storage while those from PE did not.

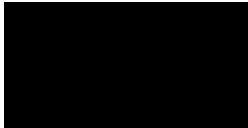
The levels of $\cdot\text{O}_2^-$ and H_2O_2 increased gradually after each cryopreparative step. Using cathodic water allowed 30% of PE explants to survive cryopreservation, while none of the St Lucia explants did. Root ultrastructure was well preserved, however, gradual ultrastructural deterioration was observed in the shoot meristem.

Seeds of *E. capensis* of temperate (PE) origin tolerate chilling temperatures well, whereas those from sub-tropical St Lucia or the tropical Tanzanian provenance, do not. Furthermore, the explants from PE could survive cryopreservation while those from St Lucia could not.

PREFACE

The experimental work described in this dissertation was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville campus, from January 2012 to December 2016, under the supervision of Professors Patricia Berjak, Norman W. Pammenter and Jennifer M. Lamb.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. The work of others, where used, has been duly acknowledged in the text.



Vishal Bharuth

DECLARATION 1 - PLAGIARISM

I, Vishal Bharuth declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
 - a. Their words have been re-written but the general information attributed to them has been referenced
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and the References sections.

Signed:

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DECLARATION 2 – PUBLICATIONS

1. Bharuth, V., and Naidoo, C. (2020). Responses to cryopreservation of recalcitrant seeds of *Ekebergia capensis* from different provenances. South African Journal of Botany, 132: 1-14
2. Bharuth, V., Naidoo, C., Pammenter, N.W., Lamb, J.M. and Naidoo, T. (2020). Responses to chilling of recalcitrant seeds of *Ekebergia capensis* from different provenances. South African Journal of Botany, 130: 8-24

CONFERENCE PRESENTATIONS

3. Bharuth, V., Berjak, P., Pammenter, P., Lamb, J. and Naidoo, T. Responses to chilling of recalcitrant seeds of *Ekebergia capensis* from different provenances. 50th Microscopy Society of Southern Africa, Cape Town 2012
4. Bharuth, V., Pammenter, N.W., Naidoo, C. and Berjak, P. Ultrastructural and biochemical analyses during procedural stages of cryopreservation of embryonic axes of *Ekebergia capensis* from different provenances. 53rd Microscopy Society of Southern Africa, Pretoria 2015
5. Bharuth, V., Pammenter, N.W., Naidoo, C. and Berjak, P. Ultrastructural and biochemical analyses during procedural stages of cryopreservation of embryonic axes of *Ekebergia capensis* from different provenances. 7th International Workshop on Desiccation Sensitivity and Tolerance across Life Forms 2016

The experimental work and analyses for publications 1 and 2 were designed and carried out by myself. These papers were a product of this thesis. I wrote both papers with input from my supervisors.

The data presented at the various conferences were my own (3-8). The posters and talks at these conferences were overseen and guided by my supervisors Prof. Berjak and Prof. Pammenter.

All authors are duly acknowledged for their role in each of the outputs listed above.

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Large plastids with starch (S) grains were also observed. The ultrastructure of the shoot meristem from this treatment (Figs. 3.53c&d) showed total destruction with no discernible organelles apart from numerous vacuoles (V) and a very granulated cytoplasm (C) – presumably remnants of cellular components. 195

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Cell walls (cw) were well-developed with distinct plasmodesmata (pd). The cells from the shoot meristem of this treatment (Figs. 3.53g&h) revealed highly vacuolated cells with no discernible organelles. 195

The ultrastructure of fresh seeds was well preserved indicating active metabolic activity (Fig. 3.54a). 262

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CHAPTER 1: INTRODUCTION

1.1 Seed categorisation and seed storage

It is necessary to store seeds not only for food, feed, and sufficient planting stock for the next season because seed production is seasonal, but not always reliable. It is also important for the preservation of genetic variability within species (Duffus and Slaughter, 1980). Dry, orthodox seeds which are desiccation tolerant can be stored at low temperature and low relative humidity (RH) (e.g. Bewley, 1986; Roos, 1986; Walters *et al.*, 2005). Although this method of storing seeds prolongs post-harvest longevity and reduces the effects of microflora that bring about seed deterioration, it is precluded for recalcitrant seeds that are desiccation sensitive and may also be chilling sensitive (Roberts, 1973; Chin and Roberts, 1980).

There are marked differences among species in the ability of recalcitrant seeds to survive desiccation, and these differences influence seed storage behaviour (Hong and Ellis, 1990). This variability in post-harvest physiology of seeds between species resulted originally in the most desiccation-sensitive types being categorised as recalcitrant and the desiccation tolerant types as orthodox (Roberts, 1973, 1979). Between these two extremes is the so-called intermediate group of seeds, which were originally defined by being able to tolerate considerable dehydration (but less than orthodox types), and being sensitive to chilling in the dehydrated state (Ellis *et al.*, 1990, 1991). However, Hong and Ellis (1996) modified this definition of intermediate post-harvest responses in associating chilling sensitivity primarily with seeds of tropical species. This categorisation of seeds was suggested to be too inflexible by Berjak and Pammenter in 1994. Those authors proposed instead that seed characteristics should be viewed as constituting a continuum across species, flanked by extreme recalcitrance (desiccation sensitivity) at one end, and the most orthodox of behaviour (in terms of desiccation tolerance) at the other. This view was later repeated by those authors (Berjak and Pammenter, 2004), and their views supported by others (e.g. Kermode and Finch-Savage, 2002).

1.2 Orthodox seeds

Orthodox seeds are those that dry down to low water contents as a developmental event and maintain viability in the desiccated state. During development, orthodox seeds virtually shut down all metabolic activities and lose water during maturation drying, generally to 0.15 –

0.08 g g⁻¹ dry mass, depending on seed composition and ambient relative humidity (Berjak and Pammenter, 2000; Hoekstra *et al.*, 2001). Such seeds can remain viable for years (Roos, 1986), decades or even centuries (depending on the species) provided that they are stored under low optimal temperature and low RH conditions (Vertucci and Farrant, 1995; Farrant *et al.*, 1993; Walters *et al.*, 2005; Berjak, 2006). The ability of orthodox seeds to tolerate desiccation is acquired during the stage when reserves in the form of starch, protein and lipid are accumulated (Bewley and Black, 1994), after which dry mass stabilises, water is lost, and as a consequence, the fresh mass of the seed declines (e.g. Bewley and Black, 1994; Wang *et al.*, 2014; Leprince *et al.*, 2017). This phase of maturation drying can proceed only because the seeds have in place a spectrum of enabling mechanisms (reviewed by Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Berjak and Pammenter, 2008).

Orthodox seeds not only tolerate desiccation, but providing they are of good quality, their longevity is predictable under specific conditions of reduced storage temperature and RH (Ellis *et al.*, 1990). The lower the storage temperature and RH, and hence seed water content (within limits [Vertucci and Roos, 1990; Walters and Engels, 1998]), the longer the successful storage lifespan of the seeds (Berjak and Pammenter, 2004). Having no freezable water (Walters *et al.*, 2005) these seeds are best stored at sub-zero temperatures.

1.3 Intermediate seeds

Intermediate seeds are those that can tolerate some water loss but not as much as orthodox seeds, and may be sensitive to chilling. Intermediate seeds constitute those of a broad group of species and their tolerance to chilling temperature varies within the group (Ellis *et al.*, 1990). These seeds appear to show some, but not all the characteristics of orthodox seeds (Hong and Ellis, 1996). However, water contents less than ~0.1 g g⁻¹ (dry mass basis) and temperatures close to 0°C appear to be deleterious (Hong and Ellis, 1996). Intermediate seeds are generally understood as being less desiccation tolerant than orthodox types, with those of many species being damaged by chilling in the dehydrated state (Hong and Ellis, 1996; Crane *et al.*, 2003).

1.4 Recalcitrant seeds

Recalcitrant seeds were originally described as being produced predominantly by tropical and sub-tropical tree species (Chin and Roberts, 1980). However, subsequently seeds of several temperate species (e.g. *Quercus* spp. [Gosling, 1989; Finch-Savage *et al.*, 1993; Bonner, 1996]; *Aesculus hippocastanum* [Tompsett and Pritchard, 1993]; *Acer* spp. [Hong and Ellis, 1990; Dickie *et al.*, 1991; Finch-Savage *et al.*, 1993; Greggains *et al.*, 2000]; *Podocarpus* spp. [Dodd *et al.*, 1989]) were discovered to be recalcitrant, as were the seeds of some herbaceous monocots (Sershen *et al.*, 2008). Recalcitrant seeds are sensitive to water loss and some are also chilling sensitive (Chin and Roberts, 1980). They are shed at high water contents (Berjak and Pammenter, 2004), and continue to be metabolically active after shedding (Berjak *et al.*, 1984; Farrant *et al.*, 1985). Recalcitrant seeds of most species, particularly those of tropical provenance, tolerate only a small proportion of water loss before potentially lethal damage occurs (e.g. Farrant *et al.*, 1989; Berjak and Pammenter, 1994, 2004; Pammenter *et al.*, 1998; Drew *et al.*, 2000; Eggers *et al.*, 2007; Umarani *et al.*, 2015).

Even when recalcitrant seeds are stored under conditions that prevent water loss, vigour and viability are lost sooner or later (Pammenter *et al.*, 1994; Berjak, 1996; Eggers *et al.*, 2007; Bharuth *et al.*, 2020), possibly as a result of the initiation of germination (Farrant *et al.*, 1988; Berjak *et al.*, 1989). The greater the desiccation sensitivity of a population of post-harvest recalcitrant seeds, the further germination is likely to have proceeded even if there is no outward sign of this occurring (Farrant *et al.*, 1988; Daws *et al.*, 2004; 2006).

However, the rate at which recalcitrant seeds lose water and the proportion of water that can be lost before lethal damage occurs, is species dependent. Species-specific characteristics of desiccation sensitivity, storability and chilling sensitivity have led to recalcitrant seed behaviour to be classified as a continuum, either highly, moderately, or minimally recalcitrant (Farrant *et al.*, 1988; Berjak and Pammenter, 1994). Since recalcitrant seeds cannot be appropriately dried, they cannot be stored at sub-zero temperatures because at the typically high water contents, lethal ice-crystal formation will occur (Chin and Roberts, 1980; Wesley-Smith *et al.*, 1992; 2014).

Recalcitrant seeds remain metabolically active throughout development to shedding, and continue to accumulate dry mass (Finch-Savage, 1992). Additionally, no significant decline

in water content occurs, nor do recalcitrant seeds undergo the dramatic metabolic shutdown that characterises orthodox seeds during the late stages of maturation (Pammenter and Berjak, 1999; Berjak and Pammenter, 2004; 2008; Kleinwächter *et al.*, 2014). Ongoing metabolism is considered ultimately to have lethal consequences as a result of the uncontrolled production of reactive oxygen species (ROS) and consequent intracellular degradation (Vertucci and Farrant, 1995; Pammenter *et al.*, 1998; Walters *et al.*, 2001; Berjak and Pammenter, 2004). It has been suggested that genetic control of some of the events culminating in desiccation tolerance is either absent or non-functional in recalcitrant seeds (e.g. Roos, 1986; Pammenter and Berjak, 1999).

There are several potential advantages associated with the storage of seed at sub-ambient temperatures, such as decreased microbial growth, lowered respiratory activity of both seeds and micro-organisms, and a reduced rate of cellular degradation (Kioko *et al.*, 2006). Low temperatures, in the region of 2°C, prove effective in preventing the premature germination of hydrated non-orthodox seeds from temperate species (e.g. *Quercus* spp.) (King and Roberts, 1980). However, generally tropical/sub-tropical recalcitrant seeds cannot be stored at such low temperatures, having to be stored hydrated and at a favourable temperature, which can be as high as 16°C for some species (Chin and Roberts, 1980). These requirements lead to various problems, particularly fungal proliferation, which rapidly becomes evident at elevated levels of humidity and temperature and is detrimental to seed survival (Berjak, 1996; Calistru *et al.*, 2000).

1.5 Problems associated with storage of recalcitrant seeds

1.5.1 Physiological:

1.5.1.1 Desiccation sensitivity

Agricultural systems rely on the ability of orthodox seeds to tolerate desiccation (Leprince *et al.*, 1993; Blackman *et al.*, 1995). Drying recalcitrant seeds to low levels of water – generally $<0.1 \text{ g g}^{-1}$ – tolerated by orthodox seeds, would result in irreversible loss of function in vegetative plant tissues. However, orthodox seeds have mechanisms that prevent or counteract damage (Blackman *et al.*, 1995; Farrant, 1995; Pammenter and Berjak, 1999;

Berjak, 2006; Berjak *et al.*, 2007), which are generally lacking, or at least non-functional in recalcitrant seeds (Pammenter and Berjak, 1999).

Deleterious events occur in the desiccation-sensitive tissues of recalcitrant seeds during dehydration (Li and Sun, 1999), the extent and severity of which depend on the rate at which water is lost (Pammenter *et al.*, 1998; Berjak and Pammenter, 2004). These result in a decline in viability (Roberts, 1973), which is a major problem for long-term conservation of the genetic resources (Li and Sun, 1999). Considerable variation in drying tolerance of recalcitrant seeds has been reported (King and Roberts, 1980; Hong and Ellis, 1990; Farrant *et al.*, 1993; Leprince *et al.*, 1993) but these studies did not consider drying rates (Pammenter *et al.*, 1998). It is necessary to consider each species individually when attempting to determine the optimal water content for maintenance of recalcitrant seed viability. Early views were that it was unlikely that a standard critical water content would emerge for the storage of recalcitrant seed of all species (King and Roberts, 1980), which is reinforced considering the range of water contents at shedding recorded across species (Berjak and Pammenter, 2004). Indeed, it has been shown that irrespective of the original water content, loss of even a small proportion of tissue water compromises seed vigour and storage longevity (Drew *et al.*, 2000; Eggers *et al.*, 2007).

Dehydration of desiccation-sensitive plant tissues results in cellular and metabolic injuries (Walters *et al.*, 2002; Corb  neau *et al.*, 2004; Arulmoorthy *et al.*, 2020). The inability of desiccation-sensitive plant tissues to maintain physiological integrity, as well as to limit the occurrence of oxidative processes or accumulation of free radicals, results in cellular deterioration (Buitink *et al.*, 2002). Dehydration is also associated with a general reduction of metabolism (Corb  neau *et al.*, 2004), but concomitantly, with injury (Corbineau *et al.*, 2004). In *Araucaria angustifolia* dehydration-induced loss of germination was characterised by a sequence of metabolic events associated with irreversible cellular damage (Salmen Espindola *et al.*, 1994). An increase in leakage of ions, sugars and proteins is associated with membrane damage (Li and Sun, 1999). This is correlated with a reduction in the ability of the embryos to convert 1-aminocyclopropane 1-carboxylic acid (ACC) to ethylene, often considered to be an early indicator of cell deterioration (Corbineau *et al.*, 1990; Matilla, 2000; Concell  n *et al.*, 2005).

As water is slowly lost, metabolism will continue, but when the seeds are still at relatively high water contents, metabolism will become unbalanced or out-of-phase, as a result of

certain processes occurring and others becoming impaired (Smith and Berjak, 1995; Pammenter and Berjak, 1999; Walters *et al.*, 2001). The most likely immediate consequence of this unregulated metabolism is thought to be the generation of free radicals and accompanying oxidative damage (Leprince *et al.*, 1990; Hendry *et al.*, 1992; Vertucci and Farrant, 1995; Walters *et al.*, 2001; Berjak, 2006).

Whatever the causes, the fact remains that recalcitrant seeds will always be naturally desiccation sensitive (Berjak and Pammenter, 2008) but on dehydration under similar conditions, the degree of water loss that is lethal appears to be species specific (Farrant *et al.*, 1989; Pammenter and Berjak, 1999; Berjak, 2006). However, within a species the extent of recalcitrance can be influenced by seed provenance, as has been shown for *Aesculus hippocastanum* and *Acer pseudoplatanus* (Daws *et al.*, 2004, 2006).

1.5.1.2 Chilling sensitivity

Plants of many species, especially those of tropical and sub-tropical origin, suffer injury when exposed to temperatures above the freezing point of tissue water but below approximately 15°C (Bedi and Basra, 1993). Sensitivity to chilling depends on species, developmental stages, organs and tissues (Levitt, 1980). Furthermore, Kratsch and Wise (2000) suggest that chilling stress is either enhanced or inhibited by other factors such as light intensity and relative humidity. Development of such injury increases with decreasing temperature and with exposure time to chilling (Posmyk *et al.*, 2001) and the longer a plant is exposed to chilling conditions, the more extensive and irreversible is the injury (Kratsch and Wise, 2000). The degree of chilling sensitivity of a plant also depends on the stage of its life cycle (Nishida and Murata, 1996).

The susceptibility of non-orthodox seeds of tropical and sub-tropical species to chilling injury is not normally a problem since temperatures below 10°C would rarely be encountered in the natural environment (King and Roberts, 1980). However, provenance may be associated with the occurrence, or not, of chilling sensitivity of non-orthodox seeds of individual species, e.g. for those of neem (*Azadirachta indica*) from coastal, equatorial Kenya (Berjak *et al.*, 1995), but apparently not from Burkina Faso (Gaméné *et al.*, 1999).

It is important that the fluidity of cell membranes be maintained. Fluidity is determined by the levels of unsaturated fatty acids of membrane phospholipids, which can change upon exposure to cold (Nishida and Murata, 1996). Chilling causes transitions in membrane lipids from the fluid, liquid crystalline phase to the gel phase that impairs their function (Lyons and Raison, 1970), and membrane re-organisation becomes impaired at low temperatures (Bramlage *et al.*, 1978). Wolfe (1978) has argued that, while the hypothesis of Lyons and Raison (1970) is correct, it makes the unwarranted assumption that there is one critical temperature for species below which a vital process fails. Furthermore, that hypothesis does not consider the effects of lipid fluidity on the integral membrane enzymic proteins (Wolfe, 1978). According to Wolfe (1978), conformation of individual integral membrane proteins is likely to be differentially affected by temperature because of differential effects resulting in lateral phase separations of the various lipids at particular temperatures. Results obtained by Bloom *et al.* (1998) suggest that phase changes within membranes are not solely responsible for chilling damage. Other possibilities are that chilling might inhibit energy metabolism or be accompanied by accumulation of deleterious by-products, as suggested by Minorsky (1985). At low temperatures, chilling damage in the vegetative tissues of plants is characterised by leaf chlorosis, a reduced growth rate, and changes in chloroplast morphology (Nishida and Murata, 1996). Other studies have demonstrated that chilling tolerance in various species is partly associated with an enhanced antioxidant defence system (Wang, 1995; Posmyk *et al.*, 2001). Despite these various observations and conjectures, the physiological basis for chilling sensitivity remain uncertain and the limiting temperature below which injury will occur in recalcitrant seeds which are metabolically active (e.g. Finch-Savage, 1992), is species dependent (Chin and Roberts, 1980; Bedi and Basra, 1993).

Tropical recalcitrant seeds generally do not tolerate chilling (Bedi and Basra, 1993), but there is only limited experimental evidence reported in the literature upon which to base this supposition. In contrast, recalcitrant seeds of species of temperate provenance generally seem to be chilling-tolerant (Daws *et al.*, 2004; Bharuth *et al.*, 2020). To achieve short- to medium-term storage, recalcitrant seeds need to be maintained at the lowest temperature that they will withstand (Berjak and Pammenter, 2008). However, this must be optimised for each species, since recalcitrant seeds of different species respond differently to storage temperatures (Berjak and Pammenter, 2008). Examples of chilling sensitive, recalcitrant seeds include those of *Theobroma cacao* which do not survive below 10°C (Chin and Roberts, 1980), while seeds of *Trichilia emetica* and *Telfairia occidentalis* perish when stored at 6°C (Kioko *et al.*,

2006; Ajayi *et al.*, 2006, respectively). Chilling injury within recalcitrant seeds is, however, generally poorly documented, and the variability of the responses to chilling (within and among species) complicates attempts at achieving short- to medium-term storage (Umarani *et al.*, 2015).

1.5.1.3 Germination during storage

Another major problem with storing hydrated recalcitrant seeds for prolonged periods is that seeds are lost through germination in storage. Upon shedding, orthodox seeds of most species have water contents considerably lower than that necessary for germination. Recalcitrant seeds, on the other hand, are generally capable of immediate germination when they are shed (King and Roberts, 1980), or will progressively germinate when stored (by necessity) at the shedding water content (Berjak *et al.*, 1990; Berjak and Pammenter, 2004; 2008).

The simplest method of slowing germination would be to reduce storage temperature (King and Roberts, 1980; Suszka and Tylkowski, 1980). However, this cannot be done if the seeds are chilling-sensitive (Umarani *et al.*, 2015). Other means of curtailing germination in storage that have been proposed, e.g. the so-called ‘sub-imbibed’ storage, in practice compromise vigour and ultimately, the viability of recalcitrant seeds (Drew *et al.*, 2000; Eggers *et al.*, 2007).

1.5.2 Microbial contamination

Contamination of stored orthodox and non-orthodox seeds is a well recognised problem (Berjak, 1996; Calistru *et al.*, 2000; Sutherland *et al.*, 2000; Anguelova-Merhar *et al.*, 2003). Generally, seeds with water contents above 10-13% (wet mass basis) – i.e. 0.1-0.15 g g⁻¹ (dry mass basis) - are either destroyed by fungal contaminants, or onset of seed death is accelerated, which is associated with activity of the mycoflora (Roberts, 1973; Calistru *et al.*, 2000, respectively). Fungal activity and proliferation increase as seeds naturally deteriorate, which in turn, further facilitates fungal metabolism often of succeeding species (Berjak, 1996).

Under normal circumstances, when plant tissues are metabolically active, they produce various precursors to defence compounds (phytoalexins) (Ebel, 1986). This has been shown to occur early during storage of recalcitrant seeds of *Avicennia marina* (Calistru *et al.*, 2000). When recalcitrant seeds deteriorate, their metabolism is affected (Berjak, 1996) and despite storage in the hydrated condition, recalcitrant seeds do deteriorate and become increasingly metabolically deranged (e.g. Pammenter *et al.*, 1994). It is at this stage that microorganisms, especially fungi, have a significant impact (Berjak, 1996). Recalcitrant and intermediate seeds generally harbour a range of fungal species and bacteria when they are harvested (Berjak, 1996; Calistru *et al.*, 2000; Sutherland *et al.*, 2000). The composition of mycoflora on, and in, recalcitrant seeds has been found to narrow with increasing storage time (Mycock and Berjak, 1990; Calistru *et al.*, 2000) with *Fusarium* spp. often becoming dominant (Calistru *et al.*, 2000; Sutherland *et al.*, 2000). In other words, a fungal succession occurs in recalcitrant seeds, with the most aggressive species out-competing the others (Berjak, 1996). When recalcitrant seed metabolism becomes unbalanced (e.g. Walters *et al.*, 2001) as suggested by Berjak (1996), the ability to generate the necessary enzymes responsible for defence is probably compromised and eventually the seeds become over-run by fungi and are destroyed. The work of Calistru *et al.* (2000) and Anguelova-Merhar *et al.* (2003) on recalcitrant *Avicennia marina* seeds supports this contention.

Reduced microbial proliferation can be achieved by lowering the temperature of the storage environment. However, because recalcitrant seeds may be chilling sensitive (Bedi and Basra, 1993), this may not be assumed a viable option for the long-term storage of recalcitrant seeds across species. Lowering the seed storage temperature to the minimum tolerated per species will delay, but not solve the fungal problem. Therefore, alternative or additional protection from fungal contamination is invariably necessary (King and Roberts, 1980). A combination of a fungicide treatment, the lowest temperature possible and inclusion of a bactericide should keep microbial activity to a minimum, prolonging the storage life of recalcitrant seeds (King and Roberts, 1980; Calistru *et al.*, 2000; Finch-Savage *et al.*, 2003). Other workers have employed different techniques which appeared to curtail fungal proliferation. For example, experiments using a crude alginate coating of seeds of *Avicennia marina* reduced the growth of fungal contaminants (Motete *et al.*, 1997), but this could have been an effect of the gel preservative, Nipastat (Berjak¹).

1.5.3 Biochemical responses of recalcitrant seeds during chilled storage:

1.5.3.1 Electrolyte Leakage

The plasma membrane separates the cell from its extracellular environment. Intracellular membranes enclose multiple compartments which include the endoplasmic reticulum (ER) and nucleus, and organelles such as the Golgi apparatus, lytic and other vacuoles, lysosomes/vacuoles, chloroplasts and mitochondria. Therefore, it is essential that cell membranes remain intact and functional within plant or animal tissue, being involved in positioning and integrating many complex, vital processes that allow the organism to function in a manner sustaining life (Bryant *et al.*, 2001).

Membrane phase behaviour during dehydration and rehydration is important to the survival of seeds and other desiccation-tolerant tissues (Bryant *et al.*, 2001; Woodenberg *et al.*, 2015). The state of membrane lipids – fluid (liquid crystalline), gel or non-lamellar – is determined by the composition of the membrane, the degree of hydration and the temperature (Bryant *et al.*, 2001; Campos *et al.*, 2003). The phase behaviour of membranes and solutions depends on general conditions additional to temperature, such as mechanical constraints, osmotic pressures and distribution of components (Bryant *et al.*, 2001). However, the situation may be dynamic: e.g. exposure to low temperature during cold acclimation is accompanied by plant cell membranes undergoing changes in lipid and fatty acid composition which maintain chloroplast function at low temperature (Campos *et al.*, 2003).

Leakage of ions as an indicator of cellular membrane integrity, measured by electrical conductivity, can be applied to predict vigour, and assess damage and deterioration of seeds, and also chilling injury of fruits (Jiang *et al.*, 2001; Arulmoorthy *et al.*, 2020). Additionally, in seeds, sugars and proteins also leak (Senaratna and McKersie, 1983; Crowe *et al.*, 1989; Mohammed *et al.*, 2001). Bewley (1979) suggested that major leakage of solutes, which

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Effective methods of microbial control are a major necessity if the storage of hydrated recalcitrant seeds is to be prolonged at ambient or, where possible, reduced temperatures.

include substances like amino acids, various sugars, phenolics, phosphates and electrolytes, is a transient phenomenon that lasts just a few minutes upon imbibition of high-quality seeds, while prolonged loss of solutes is characteristic of irreversibly damaged tissue.

For recalcitrant seeds, Salmen Espindola *et al.* (1994) showed that dehydration of the seeds of *Araucaria angustifolia* induced deterioration of cell membranes as indicated by the high increase in leakage of solutes. Similarly, when *Coffea dewevrei* seeds were exposed to chilling treatment, they maintained high leakage values and showed poor recovery when re-warmed (Campos *et al.*, 2003). The rate and extent of cytoplasmic leakage is positively correlated with the degree of damage (Senaratna and McKersie, 1983; 1986). Leakage reflects a partial loss of membrane selective permeability, suggesting that membrane dysfunction is associated with damage as a result of desiccation (Senaratna and McKersie, 1986) or chilling (Campos *et al.*, 2003).

1.5.3.2 Ethylene synthesis

Ethylene stimulates flower senescence, fruit ripening, germination, maturation and response to pathogens (Esashi, 1991; Arc *et al.*, 2013). There is also evidence that different stress-inducing factors, such as chilling, freezing, pathogen attack, salt stress and wounding, induce ethylene production (Kacperska, 1997). The bioconversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene is stimulated by auxins and cytokinins and mechanical factors such as cell damage (Matilla, 2000). Ethylene production in higher plants, which involves ACC-synthase and ACC-oxidase, is usually low but is greatly increased at certain stages of growth and development such as germination, fruit ripening or leaf abscission (Matilla, 2000).

The rate of ethylene biosynthesis increases rapidly during plant-pathogen interactions (Yang and Hoffman, 1984 in Ohme-Takagi and Shinshi, 1995), and ethylene has been shown to be associated with induction of transcription of a series of pathogenesis-related protein genes, such as those encoding class I β -1-3-glucanase and class I chitinase (Felix and Meins, 1987). Ethylene has also been implicated as the signal triggering endosperm programmed cell death (PCD) (Domínguez *et al.*, 2001). However, ethylene may not act alone, as a balance of

ethylene and ABA has been suggested to regulate endosperm PCD in maize (Young and Gallie, 2000; Domínguez *et al.*, 2001).

The production of ethylene by plant organs or tissues is widely used as an indicator of stress (Wang, 1989; Corbíneau *et al.*, 1990) and its release is temperature-dependent, being generally reduced at low temperatures (Corbíneau *et al.*, 1990). While exposure to low temperature can initially enhance ethylene production by chilling-sensitive plant tissue (Chan *et al.*, 1985 in Corbíneau *et al.*, 1990), prolonged chilling of such tissues results in a marked reduction in ACC oxidase activity and ethylene production (Corbíneau *et al.*, 1997; Concellón *et al.*, 2005). Similarly, Corbíneau *et al.* (1990) showed that the ability of somatic embryos of the highly chilling-sensitive oil palm to produce ethylene, was reduced with extended chilling treatment. However, responses to chilling are very variable, and chilling has been reported to enhance, inhibit or have no effect on ethylene production in sensitive tissues depending on the species, cultivar, developmental stage and duration of chilling treatment (Autio and Bramlage, 1986 in Corbíneau *et al.*, 1990). On the other hand, as ethylene is involved in plant growth, the production of this regulator might be a good indicator of the recovery of growing tissues after chilling exposure (Abeles, 1973 in Corbíneau *et al.*, 1990). The role of ethylene during dormancy and germination appears to be controversial among various authors (Fu and Yang, 1983; Abeles, 1986; Matilla, 2000). Despite these different data, the role of ethylene in plant tissues is certainly important in many species.

1.5.3.3 Protein synthesis

Bewley and Black (1985; 1994) divided proteins into three broad classes according to their function in seeds. The first of these classes, the structural proteins, is associated with membranes and ribosomes. The second class, according to those authors, is constituted by the enzymes, e.g., those required for mobilisation of storage reserves, and the third class is that of the storage proteins which are utilised during seed germination and seedling growth, supplying the necessary amino acids and amide nitrogen.

During germination of seeds, numerous metabolic processes are initiated. This requires storage products to be transformed to liberate energy and anabolic substrates for germination

and seedling growth (Bewley and Black, 1985; 1994). Over the course of germination, the storage proteins are degraded to peptides and free amino acids which are used for growth processes in the embryonic axes (Misra, 1994). Proteins that are required for germination (or any other process for that matter) have to be translated from messenger RNAs (mRNA) (Misra, 1994). In dry seed tissue, long-lived mRNA (Cherry and Skadsen, 1986) is stored and is immediately available when the tissue is rehydrated, for synthesis of the proteins required for early events of germination. In recalcitrant seeds, however, there is little or no drying of the seed and they are consistently metabolically active (Berjak *et al.*, 1984; Farrant *et al.*, 1985; later Farrant *et al.*, 1993). There would therefore effectively be no stored mRNA, and recalcitrant seeds could be considered comparable to growing seedlings, rather than to orthodox seeds (Berjak *et al.*, 1989).

The advent of modern techniques meant that protein synthesis could be analysed at the molecular level identifying various genes that may be up or down-regulated during stresses in recalcitrant seeds. One such study on the dehydration of recalcitrant seeds of *Camellia sinensis* showed that numerous genes were up-regulated while many others were down-regulated (Jin *et al.*, 2018). When recalcitrant seeds of *Trichilia dregeana* were dehydrated, protein synthesis in their axes was up-regulated while proteins involved with antioxidants were down-regulated during viability loss (Moothoo-Padayachie *et al.*, 2018). Similarly, alteration of proteins and protein synthesis during desiccation has been implicated in the deterioration of recalcitrant seeds of *Madhuca latifolia* (Chandra *et al.*, 2020).

1.5.3.4 The role of Heat Shock Proteins (HSPs) and Late Embryogenic Abundant (LEA) proteins

Thermal stress of plant tissues has been found to induce the synthesis of a specific group of proteins called heat shock proteins (HSPs), believed to be involved in thermal tolerance (Lafuente *et al.*, 1991), while other proteins can be downregulated. Water stress can also induce the synthesis of HSPs (Salmen Espindola *et al.* 1994). Those authors found that an early indicator of desiccation-associated damage of embryos of *Araucaria angustifolia* was a decrease in the capacity of the deteriorating cells to synthesise these and other proteins. The synthesis of HSPs during thermal stress was also suggested to play a vital role in protection against chilling stress (Kawata and Yoshida, 1988; Lafuente *et al.*, 1991).

The proteins that accumulate late in orthodox seed embryogenesis, termed late embryogenic abundant (LEA) proteins, include several groups, each having a characteristic motif in common (Close and Chandler, 1990; Cumming, 1999). The characteristics of LEA proteins and the conditions under which they appear has led to suggestions that they function in the survival of water stress by acting as protectants (Close *et al.*, 1989; Blackman *et al.*, 1995) and/or by stabilising the subcellular structures in the dry state (Lane, 1991; Chatelain *et al.*, 2012).

Dehydrins are a specific class of LEA proteins, and have been suggested to play a fundamental role in the response of plant tissues to dehydration (Close, 1996; Kleinwächter *et al.*, 2014) and drought tolerance in orthodox seeds (Azarkovich, 2015). The multiple targets of dehydrins suggest that the direct consequences of dehydrin activity are biochemically diverse (Close, 1996). The role of dehydrins in recalcitrant seeds is highly variable with their production in some species and not in others (Radwan *et al.*, 2014; Azarkovich, 2015; 2020). However, as a *caveat*, dehydrins have perhaps received disproportionate attention among the LEAs, because of the availability of antibodies (Close, 1996) which is not the case for the other groups of LEAs. Additionally, dehydrins appear to be widely distributed in plant tissues, being upregulated in response to a variety of abiotic stresses (Illing *et al.*, 2005; Dussert *et al.*, 2018). This makes it unlikely that synthesis of dehydrins is exclusively associated with dehydration.

1.6 Cryopreservation of recalcitrant seeds/ seed germplasm

1.6.1 Important factors for cryopreservation of recalcitrant seeds

1.6.1.1 Explant selection

Mature orthodox seeds can be stored at low temperatures and RH relative humidity for extended periods and still remain viable (Ellis *et al.*, 1990). However, storing recalcitrant seeds under these conditions will result in loss of seed viability (Pammenter *et al.*, 1994). Therefore, alternate methods of storage must be used to preserve these types of seeds. Attempts to store recalcitrant seeds at low temperature and high relative humidity have been unsuccessful for a wide range of species (Chin and Roberts, 1980). It is thought that

recalcitrant seed deterioration occurs during metabolic processes that continue after shedding, even at low temperature. Therefore, the only viable means of long-term storage of recalcitrant germplasm is cryopreservation (Kaviani, 2011; Berjak and Pammenter, 2014).

Whole recalcitrant seeds are simply too large to be successfully cryopreserved (Tweedle *et al.*, 2003; Daws *et al.*, 2006). Walters *et al.* (2008) showed that cooling rate decreases rapidly as a function of sample size and water content. It is therefore impossible to cool these seeds rapidly enough for them to survive cryopreservation. Various researchers showed that to cryopreserve recalcitrant seeds, explants of either zygotic embryos or embryonic axes from the seed must be used (Normah *et al.*, 1986; Pritchard and Prendergast, 1986; Chaudhury *et al.*, 1991; Vertucci *et al.*, 1991; Wen, 2009).

Ice crystal formation is probably the primary cause of cell death during freezing (Meryman, 1968; Mazur, 1984; Wesley-Smith *et al.*, 2014). It is for this reason that the embryonic axis is the best choice of explant for cryopreservation, as its small size facilitates rapid desiccation and cooling of the tissue. Such events allow removal of almost all non-freezable water which minimises tissue death via ice crystal formation upon cooling, thus improving the chances for whole plantlet regeneration upon recovery (Hor *et al.*, 1990; Chandel *et al.*, 1995; Berjak and Pammenter, 2004). However, excision of the embryonic axis may cause severe physical injury to the explant (Berjak *et al.*, 2011) resulting in abnormal root and/or shoot development after manipulations associated with cryopreservation in species such as *Quercus* (Xia *et al.*, 2014), *Trichilia dregeana* (Goveia *et al.*, 2004; Naidoo *et al.*, 2016) and *Ekebergia capensis* (Hajari *et al.*, 2011). Various researchers showed that successful cryopreservation of embryonic axes from recalcitrant seeds was possible (e.g. Pence, 1990; Chandel *et al.*, 1995; Wesley-Smith *et al.*, 2001). Based on the successes of these (and other) researchers, this study attempted to successfully cryopreserve embryonic axes from recalcitrant seeds of *Ekebergia capensis*.

1.6.1.2 Desiccation and rate of dehydration of recalcitrant germplasm

Drying recalcitrant germplasm may result in membrane disruption (Sun *et al.*, 1994; Liang and Sun, 2002), lipid peroxidation and ROS generation (Finch-Savage *et al.*, 1996; França *et al.*, 2007), eventually leading to tissue death. A rapid rate of dehydration may by-pass the

deleterious effects of drying recalcitrant germplasm and has been implicated in post-cooling survival (Pammenter *et al.*, 1991). In practice, recalcitrant germplasm has far greater desiccation tolerance following fast drying (Farrant *et al.*, 1985, 1993; Normah *et al.*, 1986; Berjak *et al.*, 1990, 1992, 1993; Pammenter *et al.*, 1991, 1999; Berjak and Pammenter, 1997; Kioko *et al.*, 1998; Pritchard and Manger, 1998; Liang and Sun, 2000). Liang and Sun (2002) suggest that if tissues are dried slowly, recalcitrant germplasm must spend longer at intermediate water contents, allowing aqueous-based deleterious processes to occur. Therefore, rapid drying lessens the time of exposure to intermediate water contents and therefore minimises deleterious events, improving viability (Pammenter *et al.*, 1991; Côme and Corbineau, 1996; Berjak and Pammenter, 1997; Pritchard and Manger, 1998). Varghese *et al.* (2011) showed that rapid drying of *T. dregeana* embryonic axes improved survival compared with slow drying. Interestingly, this is not always the case, and rapid drying was not required for successful cryopreservation of encapsulated shoots of *Begonia x erythrophylla* (Burritt, 2008).

The high water content associated with recalcitrant seeds often results in lethal ice crystal formation upon freezing of the germplasm (Hor *et al.*, 1990; Volk and Walters, 2006; Pammenter and Berjak, 2014). It then stands to reason that the explant would have to be dried to non-lethal water contents to prevent ice crystal formation during freezing. However, the range within which non-lethal water content is achieved is variable and specific to each species (Roberts, 1973; Pammenter *et al.*, 1991; Wesley-Smith *et al.*, 1992). Chandel *et al.* (1995) showed that seeds of *Camelia sinesis*, *Theobroma cacao* and *Artocarpus heterophyllus* were not germinable when dehydrated below 0.10, 0.14 and 0.12 g g⁻¹ dry mass, and therefore it is perhaps not surprising that the target water content for successful cryopreservation is between 0.25 to 0.40 g g⁻¹ dry mass (Pammenter and Berjak, 1999; Vertucci and Farrant, 1995; Walters *et al.*, 2001).

Cryopreserved recalcitrant germplasm from *Quercus robur* showed improved viability after desiccation (Chmielarz *et al.*, 2011). This was not the only the instance where improved post-cryopreservation survival was noted after desiccation. Recalcitrant embryonic axes of *Aesculus hippocastanum* (Wesley-Smith *et al.*, 2001), *Artocarpus heterophyllus* (Chandel, *et al.*, 1995), *Zizania taxana* (Walters *et al.*, 2002) and *Amaryllis belladonna* (Seršen *et al.*, 2012a) all showed improved post-freezing survival after desiccation. In an attempt to

replicate the success of those studies, in this study the embryonic axes of *E. capensis* were rapidly desiccated prior to immersion in liquid nitrogen.

1.6.1.3 Cryoprotection

Cryoprotectants are chemical compounds that prevent or reduce ice crystal formation during cooling to cryogenic temperatures by inducing vitrification (Pegg, 1995). Cryoprotectants can vitrify the cytoplasm into a concentrated viscous medium (Benson, 2008a), an event that occurs within a specified water content range. The use of cryoprotectants has been shown to significantly improve survival after cooling in embryonic axes (Hajari *et al.*, 2011; Seršen *et al.*, 2012a).

There are two classes of cryoprotectants *viz.* penetrating and non-penetrating (Finkle *et al.*, 1985; Benson, 2008a). Penetrating cryoprotectants can move across cell membranes and in so doing, prevent ice crystal formation and membrane rupture during freezing (Wolfe *et al.*, 2002; de Lara Janz *et al.*, 2012). They may also act as solutes by increasing the number of particles in solution, thereby lowering the freezing point, and can neutralise toxic compounds e.g. ROS (Berjak and Pammenter, 2014). Among others, commonly used penetrating cryoprotectants are glycerol and dimethyl sulfoxide (DMSO) (Panis and Lambardi, 2005; de Lara Janz *et al.*, 2012). In addition to osmotic balancing, DMSO may also scavenge reactive oxygen species, enhancing cellular protection and survival (Benaroudj *et al.*, 2001; Nadarajan and Pritchard, 2014).

Non-penetrating cryoprotectants e.g. sucrose, trehalose and dextrose on the other hand are unable to cross cell membranes. Instead, they improve osmotic balance during freezing (de Lara Janz *et al.*, 2012) and have been shown to maintain the liquid crystalline state of the membrane bilayers and stabilise proteins under frozen conditions (Kendall *et al.*, 1993; Helliot *et al.*, 2003). Sucrose has been shown to enhance endogenous antioxidants (particularly glutathione reductase) in olive somatic embryos (Lynch *et al.*, 2011).

Despite their ability to enhance vitrification, scavenge ROS and enhance tissue survival after cooling, cryoprotectants can also be cytotoxic to plant tissues (Kistnasamy *et al.*, 2011; Berjak and Pammenter, 2014; Best, 2015). Cell death may occur as a result of intracellular solute accumulation and/or plasma membrane collapse due to changes in osmotic pressure

associated with the dehydrating properties of cryoprotectants (Finkle *et al.*, 1985). In an attempt to cryopreserve explants from *E. capensis*, Hajari *et al.* (2011) reported improved survival after the explants were cryoprotected with a combination of penetrating cryoprotectants (DMSO and glycerol).

1.6.1.4 Cooling and rate of cooling

In theory, plant material can be stored indefinitely at ultra-low temperature in LN (-196°C) (Engelmann, 2004). At this temperature, all cellular and metabolic activity is stopped (Karthi, 1981; Chen and Karthi, 1987; Engelmann, 1991; Chandel and Pandey, 1995; Engelmann, 2004; Mandal, 2005). However, storing recalcitrant seed germplasm in LN is not as simple as plunging the explant in cryogen. As discussed above, the single most destructive component of freezing recalcitrant germplasm is ice crystal formation due to their high water content. Attempts at reducing the water content before freezing has been successful for some recalcitrant seeded species (Pence, 1990; Chandel *et al.*, 1995; Berjak, 2000; Wesley-Smith *et al.*, 2001), but not for others (Hor *et al.*, 1990; Sun, 2000).

Cooling rate is another important factor for the successful cryopreservation of recalcitrant germplasm (Berjak and Pammenter, 2014). The rate at which the explant is cooled is dependent on its thermal mass and the heat transfer properties of the cooling system (Bald, 1987). It is challenging to achieve rapid cooling with large explants because their relatively large mass requires large amount of heat to be dissipated through many layers of cells which are poor heat conductors (Bald, 1987). Therefore, to achieve the best cryopreservation conditions, it is necessary to use certain manipulations to enhance the rate of cooling of large axes. These may include either an increase in heat exchange or a reduction of the mass of the explant (by reducing water content) – or both (Wesley-Smith *et al.*, 2001, 2014). Studies on embryonic axes of *Aesculus hippocastanum* exemplified the importance of cooling rates in achieving post-cooling survival (Wesley-Smith *et al.*, 2001). It is now generally accepted that a rapid cooling rate is required for recalcitrant-seeded axes to achieve the best survival (Engelman, 2011a). In addition to rapid cooling, rapid warming of frozen material is equally important to limit intracellular damage by ice recrystallisation in rewarmed tissue (Mazur and

Schmidt, 1968; Dickinson *et al.*, 1970; Helliot *et al.*, 2003; Sakai, 2004; Wesley-Smith *et al.*, 2004).

1.6.1 5 *In vitro* regeneration

Tissue culture techniques are important for the preservation and conservation of numerous plant species (Johnson, 2002). Tissue culture involves the growth of plant material under slow growth and sterile conditions on a nutrient medium (Kaviani, 2011). Commonly used explants include shoot, leaf, embryos and cotyledons (Paunesca, 2009). *In vitro* storage is particularly useful when seed banking is not possible e.g. recalcitrant seeds (Kaviani, 2011). Important advantages of *in vitro* storage are that cultures are stored under controlled conditions (no exposure to environmental conditions) and are ideally pathogen free (Withers and Engelmann, 1997). However, *in vitro* storage may induce DNA methylation (Harding, 1994) and somaclonal variation (Kumar, 1994). This technique is also labour intensive and there is always a risk of loss due to contamination (Kaviani, 2011).

Nutrient media must contain the necessary supplements and mineral salts that will promote the development of an explant into a healthy, fully functional seedling (Grosser, 1994; Kartha and Engelmann, 1994; George, 2008). Nutrient media are species specific, and require optimisation prior to any experimental work being carried out (Engelmann, 2004; Benson *et al.*, 2007; Berjak and Pammenter, 2014). Optimisation of the medium may require addition of plant growth regulators to promote development. Some examples of plant growth regulators include 6-benzylaminopurine (Abdelnour-Esquivel and Engelmann, 2002), α -naphthaleneacetic acid (Krishnapillay, 2000), zeatin, indole-3-acetic acid (Adkins *et al.*, 1995) and N⁶-benzyl adenine (BA) (Perán *et al.*, 2006). Further optimisation may be required to promote development after recovery of explants from liquid nitrogen. These may include reduction or omission of ammonium nitrate (Decruse and Seeni, 2002), incorporation of activated charcoal (Kim *et al.*, 2002), and the addition of a haemoglobin solution (ErythrogenTM) (Al-Forkan *et al.*, 2001) or dimethyl sulfoxide (DMSO) (Naidoo *et al.*, 2016) that function as free radical scavengers. In addition to these compounds, incubating explant cultures in the dark can limit the production of ROS induced by photo-oxidation (Touchell and Walters, 2000).

Contamination of cultures by fungi and/or bacteria is a problem which may result in the loss of entire accessions (Kaviani, 2011). Without surface decontamination of explants, fungi will flourish and overrun the axes more rapidly than the explants are able to germinate (Berjak and Pammenter, 2014). Therefore, it is imperative that a suitable decontamination protocol is established for *in vitro* regeneration. In some instances, it may be helpful if anti-fungal and anti-bacterial compounds are added to the cultures (Berjak and Pammenter, 2014).

1.6.2 Reactive oxygen species (ROS)

1.6.2.1 ROS in plants and seeds

ROS which include free radicals, are produced during normal metabolic processes; in some cases they are second messengers (Smirnoff, 1993; Foyer and Noctor, 2005; Pitzschke *et al.*, 2006). Abiotic stress increases ROS formation, and these stresses include the artificial stresses that occur during cryopreservation (Dat *et al.*, 2000; Sunkar *et al.*, 2003). Among the most common direct causes of oxidative stress in plants is leakage of electrons to oxygen from electron transport chains in the chloroplast (considered to be the most powerful source of ROS in plants [Foyer *et al.*, 1994; Foyer and Shigeoka, 2011]) and mitochondria (Mittler *et al.*, 2004), and exposure to high light exacerbates production (Dat *et al.*, 2000). Another source is the so-called oxidative bursts produced by cell wall or plasma membrane NAD(P)H oxidases or peroxidases that are produced by plants in response to pathogen attack or some forms of abiotic stress (Apel and Hirt, 2004; Foyer and Noctor, 2005). Plants have evolved very efficient and complex systems to scavenge ROS to protect themselves against oxidative stress (Allen, 1995; Gest *et al.*, 2013). Increased ROS production may up-regulate genes that code for antioxidant enzymes (Baek and Skinner, 2012).

The breakdown of metabolic co-ordination in cells may initiate uncontrolled free radical activity and decrease enzymatic and non-enzymatic protection against oxidative damage (Leprince *et al.*, 1990; Gill and Tuteja, 2010). When excess ROS are not effectively scavenged, they can oxidise a wide range of biological molecules (Hendry *et al.*, 1992; Dat *et al.*, 2000). ROS scavenging mechanisms include both enzymatic and non-enzymatic components (Noctor and Foyer, 1998). The ability of antioxidants to scavenge excessive level of different ROS in various cellular locations is important, because ROS can cause considerable damage to cells (Dat *et al.*, 2000; Laloi *et al.*, 2004; Sharma *et al.*, 2012; Das *et*

al., 2015). Cellular damage manifested by ROS-mediated lipid peroxidation might be reduced or prevented by protective mechanisms involving free radical and peroxide scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and ascorbate peroxidase (APX; Posmyk *et al.*, 2001; de Tullio and Arrigoni, 2003).

Ageing of orthodox seeds (Kranter *et al.*, 2010) and dehydration of desiccation-sensitive seeds (Berjak and Pammenter, 1997), germination, ageing and pathogenesis (Hendry *et al.*, 1992; Dat *et al.*, 2000; Bailly, 2004), may lead to oxidative stress and cellular damage, which will result in seed deterioration if ROS production exceeds the capacity of ROS-scavenging reactions (Schopfer *et al.*, 2001; Bailly, 2004). In this regard, Kranter *et al.* (2006) have shown that viability was completely lost in desiccation stressed *Quercus robur* seeds when the ratio of oxidised:reduced glutathione was increased (to a half-cell reduction potential of between -180 and -160mV).

In seed physiology, ROS have conventionally been considered toxic if they accumulate to concentrations beyond those involved in normal signaling. This may lead to cell injury including oxidation of macromolecules and membrane disruption, and disturbances in seed development or germination processes (Bailly, 2004, 2008). Seeds thus rely on antioxidant machinery that should eliminate potentially toxic ROS that are produced under stressful conditions.

1.6.2.2 The role of ROS and antioxidants in the cryopreservation of recalcitrant germplasm

ROS produced by organisms are classified and summarised in the table below (Table 1.1). However, for the purposes of this study, only the superoxide ($\cdot\text{O}_2^-$) and H_2O_2 species were assessed.

As discussed above, organisms scavenge ROS using both enzymes (e.g. superoxide dismutase, catalase and ascorbate and class III peroxidases) and non-enzymatic compounds (e.g. ascorbate, glutathione, tocopherols and carotenoids) (Kuźniak and Urbanek, 2000). ROS are produced constantly due to ongoing metabolism; therefore, antioxidants must also be constantly produced/ supplied to prevent oxidative stress. Cryopreservation of recalcitrant

germplasm is fraught with many challenges. Among these is the prolonged and uncontrolled production of $\cdot\text{O}_2^-$ (Whitaker *et al.*, 2010) and an impaired anti-oxidant scavenging system (Hendry *et al.*, 1992; Côme and Corbineau, 1996; Berjak and Pammenter, 2004).

Table 1.1 The radical and non-radical ROS (from Halliwell and Gutteridge, 2007)

Radicals	Non-radicals
Superoxide ($\cdot\text{O}_2^-$)	Hydrogen peroxide (H_2O_2)
Hydroxyl ($\cdot\text{OH}$)	Peroxonitrite (ONOO^-)
Hydroperoxyl ($\cdot\text{OOH}$)	Peroxonitrous acid (ONOOH)
Peroxyl ($\cdot\text{ROO}$)	Hypochlorous acid (HOCl)
Alkoxy ($\cdot\text{RO}$)	Hypobromous acid (HOBr)
Carbonate ($\cdot\text{CO}_3^-$)	Ozone (O_3)
Carbon dioxide ($\cdot\text{CO}_2^-$)	Singlet oxygen ($^1\text{O}_2^+ \Delta g$)
Singlet oxygen ($^1\Sigma g^+$)	

As discussed above, to successfully cryopreserve recalcitrant seeds it is necessary to excise the embryonic axes from the rest of the seed (Normah *et al.*, 1986), however, excised axes of some recalcitrant-seeded species fail to produce shoots after cryopreservation. One reason for this could be that the meristematic regions are adjacent to the sites where the cotyledons have been excised from the embryonic axis (Goveia *et al.*, 2004; Hajari *et al.*, 2011). A ROS burst was implicated in apical meristem necrosis in *T. dregeana* explants (Whitaker *et al.*, 2010; Berjak *et al.*, 2011). However, it has been shown that if the axes of *T. dregeana* were excised with a piece of cotyledon still attached, then shoots developed normally after cryopreservation (Goveia *et al.*, 2004).

In some species, dehydration of recalcitrant germplasm is necessary to achieve successful cryopreservation. However, dehydrating these tissues may in itself impose stresses. Embryonic axes of recalcitrant *Castanea sativa* were shown to produce a burst of $\cdot\text{O}_2^-$ when they were excised and dehydrated (Roach *et al.*, 2008; 2010). The elevated levels of $\cdot\text{O}_2^-$ are known to react with H_2O_2 to produce highly reactive hydroxyl radicals (Halliwell, 2006), which may lead to tissue damage and possibly death. Therefore it is imperative that levels of H_2O_2 be controlled. This is achieved by the enzyme catalase, which converts H_2O_2 into water and oxygen (Arrigoni and de Tullio, 2002).

Compared with other ROS species (e.g. $\cdot\text{O}_2^-$ and $^1\text{O}_2$), hydrogen peroxide has a longer half-life, is able to penetrate membranes and travel considerable distances from its site of origin

(Wojtaszek, 1997). These properties of H_2O_2 suggest that it may function as a signalling molecule that mediates cell-to-cell communication (Kuzniak and Urbanek, 2000; Suzuki and Mittler, 2012). In response to environmental stresses, plants often show elevated levels of H_2O_2 both inter- and intracellularly (Yoshioka *et al.*, 2008).

Hydrogen peroxide has an affinity for the superoxide anion that is produced during aerobic metabolism. When these two ROS species react, they produce the highly destructive hydroxyl radical (Arrigoni and de Tullio, 2002; Halliwell 2006), therefore it is imperative that levels of H_2O_2 be controlled. This is achieved by the enzymes catalase, which converts H_2O_2 into water and oxygen (Arrigoni and de Tullio, 2002), and at lower concentrations by peroxidases, which oxidise a variety of substrates, and at the same time remove H_2O_2 (Pandey *et al.*, 2017).

For the redox state of cells to be maintained, the balance of ROS and antioxidants must also be maintained; an imbalance of which may have lethal consequences for cells (Bouayad and Bohn, 2010). This phenomenon was highlighted in the improved survival of blackberry shoot tips after cryopreservation (Uchendu *et al.*, 2010b). This study showed that exogenous application of antioxidants was able to maintain the redox balance by quenching the ROS burst associated with cryopreservation; this has also been suggested by Reed (2012, 2014).

Anti-oxidative enzymes (e.g. SOD, GR and APX) were shown to have been impaired when desiccation sensitive recalcitrant germplasm was dehydrated (Hendry *et al.*, 1992; Côme and Corbineau, 1996; Berjak and Pammenter, 2004). This can exacerbate the effects of ROS, resulting in tissue damage and eventually death (Bailly *et al.*, 2008; Kranner and Birtic, 2005).

The significance of ROS and antioxidants during cryopreservation of recalcitrant germplasm has been highlighted in the various studies listed above. Therefore, during this study, $\cdot\text{O}_2^-$, H_2O_2 and total antioxidant levels were assessed after each step of cryopreservation. These parameters were used as markers and indicators of stress.

1.6.3 Cathodic water

Application of exogenous antioxidants has been shown to significantly reduce levels of ROS in excised embryonic axes (Berjak *et al.*, 2014). One example of an antioxidant substance is “cathodic” water. Electrolysis of an aqueous salt solution will result in an anodic (oxidised) and cathodic (reduced) portion (Miyashita *et al.*, 1999; Kim and Kim, 2006; Berjak *et al.*, 2011). The reduced portion of the electrolysed solution can quench ROS but is not toxic to plant tissues (Berjak *et al.*, 2011). Cathodic water has a high pH (pH 10-12), low dissolved oxygen (1.3-3.5mg/l), high dissolved hydrogen (0.3-0.6 mg/l) and a significant negative redox potential (-400 to -900mV) (Kim and Kim, 2006). The high content of dissolved hydrogen in cathodic water is thought to be an ideal scavenger of ROS (Kim and Kim, 2006). Cathodic water has been shown to scavenge various ROS including $\cdot\text{O}_2^-$ and H_2O_2 (Shirahata *et al.*, 1997; Hanaoka, 2001; Shirahata *et al.*, 2012; Fatokun *et al.*, 2020).

To reduce the accumulation of ROS, Berjak and Pammenter (2014) suggest that sequential ameliorative steps be taken during cryopreservation of embryonic axes from recalcitrant seeds which may include explant excision with an antioxidant pre-conditioning step to counteract ROS-mediated injury. An example of the use of cathodic water is *T. dregeana* axes. Axes treated with cathodic water during the pre-cooling stages of cryopreservation produced shoots, while untreated axes produced no shoots (Naidoo *et al.*, 2016).

1.7 Phylogeny

The same plant species often grow in different geographical regions (Silvertown *et al.*, 2006), and local populations are adapted to the specific regional conditions. These local populations may differ from each other both phenotypically and genotypically (Schaal *et al.*, 1998). Quinn (1977) has suggested that growing the same plant in different environmental conditions for several generations may drastically alter the genotypic composition of the population.

The concept of phylogeography was introduced by Avise *et al.* (1987). Phylogeographic studies seek to evaluate lineage divergence and diversification in plants over long time scales (millions of years). Originally, this concept was applied more to animals rather than to plants,

but Schaal *et al.* (1998) suggested that it can also be used to study plants. Importantly, populations that have a recent common ancestry will be more similar than those that have more distant common ancestry; however, if genetic exchange between two populations or species ceases, then shared common ancestry will be the only link of genetic similarity between them (Schaal *et al.*, 1998).

Various techniques have been employed over the years to study species relatedness, including molecular genetic techniques that allow assessment of genetic diversity not only between species, but also within species (Karp *et al.*, 1997). The ITS (internal transcribed spacer) region is commonly analysed in such studies (Youngbae *et al.*, 1993; Schlötter *et al.*, 1994; Álvarez and Wendel, 2003; Muellner *et al.*, 2009) because it is highly repeated in the plant genome, thus promoting detection, amplification, cloning and sequencing of nuclear ribosomal DNA (nrDNA) (Baldwin *et al.*, 1995). The small size of the ITS region (< 700 bp) and the presence of highly conserved sequences flanking each of the two spacers make this region easy to amplify using universal eukaryotic primers (Baldwin *et al.*, 1995). This gene family undergoes rapid concerted evolution (Arnheim *et al.*, 1980; Appels and Dvorák, 1982; Arnheim, 1983) promoting intragenomic uniformity of repeat units; in general, analysis of these sequences allows accurate reconstruction of species relationships (Hamby and Zimmer, 1992; Sanderson and Doyle, 1992).

Morphological differences are now considered to be insufficient to distinguish species taxonomically, and, as an example, Muellner *et al.* (2009) evaluated taxonomic data independent of morphological data. Their study was based on maximum parsimony and Bayesian analyses of nuclear ITS and plastid *rps 16* intron sequences (TTCCGTAGGTGAACCTGCG), as well as comparison of chemical profiles observed by high performance liquid chromatography (HPLC) and gas chromatography - mass spectrometry (GC-MS). That study provided a timeline for divergence within the populations of *Aglaia eleaeagnoidea*, which is a complex and widely distributed species. In a study on *E. capensis*, analysis of the ITS region showed that there was a divergence in the genome of populations from different provenances (Bharuth *et al.*, 2020).

1.8 Ultrastructure

1.8.1 Ultrastructural changes associated with recalcitrant seeds stored at chilling temperatures

The degree of development of particular organelles is highly variable, differing not only from one cell type to another (Lee *et al.*, 2002), but also reflecting the physiological status of particular cell-types in time, and in response to particular stimuli (Berjak and Pammenter, 2000). Ultrastructural and other microscopical studies have helped understand recalcitrant seed behaviour (Berjak and Pammenter, 2000) and have contributed to our knowledge of the effects of chilling at the subcellular level in vegetative tissues, e.g. cucumber seedling root tips (Lee *et al.*, 2002).

Chilling injury causes similar ultrastructural symptoms across species (Kratsch and Wise, 2000). Such symptoms include swelling and disorganisation of both chloroplasts and mitochondria (Lee *et al.*, 2002), reduced size and number of starch grains, dilation of thylakoids and unstacking of grana, formation of small vesicles of chloroplast peripheral reticulum, lipid droplet accumulation in chloroplasts and condensation of chromatin in the nucleus (Ishikawa, 1996; Yun *et al.*, 1996; Kratsch and Wise, 2000). Chilling can cause noticeable changes in the ultrastructure after just 15 min of exposure to low temperatures (Lee *et al.*, 2002).

The degree of development of the rough endoplasmic reticulum (rER) is important in interpreting function from the ultrastructure, as it is implicated in the synthesis of all the endomembranes (Becker *et al.*, 2009). The developmental status of this organelle is thus a generally good indicator of active membrane synthesis. As an important example, specialised ER-derived membranes form new vacuoles as a response to dehydration or chilling stress culminating in abnormal vacuolation within axis cells of sensitive embryos (Berjak and Pammenter, 2000).

Lytic vacuoles, which contain hydrolases, act as turnover centres within the cell. Organelles, especially when damaged, are engulfed by the vacuoles where they are broken down. During conditions of stress, an increase in the number of vacuoles and autophagic activity has been

associated with removal of damaged or redundant organelles and cytoplasmic material in recalcitrant embryonic axes (Berjak and Pammenter, 2000).

Another organelle, from which cell developmental status can be inferred, is the Golgi body. This organelle is an important indicator of physiological status (Berjak and Pammenter, 2000) being the centre of carbohydrate metabolism, including those compounds to be added to cell walls (or in cell plate synthesis), and for compounds that will be secreted extracellularly. Furthermore, as a cell grows, the plasmalemma must be extended, which is facilitated by incorporation of membranes via Golgi-derived vesicles. A strong development of Golgi bodies will indicate a higher degree of intracellular activity, but in recalcitrant seeds these organelles are among the most sensitive to dehydration stress, when their cisternae first become swollen and then dissociated from one another (Berjak and Pammenter, 2000). This was also observed to be a symptom accompanying low-temperature storage in embryonic axes of chilling-sensitive neem seeds (Berjak *et al.*, 1995).

Deposition of starch within the plastids is also a good indicator of an enhanced metabolic state, as is its utilisation under normal circumstances. Photosynthesis is particularly sensitive to chilling stress (Taylor and Rowley, 1971), with chloroplasts usually being one of the first subcellular structures to be affected by chilling (Kimball and Salisbury, 1973). The injury manifests itself by swelling of the chloroplast and distortion of thylakoids (Kratsch and Wise, 2000). Mitochondrial development is an indicator of the status of cellular respiration. Mitochondria tend to be more resistant to chilling than chloroplasts, but plants that are hypersensitive to chilling show visible signs of mitochondrial damage on exposure to low temperatures (Kratsch and Wise, 2000).

Changes in the nucleus have also been noted in response to chilling of sensitive plants. Condensation of chromatin was observed in the nucleus of chilled tissues of *Saintpaulia ionantha* (Yun *et al.*, 1996), and other responses were recorded to include swelling and fragmented chromatin and nucleoli in cultured cells of mung bean (Ishikawa, 1996).

Ultrastructure can also be used as a diagnostic tool to determine the effect that fungi have on plant tissues. It appears that fungal hyphae probably generally penetrate recalcitrant seeds through the epidermis of cotyledons or axes (Calistru *et al.*, 2000; Anguelova-Merhar *et al.*, 2003). In these studies, a localised penetration of fungal hyphae was viewed ultrastructurally,

thus affording those workers insight into the ongoing problem of mycofloral infection. However, in terms of the visible damage to seed ultrastructure, considerable similarity was observed among the symptoms of desiccation stress and fungal effects (Calistru *et al.*, 2000) and chilling of sensitive axes (Berjak *et al.*, 1995).

The cytoskeleton is an important internal component determining cytomatrical structure. In fact, the 3-dimensional structure of the cytoskeleton organises the architecture within the cell and is responsible for maintaining the organelles in appropriate positions (Osborne and Broubiak, 1994; Vertucci and Farrant, 1995). As shown by Berjak and Mycock (2004) for recalcitrant *Trichilia dregeana* embryonic axes, even if organelles appear intact, if cellular architecture maintained by the cytoskeleton is perturbed, then cells do not function normally. Collapse of the cytoskeleton has been implicated when dehydration-sensitive axes are dehydrated or frozen under certain conditions (Berjak and Mycock, 2004; Berjak and Pammenter, 2000, respectively).

1.8.2 Ultrastructural changes associated with freezing of recalcitrant germplasm

Generally, attempts to cryopreserve recalcitrant embryonic axes are unsuccessful (Berjak and Pammenter, 2014). In addition to biochemical changes (Berjak *et al.*, 2011; Naidoo *et al.*, 2016), ultrastructural assessments can be used to locate the areas within the cytoplasm that have been damaged due to freezing (Wesley-Smith *et al.*, 1995; Kim *et al.*, 2002; Yap *et al.*, 2011). In many cases, recalcitrant embryonic axes have been reported to produce roots but no shoots after freezing (Goveia *et al.*, 2004; Berjak and Pammenter, 2014). Ultrastructural investigations of the recalcitrant embryonic axes of *Ekebergia capensis* (Perán *et al.*, 2006) after freezing showed that there was no evidence of damage in root meristem cells, while apical meristem cells were extensively damaged. Although fully hydrated embryos of *Pisum sativum* did not appear necrotic on germination medium after they were frozen, ultrastructural investigations revealed that they were in fact dead (Wesley-Smith *et al.*, 1995). In an attempt to produce virus free bananas, Helliot *et al.* (2003) attempted to cryopreserve meristem cultures (Helliot *et al.*, 2002). After freezing/ thawing, they found numerous deleterious changes in these cells which include vacuolation, nucleus and nuclear membrane anomalies, lack of membranous structures and disintegration of their cellular contents.

It is well known that ice crystal formation during freezing can be lethal to plant tissues (Helliot *et al.*, 2003). However, an ultrastructural study on ice crystal formation and localisation during freezing of embryonic axes of the recalcitrant *Acer saccharinum* showed that ice crystal formation was not necessarily lethal; in addition the distribution of ice crystals was not uniform (Wesley-Smith *et al.*, 2014). After further microscopical and ultrastructural investigations, Wesley-Smith *et al.* (2015) suggested that poor survival after freezing in plant tissues was as a result of programmed cell death and not necessarily ice crystal formation.

It is also important to analyse the ultrastructure of embryonic axes that survived freezing. This was evident in the zygotic embryos of *Amaryllis belladonna* that were successfully cryopreserved when glycerol was used as a cryoprotectant, but did not survive freezing when sucrose was used as a cryoprotectant (Sershen *et al.*, 2012a). Increased vacuolation, abnormal plastid ultrastructure and wall abnormalities were observed in the axes that didn't survive freezing. However, the ultrastructure of the axes that did survive the freezing treatment showed an intact ultrastructure without any increase in vacuolation (Sershen *et al.*, 2012a). In the current study, the analysis of ultrastructure was invaluable. This diagnostic technique (Dvorak and Monahan-Earley, 1995) was used to elucidate the effect of each of the steps required for cryopreservation of recalcitrant germplasm.

1.9 Species used in this study

The recalcitrant seeds (Pammenter *et al.*, 1998) of *Ekebergia capensis* Sparrm. (Meliaceae) (Pooley, 2003) were used in this study. *Ekebergia capensis* is a relatively fast-growing, evergreen or semi-deciduous tree, producing fruits which are 10-20 mm in diameter. The fruits are almost spherical and resemble small apples. At maturity, the fruits become bright red with the exocarp enclosing a whitish fleshy mesocarp and hard endocarp. Each fruit usually has 4-5 oval, white seeds (see Fig. 1.1 a).

In favourable conditions, *E. capensis* flowers profusely every year. In southern Africa, trees flower from September to November, and fruiting usually occurs from December to April, but may take place as late as June (Pooley, 2003). In Zambia and Tanzania, however, flowers appear between August and October and fruits from November to January (du Plessis, 1995). Flowers are pollinated by bees and ants. Fruits are eaten by monkeys and birds, which aid in seed dispersal (Sun *et al.*, 1997; Pooley, 2003). *Ekebergia capensis* has a non-aggressive root

system which makes the trees ideal for gardens and parks (World Agroforestry Centre). In South Africa, particularly in Port Elizabeth (Fig. 1.2), *E. capensis* has been extensively planted as a street tree.

Ekebergia capensis trees can tolerate mild drought conditions and very light frost, but severe frost adversely affects the tree (World Agroforestry Centre). The species is recorded as naturally occurring in a variety of habitats, including high altitude evergreen forests and coastal sandveld. It occurs both along the coast and inland in South Africa (Boucher and McDonald, 1982). *Ekebergia capensis* is found in most parts of Zambia (du Plessis, 1995) and extends in a belt down the eastern side of the continent from Ethiopia (Mengistu, 2002) through to Kenya (Hemp, 2006), Tanzania (Augustino and Gillah, 2005) and the eastern region of the Democratic Republic of Congo (Basabose, 2004) to the Cape in South Africa (Boucher and McDonald, 1982) (Fig 1.3). Trees are found in most of the regional eastern forests of South Africa, and extending into Swaziland (Boucher and McDonald, 1982) (Fig 1.2). Despite its wide distribution, *E. capensis* is a protected tree in South Africa and is a threatened species in Uganda (Hashimoto, 2000).

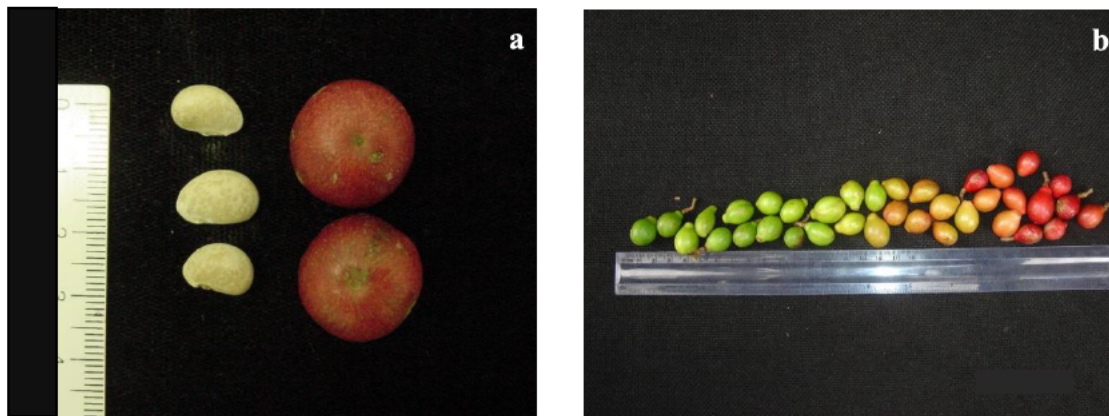


Figure 1.1 Mature seeds and fruit of *E. capensis* from PE provenance (a) and the range of fruit maturity at any one harvest (b).



Figure 1.2 Map of the Republic of South Africa (www.edst.purdue.edu/.../map-south-africa). Locations from which *E. capensis* fruit were harvested for the present study, are indicated by X.



Figure 1.3 Map of Africa (allonsy.files.wordpress.com/.../africa-map). The location of the northernmost population of *E. capensis* from which seeds were obtained for the present study, is indicated by X.

According to Grierson and Afolayan (1999), *E. capensis* has a wide range of uses, being utilised as food and fodder for livestock, firewood and charcoal production. Tannins extracted from the bark are used in the process of leather tanning. Additionally, this species also has medicinal properties (Rabe and van Staden, 1997), preparations from various parts of the tree being used to treat different ailments. Preparations from the roots are used to relieve headaches and chronic coughs, the leaves provide a remedy for intestinal worms and bark decoctions are used to treat dysentery (Rabe and van Staden, 1997; Iwalewa *et al.*, 2007). Extracts of the bark of *E. capensis* are used to treat other ailments, which include abscesses and boils (Rabe and van Staden, 1997; Grierson and Afolayan, 1999). *E. capensis* preparations have also been implicated as anti-malarial treatments (Muregi *et al.*, 2007). The combinations of extracts from *E. capensis* and other indigenous plants may have significant parasitaemia suppressions with possibilities for the treatment of drug resistant malaria (Koch *et al.*, 2005; Muregi *et al.*, 2007).

The potential medical uses, and the fact that this species is endangered - at least in Uganda - and locally protected, warrant its conservation so that another species is not lost to unsustainable utilisation. Additionally, with the likely future impact of global climate change (e.g. Rutherford *et al.*, 2000), thorough knowledge and understanding of the seed biology of *E. capensis* will be imperative to conserve the genetic diversity of species and their use in ongoing rehabilitation.

1.10 Rationale, aims and objectives of the present study

There is little information available on the impact of prolonged chilling storage of recalcitrant seeds. The aim of this aspect of the study was to determine the longevity of whole seeds of *E. capensis* in chilling storage and identify mechanisms which allowed these seeds to survive (or not).

Uncontrolled ROS generation has been identified to contribute to cell death during cryopreservation of recalcitrant-seeded germplasm across many species (Goveia *et al.*, 2004; Whitaker *et al.*, 2010 and Berjak *et al.*, 2011; Naidoo *et al.*, 2016) motivating an investigation of these parameters in this study.

The aim of this study was to find a long-term storage solution for recalcitrant seeds. An analysis of the behaviour of recalcitrant seeds of *E. capensis* from different provenances was assessed. Firstly, whole seeds were stored in a fully hydrated state at chilling temperatures, thereby reducing metabolic activity and fungal activity, treatments expected to extend seed longevity. Thereafter, explants from a fresh batch of seeds were manipulated for cryopreservation and subsequent production of viable seedlings.

1.10.1 Storage of whole seeds of *E. capensis* at chilling temperatures

This aspect of the study was designed to assess the responses of *Ekebergia capensis* seeds from selected provenances to decreased storage temperatures. The preliminary experiments showed that the responses of seeds of *E. capensis* from various provenances were, in fact, different. Various parameters were used to investigate the differences in response to reduced storage temperature.

There were several questions that needed to be answered including how behaviour differed between populations from different provenances, and whether we were actually dealing with the same species across provenances. Initially, collections were made of representative batches of seeds from each of the provenances viz. temperate and sub-tropical regions in South Africa (Fig. 1.2), and tropical Tanzania (Fig. 1.3). Seeds were collected from the different provenances when they reached maturity at different times of the year (Table 1.2). Selected viability, ultrastructural, biochemical and physiological studies were carried out on the seeds to determine the effects of chilling. Finally, molecular analysis was done to determine the lineage of the species.

Table 1.2 Average minimum and maximum temperatures in Port Elizabeth, St Lucia and Tanzania at flowering, fruiting and seed harvest of *E. capensis*

	Port Elizabeth			St. Lucia			Tanzania		
	Flower (Jan)	Fruit (Mar)	Seed harvest (June)	Flower (Aug)	Fruit (Nov)	Seed harvest (Feb)	Flower (July)	Fruit (Oct)	Seed harvest (Jan)
Min (°C)	16	15	7	14	18	18	19	20	25
Max (°C)	26	24	21	24	26	29	28	29	32

1.10.2 Cryopresevation of germplasm from recalcitrant seeds of *E. capensis*

Note: Later in the study it became impossible to collect from Tanzania, and therefore experiments were only carried out with South African material.

Embryonic axes of the recalcitrant seeds of *E. capensis* (Pammenter *et al.*, 1998) from different provenances (Fig. 1.2) were subjected to cryopreservation. Results from the first part of this study showed that seeds of *E. capensis* from different provenances showed significantly different behaviour (and survival) when stored at chilling temperatures (above 0°C). Therefore, subsequent work focused on a longer term storage strategy. Explants from the recalcitrant seeds of *E. capensis* (Pammenter *et al.*, 1998) were subject to a cryopreservation protocol for storage at sub-zero temperatures in liquid nitrogen. As part of the cryopreservation protocol, the effect of fast drying and cryoprotection were tested on their ability to maintain survival.

It has been reported that the populations of *E. capensis* from different provenances may in fact be different species or sub-species (Bharuth *et al.*, 2020). In that study, it was suggested that the lineage and perhaps genetic mutation of *E. capensis* may have had an influence on their tolerance (or lack thereof) to chilling temperatures. In this study, that hypothesis was tested by comparing explants from Port Elizabeth and St Lucia provenances subject to cryopreservation.

Uncontrolled ROS generation has been identified to contribute to cell death during cryopreservation of recalcitrant-seeded germplasm across many species (Goveia *et al.*, 2004; Whitaker *et al.*, 2010 and Berjak *et al.*, 2011; Naidoo *et al.*, 2016). In this study, the levels of ROS, specifically superoxide ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2) were assessed, together with levels of total aqueous antioxidants (TAA). These quantifications were performed at each step of cryopreservation with and without application of exogenous antioxidants and related to viability. Ultrastructural changes were investigated at each cryopreparative step of the protocol. The meristems of both root and shoot were assessed.

A novel approach of using the cathodic fraction of electrochemically reduced water was applied at each step of cryopreservation. The effect of cathodic water on *in vitro*

germinability of axes after exposure to each cryopreparative step was assessed to generate guidelines for the successful cryopreservation of explants of *E. capensis* from different provenances.

CHAPTER 2: MATERIALS AND METHODS

This chapter outlines materials and methods used in this study for (i) the effect of chilling storage of whole hydrated seeds of *Ekebergia capensis* from different provenances, and (ii) the various procedural steps and biomarkers measured/assessed during cryopreservation of zygotic explants of *E. capensis* from different provenances. All chemicals used in this study were from Sigma Aldrich (Germany), unless otherwise stated.

2.1 Hydrated, whole seed storage

2.1.1 Collection of samples

2.1.1a Fruits, Seeds

Fruits of *Ekebergia capensis* were collected from two locations in South Africa, Port Elizabeth (33° 30' S) [PE] and St. Lucia (28° 22' S). Seeds were also collected in Tanzania (6° 49' S), courtesy of Dr H.P. Msanga (Tanzania Tree Seed Agency) (Table 1.2). Fruit were randomly collected from a minimum of 10 different trees. Collections were made over two years so that storage experiments could be duplicated.

Mature fruits were collected and transported to the laboratory in the shortest time possible. Transport was directly by road from St. Lucia; by overnight road courier from Port Elizabeth; and by courier (road and then air) from Tanzania. In the laboratory, seeds were removed from the fleshy fruit and washed in cool running water to remove excess pulp and then decontaminated and stored as described in Sections 2.1.2 and 2.1.3, respectively.

2.1.1b Leaf material

Leaves for DNA analysis were also collected from each site at the same time as fruit harvest, and transported as described above. A minimum of 10 trees were harvested for leaves. The leaves collected from PE, St. Lucia and Tanzania were packaged in plastic bags containing silica gel for transport to Durban. All the samples were then sealed in Ziploc[®] bags and stored at -20°C.

2.1.2 Decontamination

Batches of seeds (approximately 500-600 seeds per batch) were weighed using a two-place balance (Denver Instrument Company DE series, model 400) before decontamination.

Seeds were submerged in 1% (v/v) solution of NaOCl (household bleach, JIK [Reckitt Benckiser, South Africa]) for 10 min, rinsed three times with distilled water and then immersed in a mixture of the fungicides, Previcur (active ingredient propamocarb-HC; RT Chemicals – Pietermaritzburg, South Africa) and Early Impact [active ingredients triazole and benzimidazole; Zeneca Agrochemicals (Pty) Ltd., South Africa] used in concentrations of 2.5 ml l⁻¹ and 0.2 ml l⁻¹, respectively, for 1 h. They were dried on paper towel (usually overnight) to the original batch fresh mass for times determined during preliminary trials.

2.1.3 Storage

Seeds were dusted with Benomyl 500 WP (active ingredient, benzimidazole; Villa Crop Protection, South Africa) and batches of 200 were stored under high relative humidity (RH) conditions (see below) in individual 5 l buckets fitted with sealing lids.

Buckets and mesh platforms (see Fig. 2.1) were thoroughly cleaned, treated with 1% NaOCl overnight, and dried before the buckets were lined with paper towel moistened with distilled water, and the seeds placed on the mesh suspended over the moist paper towel (Fig. 2.1). A layer of dry paper towel was placed on the inside surface of the lid of the bucket (to prevent condensed water dripping on to the seeds during storage) and the bucket then tightly closed. Buckets were vented at three-day intervals to prevent anoxia.

To determine the effect of storage temperature, 5 - 6 buckets (depending on size of the batch) containing seeds from each provenance were placed in incubators set at different temperatures. Seeds were sampled randomly across these buckets. The seeds from PE were stored at 1°, 3° and 6°C, and sampled at 2 week intervals while those from St. Lucia and Tanzania were stored at 3°, 6° and 16°C, and sampled every 3 d.

Preliminary experiments showed that *E. capensis* seeds from PE were chilling tolerant at 6°C; 100% viability was maintained after 12 weeks in hydrated storage. Therefore seeds from PE were stored at a lower temperature (1°C). Seeds from PE were also not stored at 16°C because germination within the storage containers occurred within the 12 week period (Fig. 2.1).

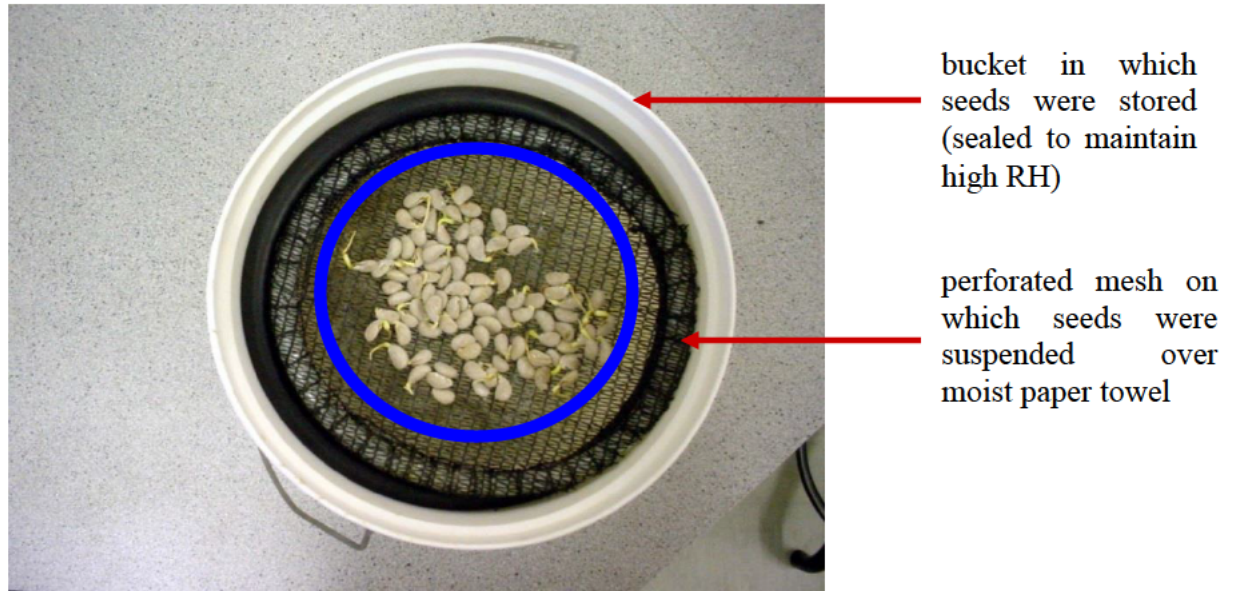


Figure 2.1 *Ekebergia capensis* seeds suspended on a mesh platform within the storage container.

2.1.4 Vigour and Viability

Rate and final % of germination of seeds from each collection were assessed before storage experiments (zero time controls), and at the specified intervals during storage. In each case, samples of 20 seeds were randomly selected across the storage containers and set to germinate. The hard endocarp was cracked with a pair of pliers and carefully removed so as not to damage the softer seed tissues. The brown, papery testa was also gently removed from the surface of the cotyledons.

Twenty 'de-coated' seeds were placed into 90 mm plastic Petri dishes lined with moist filter paper (10 per dish), and set to germinate at room temperature. Daily records of radicle emergence were taken to assess vigour.

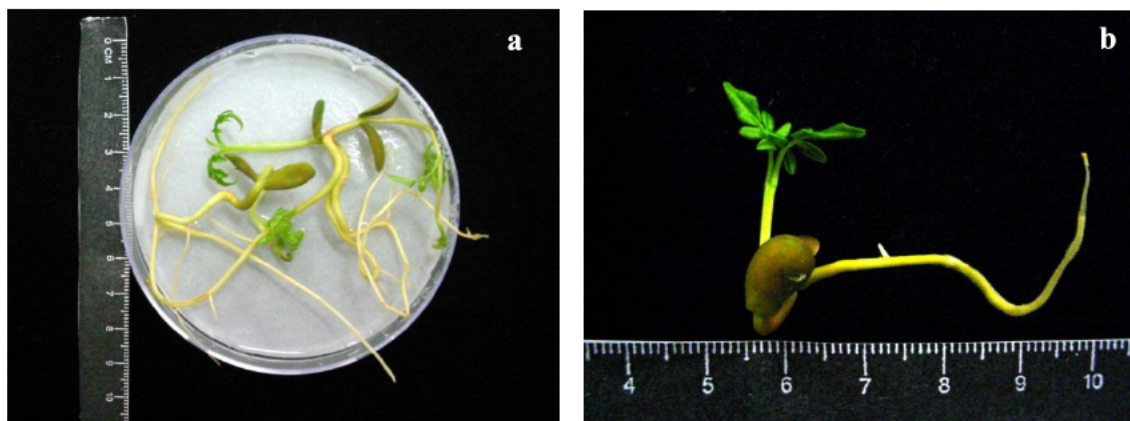


Figure 2.2 Germinated seeds of *E. capensis* in a 90 mm Petri dish lined with moist filter paper (a); and a 3-week old seedling (b).

2.1.5 Water content

Water content was assessed gravimetrically (after drying at 82°C for 48 h) at each sampling interval for five replicates of axes and cotyledons from individual seeds. Preliminary trials showed that the samples reached constant mass after 48 h drying at this temperature. The dry masses of the samples were determined after cooling to room temperature over activated silica gel within closed glass Petri dishes. In all cases a Mettler AE 240 five-place balance was used.

Water content was expressed as grams of water per gram dry mass of axis or cotyledon (g g^{-1}) and was calculated using the following equation:

$$\text{Water content (g g}^{-1}\text{)} = \frac{\text{Fresh mass (g)} - \text{Dry mass (g)}}{\text{Dry mass (g)}}$$

2.1.6 Protein synthesis

Assessment of the rate of protein synthesis of excised axes was carried out at each sampling interval, for three replicates of 20 axes excised from seeds stored at each temperature for 2, 8 and 12 weeks.

Axes were excised and maintained on moist filter paper until 20 were accumulated per replicate, after which the fresh mass was recorded. The axes were then incubated in a $1 \text{ mCi ml}^{-1} \text{ } ^3\text{H}$ amino acid mix (Amersham Biosciences, cat # TRK 440) for 4 h at 25°C in the dark. The radioactive medium was discarded and the tissue ground to a fine pulp using a mortar and pestle, and then suspended in 1 ml Tris-HCl (0.1M, pH 7.0; Merck). The suspension was centrifuged using a Spectrafuge[®] 16 M at $13\,000\times g$ for 5 min, after which 200 μl of the supernatant were spotted onto 15 mm Whatman[®] paper discs. These discs were immersed in cold (5°C) 10% trichloroacetic acid (TCA; Merck) for 1 h and then in 5% TCA for 30 min at 90°C , washed in ethanol:ether (1:1) for 30 min at 37°C , and then in 100% ether for 30 min at room temperature. Thereafter, the discs were submerged in 10 ml Ultima Gold scintillation fluid (Life and Analytical Sciences, Perkin-Elmer).

Radioactive amino acid incorporated into protein was measured in a Packard liquid scintillation analyser (Model number 1900 TR). The instrument was calibrated with a standard blank for ^3H before selecting a preset programme to assess the amount of radioactivity quantitatively (which essentially represented the amount of protein synthesised during the 4 h incubation period) in the scintillation vials. The values were expressed as disintegrations per minute (DPM) and expressed as $\text{DPM g fresh mass}^{-1} \text{ min}^{-1}$.

2.1.7 Electrolyte leakage

Membrane integrity was assessed by measuring the electrolyte conductivity of leachate from individual axes and cotyledons (matched per seed), immersed in double deionised water, using a CM 100 conductivity meter (Reid & Associates, Durban, South Africa).

Axes and cotyledons were excised from seeds after endocarp removal and a brief rinse in distilled water. Each sample was placed in 3 ml of double deionised water in an individual well of a 100-well, customised tray. Conductivity was measured per individual well for 10 replicates of axes and cotyledons. The rate of leakage was measured over a 24 h period and expressed per axis or cotyledon.

Trays were sealed with cling film and then put into Zip-Loc[®] bags. They were then frozen at -20°C overnight, and then thawed at room temperature. Each tray was then re-inserted into the conductivity meter, and ten readings at 1 min intervals taken to obtain maximum leakage. Electrolyte leakage at each sampling interval (including control) was expressed as a percentage of total electrolyte leakage after freezing and thawing the tissue.

2.1.8 Drying

To assess the desiccation sensitivity of seeds from the three provenances, batches of 25 pre-weighed endocarp-enclosed seeds were slowly dried in sealed 250 ml Ziploc[®] bags filled with either silica gel or vermiculite (the latter being used as a control throughout the experiment).

The seeds were periodically weighed on a two-place balance (Denver Instrument Company DE series, model 400) to determine water loss (in the range of 5-30%). When the required mass reduction had been achieved, 20 seeds from each batch were set out to germinate and 5 used to determine water content of the seed components. When a 30% mass loss had occurred (or when the seeds could not be dried any further) in the presence of silica gel, the germination in vermiculite of the seeds stored was determined. This data allowed estimate of the rate of water loss and water content (whole seed) below which the seeds would no longer germinate.

2.1.9 Phylogeny

2.1.9a DNA extraction

DNA was extracted from 30 mg (dry mass) leaves of *E. capensis* from the Cape (PE and surroundings), KwaZulu Natal (Durban district and Mtunzini [close to St Lucia] and Tanzania (Table 2.1). The leaves were first ground to a fine powder in liquid nitrogen (LN) before DNA was extracted using the DNeasy[®] Plant Mini Kit (Qiagen) according to manufacturer's instructions. DNA was quantified using a Nanodrop Spectrophotometer and the quality (occurrence as high molecular weight DNA) was determined by electrophoresing the DNA isolates in a 1% w/v agarose gel that contained 0.05 mg ml⁻¹ ethidium bromide in 0.5X TBE (see appendix) running buffer for 1.5 h at 120V. The bands were visualised in a UviTec BTS 1.5M and excised under ultraviolet light. The molecular weight of the bands was determined by comparing their mobility with those of the standard molecular weight markers.

2.1.9b PCR amplification

The non-coding target sequences analysed in this investigation were the internal transcribed spacer regions (ITS 1 and ITS 2) within the nuclear ribosomal DNA.

PCR reactions were run in a final volume of 25 µl. This comprised 30 ng of extracted DNA in a final volume of 9 µl and 16 µl of mastermix, containing 0.8 µl deionised water, 2.5 µl TBE (Tris-borate-EDTA) buffer (10X), 4 µl MgCl₂ (25 mM), 4 µl forward primer and 4 µl reverse primer (6.25 µM) (Table 2a), 0.5 µl deoxyribonucleotide triphosphate (dNTPs) (10 mM) and 0.2 µl *Taq* polymerase (5 units/µl) (Supertherm). Primers 1 and 2 were used for the ITS1 region and primers 3 and 4 for the ITS2 region (Table 2.2).

The thermal cycling profile used in the PCR amplification is presented in (Table 2.3). The PCR products (DNA fragments) were separated using gel electrophoresis as described previously. Bands were excised from the gel and the PCR products purified using a Qiagen Gel Extraction kit according to manufacturer's instructions.

2.1.9c DNA purification, quantification and analysis

The purified PCR products were sequenced at Inqaba Biotec (Pretoria, South Africa). Also included in the analysis were sequences of *E. capensis* derived from the Cape and KwaZulu-Natal regions of South Africa, obtained from a fellow student, Terisha Naidoo (Table 2.1). An outgroup ITS sequence of the genus *Melia* (from the same family as *Ekebergia capensis*, viz. Meliaceae) was selected after conducting a BLAST (Basic Local Alignment Search Tool) search for the closest relative. A sequence from the species *Melia azedarach* (accession no. AY695595.1) downloaded from the NCBI (National Centre for Biotechnology Information) database and was used for this purpose.

Data were analysed both phenetically by Genetic Distance Analysis (which produces neighbour-joining trees based on a measure of dissimilarity or evolutionary distance between taxa), and phylogenetically, using maximum parsimony (a cladistic method based on sharing of derived characters between compared taxa). Phylogenetic analysis using the maximum parsimony algorithm was performed on the aligned sequences using PAUP version 4.0-beta version (Swofford *et al.*, 2001).

Table 2.1 Sampling locations of leaves used in ITS sequence analysis

Species	Population designation	Origin
<i>E. capensis</i>	Springfield	Springfield, Durban
<i>E. capensis</i>	Springfield	Springfield2, Durban
<i>E. capensis</i>	Springfield	Springfield3, Durban
<i>E. capensis</i>	University of KwaZulu-Natal	Durban1
<i>E. capensis</i>	University of KwaZulu-Natal	Durban2
<i>E. capensis</i>	Mtunzini Nature Reserve	Mtunzini
<i>E. capensis</i> “hairy form”	Silverglen Nature Reserve	Chatsworth, Durban
<i>E. capensis</i>	Port Elizabeth	Port Elizabeth
<i>E. capensis</i>	Knysna	Knysna1
<i>E. capensis</i>	Knysna	Knysna2
<i>E. capensis</i>	Knysna	Knysna3
<i>E. capensis</i>	Knysna	Knysna4
<i>E. capensis</i>	Kirstenbosch National Botanical Garden	George
<i>E. capensis</i>	Harold Porter National Botanical Garden	Betty’s Bay1
<i>E. capensis</i>	Harold Porter National Botanical Garden	Betty’s Bay2
<i>E. capensis</i>	Harold Porter National Botanical Garden	Betty’s Bay3
<i>E. capensis</i>	Harold Porter National Botanical Garden	Betty’s Bay4
<i>E. capensis</i>	Harold Porter National Botanical Garden	Betty’s Bay5
<i>E. capensis</i>	Tanzania	Tanzania 1 ITS 1 2
<i>E. capensis</i>	Tanzania	<i>E. capensis</i> ex Tanzania
<i>E. pterophylla</i>	Kirstenbosch National Botanical Garden	Nelspruit
<i>M. azederach</i>	NCBI	

Table 2.2 Sequences of primers used for analysis of ITS1 (primers 1 and 2) and ITS2 (primers 3 and 4) regions of *E. capensis*

DNA marker	Primers	Target sequence (5'-3')
ITS 1	1	ttccgtaggtgaacctgcg
	2	gctgcgttcttcacgatgc
ITS2	3	gcacgatgaagaacgcagc
	4	tcctccgcttattgatatgc

Table 2.3 Thermal cycling protocol used in PCR amplification of the ITS region

Stage	Purpose	Primers 1&2 and 3&4 (ITS 1&2)	
		Temperature (°C)	Time (min)
1	Initial denaturation	94	2
*2	Denaturation	94	1
	Annealing	42	2
	Extension	72	2
3	Final Extension	72	7
4	Storage	15	∞

* Stage 2 repeated 35 times

2.1.10 Ultrastructure

At the sampling intervals (every 2 weeks for PE seeds, every 3 d up to d 18 – then at d 25 and 38 for the St Lucia seeds and every 3 d for the Tanzanian seeds), 5 radicle tips from seeds at each storage temperature regime were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 0.5% (w/v) caffeine for 24 h at 4°C. Specimens were then rinsed with phosphate buffer (3 x 5 min) and post-fixed in 0.5% (v/v) aqueous osmium tetroxide (OsO₄) for 1 h and washed again three times with 0.1 M phosphate buffer. Thereafter, the samples were dehydrated using a graded ethanol series [30%, 50% 70% and 100% (v/v)], and infiltrated in closed specimen tubes with 50:50 ethanol:epoxy resin (Spurr, 1969) for 4 h on a vertical turntable at room temperature. The ethanol/resin mixture was then replaced with full (undiluted) resin and the specimens infiltrated overnight. They were then placed in molds with fresh resin which was polymerised in an oven at 70°C for 8 h.

The resin blocks were sectioned with a Reichert-Jung Ultracut E microtome. Ultra-thin sections (60-100 nm) were post-stained with a saturated aqueous uranyl acetate solution (10 min) followed by lead citrate (Reynolds, 1963) for 10 min.

Sections were viewed using a Jeol JEM 1010 transmission electron microscope. Images of meristem cells were digitally recorded with an iTEM image analysis software package.

2.1.11 Data analysis

Data were analysed with SPSS 25.0 (version 25.0, SPSS Inc., Chicago, Illinois, USA), using critical $p < 0.05$. Normality was tested using Kolmogorov-Smirnov Test (K-S test). Thereafter, a two-way analysis of variance (ANOVA) was performed to establish if there were significant differences between storage time, storage temperature (independent variables) and water content (dependent variable). A second two-way ANOVA was also performed with storage time and storage temperature as the independent variables and protein synthesis as the dependent variable.

2.2 Procedural steps for cryopreservation

2.2.1 Seed material: procurement, sterilisation and storage

Seeds were collected, decontaminated and stored as described in Sections 2.1.1a, 2.1.2 and 2.1.3, respectively.

Various procedural steps were taken to successfully cryopreserve embryonic axes of *E. capensis* from St. Lucia and PE (Fig. 2.3). Viability was assessed after each step.

2.2.2 Excision – with cotyledon attached

The hard seed coat was carefully cracked open with a pair of pliers. This revealed the cotyledons and embryonic axes which were covered by a thin papery testa. The testa was removed exposing the “naked” seed.

Being a dicotyledonous seed, the shoot tip and hypocotyl are lodged between the cotyledons. Therefore, to minimise damage to the shoot meristem that can be caused by an oxidative burst or physical injury (Goveia, 2004), a sharp scalpel blade was used to excise the explant with a piece of cotyledon attached, thus forming what will be referred to as the “explant.” To avoid slow desiccation of explants during the experimental procedures, they were kept in a chamber with moistened filter paper that maintained high relative humidity.

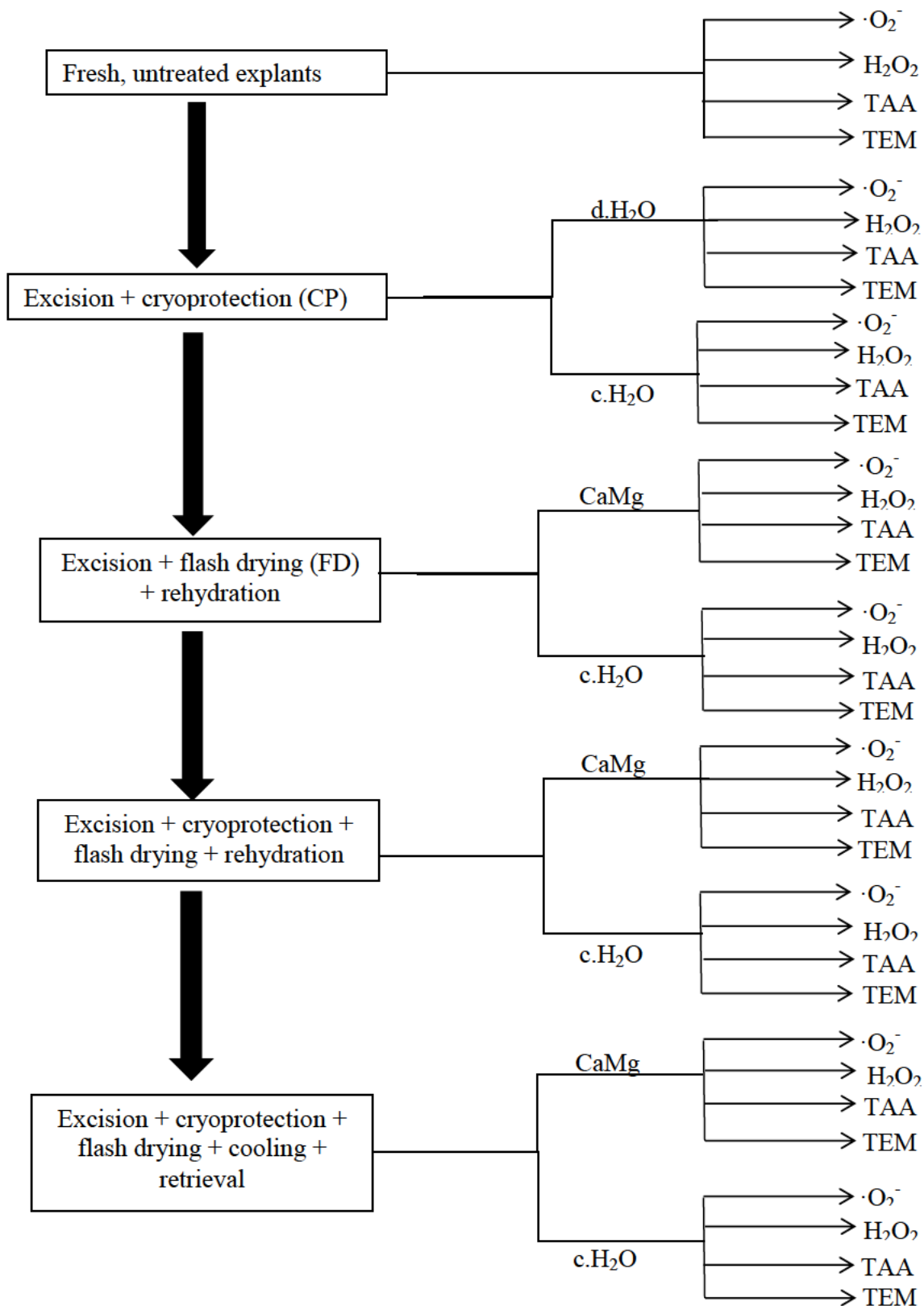


Figure 2.3 Steps followed to achieve successful cryopreservation of zygotic explants of *E. capensis* from St. Lucia and Port Elizabeth provenances.

2.2.3 Explant decontamination and germination

Prior to *in vitro* germination, explants (n=20) were decontaminated with a 1% (m/v) NaOCl solution for 10 min. This was carried out in sterile Petri[®] dishes within the confines of a darkened laminar air flow (Airvolution, Alphalab, Roodeport, South Africa). Explants were subsequently rinsed with sterile calcium magnesium solution (CaMg solution; 0.5 mM CaCl₂·2H₂O and 0.5 µM MgCl₂·6H₂O [Mycock, 1999]) and blotted dry on filter paper (Whatman No.1) before culturing them on the nutrient medium.

Hajari (2011) described a cryopreservation micropropagation protocol for *E. capensis* involving taking excised embryonic axes of *E. capensis* with pieces of cotyledon attached (as used here for cryopreservation). Using this protocol, all explants produced seedlings with roots and shoots when grown on ¼ strength MS (Murashige and Skoog, 1962) medium supplemented with 3 µM pyridoxine. The medium was comprised of MS salts (1.1 g l⁻¹), sucrose (30 g l⁻¹), agar (10g l⁻¹) and pyridoxine (0.6 mg l⁻¹) at a pH of 5.6-5.8. Therefore, this was the germination medium of choice throughout steps implemented for cryopreservation in the current study.

Cultures were incubated in a growth chamber with constant temperature of 27°C and photoperiod of 16 h and a photon flux density of 50 µmol m⁻² s⁻¹. Explants that produced both roots and shoots were deemed viable. However, for those treatments that included flash drying and cryoprotection, Petri[®] dishes were held in dark conditions for at least 7 d or until radicle protrusion was observed before being transferred to the growth chamber.

2.2.4 Water content determination

The water content of explants was determined gravimetrically as described in Section 2.1.5.

2.2.5 Flash drying

Explants were carefully placed on a wire mesh taking care to ensure that individual explants did not come into contact with each other. The mesh was placed over activated silica gel (250 g) and a stream of dry air (10 l min⁻¹) drawn from the silica gel was passed over the explants to dry them rapidly (Berjak *et al.*, 1989). This process is termed flash drying, and was carried out within a 500 ml glass bell jar.

In an attempt to increase the rate of drying, axes were flash dried in a vacuum (Fu *et al.*, 1993). For vacuum flash drying, the apparatus was placed in a desiccator with 500 g activated silica gel attached to a vacuum pump. However, no difference in the drying rate (data not shown) was found between vacuum and atmospheric pressure flash drying, therefore, explants for all experiments were dried at atmospheric pressure.

Axes were dried at 10 min intervals for 60 min (with and without exposure to cryoprotectants). Viability was checked after each time interval. The drying time was optimised for axes from both St. Lucia and Port Elizabeth provenance using a standard curve generated from the above data.

2.2.6 Production of cathodic water

Cathodic water can scavenge $\cdot\text{O}_2^-$ and H_2O_2 during cryopreservation procedures (eg. Naidoo *et al.*, 2016). The ability of cathodic water to reduce levels of ROS in explants of *E. capensis* was assessed after each step of cryopreservation. The effects of cathodic water treatment were compared with those of distilled water and a solution of Ca and Mg.

Cathodic water was produced by electrolysis of a CaMg solution that contained the electrolytes $0.5\ \mu\text{M}\ \text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $1\text{mM}\ \text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Berjak *et al.*, 2011). Two vessels (beakers), each containing 200 ml of autoclaved CaMg solution were connected by a KCl salt bridge. The salt bridge was made up of 30% KCl (w/v) and 3% agar (w/v) dissolved in 10 ml distilled water. Platinum electrodes were inserted into two beakers (200 ml per beaker) and 60 V was passed between the two vessels using a BioRad™ Powerpac (BioRad, Hercules, California, USA) (Berjak *et al.*, 2011) for 60 min at room temperature. The result was cathodic water in one beaker (pH *c.* 11.2) and anodic water (pH *c.* 2.4) in the other (Berjak *et al.*, 2011). The anodic water was discarded, while the cathodic water was used as an exogenous antioxidant to prepare cryoprotectants and rehydrate explants.

2.2.7 Cryoprotectants

Axes were immersed in solutions of cryoprotectants to promote a vitrified state of the cytoplasmic contents during cooling, thus potentially enhancing their survival after exposure to liquid nitrogen. Both penetrating (DMSO and glycerol) and non-penetrating (sucrose) cryoprotectants were used. The toxicity of these cryoprotectants were tested individually and in combination to provide maximum vitrification of the cytoplasm upon freezing.

Cryoprotectants were made up in either distilled or cathodic water. Axes were initially incubated in a 5% cryoprotectant (either w/v in the case of sucrose or v/v in the case of DMSO and glycerol) solution based on the combinations shown in Table 2.1 for 30 min and then in 10% of the respective solution for another 30 min. All the cryoprotectant incubations were carried out in the dark. Axes treated with a combination of sucrose and glycerol cryoprotectants showed higher vigour (indicated by ‘++’) than those treated with DMSO and glycerol cryoprotectant combination (indicated by ‘+’) (Table 2.4). Therefore, for cooling experiments, the sucrose and glycerol cryoprotectant combination was selected.

Table 2.4 The effect of cryoprotectants on viability when used individually and in combination. Treatments with ‘++’ had better vigour than those with ‘+’.

Cryoprotectant (s) used	Provenance	
	St. Lucia	Port Elizabeth
Glycerol	++	++
*DMSO	+	+
*DMSO+glycerol	+	+
Sucrose	++	++
*DMSO+sucrose	+	+
Sucrose+glycerol	++	++

*Reduced vigour was noted in treatments with DMSO, presumably due to toxicity.

2.2.8 Cooling

At atmospheric pressure, LN has a temperature of -196°C. When the pressure is lowered, LN forms slush which is at -210°C, offering a faster cooling rate (Wesley-Smith *et al.*, 1992). LN slush was prepared by dispensing 300 ml of LN in a polystyrene cup that was then placed in a desiccator and a vacuum subsequently introduced (Steponkus and Caldwell, 1993).

After axes were immersed in cryoprotectants and flash dried (as described in Section 2.2.5), they were plunged in nitrogen slush to cool ultra-rapidly. Explants were tumble-mixed in nitrogen slush for 1 min, thereafter, they were transferred (under LN) to cryovials (Greiner Bio-One®, Frickenhausen, Germany). The cryovials were subsequently stored in LN dewar flasks (Schorn Cryogenics, Boksburg, South Africa) for a minimum of 24 h before rewarming and recovery.

2.2.9 Warming

As discussed in Section 1.6.1.4, it is important to warm axes of recalcitrant seeds rapidly after freezing. This was done in a 40°C solution of either CaMg or cathodic water. These solutions were heated in a water bath (Juchheim Labortechnik, Germany). Axes were transferred to 65 mm Petri dishes with the heated solution for 2 min.

After rapid warming, the axes were rehydrated in either CaMg or cathodic water for 30 min at 25°C. All of the above procedures were conducted in the dark. Subsequent to rehydration, the axes were decontaminated and plated on sterile culture media (n = 20).

2.2.10 Quantification of reactive oxygen species

2.2.10a Quantification of extracellular superoxide ($\cdot\text{O}_2^-$) production

Extracellular levels of $\cdot\text{O}_2^-$ were quantified by the oxidation of epinephrine to adrenochrome. This was done in a UV-Vis spectrophotometer (Cary 50 UV Vis spectrophotometer, Varian) at $\epsilon_{490\text{ nm}} = 4.47\text{ mM}^{-1}\text{cm}^{-1}$ (Misra and Fridovich, 1972). A 1 mM epinephrine solution (pH 7.0) was prepared in the dark prior to commencement of the assay. Each of six 2 ml Eppendorf® tubes (4 explants per tube) contained 0.5 ml of the epinephrine solution and 1.5 ml distilled water (n=6). These tubes were subsequently incubated at room temperature in the dark on an orbital shaker (Labcon, Instrulab CC, Maraisburg, South Africa) set at 100 rpm. After 15 min incubation, the oxidised solution was decanted in plastic cuvettes (Sarstedt, Sarstedt AG & Company, Nümbrecht, Germany) and read at 490nm wavelength. The dry mass of the explants used were obtained by drying them in an oven set at 80°C for 48 h.

To correct for non-specific absorption, explants were incubated in distilled water and the absorbance subtracted from measured readings prior to further calculations. To test for non-specific oxidation of epinephrine (i.e. not by $\cdot\text{O}_2^-$), superoxide dismutase (SOD) at $0.1\text{ }\mu\text{g ml}^{-1}$ was added to incubation solutions. Addition of SOD to the incubation medium reduced the oxidation of epinephrine by more than 50%. While this suggests that some non-specific oxidation of epinephrine was taking place in the assays, most was a result of extracellular $\cdot\text{O}_2^-$ production (Whitaker *et al.*, 2010).

2.2.10b Quantification of extracellular hydrogen peroxide (H₂O₂) production

The xylenol orange assay (Gay and Gebicki, 2000) was used to quantify the levels of hydrogen peroxide. Each of the six replicates (n=6) contained 4 explants. These were incubated in 1 ml distilled water at room temperature for 30 min and agitated at 100 rpm on an orbital shaker (Labcon, Instrulab CC, Maraisburg, South Africa) in the dark.

A working reagent stock was prepared by mixing two solutions (solutions 1 and 2) in a 1:100 ratio. Solution 1 was comprised of 25 mM FeSO₄; 25 mM (NH₄)₂SO₄ and 2.5 M H₂SO₄ prepared in sterile, deionised water. Solution 2 was comprised of 125 mM xylenol orange (Sigma-Aldrich, Germany) and 100 mM sorbitol prepared in sterile, deionised water.

A 300 µl aliquot was removed from the distilled water (and axes) incubation and added to 1500 µl of the xylenol orange working reagent stock. This solution was incubated for 20 min at room temperature on an orbital shaker set at 100 rpm in the dark. The optical densities of these solutions (both blank and treatment) were read at 560 nm (Cary 50 Conc UV Vis spectrophotometer, Varian, Palo Alto, CA). Levels of H₂O₂ were calculated from a standard curve. The generation of ROS due to photo-oxidation was limited by working in dark conditions (Touchell and Walters, 2000). The validity of this assay was confirmed when more than 50% inhibition of H₂O₂ production occurred when 250 µml⁻¹ (2310 U mg⁻¹ protein) of catalase was added to the assay mixture.

2.2.11 Quantification of total aqueous antioxidant (TAA) capacity

The levels of enzymic and non-enzymic total aqueous antioxidant (TAA) capacity was quantified in both control and treated explants (n=8) using the ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) decolourisation assay. This assay is applicable to lipophilic and hydrophilic antioxidants (Re *et al.*, 1999). When ABTS is oxidised with potassium persulfate (K₂S₂O₈), it produces the radical monocation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS^{•+}). This monocation is thereafter reduced by hydrogen donating antioxidants. The time taken for the reaction of the radical cation with the antioxidant was assessed by this TAA assay. Initially, four batches of four explants (approximately 20 mg) were homogenised using pre-cooled pestles and mortars in liquid nitrogen with 0.1 g insoluble polyvinylpyrrolidone (PVP). Explants treated with antioxidants (cathodic water) were briefly rinsed 3 times in distilled water, blotted dry and then

homogenised. An extraction buffer (50 mM KH_2PO_4 buffer; pH 7.0, containing 1 mM CaCl_2 , 1 mM KCl and 1 mM EDTA) was prepared in advance and stored at 4°C. A micropipette was used to dispense 1 ml of extraction buffer into the homogenised explants and the contents were then mixed well. The extract was then dispensed into pre-cooled Eppendorf® (2 ml) tubes that were held on ice. The pestles and mortars were then rinsed twice with 0.5 ml of extraction buffer, bringing the final volume of the extract to 2 ml.

The Eppendorf® tubes were held on ice for 15 min, vortexing (Snijders, Holland) the solution every 5 min and then centrifuged at 4°C for 10 min at 13 000Xg (Hermle Labortechnik, Germany). After initial centrifugation, 1 ml of the supernatant was removed and centrifuged as per conditions above for a further 5 min. After the second centrifugation step, 600 µl of the supernatant was dispensed into chilled Eppendorf® tubes (on ice) for use in the ABTS assay.

The ABTS radical solution (7 mM ABTS and 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ in 1 ml of distilled water that had been incubated in the dark 12-16 h before use) was diluted with phosphate buffer saline (PBS; 5 mM Na_2HPO_4 and 37.5 mM NaCl, pH 7.4) to prepare a working solution to assess the TAA in the extracts. The working solution was considered ready for use when A_{732} was 0.70 ± 0.02 (Johnston *et al.*, 2006). The spectrophotometer was zeroed with 1 ml PBS.

After the working solution had been optimised, 1 ml of this solution was pipetted into a plastic cuvette and absorbance read at 0 min prior to the initiation of the reaction. Immediately thereafter, 100 µl of the TAA extract was added to the cuvette and inverted three times (to initiate the reaction) using a piece of Parafilm®. The absorbance was recorded after 0.5, 1 and 2 min.

A standard curve was generated for each set of experiments to calculate TAA. This was achieved by preparing a 5 mM Trolox® (6-hydroxy-2,5,7,8-tetramethylchromeane-2-carboxylic acid, 97%; Sigma-Aldrich, Germany) stock in extraction buffer. The stock was then diluted (with extraction buffer) to standard concentrations of 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 mM. The standard curve was generated by adding 100 µl of each Trolox® standard (instead of TAA extract) to the ABTS assay as described above. For each standard solution, an average of three readings was used. The ABTS assay was conducted after each cryopreparative step and expressed on a fresh mass basis.

2.2.12 Ultrastructure

The ultrastructure of root and shoot meristems of *E. capensis* from each of the two provenances were assessed. The explants from each of the cryopreparative steps (as outlined in Fig. 2.3) were recovered *in vitro* for 48 h in the dark, and then root and shoot meristems were processed as described in Section 2.1.10.

2.2.13 Statistical analysis

Data were analysed with SPSS 25.0 (version 25.0, SPSS Inc., Chicago, Illinois, USA), using critical $p < 0.05$.

Differences between various biomarkers were assessed using a one-way analysis of variance (ANOVA). Their means were compared using a Tukey *post hoc* test. The mean values were assigned with different alphabets indicating significant differences. Normally distributed data were subject to an Independent samples t-test while non-parametric data were analysed with a Mann-Whitney U test.

Total levels of $\cdot\text{O}_2^-$, H_2O_2 and TAA after each of the cryopreparative steps were compared against the controls to assess changes. The difference between the mean values of control and treatment were tested using an Independent Sample t-test for normally distributed data or Mann-Whitney U test for data that was not normally distributed. Alphabetical values were assigned to recorded mean values. Mean values with different letters were considered to be significantly different ($p < 0.05$).

CHAPTER 3: RESULTS

The data is presented in two sections, viz. section (A) which deals with the chilling storage of whole seeds and section (B) which covers attempts at cryopreserving germplasm of *E. capensis*.

A) Chilling storage of whole seeds

3.1 Water content during storage

3.1.1 Material from Port Elizabeth

An important parameter in any study of recalcitrant seeds is the water content. Therefore, unless the objective is a study of desiccation responses, it is essential that the water content is maintained relatively constant throughout a particular study. In this study, water content during storage was maintained at an average of $0.70 \pm 0.17 \text{ g g}^{-1}$ dry mass (Fig. 3.1) for the whole seeds of *E. capensis* that were sourced from Port Elizabeth (PE). The water content of the cotyledons is not presented graphically because it was essentially similar to whole seed water content at $0.68 \pm 0.17 \text{ g g}^{-1}$. Irrespective of the storage temperature, the embryonic axes of these seeds had a higher water content ($1.60 \pm 0.54 \text{ g g}^{-1}$; Fig. 3.2) than the whole seeds/cotyledons, although fluctuations in the water content occurred, and were most pronounced in the axes. According to a 2-way ANOVA, there were no significant differences in water content of either seeds or axes across storage times and temperatures ($p > 0.05$).

Axes are relatively small when compared with the cotyledons and this was evident in the fresh mass of the seed components. Cotyledons are approximately 140 times the fresh mass of the axes yet the axes had a water content (on a dry mass basis) 2 – 3 times than that of the cotyledons. Because the axis constitutes a small proportion of total seed tissue, whole seed water content reflects that of the cotyledons. There were no significant differences in water content of seeds or axes (Figs. 3.1 and 3.2) of material harvested in two consecutive fruiting seasons ($p > 0.05$; data not presented for second season).

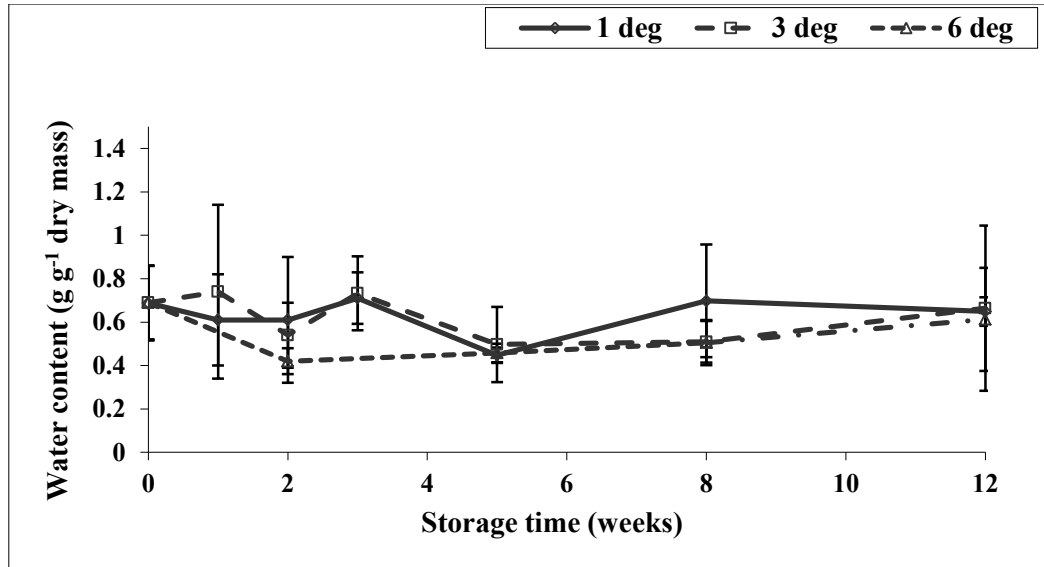


Figure 3.1 Water content (g g^{-1}) of whole seeds of *E. capensis* from PE that were stored at 1°, 3° and 6°C (n=5). Bars indicate standard deviation.

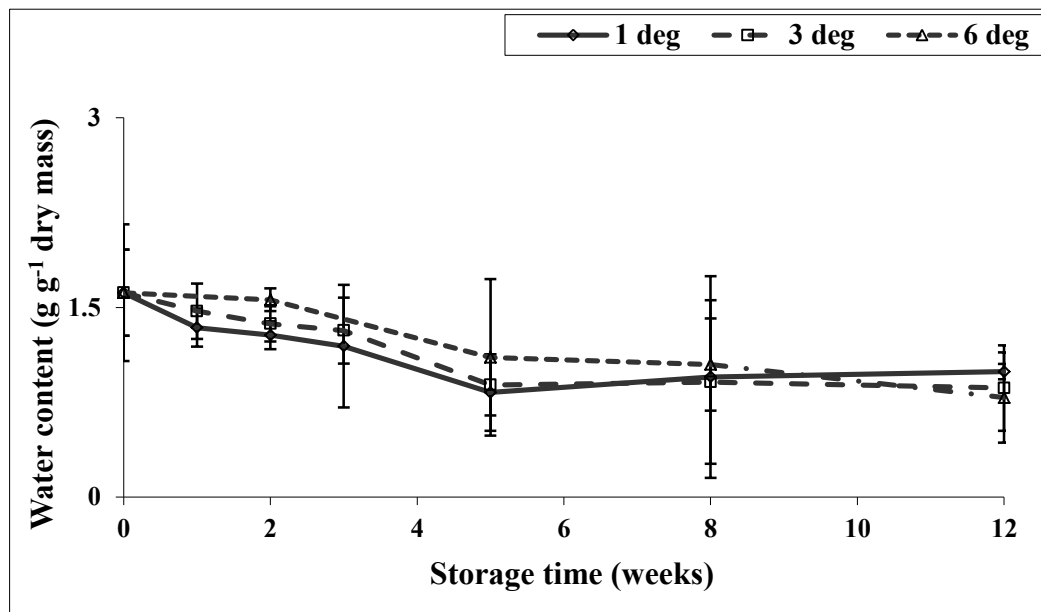


Figure 3.2 Water content (g g^{-1}) of the embryonic axes of seeds of *E. capensis* seeds from PE, stored at 1°, 3° and 6°C (n=5). Bars indicate standard deviation.

3.1.2 Material from St Lucia

The mean water content of the seeds of *E. capensis* from St. Lucia (Fig. 3.3) was $0.65 \pm 0.06 \text{ g g}^{-1}$ dry mass throughout the 38 d storage period although there were fluctuations between sampling intervals. The water content of the axes was maintained at $2.2 \pm 0.19 \text{ g g}^{-1}$ (Fig. 3.4) while the water content of the cotyledons was retained at $0.66 \pm 0.15 \text{ g g}^{-1}$, essentially reflecting whole seed water content. A 2-way ANOVA indicated that there were no significant differences in water content of either seeds or axes between storage times and temperatures ($p > 0.05$). Water content of seeds and axes was assessed over two consecutive fruiting seasons (data not presented for second season) with no significant differences between the inter-seasonal batches ($p > 0.05$).

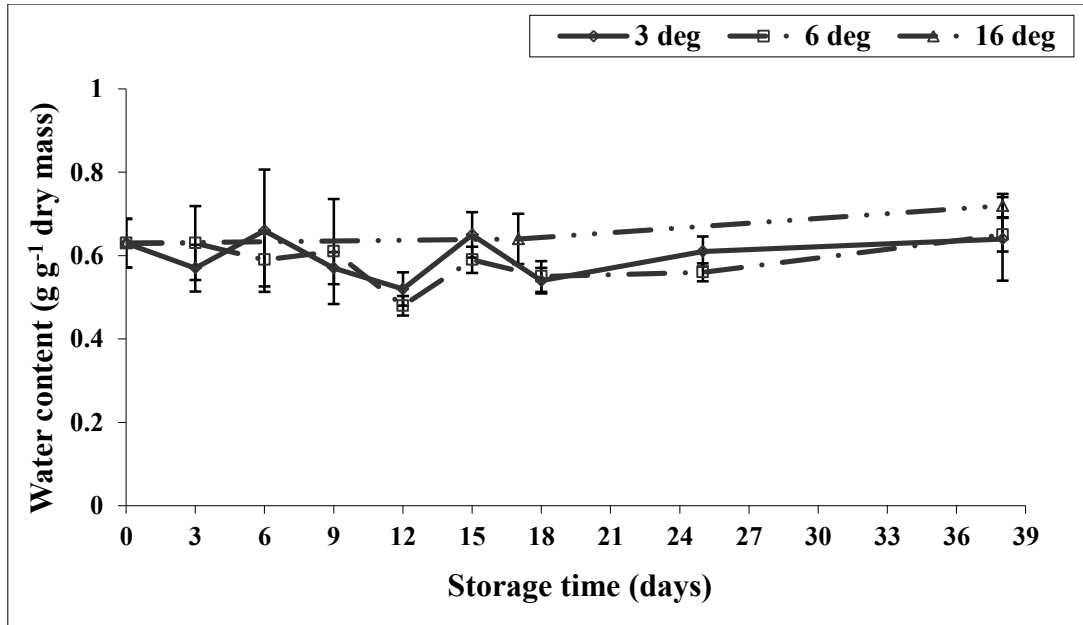


Figure 3.3 Water content (g g^{-1}) of whole seeds of *E. capensis* from St Lucia stored at 3°, 6° and 16°C (n=5). Bars indicate standard deviation.

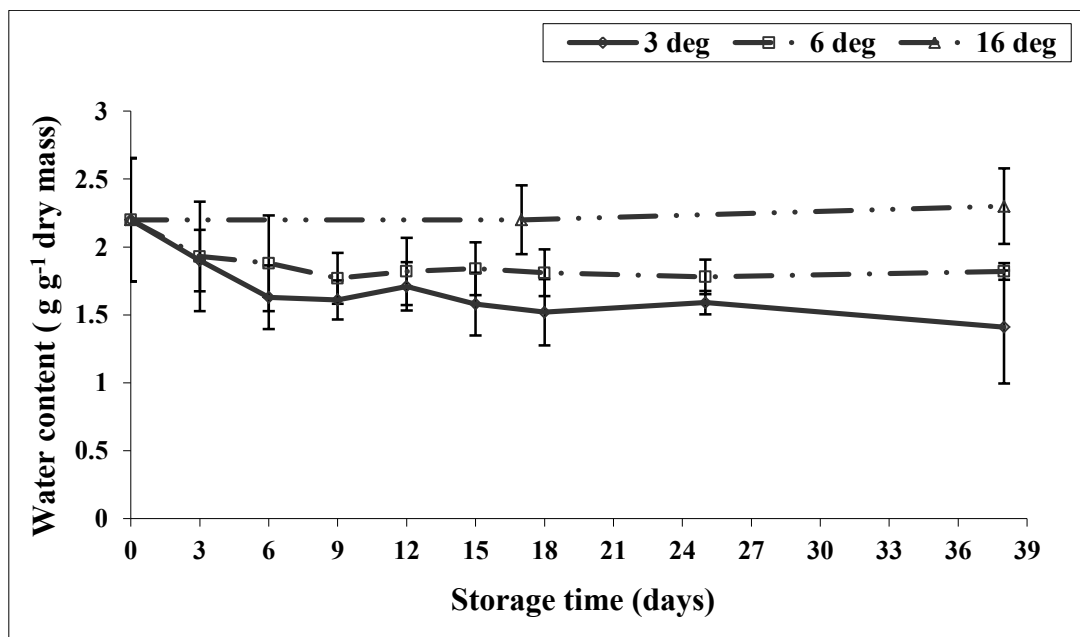


Figure 3.4 Water content (g g^{-1}) of the embryonic axes of seeds of *E. capensis* from St Lucia stored at 3°, 6° and 16°C (n=5). Bars indicate standard deviation.

3.1.3 Material from Tanzania

The mean water content of seeds of *E. capensis* from Tanzania (Fig. 3.5) was $0.60 \pm 0.08 \text{ g g}^{-1}$ throughout the sampling period of 9 d. Axis water content was maintained at $1.1 \pm 0.11 \text{ g g}^{-1}$ (Fig. 3.6), while that of the whole seeds/cotyledons was maintained at $0.53 \pm 0.14 \text{ g g}^{-1}$. This batch of seeds was sampled over a 9 d period at 3 d intervals. A 2-way ANOVA indicated there were no significant effects of storage time or temperature on seed or axis water content ($p > 0.05$). Water contents of seeds harvested in two consecutive fruiting seasons were not significantly different ($p > 0.05$) thus data not presented for the second season.

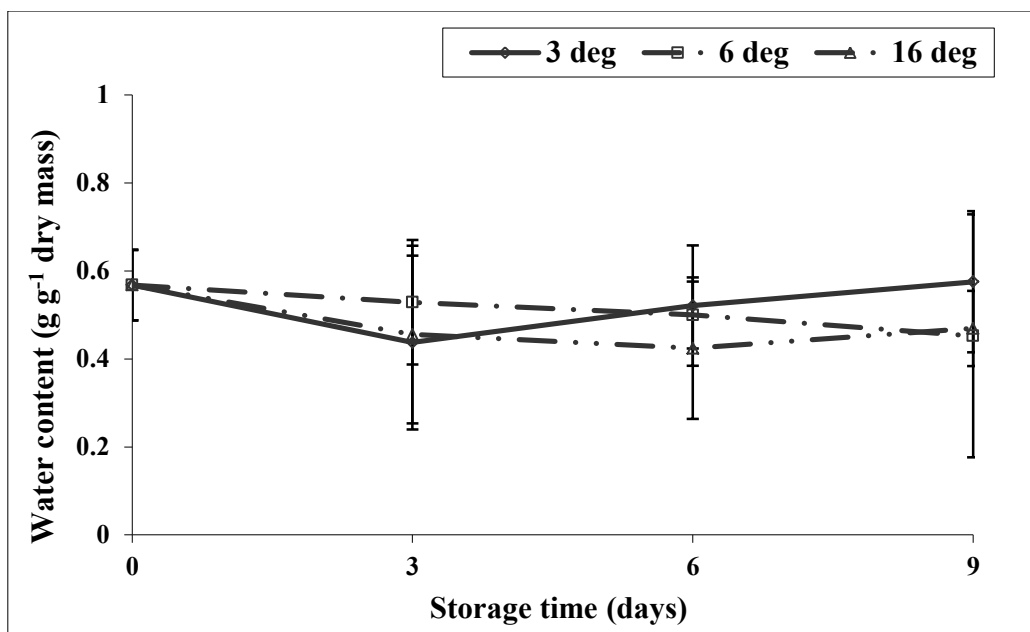


Figure 3.5 Water content (g g^{-1}) of whole seeds of *E. capensis* from Tanzania stored at 3°, 6° and 16°C ($n=5$). Bars indicate standard deviation.

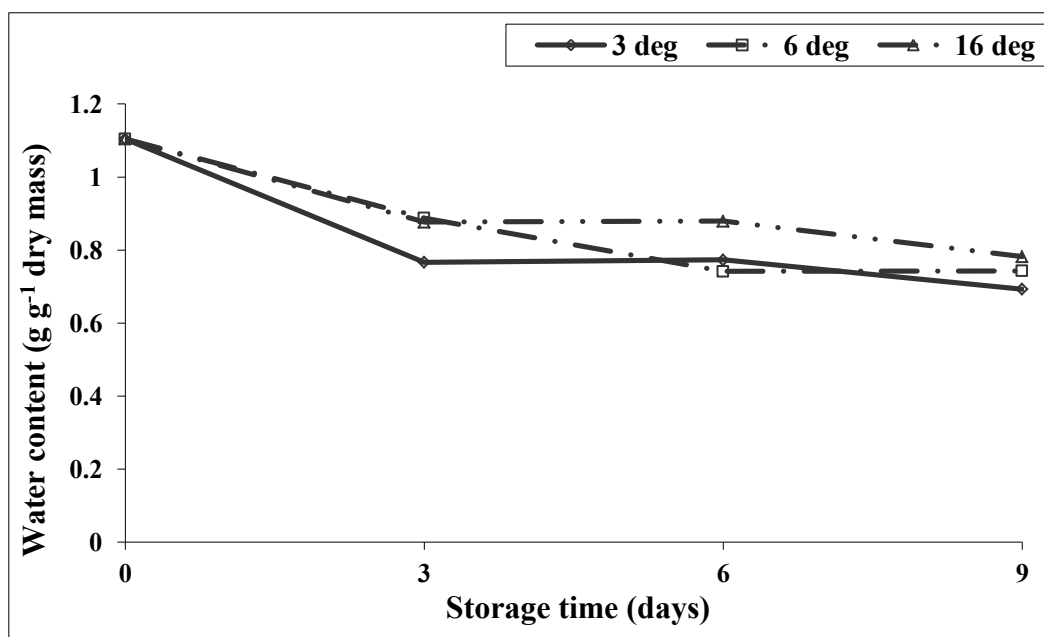


Figure 3.6 Water content (g g^{-1}) of the embryonic axes of *E. capensis* from Tanzania stored at 3°, 6° and 16°C ($n=5$). Bars indicate standard deviation.

3.2 Germination

3.2.1 Seeds from Port Elizabeth

Viability was assessed by germinating whole seeds (after removal of the closely associated endocarp and papery testa) of *E. capensis* on wet filter paper in 90 mm Petri dishes at $\pm 24^{\circ}\text{C}$.

Seeds from PE (Fig. 3.7) were sampled over 12 weeks, with samples being taken after 1, 2, 3, 5, 8 and 12 weeks. Seeds from this provenance were able to tolerate low temperatures, with viability being maintained at 100% after 8 weeks in storage at 1° , 3° and 6°C . Viability tests on batches of seeds from two successive seasons gave identical results. The seeds stored at the two lower temperatures, i.e. 1° and 3°C , showed a reduction in viability to around 80% after 8 weeks. Seeds from the warm temperate provenance thus appeared to be susceptible to chilling temperatures at 1° and 3°C , but only after storage for more than 2 months. The seeds stored at 6°C maintained viability at 100% throughout the study.

The seeds stored at 1°C (Table 3.1) took 3 d longer to germinate compared with those stored at 6°C after storage for 12 weeks (Table 3.2). Germination rate of the seeds stored at 6°C for 12 weeks (Table 3.3) appeared to be slightly affected by storage taking 4 d longer than the fresh seeds to initiate germination. Although relatively high viability was maintained (Fig. 3.7), the vigour of the seeds stored at 1° and 3°C was adversely affected; the time needed for 100% germination increased with increasing duration in storage (Tables 3.1 and 3.2).

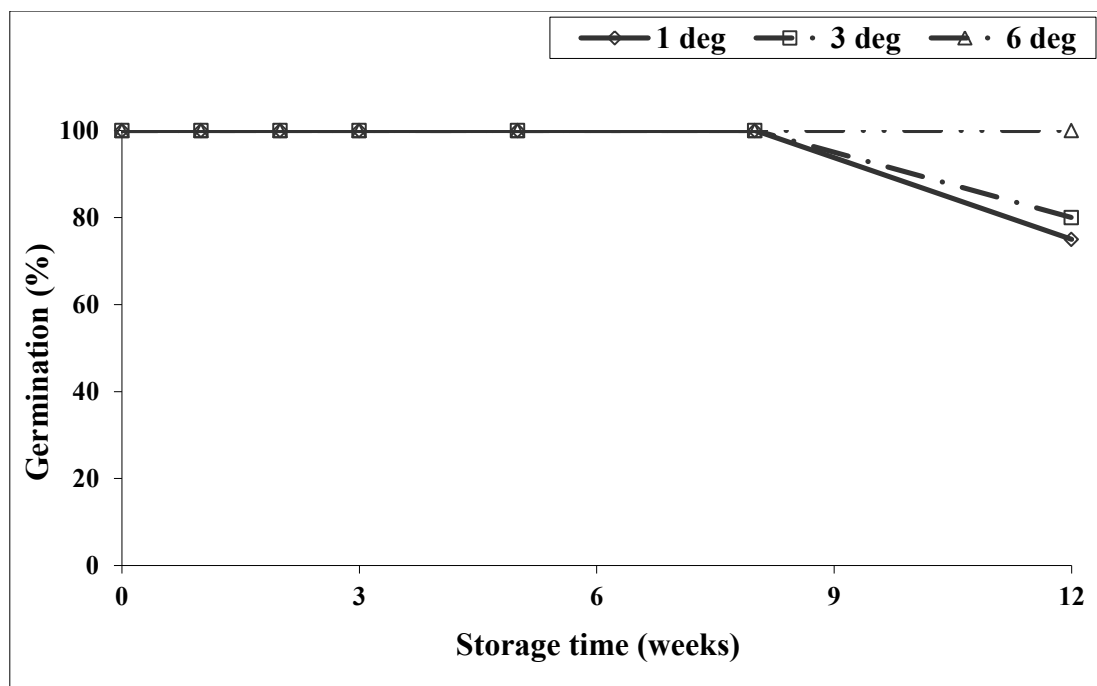


Figure 3.7 Viability of the seeds from PE sampled at intervals during storage at 1°, 3° and 6°C over a 12-week storage period (n=20).

Table 3.1 Germination rate (vigour) and totality of seeds of *E. capensis* from PE stored at 1°C (n=20).

Days to germinate	Weeks in storage						
	0	1	2	3	5	8	12
1	0						
2	80						
3	100	60	70				
4		100	90	50	25		
5			100			20	
6				70	65		
7				100		70	40
8					90		
9					100		
10							65
11						100	
12							
13							80

Table 3.2 Germination rate (vigour) of seeds of *E. capensis* from PE stored at 3°C (n=20).

Days to germinate	<u>Weeks in storage</u>						
	0	1	2	3	5	8	12
1							
2	80						
3	100	70	80	30	40		
4		100	90				
5			100	70	60	20	
6				100			
7					90	70	40
8					100	100	
9							70
10							80

Table 3.3 Germination rate (vigour) of seeds of *E. capensis* from PE stored at 6°C (n=20).

Days to germinate	<u>Weeks in storage</u>						
	0	1	2	3	5	8	12
1					20		
2	80	60	70	60	50	40	
3	100	100	80	75	80	70	30
4			95	100	90	90	50
5			100		100	100	80
6							90
7							100

3.2.2 Seeds from St. Lucia

The seeds from St Lucia were sampled over 38 d at 3 d intervals for 18 d and then after 25 and 38 d (Fig. 3.8). Those stored at 6° and 16°C were not adversely affected by the sub-ambient temperatures. However, 16°C may not constitute a chilling temperature for seeds of this provenance, as the region is sub-tropical rather than tropical. The 16°C-stored seeds retained 100% viability. There was only a slight decline in viability for those stored at 6°C after 38 d which was unexpected, as such low ambient temperatures are not encountered at St Lucia.

The lowest storage temperature, 3°C, reduced the viability of the seeds, which, after only 3 d was 40% (Fig. 3.8). Fungal growth was evident when these seeds germinated. After a further 15 d of storage, germinability was reduced to 10% and fungal growth was prolific (see Fig.

3.9). The fungal proliferation seen suggests that this occurred when seed vigour had declined, especially as proliferation was not seen when fresh seeds (or presumably, those stored at 6 and 16°C) were set to germinate. It is extremely unlikely that the 6 and 16°C-stored seeds did not harbour fungal inoculum, but that they remained vigorous enough for proliferation to be counteracted. A reduction in vigour was also evident. Fresh seeds germinated within two days under favourable conditions, but the seeds that had been adversely affected by the chilling treatment (3°C) took up to 20 d to achieve 5% germination after 25 d in storage (Table 3.4). After 38 d in storage, none of the 3°C stored seeds germinated. Seeds stored at 6° and 16°C for 38 d took 7 and 4 d, respectively, to achieve 100% germination (Tables 3.5 and 3.6). Results of vigour and viability tests on batches of seeds from successive seasons were identical.

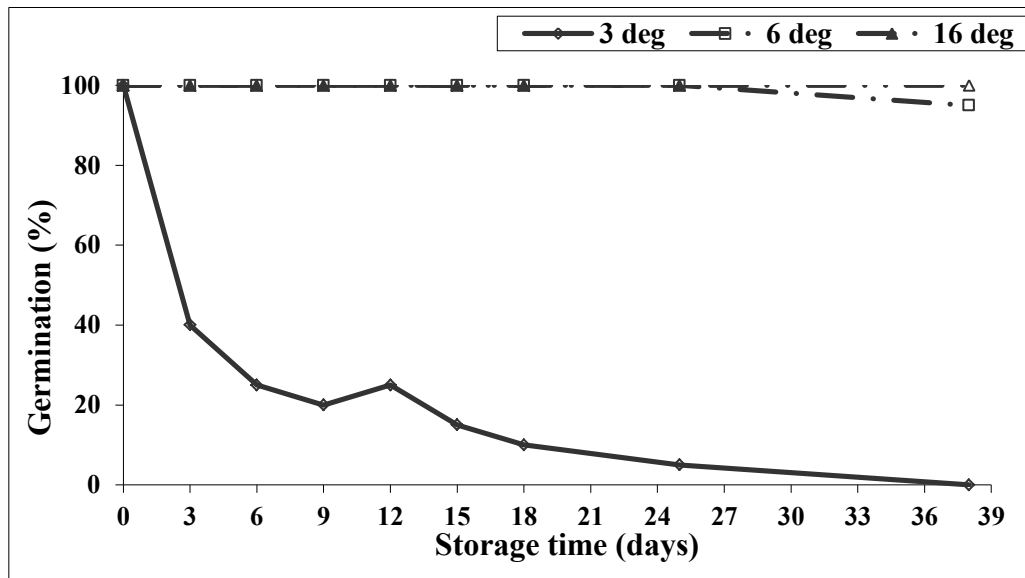


Figure 3.8 Viability of the seeds from St Lucia sampled at intervals during storage at 3°, 6° and 16°C over 38 d (n=20).

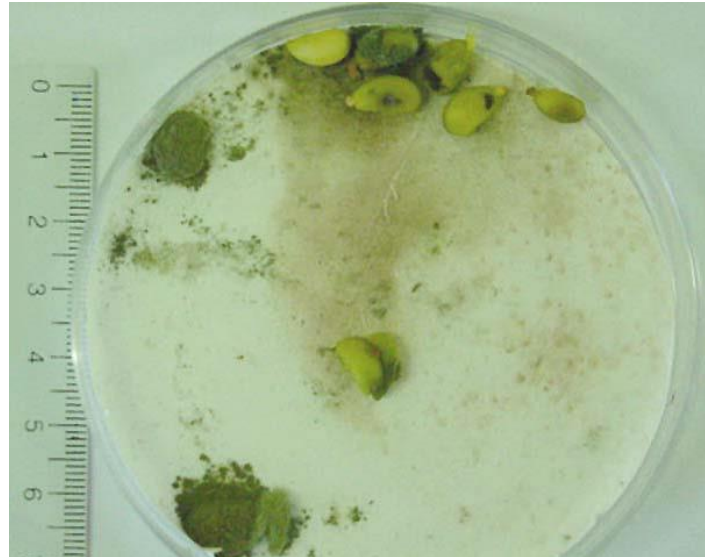


Figure 3.9 Fungal proliferation occurring from some of the seeds from St Lucia when they were germinated after storage for 18 d at 3°C.

Table 3.4 Germination rate (vigour) of seeds of *E. capensis* from St Lucia stored for different periods at 3°C (n=20).

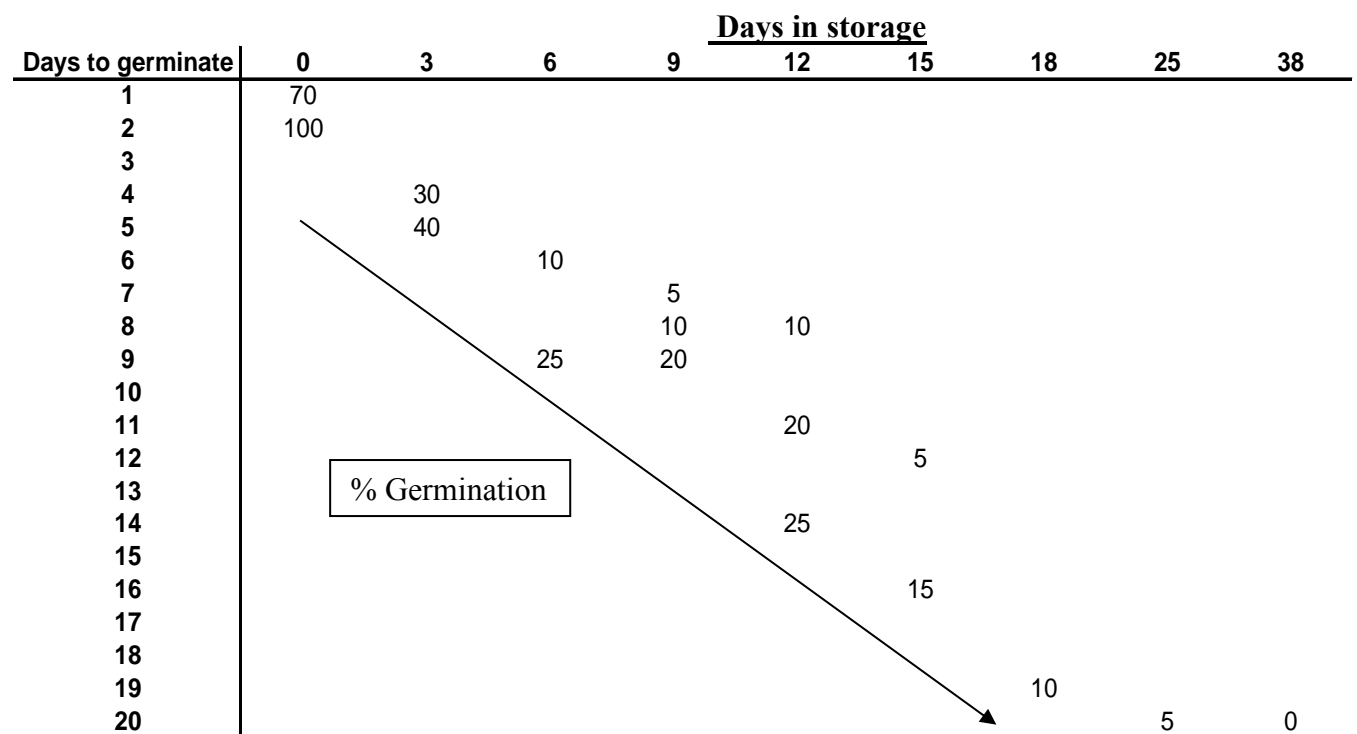


Table 3.5 Germination rate (vigour) of seeds of *E. capensis* from St Lucia stored at 6°C (n=20).

Days to germinate	<u>Days in storage</u>								
	0	3	6	9	12	15	18	25	38
1	70		20						
2	100	30	70		20		10		
3		80	100	30	50	30	30	30	
4		100		75	90	70	80	50	20
5				100	100	100	100	70	40
6		<div>% Germination</div>						90	80
7								100	100

Table 3.6 Germination rate (vigour) of seeds of *E. capensis* from St Lucia stored at 16°C (n=20).

Days to germinate	<u>Days in storage</u>								
	0	3	6	9	12	15	18	25	38
1	70	50	60	70	50	70	40	20	40
2	100	90	70	90	60	80	60	40	60
3		100	80	100	80	100	80	50	90
4			100		100		100	80	100
5		<div>% Germination</div>						100	

3.2.3 Seeds from Tanzania

The seeds from Tanzania were sampled over a period of 9 d at 3 d intervals (Fig. 3.9) during storage at 3°, 6° and 16°C. The seeds stored at 3°C deteriorated rapidly with only 10% survival after 3 d, while those stored at 6°C retained 25% viability and 65% of those stored at 16°C remained viable after 3 d. After 6 d in storage at 3°C, viability remained unchanged at 10%, but was further reduced to 20% and 40% at 6°C and 16°C, respectively. Three days later (9 d), all seeds stored at 3°C were dead, while viability in those seeds stored at 6°C was reduced to 10% and to 40% in those stored at 16°C. Results of viability tests on batches of seeds from the two successive seasons were identical.

Vigour was severely affected by low storage temperatures. After only 3 d in storage at 3°C, it took 12 d for the seeds to achieve 10% germination compared with only 2 d for the fresh seeds to achieve 90% germination (Table 3.7). Germination rate continued to decline the

longer the seeds were in storage. A similar pattern emerged when the seeds were stored at 6°C (Table 3.8). From the increasingly long time taken to germinate after 3 d in storage the seeds stored at 16°C were presumed also to have lost vigour (Table 3.9). Germination data collection was not taken beyond 20 d because the seeds had either become over-run with fungi or were necrotic.

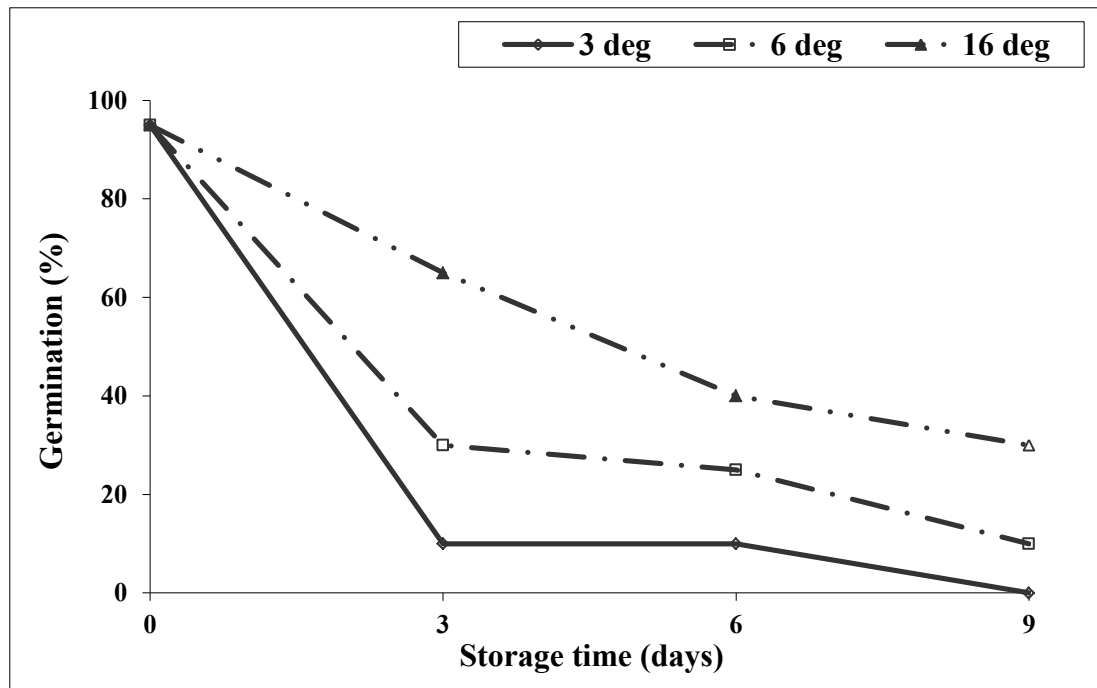


Figure 3.9 Viability of the seeds from Tanzania sampled during storage at 3°, 6° and 16°C, over a 9 d period (n=20).

Table 3.7 Germination rate (vigour) of seeds of *E. capensis* of Tanzanian origin stored at 3°C (n=20).

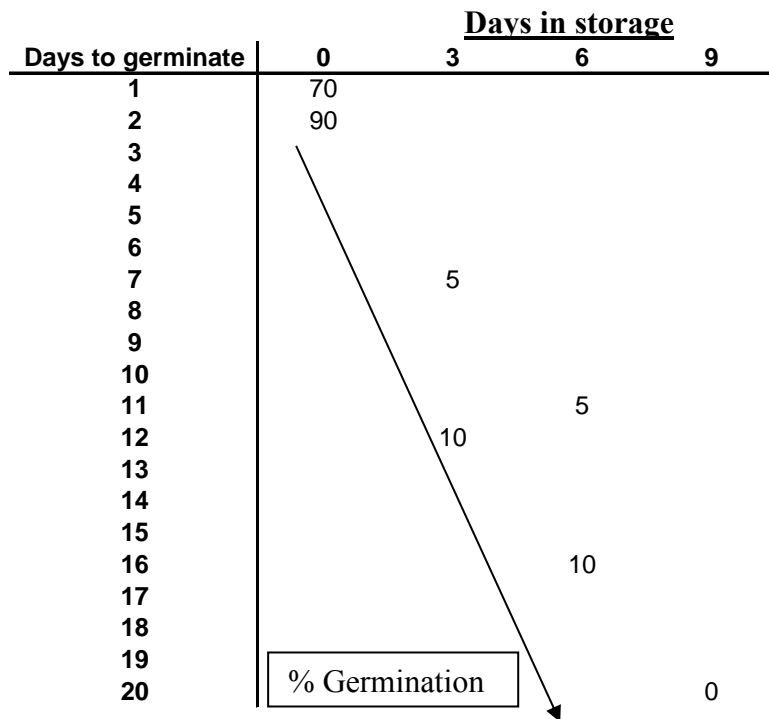


Table 3.8 Germination rate (vigour) of seeds of *E. capensis* of Tanzanian origin stored at 6°C (n=20).

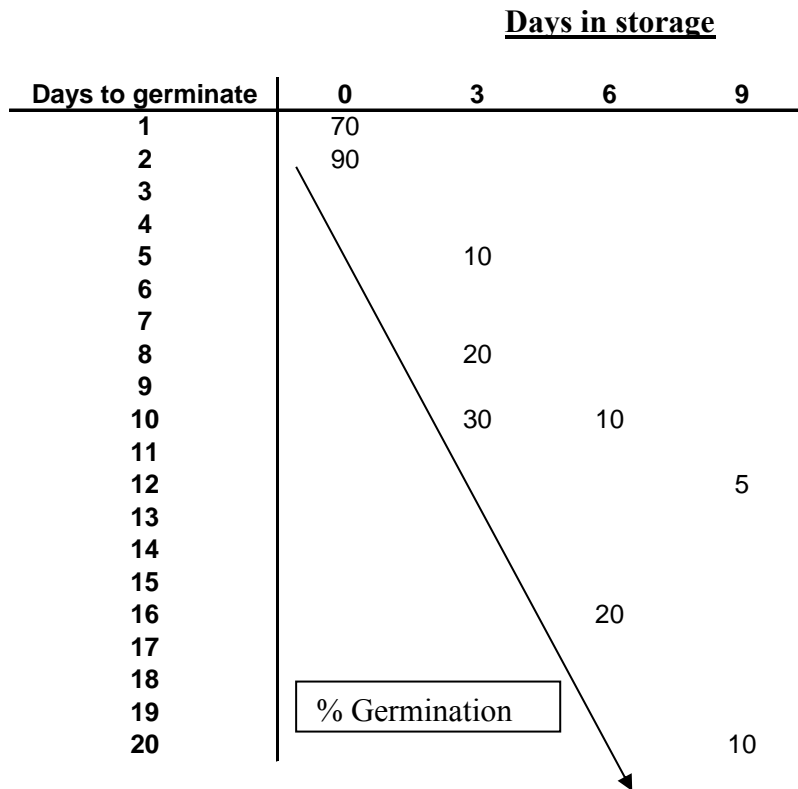
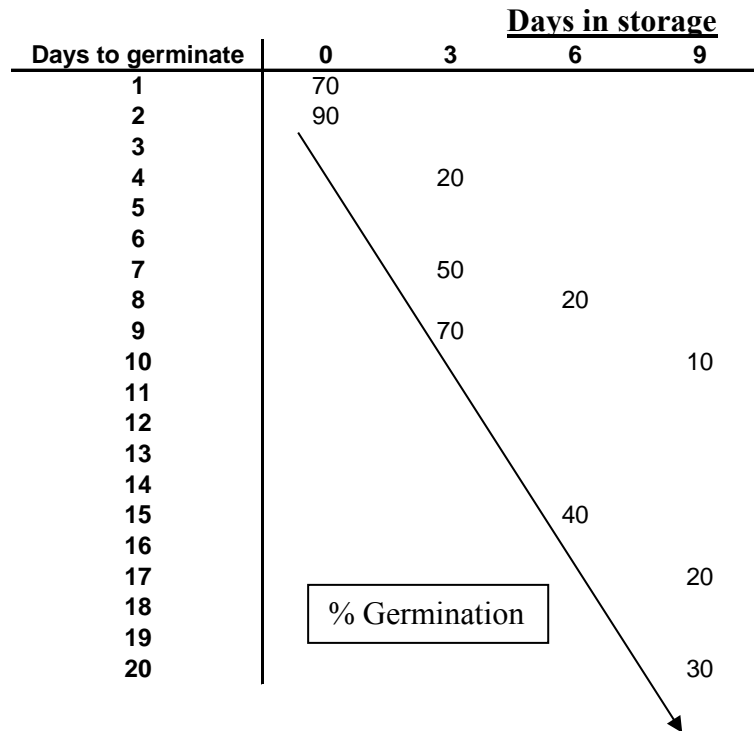


Table 3.9 Germination rate (vigour) of seeds of *E. capensis* of Tanzanian origin stored at 16°C (n=20).



3.3 Rate of protein synthesis

3.3.1 Material from Port Elizabeth

The rate of protein synthesis following 4 h incubation in a tritiated amino acid mix was measured for excised embryonic axes from seeds sampled after 0, 2, 8 and 12 weeks of storage (as there was a limitation in seed numbers, sampling could be carried out only at zero time and three intervals over the 12 week storage period at each temperature). The results (Fig. 3.10) shows that a steady decline in the rate of protein synthesis occurred across all three treatments i.e. with seed storage at 1°, 3° and 6°C over 12 weeks.

The rate of protein synthesis for axes from fresh seeds was 253 ± 29 DPM $\text{g}^{-1} \text{min}^{-1}$. After 2 weeks in storage, the rate had dropped to 203 ± 25 , 106 ± 19 and 160 ± 42 DPM $\text{g}^{-1} \text{min}^{-1}$ for material stored at 1°, 3° and 6°C, respectively, and interestingly was highest in the seeds that were stored at 1°C. However, over the first 6 weeks protein synthesis rate was apparently

lower for axes from seeds stored at 3°C, than for those at 6°C, although this cannot be stated unequivocally because of the long intervals between sampling. The rate of protein synthesis remained essentially similar for axes excised from seeds at all three storage temperatures after 8 weeks in storage (Fig. 3.10) when viability was still 100% for all (Fig. 3.7).

After 12 weeks in storage, the rate of protein synthesis varied between storage temperatures (Fig. 3.10) being 78 ± 8 , 97 ± 20 and 115 ± 12 DPM g⁻¹ min⁻¹ at 1°, 3°C and 6°C respectively ($p < 0.05$). At this point, the rate was slowest for axes from seeds stored at 1°C and highest in those stored at 6°C, when only the seeds stored at 6°C had maintained 100% viability. By the end of the study, the rate of protein synthesis was reduced to approximately one- third in the seeds stored at 1°C; i.e. they would take three times as long to produce the same amount of protein that was produced in the fresh seeds. There was a similar trend in the other two treatments, i.e. those stored at 3° and 6°C would take about 2.6 times and 2.2 times longer, respectively, to produce the same amount of protein as the fresh seeds. A 2-way ANOVA showed that there was a significant effect of both storage temperature and storage time on rate of protein synthesis ($p < 0.05$).

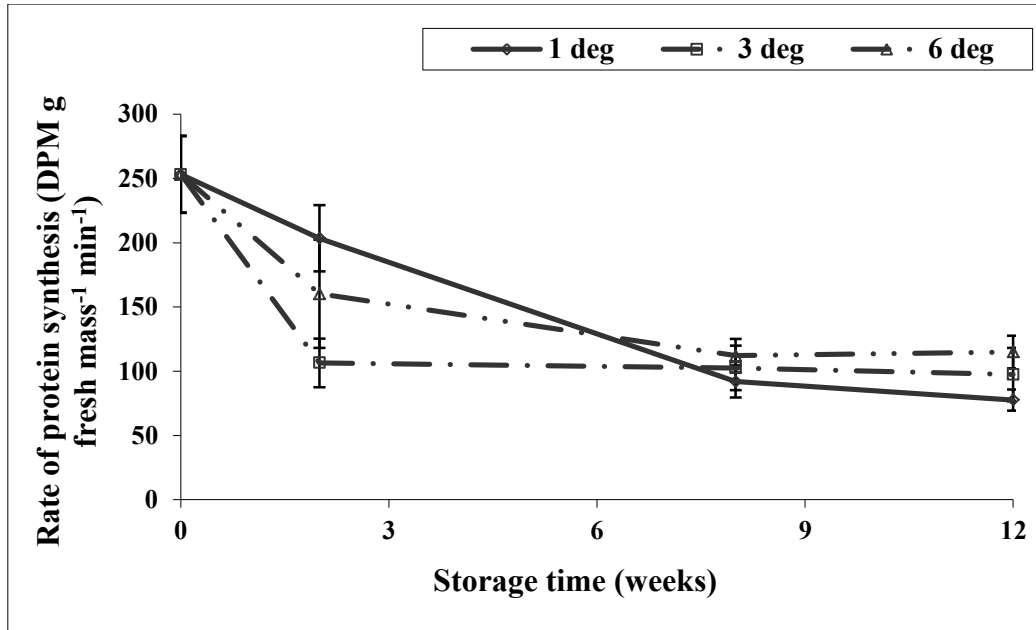


Figure 3.10 Rate of protein synthesis following 4 h incubation in a tritiated amino acid mix, for axes of *E. capensis* from PE. Seeds were stored at 1°, 3° and 6°C and sampled after 2, 8 and 12 weeks (n=20). Bars indicate standard deviation.

3.3.2 Material from St Lucia

In the material from St Lucia, the rate of protein synthesis was measured at each sampling point used for germination trials. Seeds were stored at 3°, 6° and 16°C and sampled at 3 d intervals (Fig. 3.11).

Axes from fresh seeds had an initial rate of protein synthesis of 200 ± 9 DPM g⁻¹ min⁻¹ increasing to 230 ± 16 DPM g⁻¹ min⁻¹ after storage at 16°C for 3 d. The rate of protein synthesis decreased gradually to 150 ± 15 DPM g⁻¹ min⁻¹ in the 16°C batch after 9 d, and then declined steadily to 66 ± 7 DPM g⁻¹ min⁻¹ after 38 d.

Axes from seeds stored at 3° and 6°C showed a similar trend, but without the initial peak shown early in storage at 16°C. The rate of protein synthesis in the axes of seeds stored at 3°C declined to 86 ± 9 DPM g⁻¹ min⁻¹ after 9 d in storage declining further to 47 ± 2 DPM g⁻¹ min⁻¹ after 15 d (Fig. 3.11) and continued to decrease to 34 ± 2 DPM g⁻¹ min⁻¹ after 38 d. Protein synthesis rate of axes from seeds stored at 6°C displayed a similar pattern to those stored at 3°C. The rate of protein synthesis declined to

120 ± 22 DPM $\text{g}^{-1} \text{min}^{-1}$ after 3 d in storage and maintained that level up until 9 d storage. The rate decreased further to 84 ± 19 DPM $\text{g}^{-1} \text{min}^{-1}$ after 12 d in storage and continued to decrease. After 38 d in storage, the rate of protein synthesis had slowed to 54 ± 13 DPM $\text{g}^{-1} \text{min}^{-1}$ (Fig. 3.11).

Not surprisingly, the decrease in the rate of protein synthesis corresponded to the loss in viability of the seeds stored at 3°C (Fig. 3.8) where the lowest rate in protein synthesis was recorded when all the seeds lost their ability to germinate, i.e. after 38 d. Although viability was maintained at 100% for the seeds stored at 6° and 16°C, a 2-way ANOVA showed that there was a significant effect of both storage temperature and storage time on the rate of protein synthesis ($p < 0.05$), presumably as a result of the slowdown in metabolism which could be associated with lower storage temperatures for seeds of sub-tropical provenance.

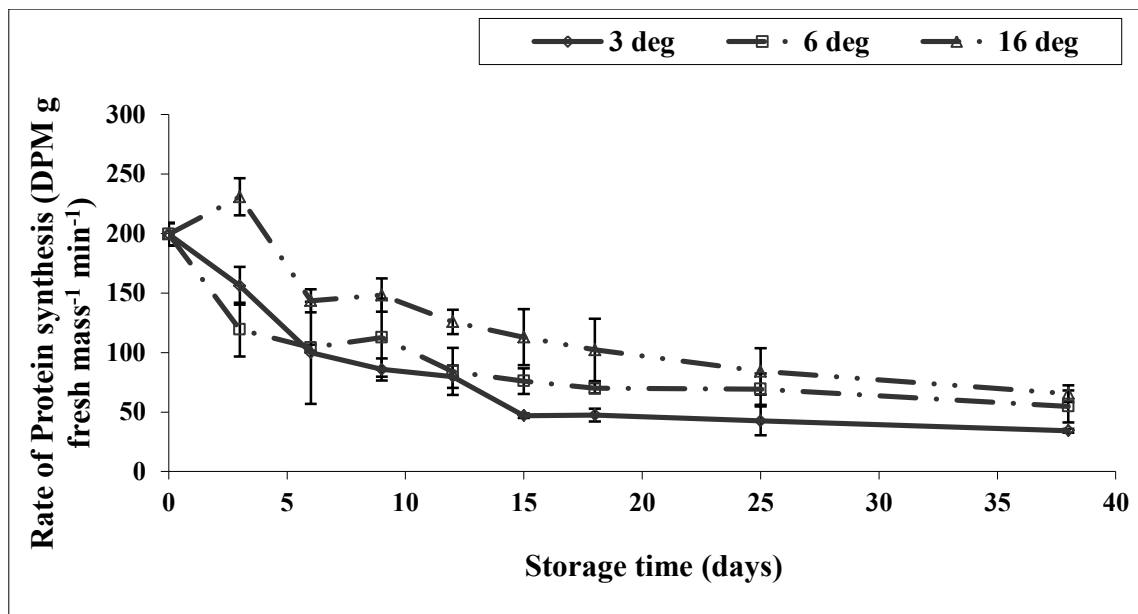


Figure 3.11 Rate of protein synthesis measured periodically over 38 d for axes excised from seeds of *E. capensis* from St Lucia sampled at intervals during storage at 3°, 6° and 16°C ($n=20$). Bars indicate standard deviation.

3.3.3 Material from Tanzania

The seeds of Tanzanian origin were stored in batches at 3°, 6° and 16°C and were sampled at 3 d intervals (Fig. 3.12). These seeds had an initial rate of protein synthesis of 207 ± 39 DPM

$\text{g}^{-1} \text{min}^{-1}$ (Fig. 3.12). After 3 d the storage reduced temperatures reduced protein synthesis to 108 ± 11 , 118 ± 14 and $150 \pm 35 \text{ DPM g}^{-1} \text{min}^{-1}$ at 3° , 6° and 16°C . The rate slowed further to $27 \pm 1 \text{ DPM g}^{-1} \text{min}^{-1}$, $39 \pm 9 \text{ DPM g}^{-1} \text{min}^{-1}$ and $48 \pm 7 \text{ DPM g}^{-1} \text{min}^{-1}$ in material stored at 3° , 6°C and 16°C , respectively, for 9 d (Fig. 3.12). A 2-way ANOVA showed that storage time and storage temperature had a significant effect on the rate of protein synthesis ($p < 0.05$).

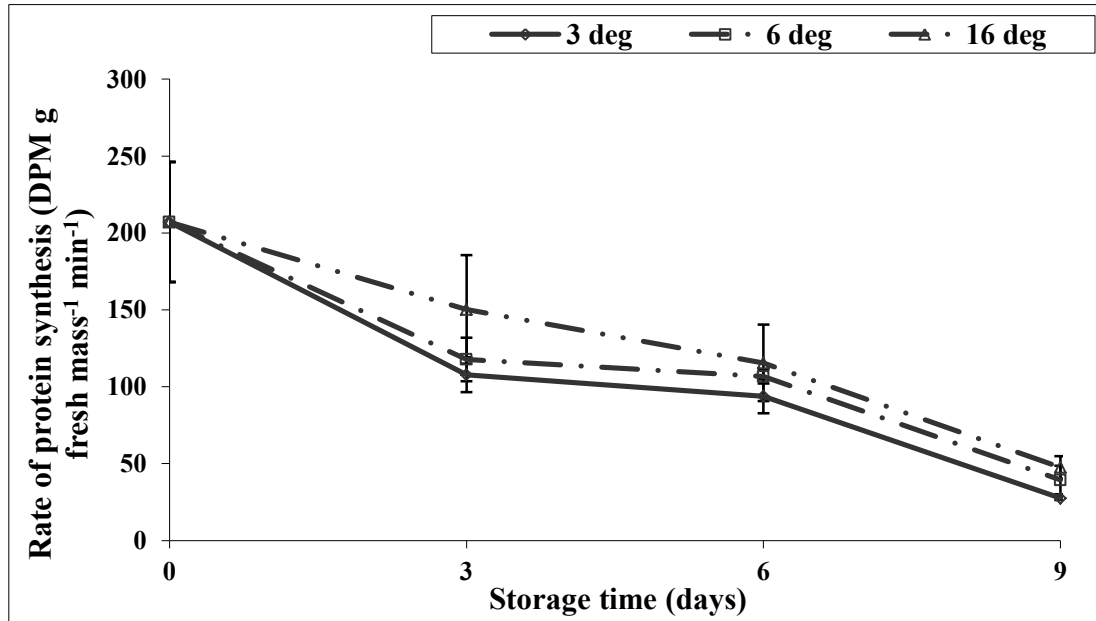


Figure 3.12 Rate of axis protein synthesis measured at 3 d intervals over a 9 d period for seeds of *E. capensis* from Tanzania stored at 3° , 6° and 16°C ($n=20$). Bars indicate standard deviation.

3.4 Electrolyte leakage

3.4.1 Material from Port Elizabeth

Electrolyte leakage is generally taken as a measure of membrane integrity (e.g. Bryant *et al.*, 2001; Campos *et al.*, 2003). An increase in solute leakage is usually indicative of a deterioration of cell membranes, particularly the plasmalemma. This has implications in maintenance of cellular structure and metabolism and thus on maintenance (or loss) of seed/axis vigour and viability. Initial leakage was measured at $58 \pm 22\%$ of total electrolytes (Fig. 3.13). Leakage from axes of the seeds that were stored at 1°C peaked at $92 \pm 18\%$ of total leakage after 2 weeks. Thereafter, leakage decreased progressively to $48 \pm 12\%$ of total

leakage by the end of 12 weeks in storage. A similar pattern was observed for the axes from seeds that were stored at 3°C, with a peak of electrolyte leakage at $75 \pm 15\%$ of total leakage after 2 weeks after which lower values were recorded (Fig. 3.13). After 12 weeks in storage, this was reduced to $46 \pm 10\%$ of total leakage. Electrolyte leakage from axes of seeds stored at 6°C peaked at $75 \pm 11\%$ of total leakage after 2 weeks in storage (Fig. 3.13) with the concentration of electrolytes in the leachate gradually reducing to $47 \pm 8\%$ of total leakage after 12 weeks.

The highest percentage leakage occurred from the seeds that were stored at 1°C. It is possible that the stress of the low temperature initially had a deleterious impact on the membranes of the axis cells. However, it seems that the axes were able to compensate for the initial damage as indicated by the reduction in electrolyte leakage. Similarly, for the other two treatments, viz. storage at 3° and 6°C, the seeds appeared to overcome the early adverse effects on the membranes of low temperature, showing a reduction in electrolyte leakage that peaked after 2 weeks in storage. A 2-way ANOVA showed that storage time and storage temperature had a significant effect on both total electrolyte leakage and the rate of electrolyte leakage ($p < 0.05$).

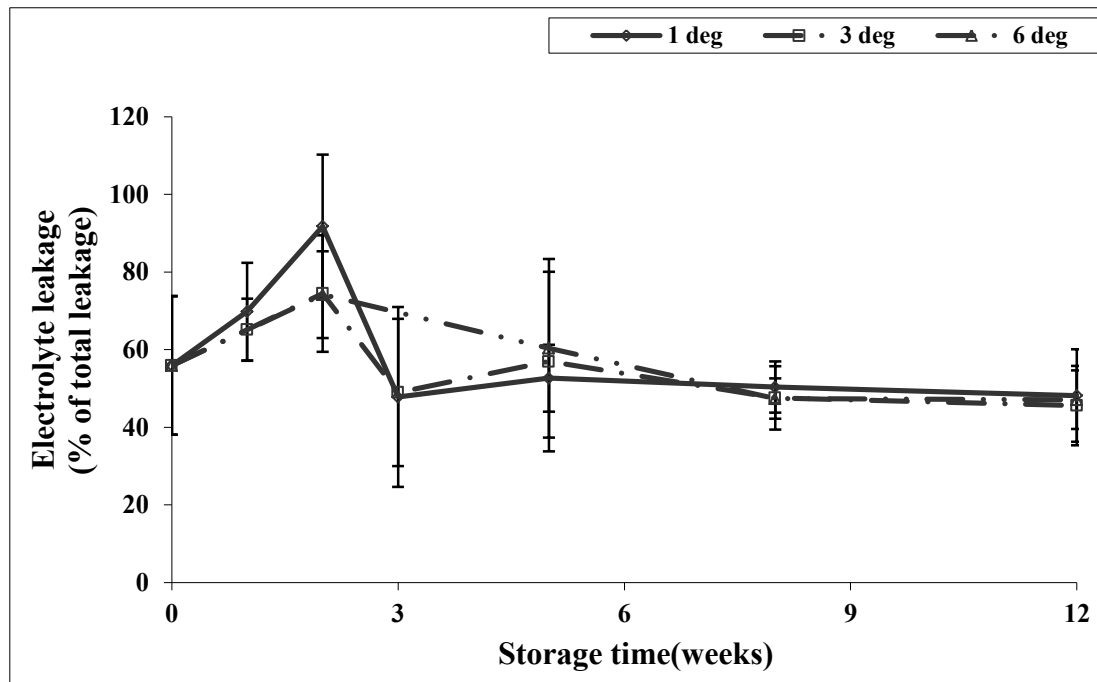


Figure 3.13 Electrolyte leakage (as a proportion of the maximum after freezing and thawing) after 24 h from axes from seeds of *E. capensis* from the PE provenance during storage at 1°, 3° and 6°C over a period of 12 weeks (n=10). Bars indicate standard deviation.

3.4.2 Material from St Lucia

An increase to $81 \pm 27\%$ of total leakage was observed after 9 d in storage at 3°C and thereafter remained relatively constant. High levels of leakage are consistent with both the decline in viability (Fig. 3.8) and obvious fungal contamination (Fig. 3.9).

Electrolyte leakage from axes of seeds stored at 6°C remained relatively constant for 25 d in storage, at approximately 58% of total leakage, implying no major structural changes or alterations of the membranes. However, a sharp increase to $81 \pm 26\%$ of total electrolyte leakage occurred between 25 and 38 d, suggesting a decline in membrane integrity in some specimens with the potential to affect overall viability; viability did show a slight decline over this period (Fig. 3.14). A similar trend was noted for axes of seeds that were stored at 16°C (Fig. 3.14). Electrolyte leakage measurable in solution remained fairly constant at approximately 58% of total leakage up to 25 d in storage. Thereafter, the leachate concentration increased sharply to $90 \pm 13\%$ total leakage after 38 d in storage.

The present results for the *E. capensis* seeds from St Lucia suggest that sustained storage at this temperature could ultimately have damaging effects. Hence, even 16°C may represent cold stress for seeds of sub-tropical provenance. Storage at 16°C and below was associated with an increase of electrolyte leakage across all treatments. However, the sharp early increase in leakage from axes of seeds stored at 3°C compared with the other two treatments may be an indicator of different levels of cold stress. A 2-way ANOVA showed that storage time and storage temperature had a significant effect on both total leakage and rate of electrolyte leakage ($p < 0.05$).

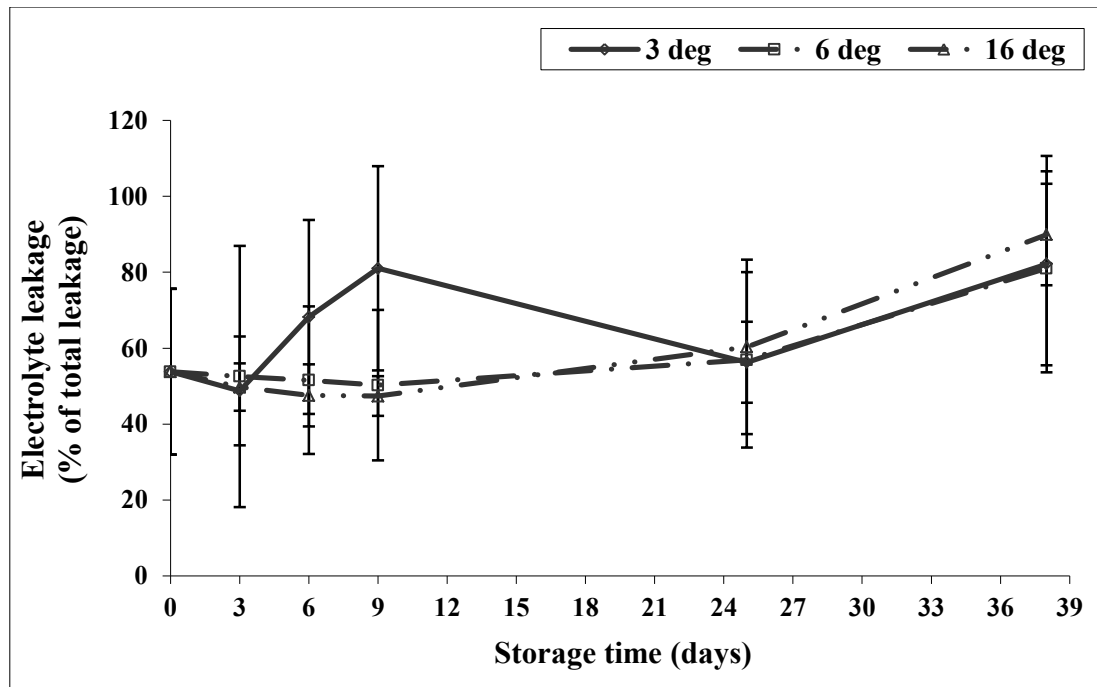


Figure 3.14 Electrolyte leakage (as a proportion of the maximum after freezing and thawing) from axes from seeds of *E. capensis* from the St Lucia provenance. The seeds were stored at 3°, 6° and 16°C for a period of 38 d (n=10). Bars indicate standard deviation.

3.4.3 Material from Tanzania

Electrolyte leakage from axes of the seeds stored at 3°C remained essentially the same as the initial value at $40 \pm 18\%$ of total leakage after 3 d. However, thereafter there was a sharp rise to $89 \pm 10\%$ of total leakage (Fig. 3.15). A similar trend was observed for axes from the seeds that were stored at 6° and 16°C. A similar pattern of electrolyte leakage was noted across all three treatments, viz. an initial lag followed by sharp increase to a plateau. At this stage, membrane integrity had been lost completely allowing the loss of virtually all the solutes from the axis cells. The amount of leakage increased rapidly to far higher levels compared with the PE and St Lucia batches before falling. A 2-way ANOVA showed that storage time and storage temperature had a significant effect on both total leakage and the rate of electrolyte leakage ($p < 0.05$).

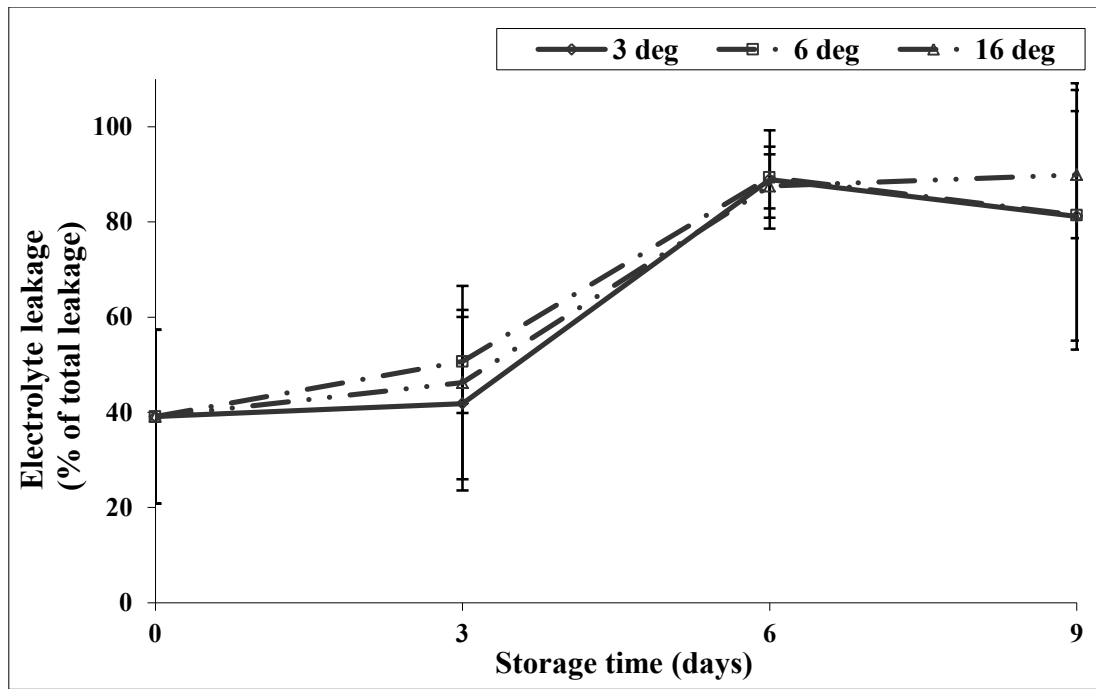


Figure 3.15 Electrolyte leakage (as a proportion of the maximum after freezing and thawing) from axes from seeds of *E. capensis* from the Tanzanian provenance. The seeds were stored at 3°, 6° and 16°C for a period of 9 d (n=10). Bars indicate standard deviation.

3.5 Drying

3.5.1 Material from Port Elizabeth

It was necessary to determine the response to dehydration of *E. capensis* seeds from the different provenances, when a slow drying technique was employed. To achieve this, seeds were left enclosed by the endocarp (Pammenter *et al.* 1998). Those authors showed that *E. capensis* seeds would lose water relatively slowly if the endocarp is left intact.

The whole seed water content decreased from an initial $0.63 \pm 0.09 \text{ g g}^{-1}$ to $0.58 \pm 0.004 \text{ g g}^{-1}$ within 1 h of slow drying, corresponding to 5% decrease in mass. This relatively rapid initial decrease in water content was accompanied by a reduction in viability from 100% (fresh seeds) to 80% (Fig. 3.16).

From this point, the seeds continued to lose water but at a much slower rate as indicated in Fig. 3.16. A 15% decrease in mass occurred after 30 d with a corresponding mean whole seed water content of $0.030 \pm 0.003 \text{ g g}^{-1}$. Only 40% of the seeds had retained viability at this

juncture. The water content remained constant at $0.03 \pm 0.002 \text{ g g}^{-1}$, while viability decreased to 5%.

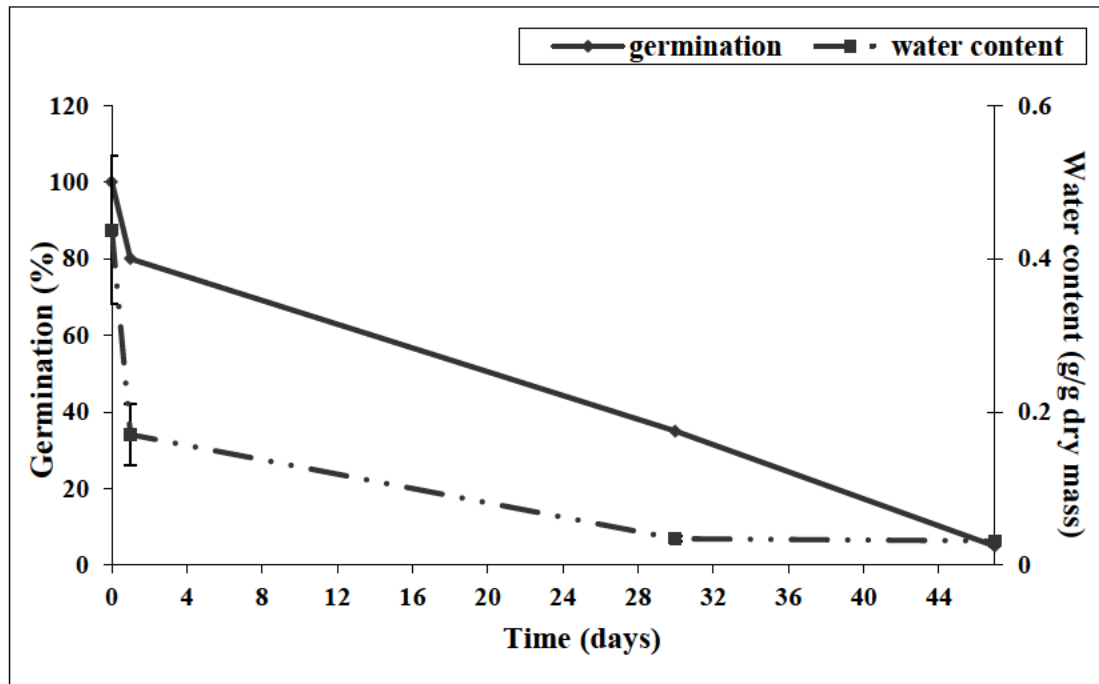


Figure 3.16 Viability and corresponding whole seed water content for seeds of *E. capensis* from the PE provenance (n=5 for water content; n=20 for viability). Bars indicate standard deviation.

3.5.2 Material from St Lucia

Initial water content and viability were $0.68 \pm 0.08 \text{ g g}^{-1}$ and 100% respectively (Fig. 3.17). Within 8 h, seed water content decreased to $0.47 \pm 0.01 \text{ g g}^{-1}$. Despite this initial relatively rapid decrease in water content, viability was maintained at 90%. Water content then decreased more slowly, and with a 15% fresh mass being lost after 78 h. The water content decreased from $0.47 \pm 0.01 \text{ g g}^{-1}$ to $0.32 \pm 0.05 \text{ g g}^{-1}$. During drying, the percentage of viable seeds declined progressively from 90% to 45%. Water content continued to decrease, and after 216 h 30% loss in fresh mass occurred corresponding to a water content of $0.15 \pm 0.01 \text{ g g}^{-1}$ and a seed viability of 10%.

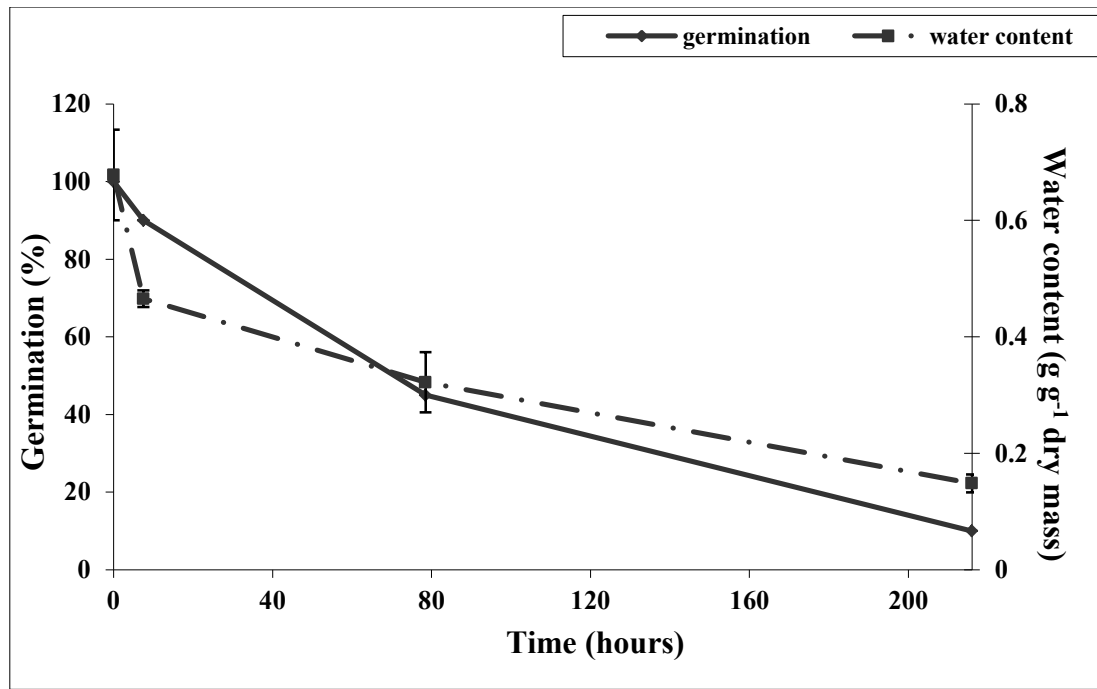


Figure 3.17 Viability curve of seeds of *E. capensis* from the St Lucia provenance. The corresponding points of water content are also represented on the graph (n=5 for water content; n=20 for viability). Bars indicate standard deviation.

3.5.3 Material from Tanzania

Initial water content and germinability were $0.57 \pm 0.08 \text{ g g}^{-1}$ and 90% respectively (Fig. 3.18). A 5% mass decrease was achieved in 3.5 h. Water content decreased to $0.46 \pm 0.09 \text{ g g}^{-1}$, and this relatively small decrease in water content was accompanied by rapid loss of viability (from 90% to 40%).

The mass of the seeds declined by 15% after 4.5 h, corresponding to a drop in water content from $0.46 \pm 0.09 \text{ g g}^{-1}$ to $0.38 \pm 0.07 \text{ g g}^{-1}$ and a reduction in viability from 40% to 15%. Both water content and viability then changed rapidly (Fig 3.18). After 21 h, the mass dropped by 30% and the water content was reduced to $0.06 \pm 0.01 \text{ g g}^{-1}$; none of the seeds remained viable (Fig 3.18). It appears that the seeds from the Tanzanian provenance are most susceptible to dehydration and the seeds from PE are least so. However, interpreting this result is confounded by the fact that for no obvious reason seeds from PE dried much slower than those from St Lucia and Tanzania. The target 30% mass decrease was achieved in only 21 h for the Tanzanian seeds, while it took 47 d (1128 h) to achieve the same target mass

decrease in the seeds from PE. In comparison, it took 216 h to reach the 30% mass decrease in the seeds from St Lucia. Taken together, results indicated that seeds from PE are least recalcitrant and those from tropical Tanzania are most recalcitrant with seeds from St Lucia showing intermediate characteristics. Susceptibility to dehydration was estimated between the seeds of *E. capensis* from the three provenances. Fig. 3.16 shows that the seeds from PE provenance were most tolerant to dehydration maintaining 80% viability at a water content of $\pm 0.2 \text{ g g}^{-1}$, while those of St Lucia and Tanzanian provenance required their water contents to be maintained at ± 0.5 and $\pm 0.9 \text{ g g}^{-1}$ respectively to retain 80% viability. To retain 60% viability, water contents of ± 0.16 , ± 0.5 and $\pm 0.65 \text{ g g}^{-1}$ were necessary for the seeds from PE, St Lucia and Tanzania respectively.

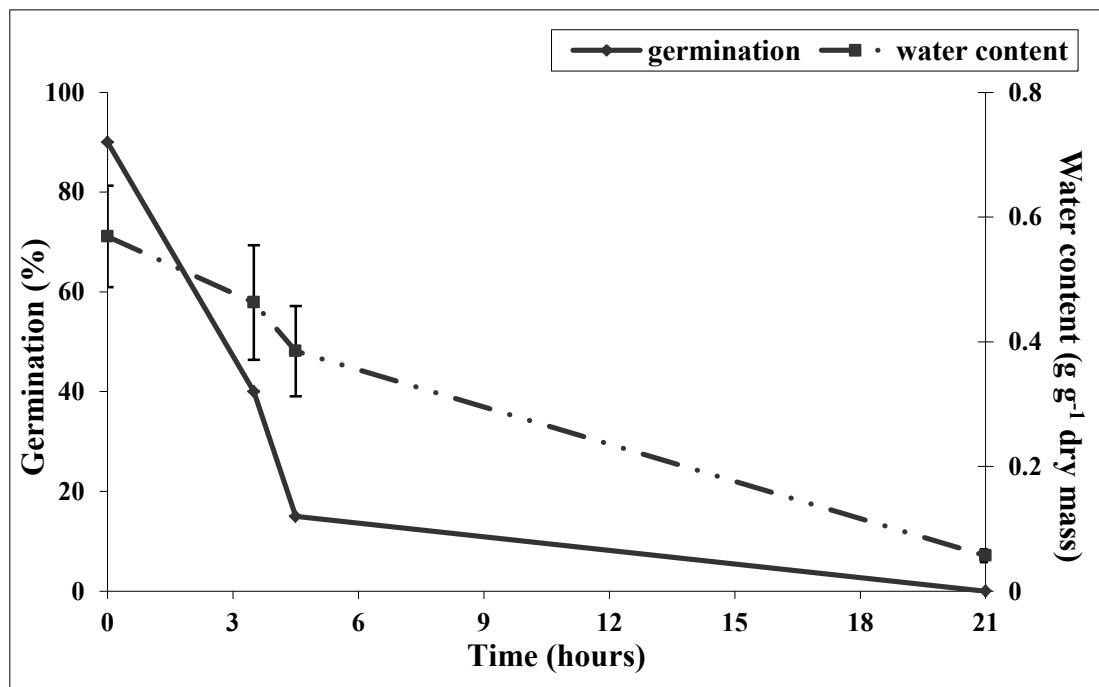


Figure 3.18 Viability curve of seeds of *E. capensis* from the Tanzania provenance. The corresponding points of water content are also represented on the graph (n=5 for water content; n=20 for viability). Bars indicate standard deviation.

3.6 Phylogeny

These analyses were carried out in collaboration with Ms Terisha Naidoo, who acquired and sequenced the South African material. The material included several sources around Port Elizabeth, Knysna, George and Betty's Bay in the Cape (called the Cape population here) as

well as from the Durban to Mtunzini region, the latter being in the proximity of St Lucia (ref. Fig. 1.2), which constituted the KZN population. Material from the same population as the seeds was also sourced and analysed by the present author.

Analysis of the ITS sequences revealed some striking variations within *E. capensis*. The neighbour joining tree (Fig. 3.19) showed that samples located in different geographical regions (Cape, KZN and Tanzania) formed distinct monophyletic clades with moderate to high levels of bootstrap support (72, 100 and 87%, respectively). Further, the Tanzanian and KZN samples form a strongly-supported monophyletic clade (93%) which is sister to the moderately-supported Cape clade (87%).

Genetic distance analysis showed that the Tanzanian population was more similar to the KZN (3.1%) than the Cape (4.6%) population (Table 3.10). The greatest genetic distance within *Ekebergia* was between the geographically-intermediate KZN population and the more southerly Cape population (6.0%). Distances between the three *Ekebergia* groups and the outgroup, *Melia azedarach*, a different genus within the Meliaceae family, ranged from 28.3% to 31.7%. Although these data show a distinct geographically-based separation of populations, it must be noted that only two Tanzanian samples (from the same region) are represented.

Table 3.10 Net between-groups genetics distances. Groups are defined in Figure 3.19. Lower left matrix; units=substitutions per site. Upper right matrix; units=percent.

Group	1	2	3	4
1	-	3.1	4.6	31.7
2	0.031	-	6.0	30.8
3	0.046	0.060	-	28.3
4	0.317	0.308	0.283	-

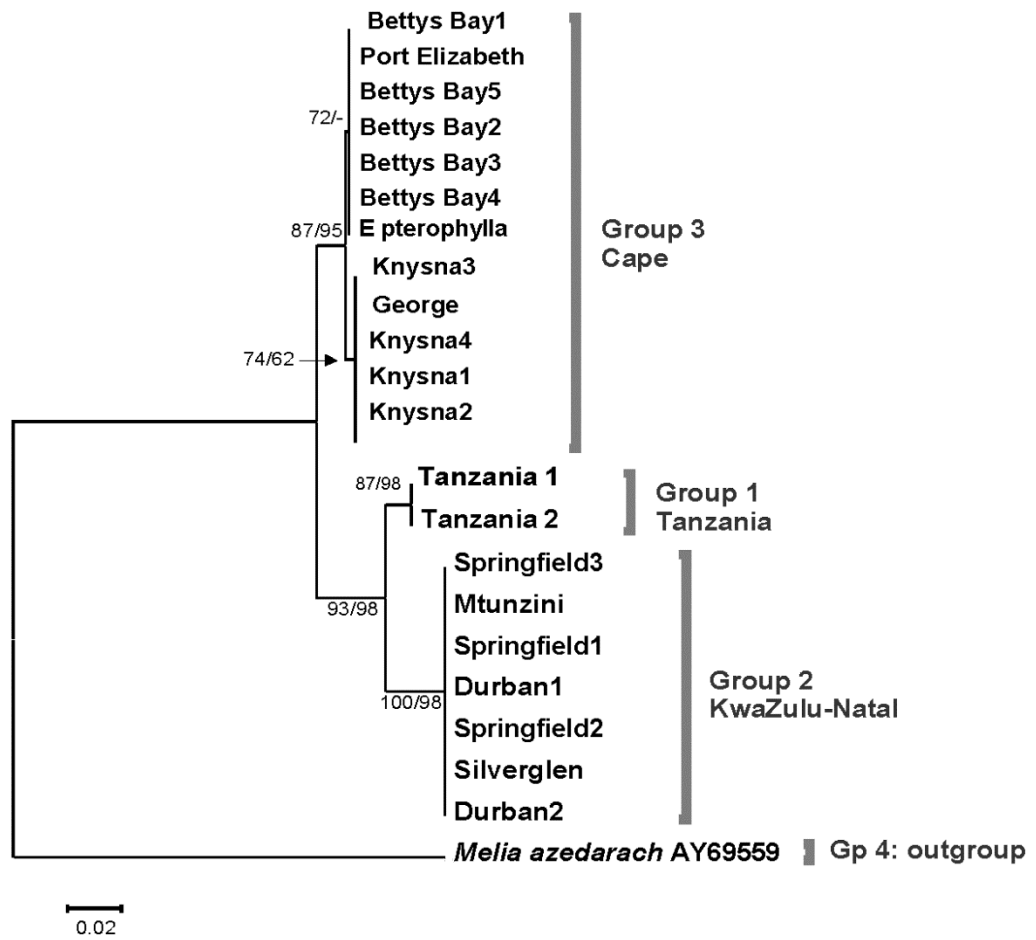


Figure 3.19 Bootstrap values (BV) from congruent maximum parsimony analyses are provided. Nodal support is indicated as (neighbour-joining BV (%) / maximum parsimony BV (%)).

3.7 Ultrastructure

3.7.1 Axes of seeds from Port Elizabeth

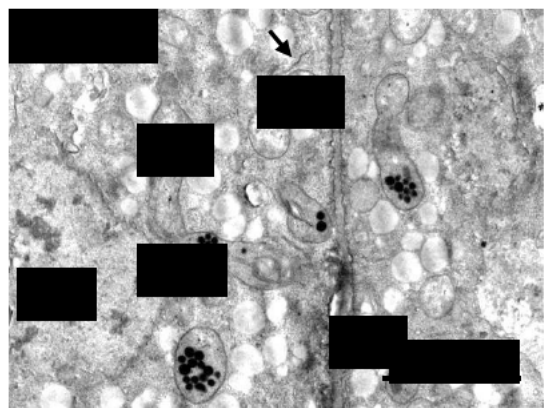
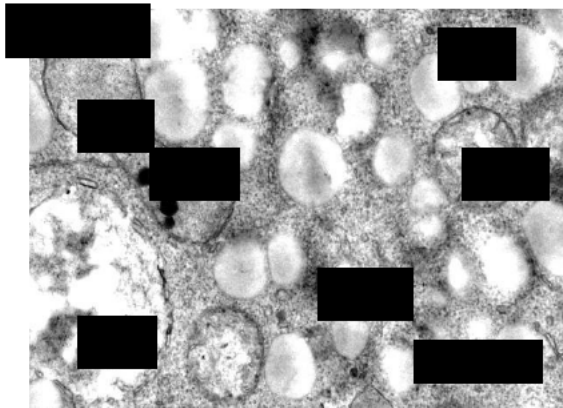
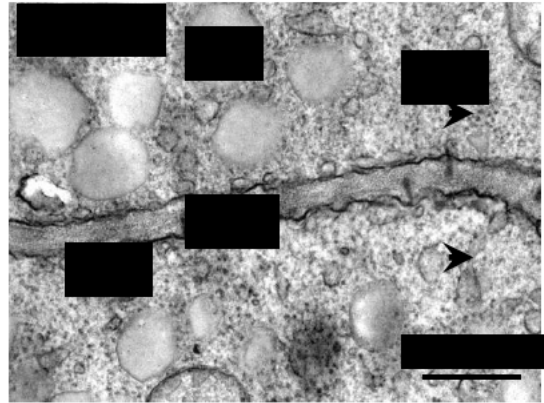
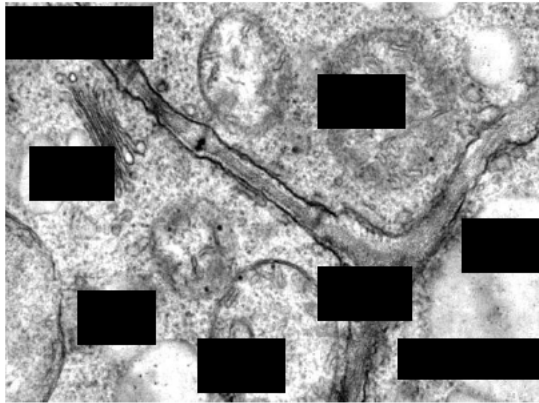
3.7.1.1 Fresh seeds

Transmission electron microscopical (TEM) investigation of the root meristematic region (meristem and closely associated cells) of axes from newly-harvested seeds revealed an ultrastructure characterised by cells with well-defined walls, membranes (Fig. 3.20a) and nuclei (Fig. 3.20d). There was evidence of vacuole formation, taken to indicate the ontogeny of lytic compartmentation in these meristematic cells (Fig. 3.20c) (Lamb and Berjak, 1981). Mitochondria (Figs. 3.20a, c & d) showing short, but well-defined cristae were clearly resolved. The plastids generally lacked inner membrane (thylakoid) development, but contained prominent plastoglobuli (Figs. 3.20c & d). The latter have been suggested to represent pools of membrane lipid within plastids, with their occurrence being inversely proportional to inner membrane development (Borisjuk *et al.*, 2005).

The occurrence of numerous cytoplasmic polysomes (examples are indicated by arrow heads in Fig. 3.20b) is evidence of active protein synthesis in the axes of fresh seeds, implying metabolically active tissue. The presence of Golgi bodies (Fig. 3.20a) supports this observation (Farrant *et al.*, 1997). Surprisingly though, only occasional, short profiles of endoplasmic reticulum were encountered (Fig. 3.20c), although this may have been because of targeting of specialised ER derivatives to vacuolation (Fig. 3.20c).

Lipid bodies were located throughout the cytomatrix (Figs. 3.20b, c & d), presumably representing a primary nutrient reserve utilised by the axes during the early stages of germination. This contention is supported by the absence of plastidial starch in axis cells of newly harvested seeds (Figs. 3.20c & d). A feature of the cells was the occurrence of many small vesicles, particularly in the vicinity of the plasmalemma (Fig. 3.20b). It is clear from the ultrastructural observations that fresh *E. capensis* seeds of PE provenance were metabolically active.

Figures 3.20a – d Ultrastructure of axes from fresh seeds of PE provenance. Fig. 3.20a shows well developed mitochondria (M) and a Golgi body (Gb). Cell walls (cw) traversed by plasmodesmata separate adjacent cells. Fig. 3.20b illustrates a collection of the many lipid bodies (L) and vesicles (ve), particularly in the vicinity of the plasmalemma present within the cytomatrix. Numerous polysomes (examples indicated by arrow heads) indicative of cytoplasmic protein synthesis are evident. Fig. 3.20c illustrates the typical appearance of plastids (P) with sparse inner membrane formations; nascent vacuole (V) formation was evident, and only occasional profiles of endoplasmic reticulum (ER) were seen. Fig. 3.20d illustrates a nucleus (N) with small patches of heterochromatin; plastids (P) containing prominent plastoglobuli (pg) and numerous lipid bodies. However, profiles of ER (arrow) appeared to be implicated in vacuole (V) formation.

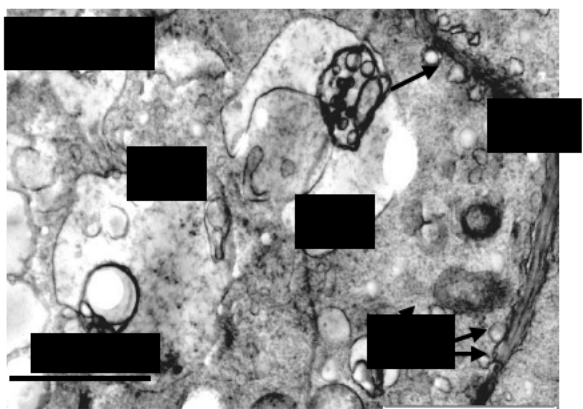
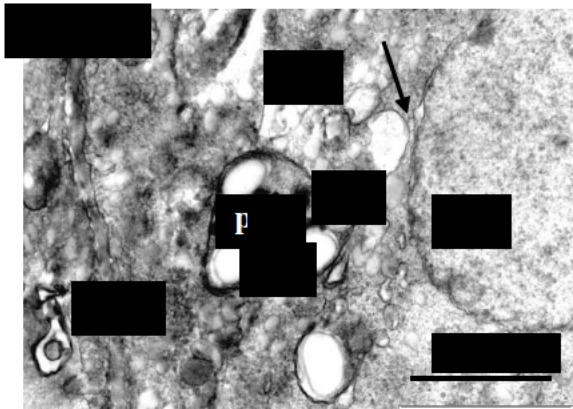
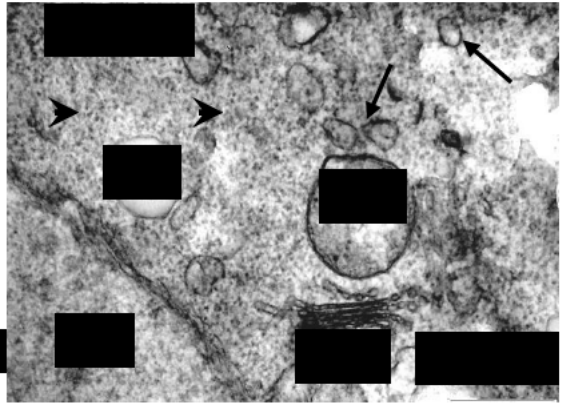
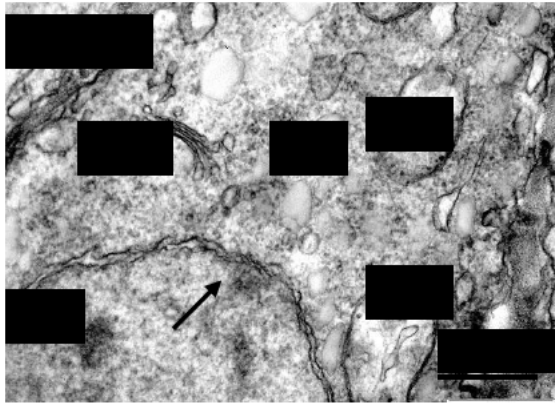


3.7.1.2 Seeds from PE stored at 1°C

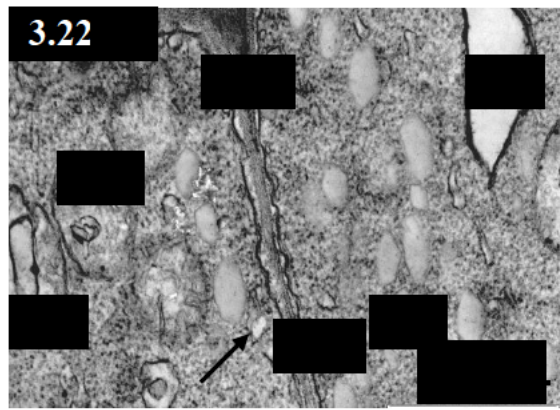
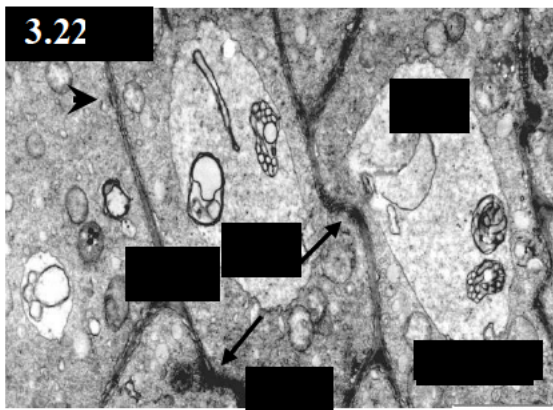
The seeds that were stored for 2 weeks at 1°C did not show any deterioration in their ultrastructure. There were numerous relatively well-developed mitochondria (Figs. 3.21a & b) suggesting active energy metabolism to drive the progression towards germination. Nuclei (Figs. 3.21a & c) were well developed and cell walls (Figs. 3.21c & d) were regular. Starch grains (Fig. 3.21c) were present in plastids apparently having been formed during low temperature storage. Golgi bodies (Figs. 3.21a & b) were among the organelles that were identified within the cytomatrix. Polysomes (indicated by arrow heads in Fig. 3.21b) imply active protein synthesis was underway. Intravacuolar membranous inclusions suggested that autophagic activity had taken place (Fig. 3.21d), which could have been metabolism or stress related.

The seeds stored for 12 weeks retained a generally organised ultrastructure. However, subtle changes were noted in the ultrastructure of the representative sample of the 80% which were presumed to have remained viable after 1°C storage (this assumption is based on other representative specimens showing a degree of intracellular disorder in keeping with viability loss). After 12 weeks storage at 1°C, localised wall deformation (buckling) was apparent in some cells (Fig. 3.22a). Mitochondrial matrices were dense, and cristae could be discerned (Figs. 3.22b & c). Plastids (Fig. 3.22c) were noted in the cytoplasm; however, these showed a lack of plastoglobuli and little evidence of starch compared with those of the 2-week stored material. Lipid bodies (Fig. 3.22b) appeared diminished in size compared with those of fresh material (Fig. 3.22b, cf. Fig. 3.20c) implying utilisation of storage reserves. Golgi bodies were not evident. The most striking change, however, was the increased degree of vacuolation and associated autophagy, already occurring after 2 weeks storage and prominent after the seeds had been stored for 12 weeks at 1°C (Figs. 3.21a; 3.22a & c). When the seeds were stored at 1°C, some ultrastructural differences and abnormalities were evident compared with the fresh condition. However, the general degree of organisation indicates that metabolic potential was likely to have been retained.

Figures 3.21a – d Root apex ultrastructure after PE seeds had been stored at 1°C for 2 weeks. Fig. 3.21a shows mitochondria (M) which have retained internal structure and a Golgi body (Gb). Lipid bodies (L) are scattered within the cytomatrix. Nuclear morphology can be seen to appear unchanged (Fig. 3.21a) with visible regions of heterochromatin (arrow). The double membrane of the nuclear envelope is clearly visible. Fig. 3.21b shows a Golgi body and several vesicles (indicated by arrows) which could be ER derived. Examples of polysomes are indicated by arrow heads. Fig. 3.21c shows a well developed plastid (P) with plastoglobuli (pg) and starch (S). The nucleus (N) illustrated has retained a normal profile, although slight irregularities of the envelope are apparent (arrow). Fig. 3.21d shows vacuoles (V) with membranous inclusions and peripheral vesicles (ve), indicated by arrows.



Figures 3.22a – c Ultrastructural situation after 12 weeks storage of PE seeds at 1°C. Fig. 3.22a shows large, well developed vacuoles (V) with numerous membranous inclusions. Cell walls (cw) were generally well developed although showing a localised tendency to buckle (arrows). Vesicles are associated with the cell wall (indicated by arrow head). Fig. 3.22b shows well developed, cell walls (cw) and the typical undulating appearance of the plasma membrane. Mitochondria (M) appear large with clearly resolved cristae. Lipid bodies (L) are scattered throughout the cytomatrix. Vesicles (indicated by arrow) appear to be associated with the plasma membrane. Probable vacuole (V) ontogeny via ER dilation is evident. Fig. 3.22c illustrates a large, well developed vacuole (V) with numerous membranous inclusions. Mitochondrial (M) elongation can be seen while vesicle activity is evident in the vicinity of the plasma membrane (indicated by arrows). An elongated plastid (P) is clearly visible.

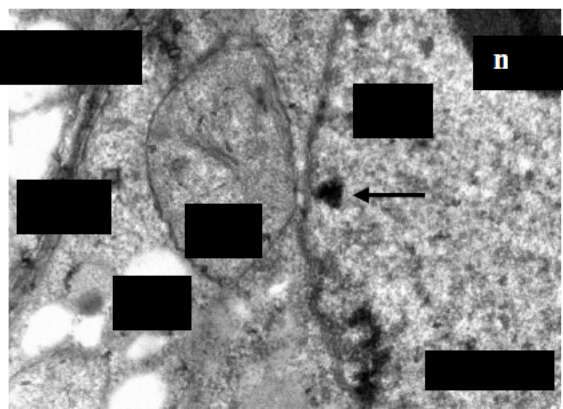
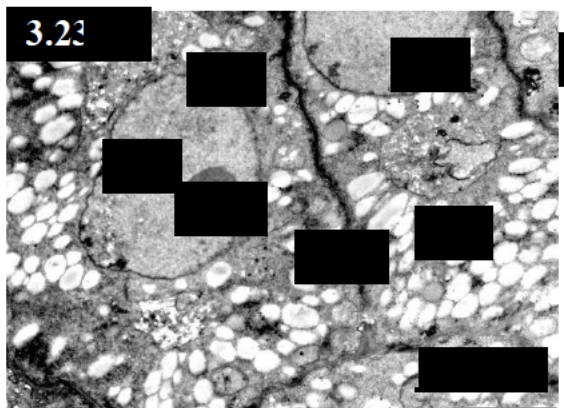
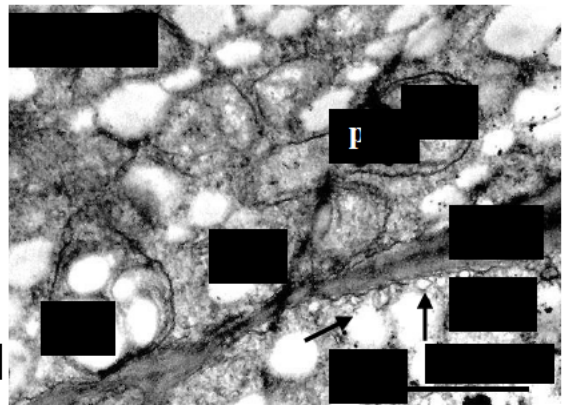
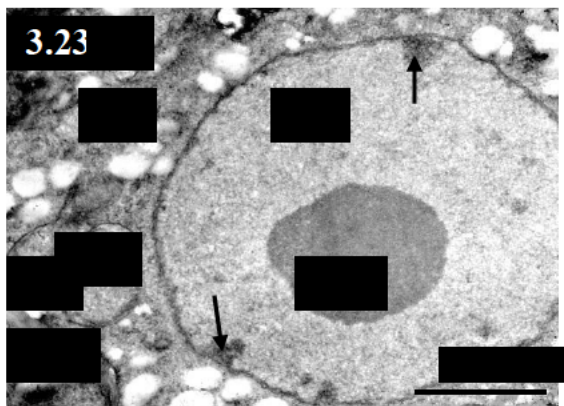


3.7.1.3 Seeds from PE stored at 3°C

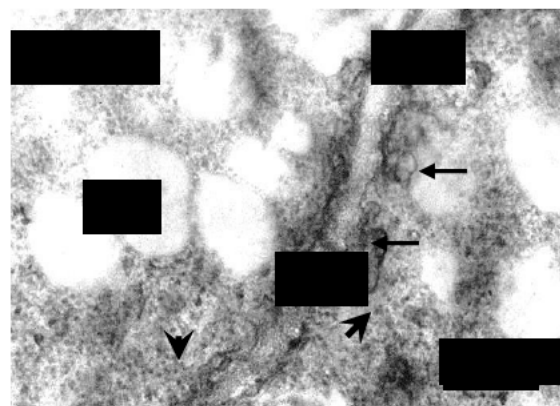
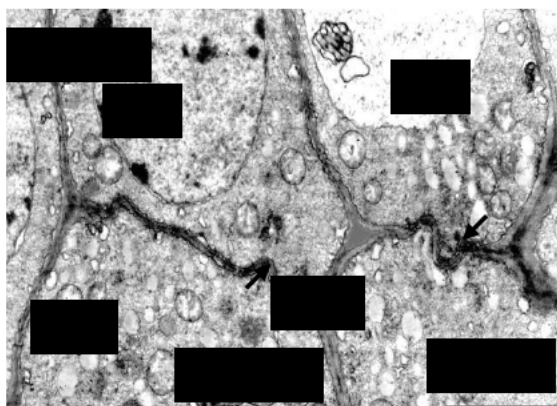
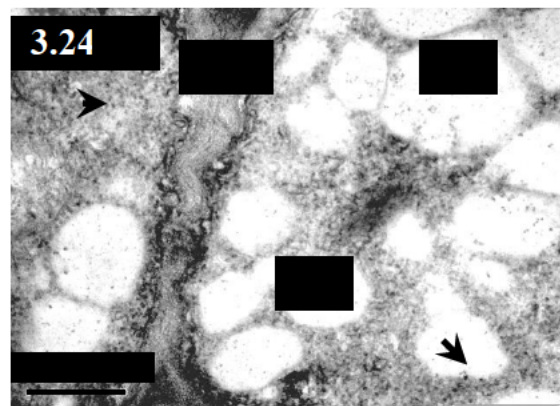
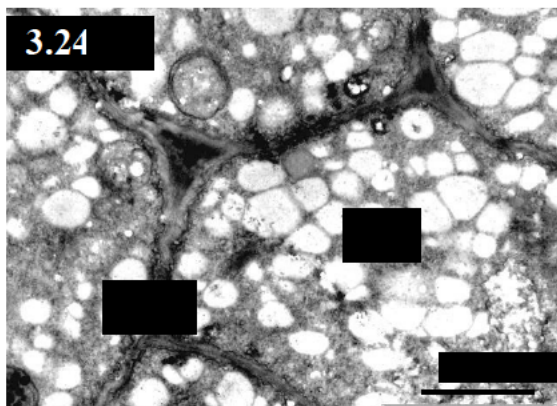
Seeds stored for 2 weeks at 3°C showed well defined organelles in the cells of the meristematic region. Nuclei and nucleoli (Figs. 3.23a & c) were clearly visible with patches of heterochromatin in the matrix and closely associated with the inner membrane of the nuclear envelope (indicated by arrow in Fig. 3.23a). Mitochondria (Fig. 3.23a) and prominent plastids (Figs. 3.23a, b & d), many with visible starch grains and plastoglobuli, and some internal membrane development were distributed throughout the cytoplasm. Lipid bodies (Figs. 3.23a, b, c & d) were located throughout the cytomatrix. There was evidence of *de novo* vacuole formation, as illustrated in Fig. 3.23c. Cell walls showed no apparent ultrastructural abnormality (Figs. 3.23a, b & d). The ultrastructure described below typified the 80% of *E. capensis* seeds from PE which could germinate after storage for 12 weeks.

Many lipid bodies were present, particularly peripherally in the cytomatrix (Figs. 3.24a, b & d) in the seeds stored at 3°C for 12 weeks. The meristem cells had nuclei with clearly delineated heterochromatin (Fig. 3.24c). Numerous mitochondria (Fig. 3.24c) were located throughout the cytomatrix, and plastids containing a few plastoglobuli but little starch were visible (Fig. 3.24c). Each section contained one or two vacuoles (Fig. 3.24c), that comprised cellular material (vacuolar inclusions) indicating either their ontogeny via cytolysosome formation (ref. Fig. 3.23c) and/or a measure of autolytic activity having occurred during seed storage. Vesicular formations (indicated by arrows in Fig. 3.24d) were associated with the plasma membrane, suggesting endocytic (or, less likely, exocytic) activity. The presence of many polysomes (examples indicated by arrow heads in Figs. 3.24b & d) implies ongoing protein synthesis in axes during seed storage despite the low temperature of 3°C. The axis root meristem and contiguous cells generally appeared to have retained ultrastructural integrity throughout the 12 week storage period. The only feature observed which could have indicated that prolonged storage at 3°C was stressful was a tendency for cell wall buckling in places (arrowed in Fig. 3.24c).

Figures 3.23a – d Root apical cell ultrastructure of axes from PE seeds that had been stored for 2 weeks at 3°C. Fig. 3.23a shows a prominent, well developed nucleus (N) and nucleolus (nu) with peripheral patches of heterochromatin (indicated by arrows), mitochondria (M) and lipid bodies (L). Fig. 3.23b illustrates two well developed plastids (P) one showing plastoglobuli (pg) and the other starch (S). Vesicles (ve) associated with the plasma membrane are indicated by arrows. Fig. 3.23c illustrates two nuclei (N), one showing a prominent nucleolus (nu), and numerous lipid bodies (L). Nascent vacuole formation (V) was apparent. Fig. 3.23d shows the cell wall (cw), portion of the nucleus with peripherally located heterochromatin (indicated by arrow) and a plastid (P) with some inner membrane development.



Figures 3.24 a – d Ultrastructure of root apex cells after 12 weeks storage of PE seeds at 3°C. Fig. 3.24a shows contiguous cells separated by regular walls (cw); numerous lipid bodies (L) are apparent in the cytomatrix. Fig. 3.24b shows lipid bodies distributed in the cytomatrix in which polysomes are abundant (arrow heads). Fig. 3.24c illustrates cells which contained large vacuoles (V) which, in turn contained membranous and granular inclusions. Numerous mitochondria (M) were noted within these cells. Plastids (P) contained plastoglobuli (pg), but apparently little starch occurred in these cells. The nuclei tended to be oval and showed patches of heterochromatin. There was, however, localised, deformation of wall structure (arrows). Fig. 3.24d shows large, opaque lipid bodies. Vesicles (indicated by arrows) occurred in association with the plasmalemma. Plasmodesmata (pd) were clearly visible traversing the cell wall (cw).



3.7.1.4 Seeds from PE stored at 6°C

Storage of seeds for 2 weeks at 6°C did not result in any adverse changes in the axis ultrastructure. The plastoglobuli-containing plastids appeared unchanged in lacking in internal membrane formations (Fig. 3.25a). Mitochondrial matrices were denser than in axes from newly-shed seeds (Figs. 3.25b & c) and. Lipid bodies (Figs. 3.26a, b&c) were distributed throughout the cytomatrix. Apparent vacuole formation was observed (Fig. 3.25c).

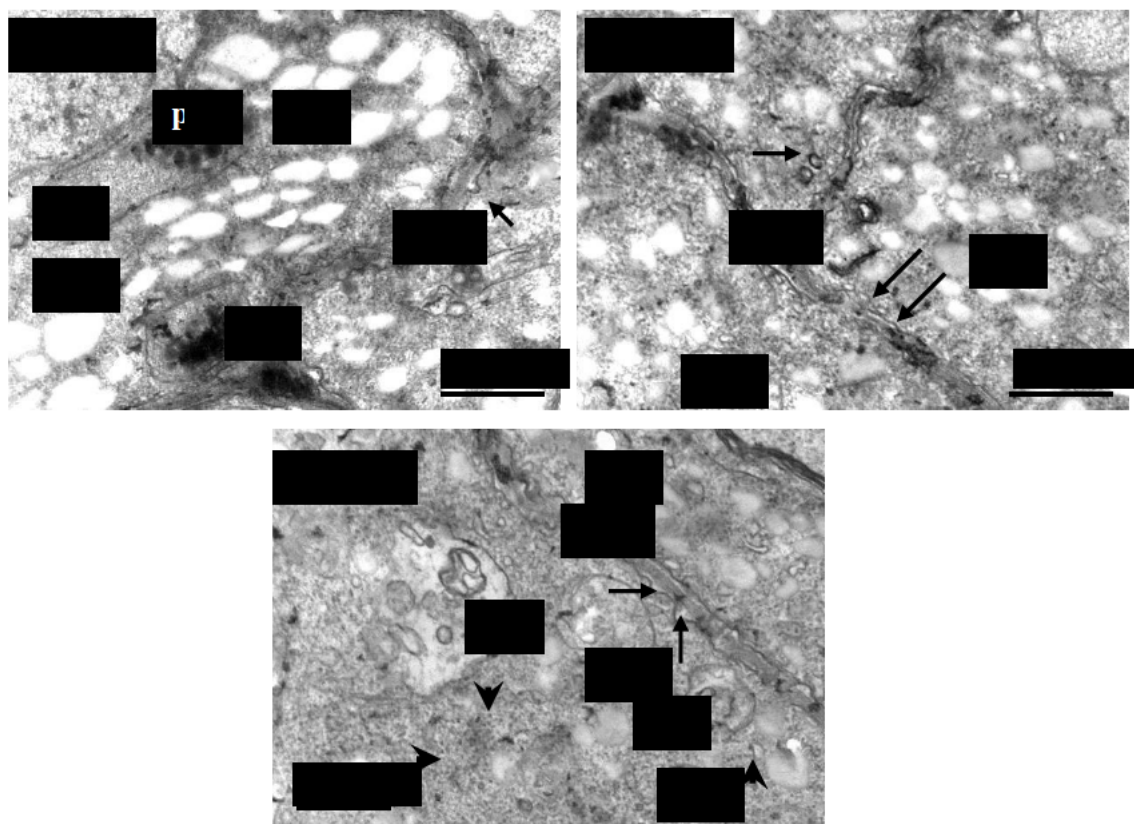
Vesiculation was apparent in close association or continuous with the plasma membrane (indicated by arrows in Figs. 3.25a, b & c). It is uncertain whether these are endocytic or exocytic vesicles. However, as the organelles associated with vesicle formation *viz.* the ER and Golgi bodies were not prominent, it is more probable that the vesicles observed were endocytotic.

The axis ultrastructure after seed storage at 6°C for 12 weeks indicated markedly increased metabolic activity (compared with the 1° and 3°C treatment after 12 weeks, and the intracellular status after 2 weeks storage at 6°C). The two membranes comprising the nuclear envelope were well defined and parallel with each other (Fig. 3.26a). Vesicles (Figs. 3.26a, b & c), some of which were clearly associated with well-developed Golgi bodies (Fig. 3.26b) were prominent in the cytoplasm. The close association of vesicles with the plasma membrane was a consistent feature. Considered in conjunction with the occurrence of Golgi bodies, it is likely that exocytosis was occurring, in addition to endocytosis, as suggested above. Most mitochondria had differentiated showing clearly resolved cristae (Fig. 3.26b). Plastids (Fig. 3.26b) showed some inner membrane elaborations and fewer plastoglobuli (Fig. 3.26b) while there was also evidence of starch deposition (Fig. 3.26c). Well defined plasmodesmata (Fig. 3.26a) were clearly visible traversing the wall (in cells peripheral to the meristem). Most vacuoles contained material of cytomatrix origin, suggesting autophagy and turnover (Figs. 3.26a, b & c).

Cytomatrix polysomes were abundant (Figs. 3.26a & b). Parallel ranks of ER were not seen, but single cisternae were encountered in close juxtaposition to Golgi bodies (Fig. 3.26b). The seeds stored at 6°C had undergone further development progressing toward germination, as indicated by the state of activity revealed by the ultrastructure after 12 weeks in storage. Generally, retention of good ultrastructural integrity throughout the storage treatment is

consistent with the ability of *E. capensis* seeds of PE provenance to tolerate chilling temperatures (Table 3.11).

Figures 3.25a – c Ultrastructure of axis apex cells after 2 weeks storage at 6°C of seeds from PE. Fig. 3.25a shows plastids (P) with plastoglobuli (pg), but no evidence of starch accumulation. Vesicles (indicated by arrows here, and in Figs. 3.25 b&c) were associated with the plasmalemma. Numerous lipid bodies (L) were present throughout the tissue. Fig. 3.25b illustrates the association of small vesicles (arrows) with the cell periphery. Note the undulating nature of the cell wall (cw). Polysomes are indicated by arrowheads. Fig. 3.25c shows what maybe a stage in vacuole ontogeny, and mitochondria (M).



Figures 3.26a – c Ultrastructure typical of axis root tips from PE seeds stored for 12 weeks at 6°C. Fig. 3.26a shows the nucleus with clear definition of the parallel membranes of the nuclear envelope. Sparse crista development was evident in occasional mitochondria (M), as illustrated. Plasmodesmata (pd) are clearly visible traversing the cell walls (cw). Tubular vesicular structures (arrowed) suggest that active membrane turnover thought to be via endocytosis, was occurring. A vacuole (V) containing membranous inclusions, is illustrated, as are cytomatrical polysomes (arrow heads). Fig. 3.26b shows rER closely associated with well developed Golgi bodies (Gb). As distinct from the larger plasmalemma-associated vesiculations (arrows) which may be indicative of endocytosis, numerous small vesicles presumed to be Golgi-derived are apparent in the vicinity of, and conjectured to fuse with the plasmalemma. Plastids (P) with a degree of inner membrane elaboration and plastoglobuli (pg) and a well developed mitochondrion (M) can be seen. Arrowhead indicates a polysome formation. Fig. 3.26c illustrates lipid bodies (L). A well developed nucleus (N) with peripheral patches of heterochromatin (indicated by arrow) is also visible. Plastids (P) and mitochondria (M), the latter in close proximity to the many lipid bodies, can also be seen.

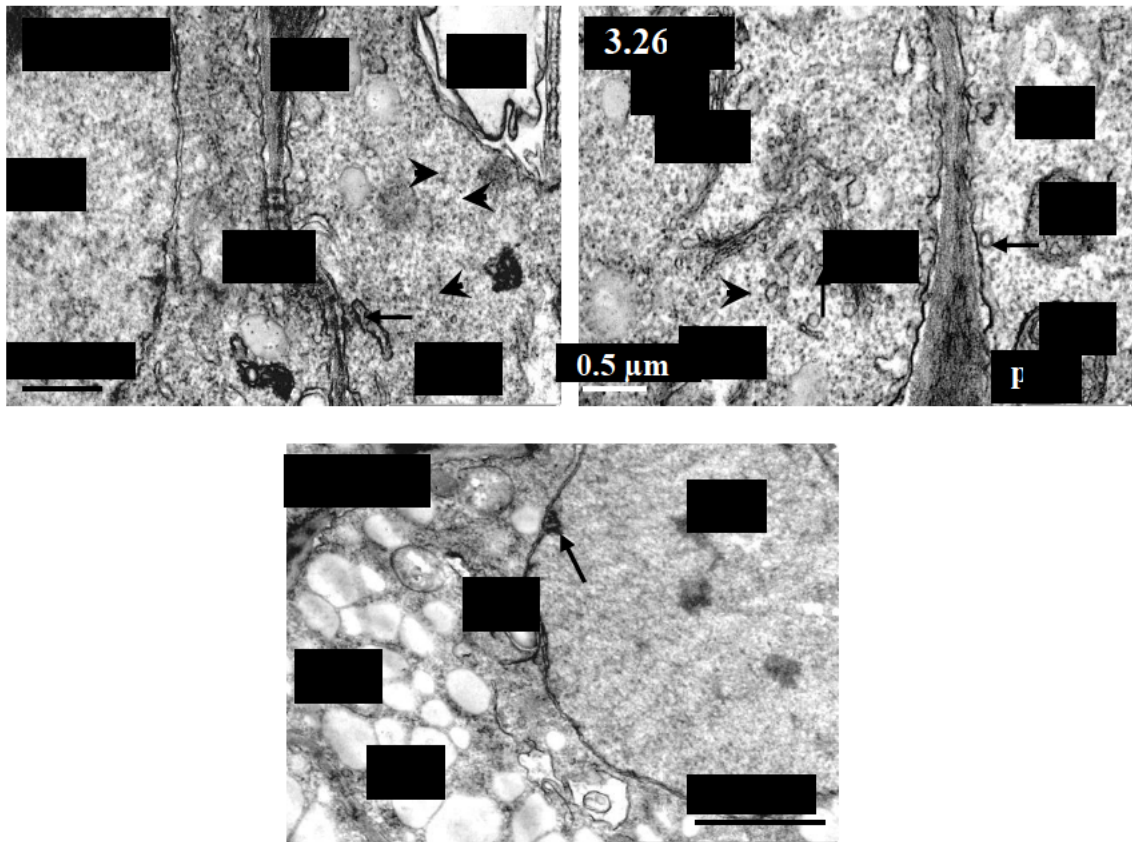


Table 3.11 The effect of temperature on root ultrastructure in seeds from Port Elizabeth (PE) provenance after hydrated storage.

Fresh	1°C	3°C	6°C
Ultrastructure had well developed nuclei, mitochondria and lipid bodies. Numerous vesicles associated with the plasmalemma. Generally, ultrastructure indicated metabolically active tissue.	After 2 weeks in storage, numerous well-developed organelles were observed indicating progression towards germination. After 12 weeks in storage, the ultrastructure was generally well maintained with subtle abnormalities.	Metabolically active tissue with well-developed organelles was observed throughout the ultrastructure after 2 weeks in storage. After 12 weeks, enhanced vesicle activity and increased vacuolar autophagic was observed. Numerous polysomes observed indicating protein synthesis.	Organelles were well-developed and metabolically active after storage for 2 weeks. Enhanced vesicle activity was noted. After 12 weeks in storage, there was a marked enhancement of organelle development. The ultrastructure was typical of highly metabolically active cells.

3.7.2 Axes of seeds from St Lucia

3.7.2.1 Fresh seeds

The evidence presented by the ultrastructure of these root meristems indicated a considerably greater degree of metabolic activity, and/or a more developed status in axes from fresh seeds of the St Lucia provenance, compared with those from PE. Relatively small vacuoles with scattered granular contents were a consistent feature of the cells (Figs. 3.27a & c). Root meristem and immediately neighbouring cells of axes from fresh seeds had oval nuclei, characterised by small patches of heterochromatin mainly located peripherally (Figs. 3.27b & e) and a dense nucleolus (Fig. 3.27e).

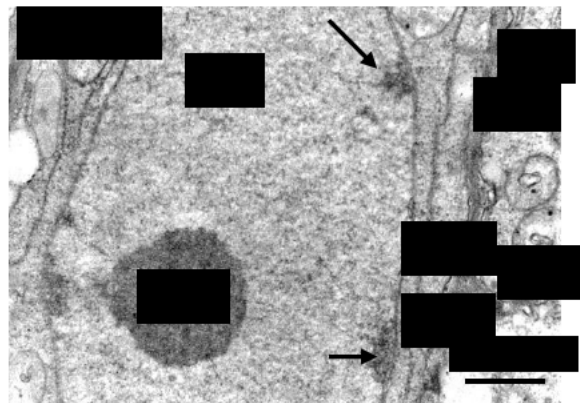
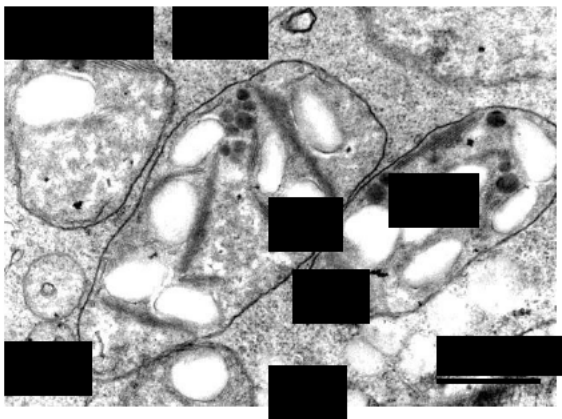
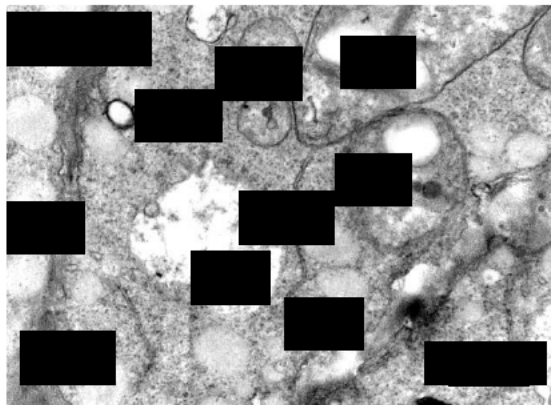
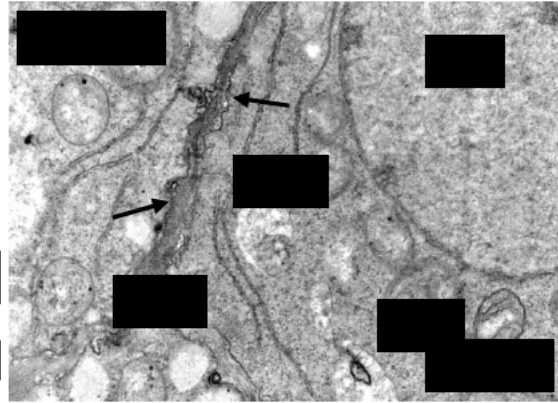
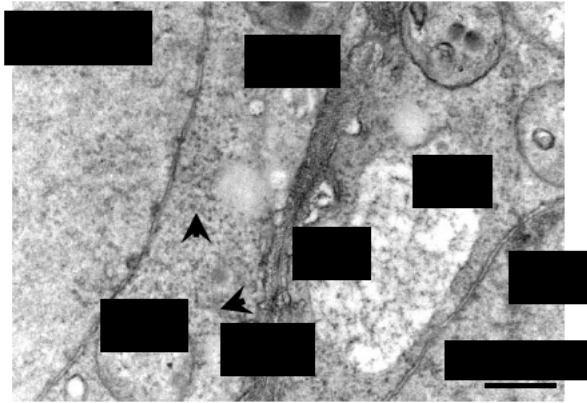
The shape of the mitochondrial profiles suggests these organelles were spherical to oval with characteristically short cristae (Figs. 3.27a & e). In some mitochondria vesicular inner membrane formations were apparent (Figs. 3.27c & d). Such formations have been relatively regularly observed in other non-stressed recalcitrant material, but their significance is not known (Berjak, pers. comm.²).

Long profiles of rough endoplasmic reticulum (rER) (Figs. 3.27b & e) with associated polysomes, as well as cytomatrical polysomes (indicated by arrow heads in Figs. 3.27c & d) indicate that active protein synthesis was occurring. Vesicles and tubular structures thought to indicate endocytosis, were frequent at the cell periphery, and in some cases could be seen to be continuous with the plasmalemma (Figs. 3.27a, b & c). However, it cannot be unequivocally concluded that these vesicles represent endocytosis, although their peripheral location in conjunction with the only sparse occurrence of Golgi bodies suggest their endocytic rather than exocytic role (Fig. 3.27a). Furthermore, the appearance of the invaginated membrane vesiculations/tubules, suggested their endocytotic origin as coated vesicles (Becker *et al.*, 2009). The tubular shape of peripheral vesiculations (illustrated in Fig. 3.27a) has been suggested by Staehelin (1997) to be caused by the turgidity of plant cells.

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The tissue had storage reserves in the form of both lipid and starch (Figs. 3.27a, c & d). Generally, lipid bodies were both peripherally located and scattered in the cytomatrix (Figs. 3.27a & c), with the starch occurring in plastids which showed clear internal membrane development and relatively few plastoglobuli (Figs. 3.27c & d).

Figures 3.27a-e Axis root apex ultrastructure in fresh material. Fig. 3.27a shows a mitochondrion (M) exhibiting short cristae. The presence of relatively small vacuoles (V) was a common feature. Note the tubular vesicle (tve) showing distinct continuity with the plasmalemma. Examples of polysomes are indicated by arrow heads. Fig. 3.27b shows long profiles of rough endoplasmic reticulum (rER) and mitochondria (M) with dense matrices and short cristae. Typical plasmalemma undulations (arrowed) suggesting endo and/or exocytosis are evident. Fig. 3.27c shows the association of vesicles (ve) with the plasmamembrane suggesting active endocytosis. Fig. 3.27d illustrates the presence of storage material in the form of starch (S) and lipid (L); note the development of the plastidial internal membranes. Fig. 3.27e shows the typical oval nuclear shape (N), the nucleolus (nu) and heterochromatin (indicated by arrow heads in Fig. 3.27e) present in close proximity to the inner membrane of the nuclear envelope.



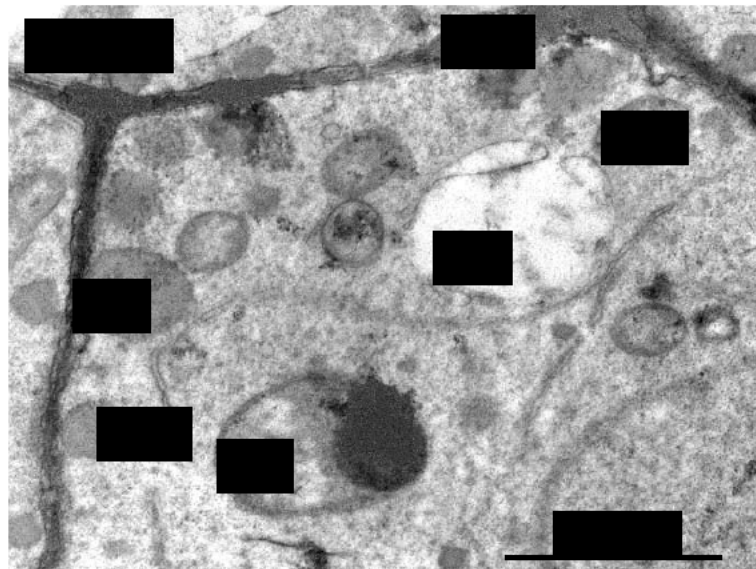
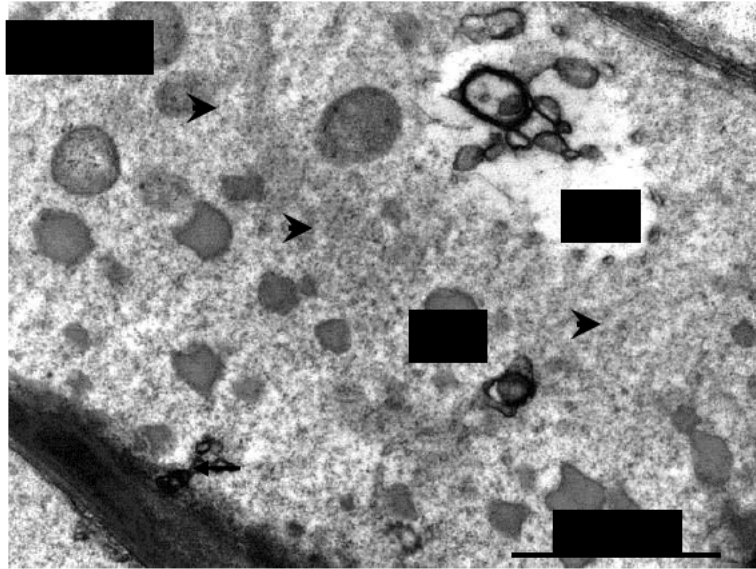
3.7.2.2 Seeds from St Lucia stored at 3°C

The axes excised from these seeds stored at 3°C showed gradual deterioration of the ultrastructure with time to 38 d. After 3 d in storage, irregularly shaped lipid bodies of increased density compared with the fresh condition were scattered throughout the cytomatrix. Vesicle association (indicated by arrows in Fig. 3.28a) with the plasmalemma was evident; however, a lack of Golgi bodies suggests that these vesicles were probably endocytotic, perhaps having formed prior to or early during storage at 3°C. Long profiles of rough endoplasmic reticulum (Fig. 3.28b) with associated polysomes (indicated by arrow heads in Fig. 3.28a), as well as non-membrane-bound, cytomatrical polysomes still persisted. There was evidence of loss of continuity of tonoplasts, indicating incipient leakage of vacuolar contents (including lytic enzymes) and thus incipient autolysis (Figs. 3.28a & b) (Pammenter *et al.*, 1998). Although mitochondria (and plastids) remained intact, there appeared to have been regression of cristae, compared with the fresh material (Fig. 3.28b).

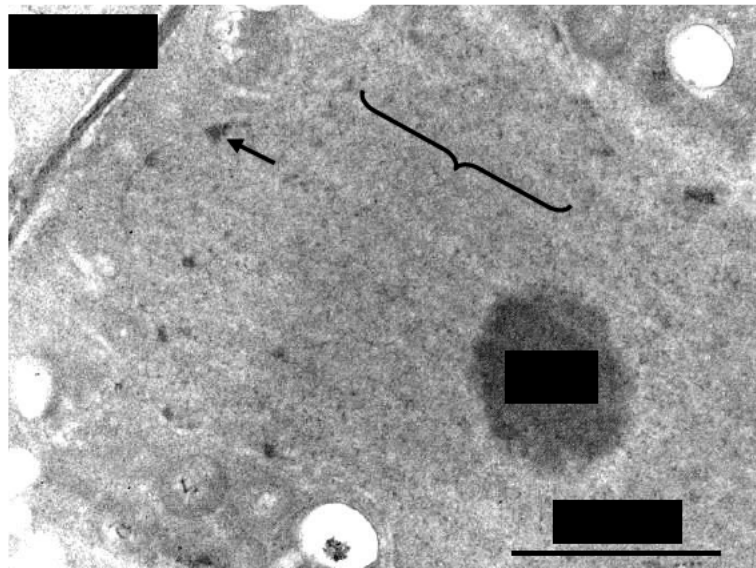
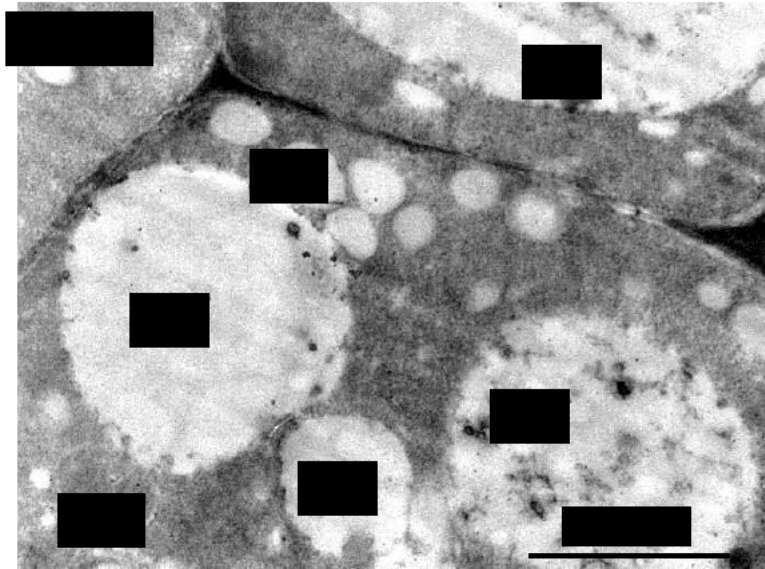
After 9 d in storage, extensive ultrastructural deterioration was evident. Lipid bodies (Fig. 3.29a) persisted, but appeared less dense compared with those in axes from 3 d stored seeds. The cytomatrix was very dense and generally, organelles were not clearly resolved. Mitochondria appeared very dense, but could not be resolved (Fig. 3.29a). Vacuoles were evident but their fuzzy boundaries suggested loss of tonoplast integrity (Fig. 3.29a). Deterioration to the point of disintegration of the nuclear envelope was evident (Fig. 3.29b). However, patches of heterochromatin were still present in association with nuclear envelope fragments (indicated by arrow, Fig. 3.29b).

The ultrastructure continued to deteriorate with prolonged exposure to the chilling treatment. After 38 d in storage, the intracellular structure had completely disintegrated with no organelles discernable (Figs. 3.30a & b). From these observations, it can be concluded that when the sub-tropical seeds of St Lucia provenance are stored at 3°C, a loss of ultrastructural integrity is initiated relatively soon with deterioration proceeding to the point that no recognisable organelles could be identified.

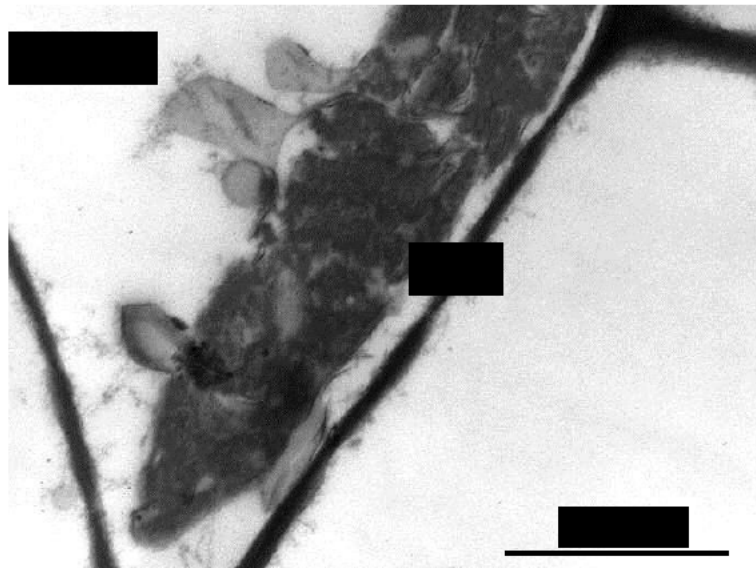
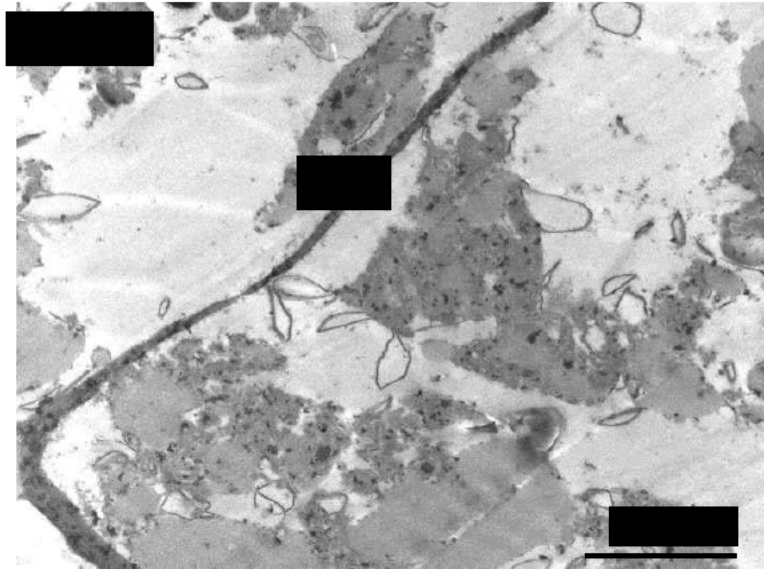
Figures 3.28a & b Ultrastructural situation in root meristem cells after 3 d storage at 3°C of seeds from St Lucia. Figs. 3.29a & b represent the situation after 9 d, and 3.30a & b the total intracellular deterioration after 38 d at 3°C. Fig. 3.28a shows apparent disintegration of the bounding membrane (tonoplast) of a vacuole (V). Lipid bodies (L) are seen to be irregular, dense and scattered in the cytomatrix. Some vesicles (indicated by arrows) persisted peripherally. Polysomes associated with the surfaces of ER cisternae are indicated by arrow heads. Fig. 3.28b shows mitochondria (M) and a plastid (P) in which structure appears to have regressed as well as persisting ER cisternae, as well as a vacuole (V), which is suggested to be nascent.



The ultrastructure in root meristem cells after 9 d storage at 3°C is represented in Fig. 3.29a & b. Fig. 3.29a shows vacuoles (V) without resolvable boundary (tonoplast), and persistent lipid bodies (L) in the cytomatrix. Fig. 3.29b shows a nucleolus (nu) and heterochromatin patches (indicated by arrows) associated with nuclear envelope, which appears largely disintegrated (indicated by bracket).



Figures 3.30a & b Successive stages of intracellular deterioration after 38 d of storage of seeds from St Lucia at 3°C. Cell walls (cw) are virtually the only identifiable structures.



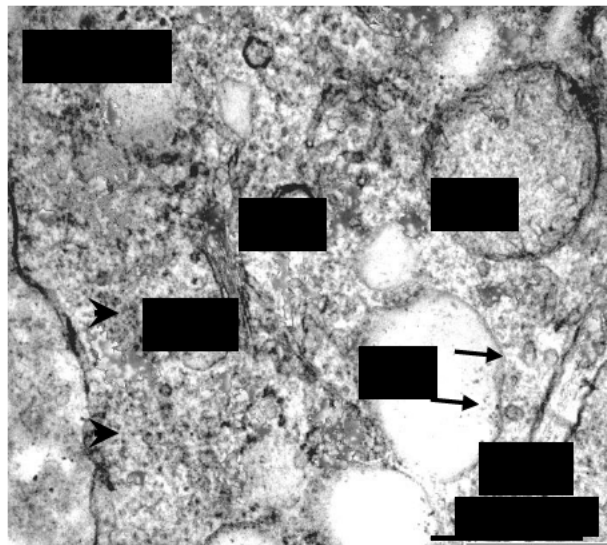
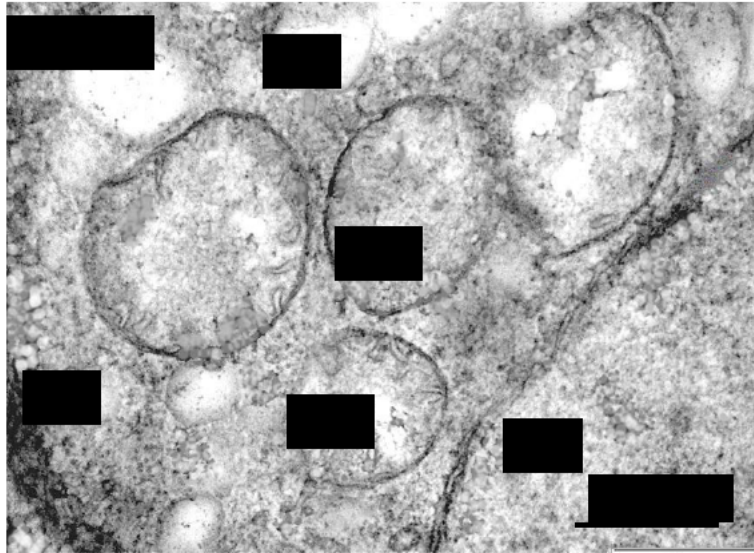
3.7.2.3 Seeds from St Lucia stored at 6°C

There was a dramatic difference in axis ultrastructure when seeds were stored at 6°C, compared with the situation at 3°C. The ultrastructure of the seeds stored at 6°C indicated ongoing, vigorous metabolism. After 18 d in storage, the mitochondria (Figs. 3.31a & b) were well developed with the cristae being clearly resolved. Lipid bodies (Figs. 3.31a & b) were relatively translucent implying a utilisation of reserves presumably to sustain the metabolic activity. Although not frequently encountered, well developed Golgi bodies (Fig. 3.31b) were clearly resolved. The plasmalemma which was closely appressed to the regular cell wall (Fig. 3.31b) showed associated vesicles (indicated by arrows in Fig. 3.31b). Despite the apparent level of metabolic activity and the presence of polysomes, there was a surprising lack of long rER cisternae. However, it appeared that specialised rER derivatives might have been widely implicated in *de novo* vacuolation by encapsulating discrete volumes of cleared cytomatrix (Fig. 3.31a) as described by Lamb and Berjak (1981). Vesicles with a densely-staining membrane were apparent, as shown in Fig. 3.31b. It is probable that this vesicle type was clathrin-coated and derived endocytotically (Becker *et al.*, 2009).

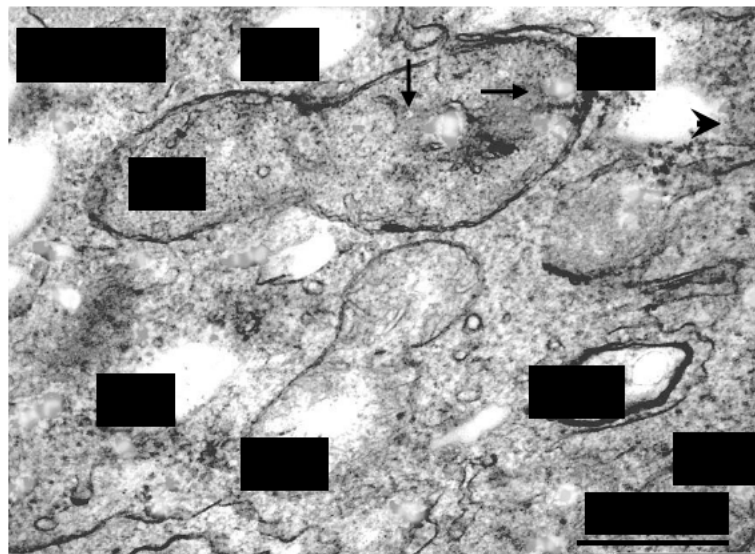
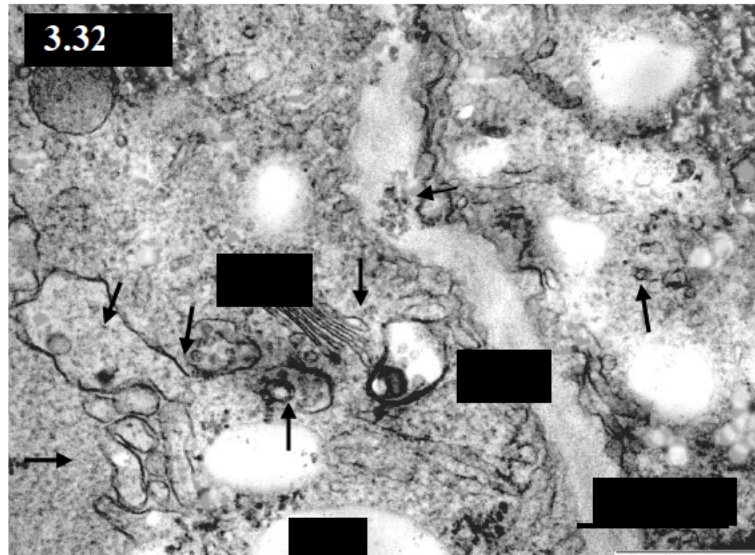
After 38 d in storage, the ultrastructure was well maintained. The lipid bodies (Figs. 3.32a & b) appeared more transparent compared with the control seeds. Figure 3.32a also shows a vesicular progression (arrows) suggested to illustrate a pathway of endocytosis to the interior of the cell. Mitochondria exhibiting clearly defined cristae had elongated (Fig. 3.32b). The presence of numerous polysomes (indicated by arrow heads in Fig. 3.32b) reinforced the impression of metabolic activity.

The retention and amplification of the ultrastructure of the root meristematic and neighbouring cells of axes from these seeds indicates their state of increasing metabolic activity throughout the storage period at 6°C. This conclusion is supported by the viability data reported previously.

Figures 3.31a & b Ultrastructure of root meristem cells after storage of seeds from St Lucia at 6°C for 18 d. Fig.3.31a shows well-developed mitochondria (M) with many short cristae. Lipid bodies (L) were generally translucent and present throughout the cytomatrix. The nuclear envelope was clearly delineated (N, nucleus). Evidence of *de novo* vacuole formation as seen (arrowed region, upper right) where (specialised) ER cisternae were apparently involved in sequestering a discrete portion of cytoplasm cleared of organelles. Fig. 3.31b shows Golgi bodies (Gb) with associated vesicles. Polysomes were abundant (indicated by arrow heads). A coated vesicle is apparent (ve, midfield), as are small vesicles (arrows) in the vicinity of the plasmalemma. The structure at the lower left segment of the micrograph is suggestedly a nascent vacuole (cf. arrowed region, Fig. 3.31a).



These figures illustrate the root apical cell situation after seeds were stored for 38 d at 6°C. Fig. 3.32a shows a well - developed Golgi body (Gb) with vesicles of increasing density from the *cis* to the *trans* Golgi (face). Note the series of enlarging vesicles (arrowed centre – field, near Golgi body) which are suggested to convey endocytotically– derived material to the interior of the cell. Fig. 3.32b illustrates a large mitochondrion (M) with well- developed cristae. Polysomes are indicated by arrow heads. A plastid (P) showing some elaboration of inner membrane and what possibly might be starch remains (arrows; cf. Fig. 3.27d [newly-harvested condition]) are also illustrated. The structure marked * is suggested to be a stage in the endocytotic vesicle progression shown in the micrograph above.



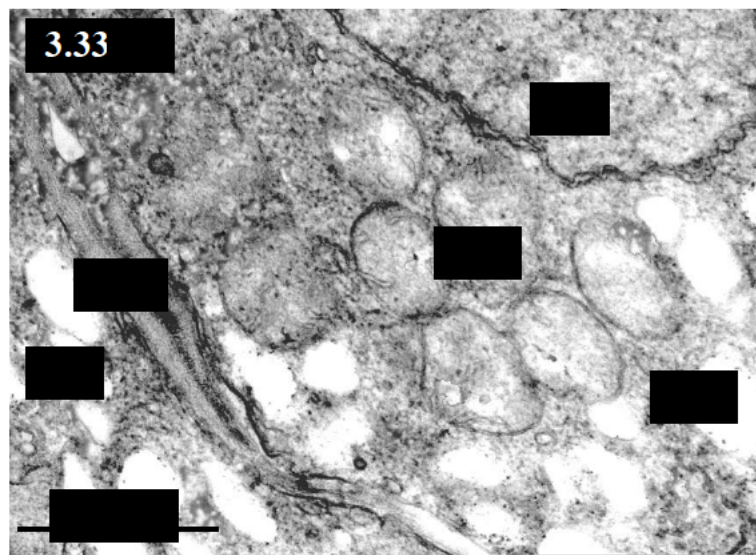
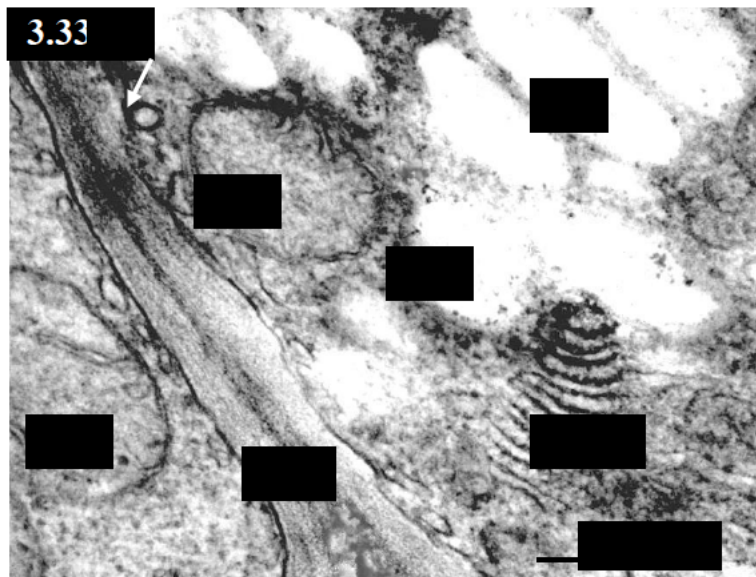
3.7.2.4 Seeds from St Lucia stored at 16°C

The seeds stored at 16°C did not show any ultrastructural signs of deterioration. After 18 d in storage, mitochondria (Figs. 3.33a & b) with well-developed cristae were observed. Rough endoplasmic reticulum (Fig. 3.33a) with associated (membrane bound) polysomes, and non-membrane bound polysomes are indicators of active protein synthesis. As observed in root tip cells of axes stored at 6°C, coated vesicles were observed peripherally. However, there were few Golgi bodies observed after 18 d storage of the seeds which might explain the scarcity of small plasmalemma-associated vesicles (Fig. 3.34c), which are presumed to be exocytotic. Lipid bodies (Figs. 3.33a & b) were omnipresent, but they lacked the density seen in the fresh material probably indicating utilisation of the contents during storage, commensurate with the general indication of heightened metabolism.

After 38 d in storage, polysome formation appeared to be enhanced and generally the ultrastructure indicated a state of intensified metabolic activity typifying axes of seeds progressing toward germination (Fig. 3.34a & b) (Pammenter *et al.*, 1984). This is evident in the appearance of Golgi bodies (Fig. 3.34b) that were not seen in the fresh material or after 18 d storage, and the prominent mitochondria showing strong development of cristae (Fig. 3.34a). Lipid bodies (Figs. 3.34a, b & c) with apparently partially depleted contents remained a consistent feature of the root tip cells. Plastids (Fig. 3.34c) showed occasional plastoglobuli, but little inner membrane formation, or starch, which had been prominent in the fresh material, suggesting its metabolic utilisation during the 38 d of storage at 16°C.

Ultrastructural observations thus revealed that when the seeds were stored at 16°C, they showed many features of a progression toward germination. When these seeds (St Lucia provenance) were maintained at 6°C, although there were indications of enhanced metabolic activity, these were not of the (qualitative) intensity of those occurring during storage at 16°C (Table 3.12).

Figures 3.33a & b Ultrastructural situation of axis root tip cells from seeds of St Lucia provenance after storage for 18 d at 16°C. Fig. 3.33a shows a cell in the vicinity of the meristem. Visible are well developed mitochondria (M) and some of the numerous lipid bodies (L) which were scattered throughout the cytomatrix. An array of apparently short profiles of rough endoplasmic reticulum (rER) is clearly visible. A coated vesicle adjacent to the plasmalemma (top left) is indicated by an arrow. Fig. 3.33b illustrates a group of mitochondria (M) in the vicinity of the nucleus (N).



Figures 3.34a – c The situation of axis apex cells after seeds had been stored for 38 d at 16°C. Fig. 3.34a shows lipid bodies and mitochondria (M) with clearly defined cristae. Coated vesicles situated peripherally are indicated by arrows in the upper left segment of the micrograph. Clearly defined plasmodesmata (pd) occur between these adjacent cells (which are recent meristem derivatives). Polysomes are indicated by arrow heads. Fig. 3.34b shows mitochondria (M) and lipid bodies (L), and an example of a Golgi body (Gb), and is characteristic of root meristem derivatives after 38 d storage at 16°C. Fig. 3.34c shows a typical plastid (P), at this stage not containing substantial starch deposits; numerous lipid bodies (L) remained present within the cytomatrix.

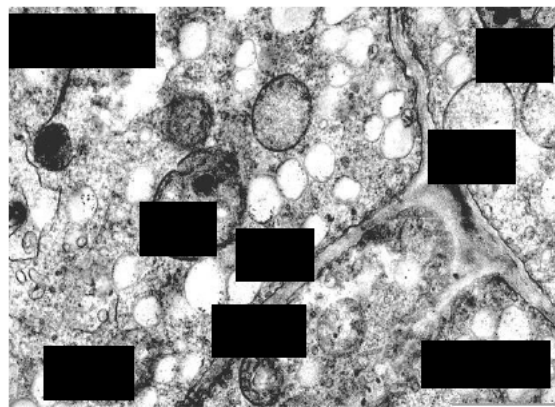
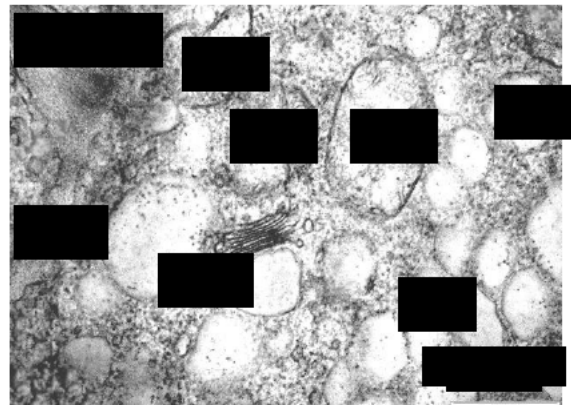
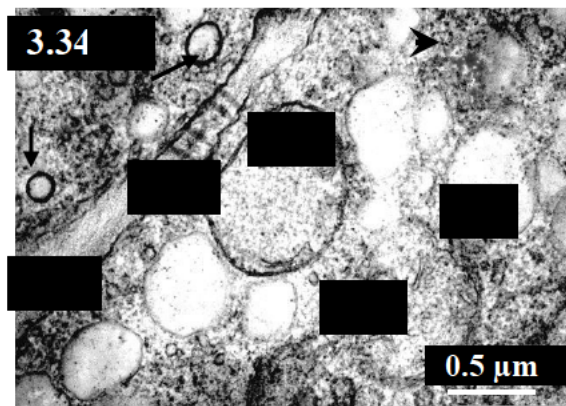


Table 3.12 The effect of temperature on root ultrastructure in seeds of St. Lucia provenance after hydrated storage.

Fresh	3°C	6°C	16°C
Organelles, particularly nuclei and mitochondria were well-developed. Long profiles of rER with associated polysomes indicated active protein synthesis. The ultrastructure was characterised by numerous lipid bodies.	Long profiles of ER, polysomes and vesicles were evident after 3 d in storage. There was evidence of regression in mitochondrial development. After 9 d in storage, the ultrastructure was dense with poor resolution of organelles. After 38 d in storage, the ultrastructure had disintegrated with no discernible organelles.	After 18 d in storage, the ultrastructure showed ongoing, vigorous metabolism. Well-developed mitochondria and Golgi bodies were observed. After 38 d, mitochondria were well-developed. Numerous polysomes and vesicles were observed.	The ultrastructure showed enhanced metabolic activity after storage for 18 d. Active protein synthesis was ongoing suggested by well-developed rER and polysomes. Metabolic activity had intensified after 38 d in storage. Golgi bodies were observed in the cytoplasm.

3.7.3 Axes of seeds from Tanzania

3.7.3.1 Fresh seeds

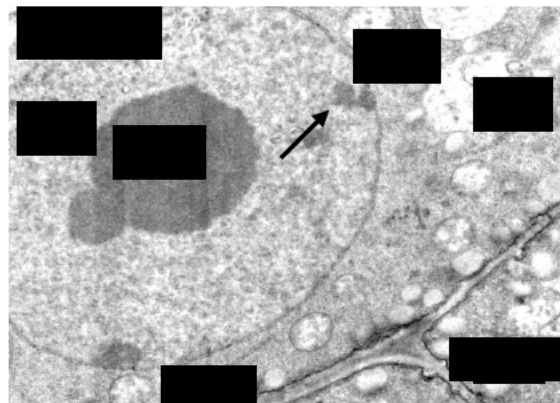
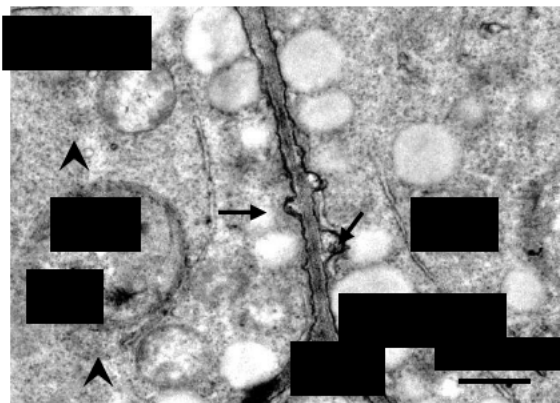
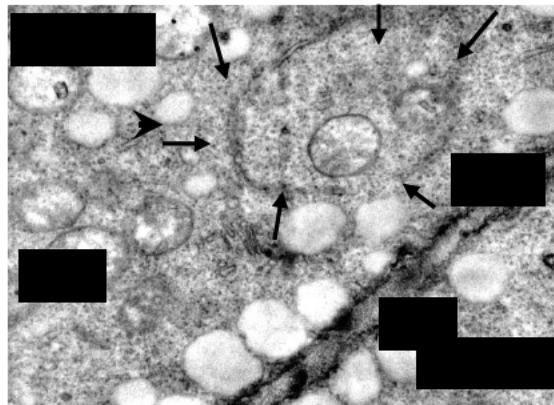
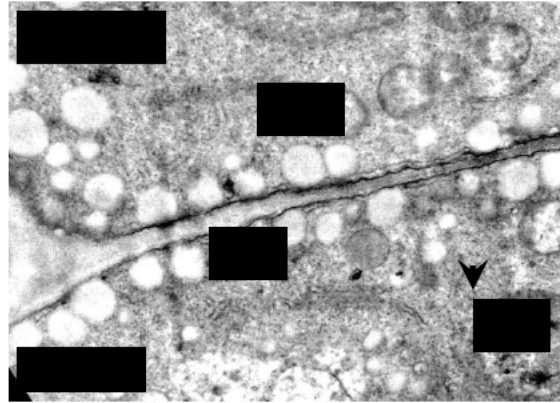
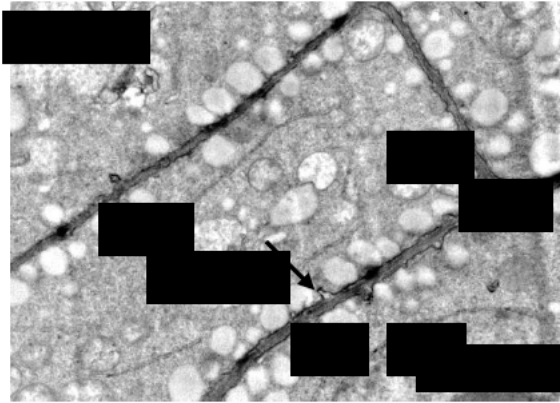
Newly excised axes displayed well developed root tip cells with regular walls (Fig. 3.35a), many mitochondria (Figs. 3.35a, b & e) and spherical to oval nuclei (Fig. 3.35e). Nuclei contained well-developed nucleoli (Fig. 3.35e). The disposition of heterochromatin was essentially similar to that seen in the PE and St Lucia specimens. There was only sparse development of internal membranes and a few plastoglobuli in the plastids (Figs. 3.35b & d). The association of vesicles with the plasmalemma is indicated by arrows in Figs. 3.35a & d. As illustrated in Fig. 3.35d, these appeared to be endocytotic coated vesicles (cf St Lucia material). Numerous mitochondria were visible, but appeared less well developed than those in equivalent cells of the axes from fresh seeds of PE and St Lucia provenance, as indicated by the sparseness of crista formation. However, there was occasional evidence of somewhat elongated mitochondria (Fig. 3.35b).

Profiles of rough endoplasmic reticulum (rER) with associated polysomes (Figs. 3.35a & d) were apparent as were non-membrane-associated polysomes, attesting to active membrane-associated and cytomatrix protein synthesis. Fig. 3.35c shows that certain ER-derived cisternae were involved in incipient vacuolation via encapsulation of discrete regions of the cytoplasm which contained few organelles (Lamb and Berjak, 1981), while vacuolar autophagy was also apparent (Fig. 3.35a).

Lipid bodies occurred as a common feature in the cells (Figs. 3.35a, b & d). However, their disposition was markedly different from that in either the PE or the St Lucia material, being primarily located peripherally, contiguous with the inner face of the plasmalemma. There was little evidence of plastidial starch (Figs. 3.35b & d). The relative absence of lipid bodies deeper within the cytomatrix, and the absence of starch, compared with axis cells of seeds of St Lucia provenance, suggests that the seeds from Tanzania were in a different developmental phase than those from the more southerly provenances. However, the ultrastructure presented by newly-excised axes from the Tanzanian seeds was different in several respects, and it is difficult to say whether the axes from the Tanzanian seeds were relatively less, or more developed at fruit harvest, than those from the PE or St Lucia seeds. Nevertheless, the incidence of polysomes, the rate of protein synthesis (Fig. 3.12) and the evidence of both

incipient vacuolation (Fig. 3.35c) and autophagy (Fig. 3.35a) were clear indications of the metabolically active state of the axes from the seeds of Tanzanian origin.

Figures 3.35a – e Ultrastructural situation of axis root tip (meristem and immediate derivative) cells of fresh seeds from Tanzania. Fig. 3.35a shows regular cell walls (cw) and the typical disposition of the nucleus (N) and organelles. Vesicles were associated with the plasmalemma (arrowed). Apparent autophagy of vesicles (*; possibly of endocytotic origin) by a vacuole (V) is illustrated. Fig. 3.35b shows lipid bodies (L) located predominantly as a monolayer peripherally along the plasma membrane. Mitochondria (M) were present and there was some evidence of elongation; however, cristae were poorly developed (Fig. 3.35c). Incipient vacuolation initiated by ER- derived membrane encapsulation of a discrete region of cytoplasm (arrows) was apparent. Fig. 3.35d shows profiles of rough endoplasmic reticulum (rER) and cytomatrical polysomes (arrowheads) scattered within the cytomatrix. A typical plastid (P) with sparse internal membrane formations and a few plastoglobuli (pg) is illustrated. Arrows indicate plasmalemma-associated vesiculation (cf Fig. 3.31b [18 d, 6°C stored St Lucia material]). Fig. 3.35e shows a nucleus (N) that contained prominent nucleolus (nu) and peripheral patches of heterochromatin (arrow).

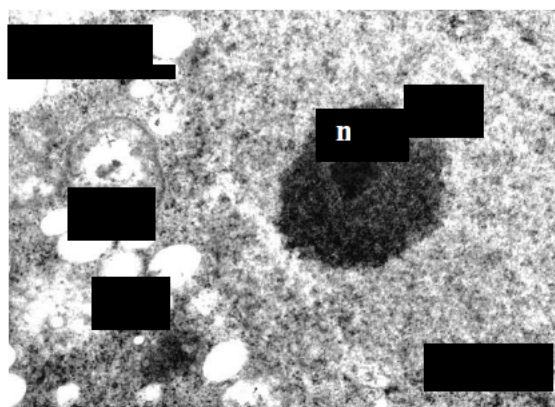
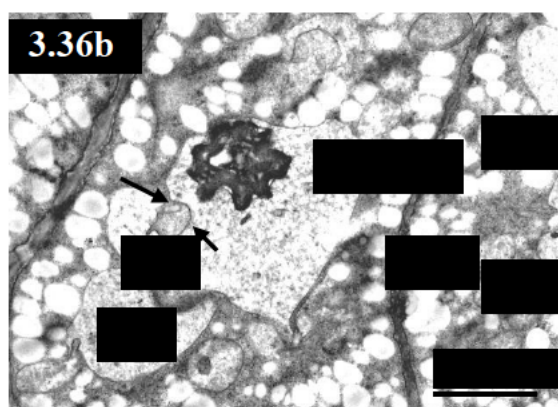
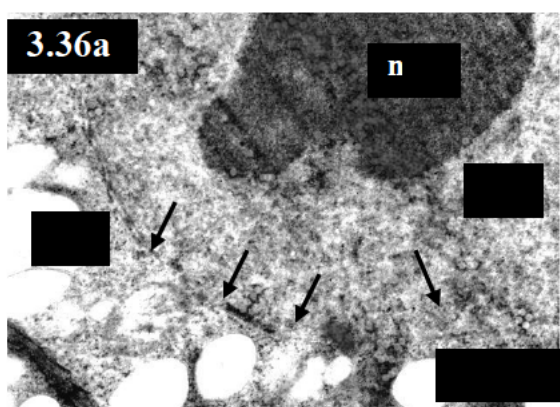


3.7.3.2 Seeds from Tanzania stored at 3°C

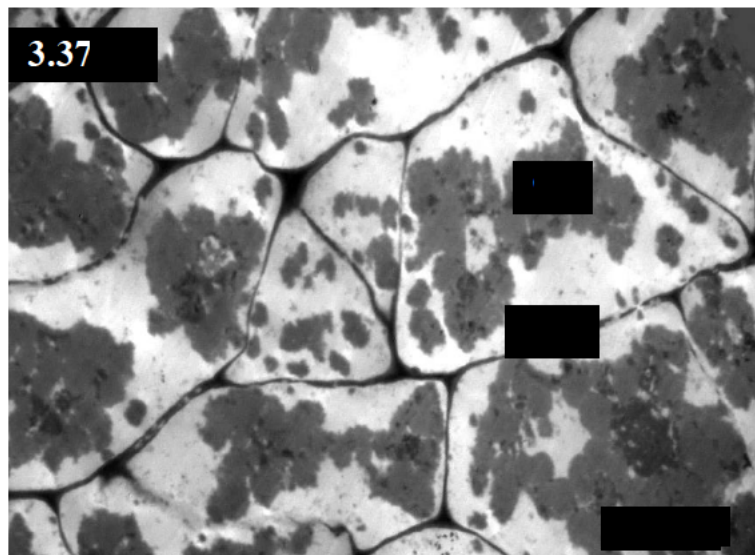
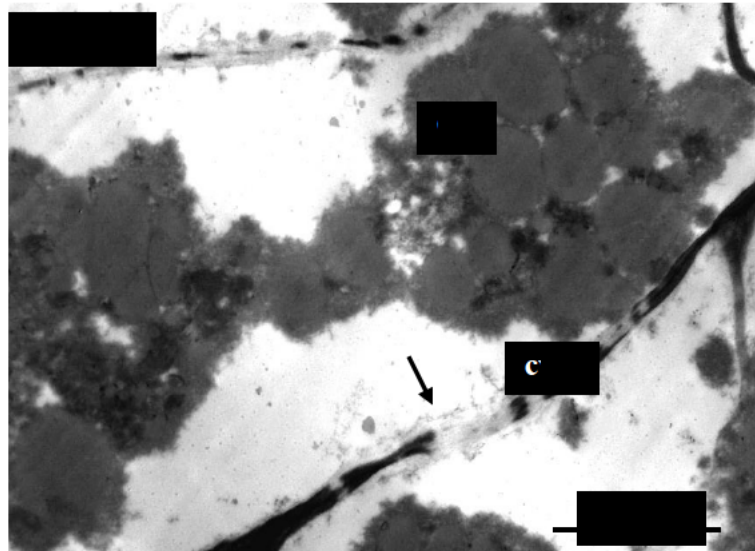
An examination of the ultrastructure revealed the detrimental effect of storage at 3°C on these seeds. Well defined nuclei and membranes constituting the envelope were observed in the control seeds; however, the nuclear envelopes of the seeds stored for 3 d appear discontinuous and poorly defined (Figs. 3.36a & c). Despite this, the nucleoli had persisted and appeared little changed morphologically. Vacuoles had developed (Fig. 3.36b), the initiation of some of which was already apparent in the fresh material (Fig. 3.35a). It is likely that this response, which facilitated subsequent autophagy (Fig. 3.36b), was accelerated as a result of stress rather than by normal metabolic activity as might have occurred at ambient temperature in Tanzania. Mitochondrial structure had showed a deterioration compared with that seen in the control material (Figs. 3.36b & c). Most of the lipid bodies (Figs. 3.36a, b & c) had retained the original peripheral orientation, but some were (Fig. 3.36b) displaced towards the interior in the more degraded cells (Fig. 3.37c). Cell walls appeared to be intact (Fig. 3.36b). The ER seen in fresh material had disappeared, and absence of any vesicles associated with the plasma membrane attested to loss of endocytotic (or exocytotic) ability at the cell surface, which is indirectly taken to indicate breakdown of intracellular structures (particularly cytoskeletal elements) involved in their organisation and movement (Becker *et al.*, 2009).

When the seeds had been stored for 9 d at 3°C, complete deterioration of axis ultrastructure was evident and no discernible organelles could be identified. The cell contents were aggregated into an undifferentiated mass (Figs. 3.37a & b), similar to the situation observed for axes of St Lucia seeds stored at 3°C for 38 d (Figs. 3.30a & b). Although cell walls still persisted, they appeared almost completely disintegrated in places (arrows, Fig. 3.37a). The axis ultrastructure of the seeds from Tanzania stored at 3°C deteriorated rapidly. The fresh seeds showed metabolically active axis cells; after only 3 d in storage deterioration of organelles was evident and total loss of ultrastructural integrity occurred after 9 d in storage.

Figures 3.36a – c Ultrastructural features of axis degradation which accompanied loss of seed viability after 3 d storage at 3°C. Fig. 3.36a shows a clearly recognisable nucleolus; however, damage to the nuclear envelope (indicated by arrows) is apparent. There are numerous transparent lipid bodies (L). Fig. 3.36b shows some of the frequently occurring vacuoles (V) with clear evidence of autophagy (arrowed). Fig. 3.36c shows a persistent nucleolus (nu) within the nucleus of which the nuclear envelope is largely fragmented. Mitochondria (M) were undifferentiated with substantial electron translucent areas.



Figures 3.37a & b The remains of root apex cells of axes from seeds from Tanzania, after 9 d storage at 3°C. Figs. 3.37a and 3.37b show the complete deterioration which had occurred, the cytoplasm being condensed into an undifferentiated mass, with no organelles identifiable. Damage to the cell wall (cw) (in Fig. 3.37a) was also evident (indicated by arrow).



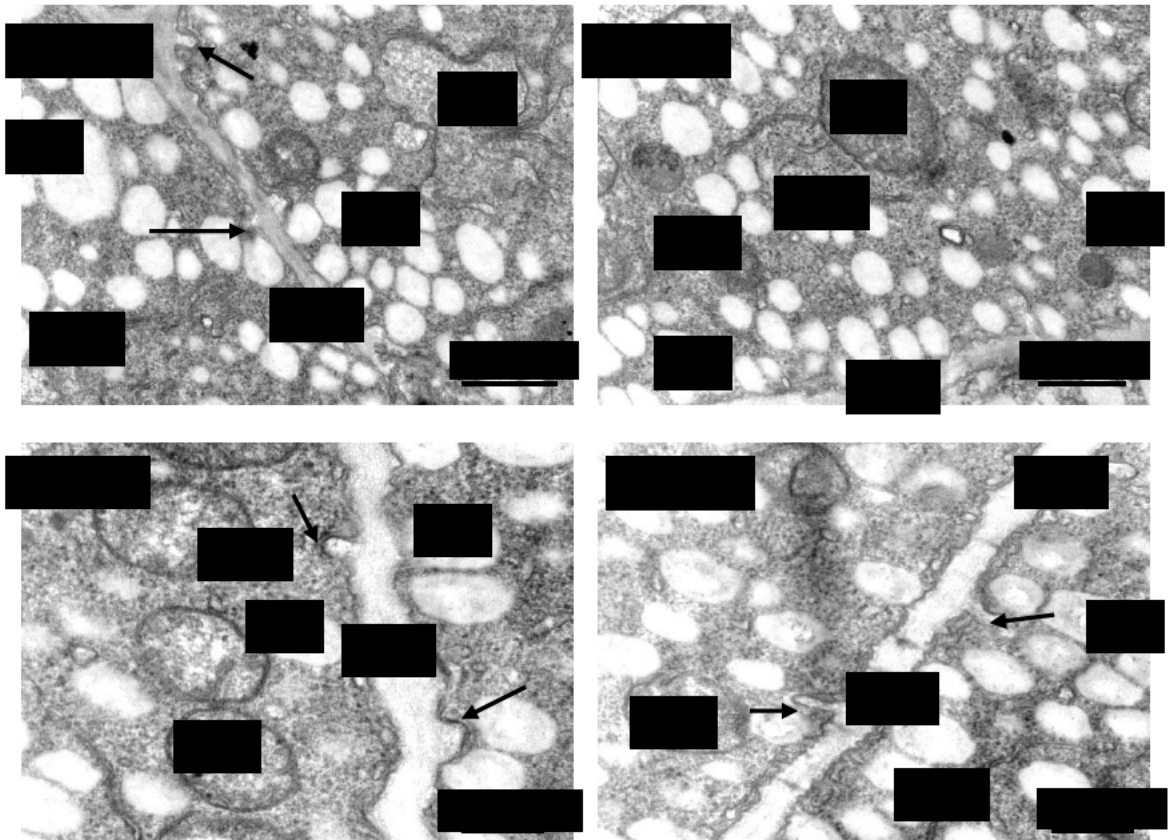
3.7.3.3 Seeds from Tanzania stored at 6°C

The ultrastructure of the seeds stored at 6°C deteriorated over the storage time, but the subcellular changes occurred more slowly and were not as drastic after 9 d as those seen in the seeds that were stored at 3°C. After 3 d in storage at 6°C, cells of axes presumed to represent surviving seeds, showed indications of metabolic activity. The cell walls had remained essentially regular (Figs. 3.38a, b, c & d). Plasmalemma associated vesicles, suggested to be of the endocytotic coated type, (indicated by arrows in Figs. 3.38a, c & d) were a good indicator of metabolic activity having continued over the 3 d. This observation was supported by the visualisation of short profiles of rough ER (Fig. 3.38b), polysomes (Figs. 3.38a, c & d) and Golgi bodies (Fig. 3.38a). Mitochondria (Figs. 3.38b, c & d) with some resolvable cristae were present, although the occasional mitochondrion showed abnormality (Fig. 3.38c). The mitochondria appeared to be more differentiated compared with those seen in the control material. Numerous lipid bodies (Figs. 3.38a, b, c & d) were distributed throughout the cytomatrix.

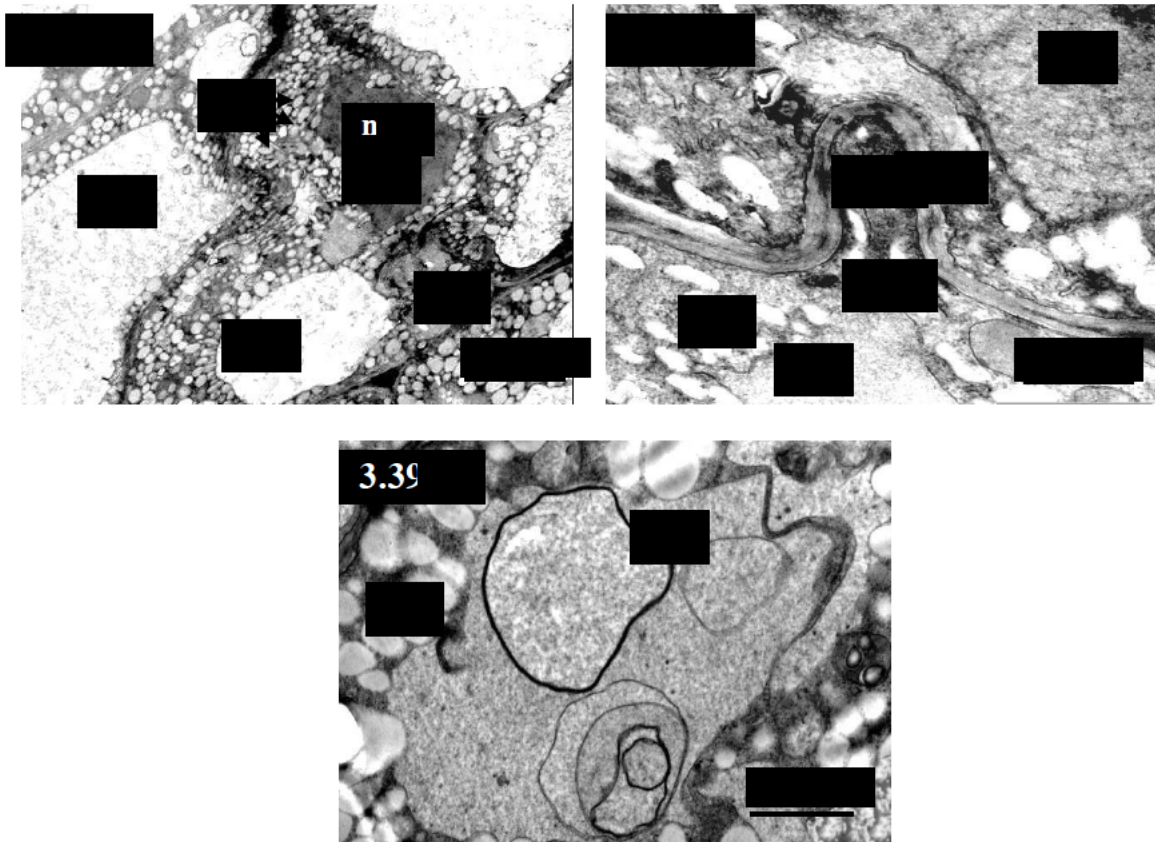
After 9 d in storage, there was evidence of ultrastructural deterioration in axis root tip cells of seeds of Tanzanian provenance. However, this was not as advanced as that seen in the seeds stored for 9 d at 3°C. Distorted nuclei and nucleoli (Fig. 3.39a) were observed in a cytomatrix in which numerous small lipid bodies were compressed. Abnormally distended vacuoles, some with membranous inclusions were present within the cells (Figs. 3.39a, b & c). The close packing of the lipid bodies is suggested to be the consequence of pressure exerted by large vacuoles (Figs. 3.39a, b & c). Cell walls appeared distorted forming marked folds in places (Fig. 3.39b), the buckling being suggested to be a response to the stress imposed by the chilling treatment. Aside from the occasional plastid (Fig. 3.39c) other organelles could not be identified in the cytomatrix.

These seeds showed adverse reactions when exposed to chilling temperatures. Ultrastructural deterioration was evident after 9 d in storage; however, the degree of deterioration was less extreme than that observed in the 3°C treatment where there were no organelles that could be identified. Nevertheless, only 10% of the seeds from Tanzania had retained viability after 9 d at 6°C.

Figures 3.38a – d Ultrastructure of axis root tip cells from seeds of Tanzanian origin representing the survivors after storage at 6°C for 3 d. Fig. 3.38a shows the normal appearance of the cell wall (cw) and plasmalemma-associated vesicles (indicated by arrows). There were numerous lipid bodies (L) dispersed through the cytomatrix. Vacuoles (V) appeared to be in the initial stages of development, the one illustrated appearing to originate by ER dilation, and Golgi bodies (Gb) were apparent. Fig. 3.38b shows endoplasmic reticulum (ER), an undifferentiated plastid (P) and mitochondria (M). Fig. 3.38c shows lipid bodies (L) with partially depleted contents (see also Fig. 3.38d). Mitochondria with dense matrices and some clearly defined cristae are visible, with one of these organelles showing an abnormal attenuation (*) Evidence of the association of what appear to be coated vesicles with the plasmalemma is indicated by arrows. Fig. 3.38d shows tubular vesicles with clear connection to the plasma membrane (arrowed), and lipid bodies (L) the contents of which appear to be partially depleted. Both rough ER and cytomatrical polysomes (arrowheads) can be seen.



Figures 3.39a – c Aspects of the cellular and intracellular situation in axis root tip cells of seeds from Tanzania after 9 d in storage at 6°C. Fig. 3.39a shows the abnormally distended vacuoles (V) within the cytomatrix which is otherwise dominated by lipid bodies (L). The nucleus (N) can be seen essentially to have collapsed, degenerated plastids (P) are apparent, and cell walls (cw) are distorted. Fig. 3.39b shows a cell wall (cw) that has been adversely affected to form folds into which an attenuated extension of a distorted vacuole has protruded (*). Fig. 3.39c shows dense lipid bodies (L) and a vacuole (V) with membranous inclusions and considerably finer granular material; many lipid bodies (L) are crowded in the surrounding cytomatrix, in which plastids (P) can also be discerned.



3.7.3.4 Seeds from Tanzania stored at 16°C

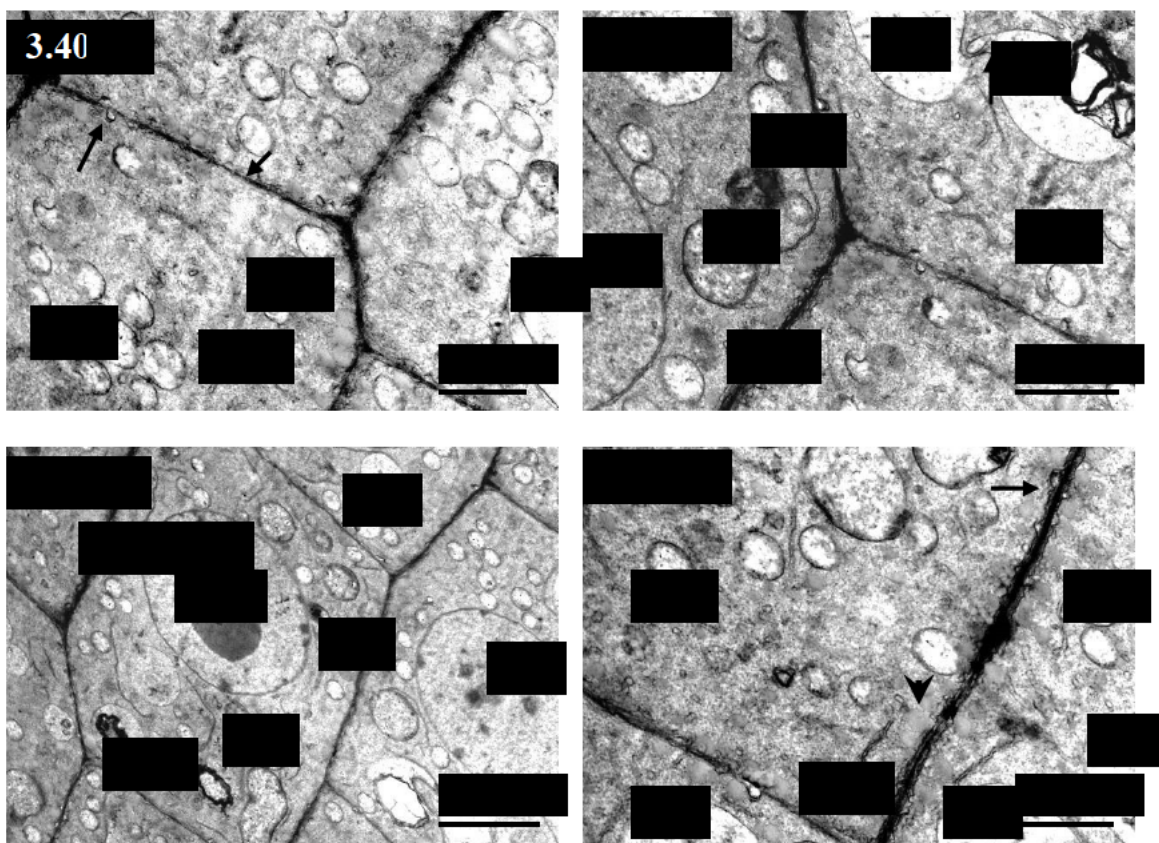
After 3 d in storage at 16°C, 65% of the seeds from Tanzania were still able to germinate. Figs. 3.40 a-d show the axis ultrastructure typically visualised. Vesicle association with the plasmalemma (indicated by arrows in Figs. 3.40a & d) was evident; however, the degree of vesiculation appeared to be reduced compared with that observed in the control tissue (ref. Fig. 3.35d). As was suggested previously, lack of Golgi bodies and prevalence of vesiculation suggest that they are endocytotic coated vesicles. Profiles of rough ER (Figs. 3.40a, b, c & d) were observed in the cytomatrix, and vacuoles (Figs. 3.40b & c) showed evidence of autophagic activity. Vacuolar inclusions could indicate an active turnover of cytomatrix components, and/or concentric membranous remains (Fig. 3.40b) might be 'left-overs' from vacuolar ontogeny via cytolysosomes (Lamb and Berjak, 1981). Incipient vacuolar fusion, which could be a normal aspect of intracellular development was also observed (Fig. 3.40b). There were numerous mitochondria (Figs. 3.40a, b & d) distributed throughout the cytomatrix, but these were surprisingly poorly developed, lacking any internal definition in terms of crista development. Nuclei and nucleoli were structurally intact, and patches of heterochromatin were associated with the nuclear envelope (Fig. 3.40c). There was only sparse occurrence of lipid bodies suggesting that metabolic utilisation had occurred during storage (Fig. 3.40c). The plastids (Figs. 3.40b & c) were small and poorly developed, generally lacking internal membrane formations, plastoglobuli or starch.

After 9 d in storage, corresponding to the time when 40% of the seeds retained viability, there was evidence of deterioration in the ultrastructure of some cells. Fig. 3.41a shows a nucleolus with an abnormal, relatively clear area, while the nuclear envelope as well as certain portions of cell wall appear to be disintegrating (brackets, Fig. 3.41a). However, such damage was not generally the case and in many cells, walls were ultrastructurally intact and there was evidence of plasmalemma-associated vesicles (assumed to be the coated type) (Figs. 3.41b & d). A lack of Golgi bodies suggests that no other vesicles would have been implicated. Numerous mitochondria (Fig. 3.41b) persisted in the root tip cells after 9 d of seed storage at 16°C, but these were generally without discernible crista development and lacked internal density (Fig. 3.41b). There were few lipid bodies, supporting the assumption that their content had been metabolically depleted earlier during storage at 16°C (Figs. 3.41c & d). Plastids with plastoglobuli and/or internal membrane formations (Figs. 3.41b & c) were rarely observed. Compared with those seen in the control tissue, it is suggested that

deterioration of these organelles had occurred.

Generally, the ultrastructure deteriorated during storage of the seeds at 16°C. Nevertheless, seed viability and the intracellular structure was best maintained at this temperature compared with the other two storage temperatures viz. 3° and 6°C (Table 3.13). Considering, however, that these Tanzanian seeds were from a tropical provenance, their storage at a sustained temperature of 16°C for 9 d, might well have imposed a mild, but prolonged chilling stress, manifested by the various intracellular abnormalities observed. This is supported by the survival data (Fig. 3.9) showing germinability to have dropped to <40% after 9 d of storage at 16°C. However, repair prior to germination must have been possible on the assumption that the best ultrastructure (as illustrated) characterised axes from surviving seeds.

Figures 3.40a – d Typical axis root tip ultrastructure after storage for 3 d at 16°C of seeds from Tanzania. Fig. 3.40a shows a dense cytomatrix with numerous electron translucent mitochondria (M) which show little crista development. Vesicles associated with the plasma membrane are indicated by arrows here (and in Fig. 3.40d). Fig. 3.40b shows vacuoles (V), some containing membranous inclusions, and an indication of incipient vacuolar fusion (arrow). Plastids (P) were present and short, scattered profiles of rough endoplasmic reticulum (ER) can be seen. Fig. 3.40c shows normally shaped nuclei (N) and a nucleolus (nu). Plastids (P) and short profiles of endoplasmic reticulum (ER) are also illustrated. Fig. 3.40d shows the relatively undifferentiated mitochondria (M), and vacuoles.



Figures 3.41a – d Axis root tip ultrastructural situation after seeds from Tanzania had been stored for 9 d at 16°C. Fig. 3.41a the nucleolus (nu) in the cell illustrated, showing an abnormal cleared patch (*) is illustrated and the nuclear envelope shows a disintegrated region (indicated by bracket). Fig. 3.41b shows numerous electron - translucent mitochondria (M) with little or no evidence of cristae, distributed throughout the cytomatrix. Vesicles in the region of the plasmalemma are apparent (indicated by arrow). Fig. 3.41c shows a plastid (P) with an aggregate of plastoglobuli (pg) and little internal membrane structure. Lipid bodies (L) can be seen to be located peripherally. The cell wall (cw) appears to have lost some integrity (along the portion indicated by the bracket). Fig. 3.41d shows vesicles associated with the plasmalemma (indicated by arrows), and profiles of rough endoplasmic reticulum (ER) distributed within the cytomatrix.

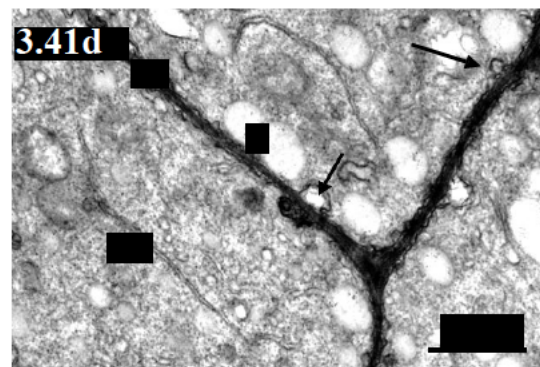
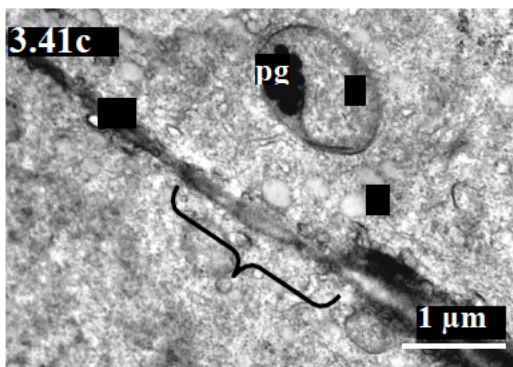
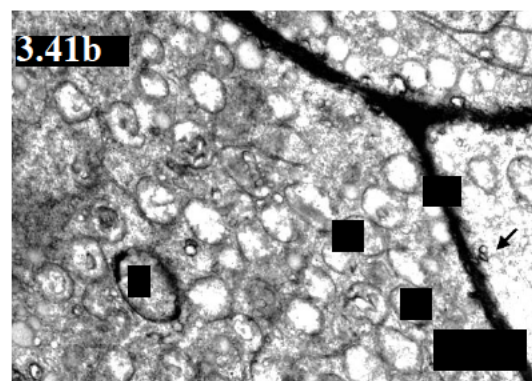
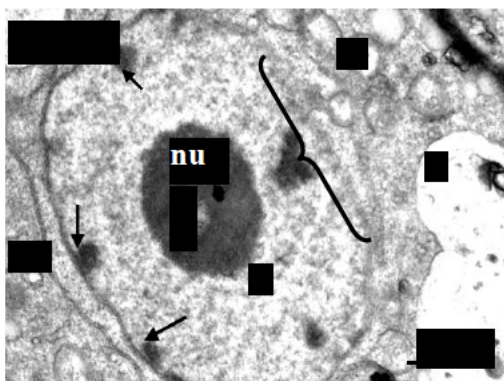


Table 3.13 The effect of temperature on root ultrastructure in seeds of Tanzania provenance after hydrated storage.

Fresh	3°C	6°C	16°C
Nuclei and nucleoli were well-developed. Numerous mitochondria with poorly developed cristae were observed. The presence of rER and polysomes indicated active protein synthesis. Numerous lipid bodies were arranged peripherally along the plasma membrane.	The ultrastructure deteriorated after 3 d in storage. The nuclear envelope was discontinuous and poorly defined. Mitochondria were poorly developed. There was complete disintegration of the ultrastructure after storage for 9 d. There were no discernible organelles.	Cells were metabolically active after storage for 3 d with numerous vesicles, polysomes and short profiles of rER. After 9 d in storage, organelles deteriorated. Abnormally distended vacuoles and distorted nuclei and nucleoli were observed.	The ultrastructure was intact after 3 d in storage. Vesicles, profiles of rER and vacuolar inclusions were observed. Ultrastructural deterioration was evident after 9 d. Numerous poorly developed mitochondria and damaged nuclei were observed.

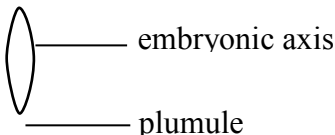
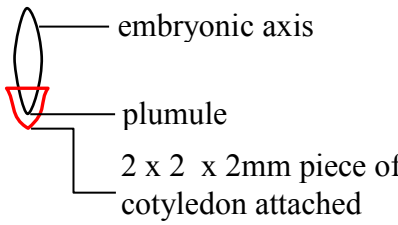
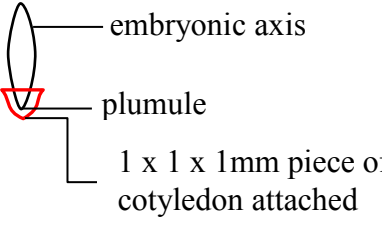
B) Cryopreservation of germplasm

3.8 Explant selection

It is well known that whole recalcitrant seeds cannot be successfully cryopreserved (Tweddle *et al.*, 2003; Daws *et al.*, 2006). Therefore, a suitable explant must be selected for this process. The embryonic axis was selected as the explant of preference for all experiments. However, improved survival of axes was noted when these were excised with a piece of cotyledon attached (Goveia *et al.*, 2004; Whitaker *et al.*, 2010).

To determine the smallest possible explant, three different types were tested (Table 3.14 below). Seventy percent of the axes without cotyledon produced seedlings, while all the axes that had a piece of cotyledon attached (2 x 2 x 2 mm) germinated on growth medium ($\frac{1}{4}$ MS salts with 3 μ M pyridoxine). In an attempt to reduce the explant size, the attachments of parts of the cotyledon were further trimmed to approximately 1 x 1 x 1 mm. Reducing the size of the attachments of parts of the cotyledons did not have an adverse effect on the explant viability (Table 3.14). Therefore, explants with the smaller attachments of cotyledons were selected as the explant of choice (for explants from both PE and St Lucia provenance).

Table 3.14 Viability of the embryonic axis with and without cotyledon attached (n=20).

Explant type	Media	Germination (%)
<p>1. Embryonic axis only (no cotyledon attached)</p>  <p>embryonic axis</p> <p>plumule</p>	$\frac{1}{4}$ strength MS (Murashige and Skoog, 1962) salts supplemented with 3 μ M pyridoxine	70
<p>2. Embryonic axis with 2 x 2 x 2mm piece of cotyledon attached</p>  <p>embryonic axis</p> <p>plumule</p> <p>2 x 2 x 2mm piece of cotyledon attached</p>		100
<p>3. Embryonic axis with 1 x 1 x 1mm piece of cotyledon attached</p>  <p>embryonic axis</p> <p>plumule</p> <p>1 x 1 x 1mm piece of cotyledon attached</p>		100

3.9 Optimisation of water content and drying time for cryopreservation

3.9.1 Explants from PE provenance

Currently, the only way to dehydrate recalcitrant seed germplasm while still maintaining viability is by flash drying (Berjak *et al.*, 1989; 1990; Walters *et al.*, 2008). Explants were flash dried at 5 or 10 min intervals for up to 60 min. At each 10 min interval, the explants were assessed for water content and viability. After flash drying for 10 min, the explants reached the acceptable target water content range (for cryopreservation) of $0.39 \pm 0.05 \text{ g g}^{-1}$ while retaining 70% viability (Fig. 3.42a). After 60 min, the water content dropped to $0.16 \pm 0.03 \text{ g g}^{-1}$ with 5% viability.

Explants were also exposed to cryoprotectants (sucrose and glycerol combination), and then flash dried at 5 min intervals for 20 min (Fig. 3.42b). At each time interval, water content and viability were measured. From Fig. 3.42b, it was noted that after 10 min of flash drying, the explants reached the target water content range of $0.32 \pm 0.09 \text{ g g}^{-1}$ and retained viability of 70%. The dehydrating effect of the cryoprotectants is evident because the explants retained a higher water content when flash dried without cryoprotectants for the same time (with the same viability).

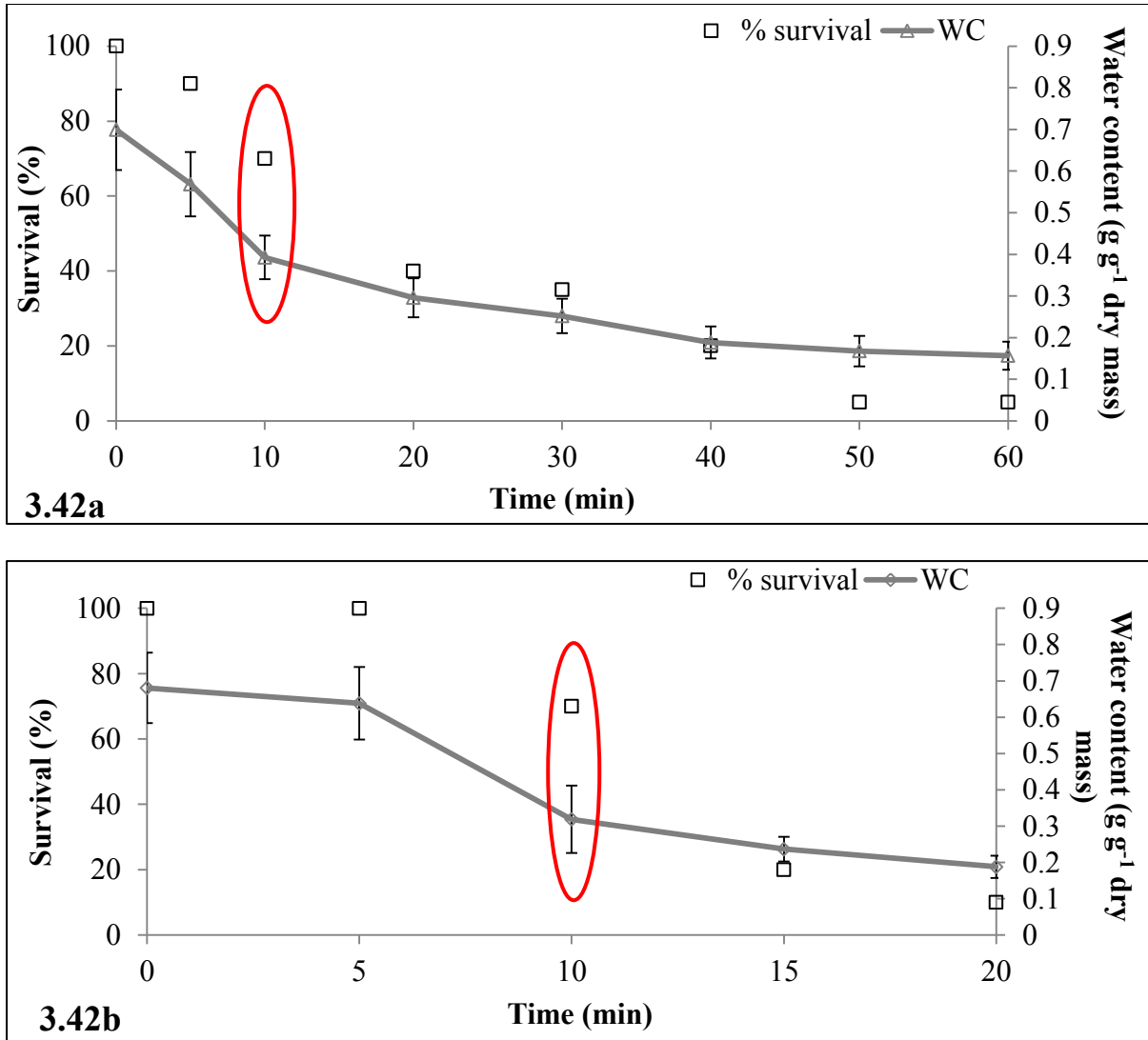


Figure 3.42 Water content (WC) and seedling production (n=20) in explants of *E. capensis* from PE that were flash dried without (a) and with cryoprotectants (b). WC values represent mean \pm SD (n=10). Red circles indicate explant WC, drying time and associated viability (considered to be suitable for partial dehydration prior to cooling).

3.9.2 Explants from St Lucia provenance

The target water content suitable for cryopreservation was attained after 20 min of drying (Fig. 3.43a). At this point, water content of the explants was 0.39 ± 0.23 g g⁻¹ with 75% of the axes remaining viable. After 30 min of drying, water content dropped slightly to 0.37 ± 0.19 g g⁻¹ but more significantly, viability dropped to 50%. After 60 min of drying, water content was reduced to 0.15 ± 0.15 g g⁻¹ with only 10% of the explants retaining viability.

The explants were immersed in cryoprotectants (sucrose and glycerol combination) and flash dried at 10 min intervals for 40 min (Fig. 3.43b). The target water content of $0.32 \pm 0.10 \text{ g g}^{-1}$ was achieved after 20 min of flash drying, with 75% of the axes retaining viability. Drying the axes for a further 10 min resulted in the water content and viability dropping to $0.21 \pm 0.10 \text{ g g}^{-1}$ and 35%, respectively.

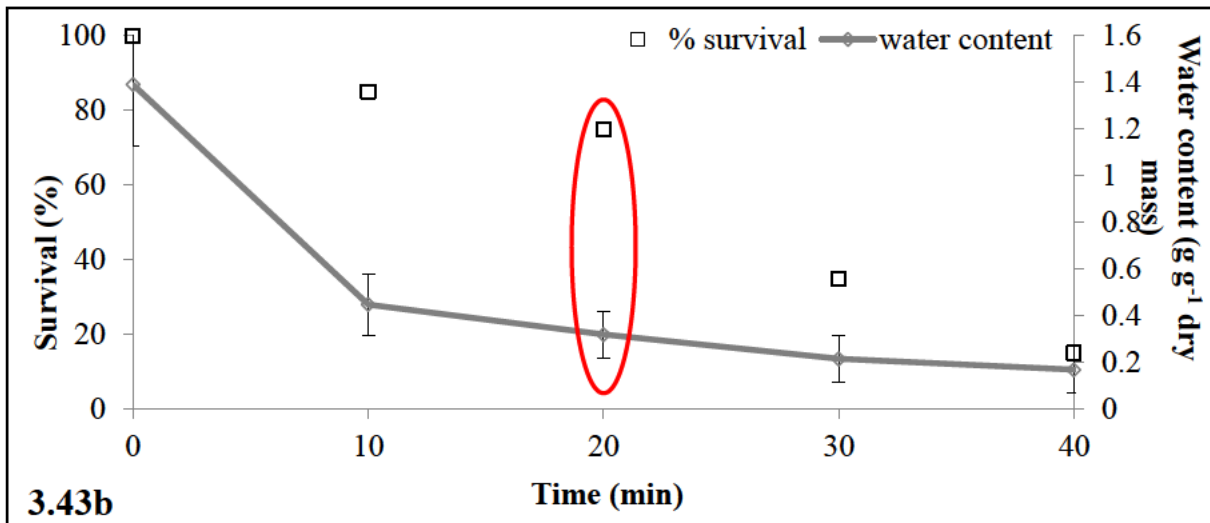
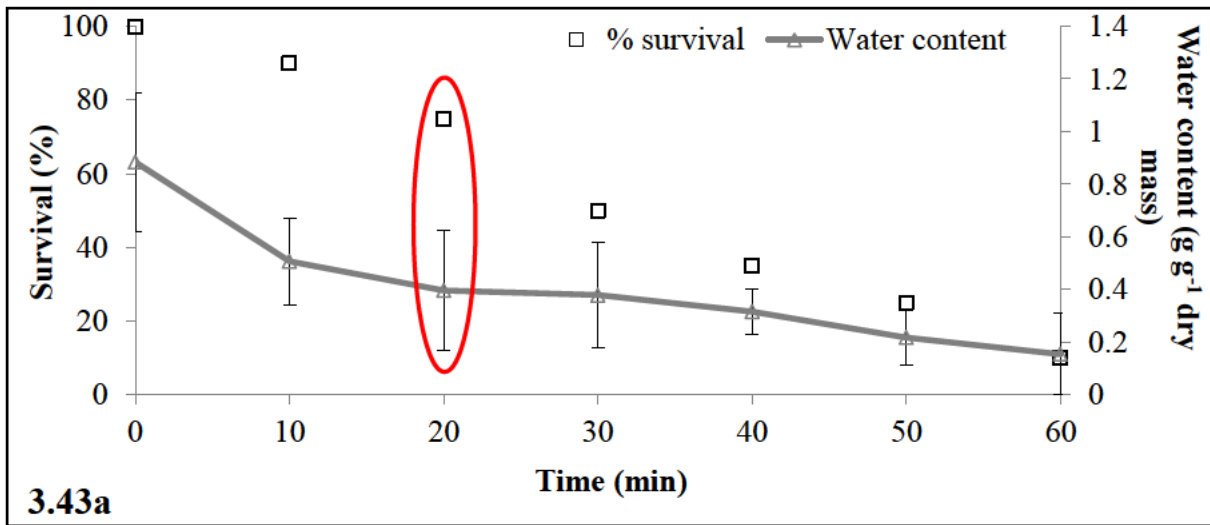


Figure 3.43 Water content (WC) and seedling production (n=20) in explants of *E. capensis* from St Lucia that were flash dried without (a) and with cryoprotectants (b). WC values represent mean \pm SD (n=10). Red circles indicate explant WC, drying time and associated viability (considered to be suitable for partial dehydration prior to cooling).

3.10. Effect on extracellular ROS and TAA levels after treatment of explants with and without cathodic water

3.10.1 Untreated explants

Freshly excised explants from both provenances were assessed for superoxide ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2) and total aqueous antioxidants (TAA) (Table 3.15 [PE] and 3.16 [St Lucia]) to assess the levels of these molecules before the various steps of cryopreservation. Those from PE showed rates of $\cdot\text{O}_2^-$ and H_2O_2 production of 4.85 ± 0.74 nmol g DM⁻¹ s⁻¹ and 0.23 ± 0.09 nmol g DM⁻¹ s⁻¹, respectively (Table 3.15). The TAA level was 11.15 ± 1.99 $\mu\text{mol g}^{-1}$ FM (Table 3.15). Compared with the PE explants, after excision, the explants of St Lucia provenance (Table 3.16) showed 2.2X higher rate of $\cdot\text{O}_2^-$ production ($p < 0.05$), 1.65X slower rate of H_2O_2 production ($p > 0.05$) and 1.1X higher levels of TAA ($p > 0.05$). However, fresh explants from both provenances exhibited 100 % viability (Table 3.15 and 3.16).

3.10.2 The effect of CaMg or cathodic water on ROS and total antioxidant capacity during cryopreservation of zygotic explants from PE and St Lucia provenances

Excised explants of *E. capensis* from both PE and St Lucia provenance were subsequently manipulated in preparation for cryopreservation. At each stage of these manipulations (treatments), the explants were assessed for rates of ROS production, specifically $\cdot\text{O}_2^-$, H_2O_2 and their TAA contents. The potentially ameliorative effect of cathodic water was tested during each of the treatments. The results from these treatments comparing CaMg and cathodic water treated explants from each of the provenances are presented below (Tables 3.15 and 3.16). Excised explants were exposed to a cryopreservation protocol as described in Fig. 2.3. The levels of ROS and TAA are presented in Tables 3.15 and 3.16 for PE and St Lucia explants, respectively.

When the cooled explants from PE were retrieved in CaMg, levels of exogenous $\cdot\text{O}_2^-$ and H_2O_2 were higher than in those retrieved in cathodic water (Table 3.15). None of the explants survived when they were retrieved in CaMg. Retrieval of the explants in cathodic water resulted in elevated levels of total antioxidant activity compared with those retrieved in

CaMg. Although viability was adversely affected, 30% explant survival was achieved after recovery in cathodic water (root and shoot production; Table 3.15).

The explants from St Lucia provenance that were cooled and retrieved in CaMg exhibited higher levels of ROS and lower levels of TAA than those retrieved in cathodic water (Table 3.16). Root production only was observed in 20% of the CaMg retrieved explants, while 30% of the explants retrieved in cathodic water produced roots (data not shown). None of the explants from St Lucia provenance produced shoots after cooling (Table 3.16).

Once the ameliorative effect of cathodic water had been established via full seedling development in cryopreserved explants receiving exogenous treatment, comparisons between ROS and TAA parameters were assessed. The effect of cathodic water on the production of $\cdot\text{O}_2^-$, H_2O_2 and TAA was compared between explants of PE and St Lucia provenance after they were subjected to a cryopreservation protocol. The effect of cathodic water on explant viability at each cryopreparative step is described in Tables 3.15 and 3.16 below.

3.10.3 Comparisons of explant $\cdot\text{O}_2^-$ production across procedural steps of cryopreservation within and across provenances after recovery in cathodic water

Rates of $\cdot\text{O}_2^-$ production were compared within and across provenances after successive steps of cryopreservation. All treatments involved exogenous application of cathodic water. The explants from PE showed relatively high rates of $\cdot\text{O}_2^-$ production in fresh material (Table 3.15). Material taken through subsequent steps of the cryopreservation protocol showed significantly ($p < 0.05$) lower rates of $\cdot\text{O}_2^-$ production compared with fresh explants (Table 3.15). Although rates of $\cdot\text{O}_2^-$ production after cooling and retrieval (CP+FD+LN+Ret) were significantly lower than those of fresh material, they were significantly higher than those subjected to cryoprotectants (CP), flash drying and rehydration (FD+R), and cryoprotectant, flash drying and rehydration (CP+FD+R) treatments (Table 3.15; $p < 0.05$). Although the rates of $\cdot\text{O}_2^-$ production were higher after cooling (CP+FD+LN+Ret) compared with the other steps during cryopreservation [cryoprotection (CP), flash drying and rehydration (FD+R), and cryoprotection, flash drying and rehydration (CP+FD+R)], 30% of the explants survived the cooling treatment (Table 3.15).

A similar trend was observed in the explants from St Lucia. The subsequent steps required for cryopreservation showed significantly lower levels of $\cdot\text{O}_2^-$ in the treated compared with fresh explants (Table 3.16) ($p < 0.05$). Although there was no significant difference in $\cdot\text{O}_2^-$ levels between flash drying and rehydration (FD+R), cryoprotection, flash drying and retrieval (CP+FD+R) and cooling and retrieval (CP+FD+LN+Ret) ($p > 0.05$), a steady decline in viability was noted (Table 3.16). However, despite significantly lower rates of $\cdot\text{O}_2^-$ production after cooling and retrieval (CP+FD+LN+Ret) compared with fresh explants ($p < 0.05$), none of the explants survived the cooling treatment.

Freshly excised explants (fresh) from PE showed considerably lower rates of $\cdot\text{O}_2^-$ production compared with those of St Lucia provenance ($p < 0.05$; Table 3.17). Explant viability from both provenances was 100% (Table 3.17). Rates of $\cdot\text{O}_2^-$ production in the explants of PE provenance were higher than those from St Lucia after they were flash dried and rehydrated (FD+R) (Table 3.17) rendering 60% of the explants from PE viable, compared with 70% in their St Lucia counterparts (Table 3.8). Excised explants were then subject to cryoprotection, flash drying and rehydration (CP+FD+R). This treatment resulted in a four-fold higher rate of $\cdot\text{O}_2^-$ production in the PE explants compared with those from St Lucia ($p < 0.05$). The explants from both provenances retained 50% viability after this treatment (Table 3.17). Compared with the explants from St Lucia, the rate of $\cdot\text{O}_2^-$ production was 1.8X higher in the PE explants ($p > 0.05$) after they were cryoprotected, flash dried, plunged in LN slush and thawed in warm (40°C) cathodic water (CP+FD+LN+Ret). After this treatment, none of the explants from St Lucia survived, while only 30% of the explants from PE were viable.

3.10.4 Comparisons of explant H_2O_2 production across procedural steps of cryopreservation within and across provenances after recovery in cathodic water

The rates of H_2O_2 production in fresh explants of PE origin were relatively low compared with those following the procedural steps required for cryopreservation (Table 3.15). The rates of H_2O_2 production increased gradually after these steps, although the increase in rates of H_2O_2 production was not significant following cryoprotection (CP) and flash drying and rehydration (FD+R) treatments ($p > 0.05$). A significant increase in rates of H_2O_2 production was observed after cryoprotection, flash drying and rehydration (CP+FD+R) and cooling (CP+FD+LN+Ret) treatments ($p < 0.05$) compared with fresh explants. The elevated rates of

H₂O₂ production resulted in 30% of the explants retaining viability after cooling (Table 3.15). Increased levels of H₂O₂ after each cryopreparative step coincided with a gradual loss of viability after each of these steps.

The explants from St Lucia exhibited a similar trend to those of their PE counterparts. The levels of H₂O₂ were relatively low in the fresh explants compared with those subjected to the steps in cryopreservation (Table 3.16). Although the rates of H₂O₂ production were higher after cryoprotection (CP) and flash drying and rehydration (FD+R), the increase was not significant. The highest rates of H₂O₂ production was observed after the explants were cryoprotected, flash dried and rehydrated (CP+FD+R). The rates of H₂O₂ production after this (CP+FD+R) and cooling (CP+FD+LN+Ret) treatments were significantly higher than those observed in fresh explants ($p<0.05$; Table 3.16). The elevated rates of H₂O₂ after cooling coincided with none of the explants surviving this treatment (Table 3.16). A steady increase in the rates of H₂O₂ production after each cryopreparative step was correlated with a gradual decline in viability.

The rates of H₂O₂ production in freshly excised PE explants (fresh) were higher than those of St Lucia (Table 3.17). All explants from this treatment placed on nutrient medium had germinated (100% viability). In subsequent manipulations for cryopreservation, the differences in rates of H₂O₂ production were not significant between the two provenances but there was a gradual loss of viability (Table 3.17). However, the final step of the cryopreservation protocol (CP+FD+LN+Ret) showed that the rate of H₂O₂ production in the explants of PE provenance was twice that of the St Lucia explants ($p<0.05$; Table 3.17). Viability was adversely affected after this treatment. None of the explants from St Lucia survived, while 30% of those from PE did (Table 3.17).

3.10.5 Comparisons of explant TAA capacity across procedural steps of cryopreservation within and across provenances after recovery in cathodic water

Total aqueous antioxidants (TAA) content was assessed at each of the steps of cryopreservation after exogenous application of cathodic water. The TAA contents of *E. capensis* explants from both PE and St Lucia were measured.

The TAA content of explants of PE origin fluctuated in a narrow band ranging from 9.01 ± 1.81 (FD+R) to 11.15 ± 1.99 (fresh) $\mu\text{mol g}^{-1}$ FM (Table 3.15) and did not differ significantly (Table 3.15). However, viability declined gradually presumably as a result of the cumulative effects of stress imposed by cryopreservation (Table 3.15) with 30% of the explants surviving cooling (CP+FD+LN+Ret; Table 3.15).

The differences in TAA content in the St Lucia explants were not statistically significant across fresh, cryoprotection (CP), flash drying and rehydration (FD+R) and cryoprotection, flash drying and rehydration (CP+FD+R; Table 3.16). In each of these treatments, viability retention was at least 50% (Table 3.16). The reduced TAA content observed after the explants were cooled in LN (CP+FD+LN+Ret) was significantly different ($p < 0.05$) compared with initial cryopreparative steps (CP, FD+R, CP+FD+R; Table 3.16). None of the explants were viable after cooling (CP+FD+LN+Ret; Table 3.16).

The levels of TAA in the explants between PE and St Lucia provenances were significantly different after cryoprotection (CP), flash drying and rehydration (FD+R) and flash drying and rehydration (CP+FD+R). After cryoprotection, the levels of TAA in the St Lucia explants were 1.3X higher compared with those from PE ($p < 0.05$; Table 3.17). Similarly, after flash drying and rehydration (FD+R), TAA levels were 1.3X higher in the St Lucia explants compared with those from PE ($p < 0.05$; Table 3.17). After cryoprotection, flash drying and rehydration (CP+FD+R), the levels of TAA in the St Lucia explants were 1.2X higher than the PE explants ($p < 0.05$). Despite the elevated levels of TAA, reduced viability was observed in explants from both PE (50%) and St Lucia (50%) provenance (Table 3.17). The final step towards successful cryopreservation required the explants to be immersed in LN slush. After cryoprotection and flash drying, the explants were plunged in supercooled LN slush and subsequently thawed in warm cathodic water (CP+FD+LN+Ret). Although the explants from St Lucia had a higher level of TAA, this difference was insignificant ($p > 0.05$). After this treatment, 30% of the explants from PE remained viable, while none of the explants of St Lucia provenance survived (Table 3.17).

Table 3.15 The effect of cathodic water on extracellular production of superoxide ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2), total aqueous antioxidant (TAA) levels and viability in fresh explants of Port Elizabeth (PE) provenance, and after the procedural steps of cryopreservation (E: excision; CP: cryoprotection; FD: flash drying; R: rehydration; C: cooling; Ret.: retrieval). Values represent mean \pm SD (n=6 for $\cdot\text{O}_2^-$ and H_2O_2 ; n=8 for TAA and n=20 for viability). Values labelled with different letters are significantly different when compared between treatments ($p < 0.05$, independent sample t-test).

	Fresh	E+CP		E+FD+R		E+CP+FD+R		E+CP+FD+C+Ret	
		d. H_2O	c. H_2O	CaMg	c. H_2O	CaMg	c. H_2O	CaMg	c. H_2O
Superoxide ($\cdot\text{O}_2^-$) (nmol g DM^{-1} s $^{-1}$)	4.85 \pm 0.74 ^a	2.54 \pm 0.99 ^a	1.04 \pm 0.08 ^c	2.39 \pm 0.44 ^a	1.37 \pm 0.36 ^c	2.34 \pm 0.79 ^a	0.83 \pm 0.27 ^c	4.95 \pm 1.6 ^a	2.68 \pm 1.11 ^b
Hydrogen peroxide (H_2O_2) (nmol g DM^{-1} s $^{-1}$)	0.23 \pm 0.99 ^c	0.52 \pm 0.18 ^a	0.40 \pm 0.12 ^{bc}	0.40 \pm 0.14 ^a	0.27 \pm 0.06 ^c	0.69 \pm 0.23 ^a	0.62 \pm 0.22 ^{ab}	1.19 \pm 0.28 ^a	0.75 \pm 0.28 ^b
Total aqueous antioxidants (TAA) ($\mu\text{mol g}^{-1}$ FM)	11.15 \pm 1.99 ^a	11.08 \pm 2.21 ^a	10.78 \pm 1.8 ^a	8.69 \pm 1.63 ^a	9.01 \pm 1.81 ^a	10.74 \pm 1.58 ^a	11.06 \pm 1.3 ^a	9.39 \pm 0.86 ^a	9.59 \pm 1.42 ^a
Viability (%)	100	90	90	50	60	30	50	0	30

Table 3.16 The effect of cathodic water on extracellular production of superoxide ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2), total aqueous antioxidant (TAA) levels and viability in fresh explants of St Lucia provenance, and after the procedural steps of cryopreservation (E: excision; CP: cryoprotection; FD: flash drying; R: rehydration; C: cooling; Ret.: retrieval). Values represent mean \pm SD (n=6 for $\cdot\text{O}_2^-$ and H_2O_2 ; n=8 for TAA and n=20 for viability). Values labelled with different letters are significantly different when compared between treatments ($p < 0.05$, independent sample t-test).

	Fresh	E+CP		E+FD+R		E+CP+FD+R		E+CP+FD+C+Ret	
		d.H ₂ O	c.H ₂ O	CaMg	c.H ₂ O	CaMg	c.H ₂ O	CaMg	c.H ₂ O
Superoxide ($\cdot\text{O}_2^-$) (nmol g DM ⁻¹ s ⁻¹)	10.88 \pm 2.49 ^a	5.1 \pm 2.02 ^a	4.63 \pm 0.67 ^b	5.57 \pm 0.7 ^a	1.05 \pm 0.26 ^c	6.46 \pm 0.74 ^a	0.21 \pm 0.09 ^c	3.57 \pm 0.6 ^a	1.46 \pm 0.4 ^c
Hydrogen peroxide (H_2O_2) (nmol g DM ⁻¹ s ⁻¹)	0.14 \pm 0.05 ^c	0.37 \pm 0.09 ^a	0.25 \pm 0.07 ^{bc}	0.27 \pm 0.02 ^a	0.28 \pm 0.07 ^{bc}	0.43 \pm 0.1 ^a	0.41 \pm 0.08 ^a	0.67 \pm 0.15 ^a	0.37 \pm 0.09 ^{ab}
Total aqueous antioxidants (TAA) ($\mu\text{mol g}^{-1}$ FM)	12.33 \pm 1.60 ^a	12.41 \pm 0.67 ^b	13.92 \pm 1.68 ^a	10.95 \pm 1.53 ^a	12.18 \pm 1.46 ^a	13.15 \pm 1.25 ^a	13.71 \pm 2.03 ^a	8.68 \pm 1.05 ^b	9.77 \pm 0.59 ^c
Viability (%)	100	90	90	60	70	40	50	0	0

Table 3.17 The extracellular $\cdot\text{O}_2^-$, H_2O_2 production, total aqueous antioxidant capacity and viability for excised explants of *E.capensis* from Port Elizabeth (PE) and St Lucia (SL) at each of the steps towards cryopreservation. Cathodic water was used for the purposes of cryoprotectant preparation and explant rehydration. Treatments correspond to the various procedural steps applied during cryopreservation: cryoprotection (CP), flash drying and rehydration (FD+R), cryoprotection, flash drying and rehydration (CP+FD+R), and cryoprotection, flash drying, cooling and rewarming (CP+FD+LN+Ret). Values represent mean \pm SD (n=6 for $\cdot\text{O}_2^-$; n=6 for H_2O_2 ; n=8 for TAA and n=20 for viability). Significant differences between treatments across provenances are denoted by different letters (p<0.05, ANOVA).

	Fresh	CP	FD+R	CP+FD+R	CP+FD+LN+Ret
$\cdot\text{O}_2^-$ (nmol g DM ⁻¹ s ⁻¹)					
PE	4.85 \pm 0.74 ^b	1.04 \pm 0.08 ^b	1.37 \pm .36 ^a	0.83 \pm 0.27 ^a	2.68 \pm 1.11 ^a
St Lucia	10.88 \pm 2.49 ^a	4.36 \pm 0.67 ^a	1.05 \pm 0.26 ^a	0.21 \pm 0.09 ^b	1.46 \pm 0.40 ^a
H_2O_2 (nmol g DM ⁻¹ s ⁻¹)					
PE	0.23 \pm 0.99 ^a	0.40 \pm 0.12 ^a	0.27 \pm 0.06 ^a	0.62 \pm 0.22 ^a	0.75 \pm 0.28 ^a
St Lucia	0.14 \pm 0.05 ^a	0.25 \pm 0.08 ^a	0.28 \pm 0.07 ^a	0.43 \pm 0.08 ^a	0.37 \pm 0.09 ^b
TAA (Trolox equivalent μmol g ⁻¹ FM)					
PE	11.15 \pm 1.99 ^a	10.78 \pm 1.99 ^b	9.01 \pm 1.81 ^b	11.06 \pm 1.30 ^b	9.59 \pm 1.42 ^a
St Lucia	12.33 \pm 1.60 ^a	13.92 \pm 1.68 ^a	12.18 \pm 1.46 ^a	13.71 \pm 2.03 ^a	9.77 \pm 0.59 ^a
Viability (%)					
PE	100	90	60	50	30
St Lucia	100	90	70	50	0

3.11 Ultrastructure

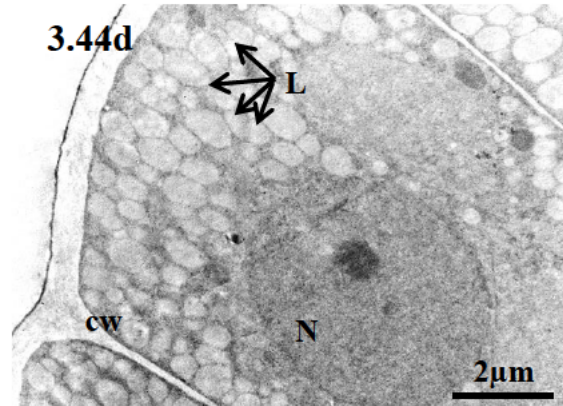
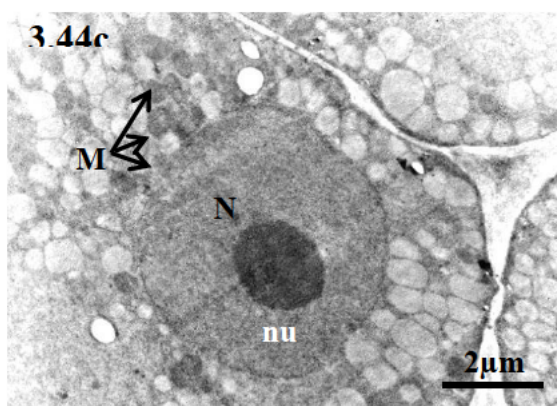
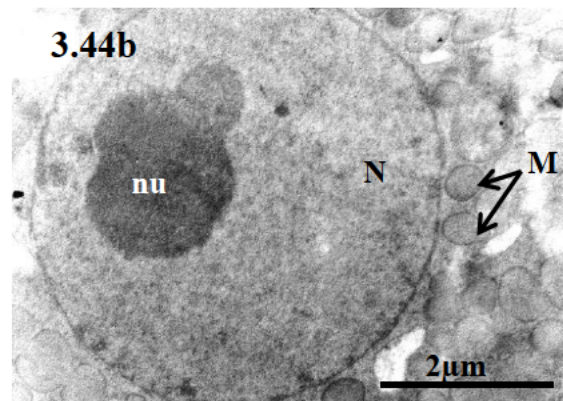
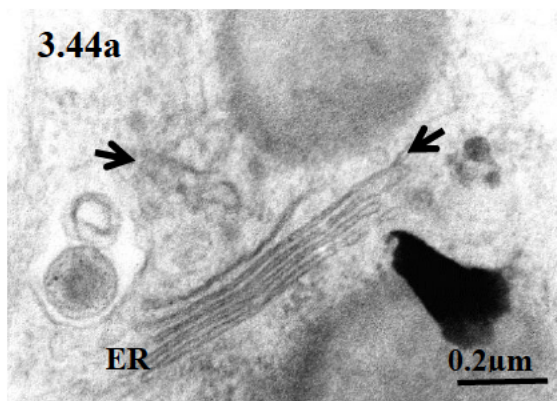
3.11.1 PE

3.11.1.1 Ultrastructure of fresh (untreated), excised explants

The ultrastructure of root and shoot meristem (and closely associated cells) of fresh axes of *E. capensis* from PE were assessed. The ultrastructure of root meristem cells was indicative of metabolically active tissue with well-developed organelles (Figs. 3.44a&b). The presence of long profiles of ER (Fig. 3.44a) is typical of ongoing protein synthesis in metabolically active tissue. As is normal in the root meristem, prominent nuclei and nucleoli (Fig. 3.44b) occurred in most cells. Although poorly developed, numerous mitochondria were observed throughout the cytomatrix. The cells consisted of single, large vacuoles. Numerous vesicles (indicated by arrows) were noted within the cytoplasm and were associated with the plasma membrane indicating active signal transduction (which would have been either endocytotic or exocytotic) and active respiration.

Shoot meristem (tunica cells) displayed well preserved ultrastructure with well-developed organelles (Figs. 3.44c&d). Well-developed cell walls, nuclei and nucleoli (Fig. 3.44c&d) were clearly visible throughout the tunica cells. Numerous mitochondria were observed throughout the cytomatrix, however, they were poorly developed with no discernible cristae (Fig. 3.44c).

Figures 3.44a&b Ultrastructure of untreated (fresh) root meristem. Well developed nuclei (N), nucleoli, endoplasmic reticulum (ER), numerous mitochondria (M) and vesicles (ve) (indicated by arrows) were observed in the cytoplasm. Figs. 3.44c&d shows the ultrastructure of untreated (fresh) shoot meristem. Well developed nuclei (N), nucleoli (nu) and cell walls (cw) were observed. Numerous, poorly developed mitochondria (M) were evident. The cytoplasm contained numerous lipid bodies (L).



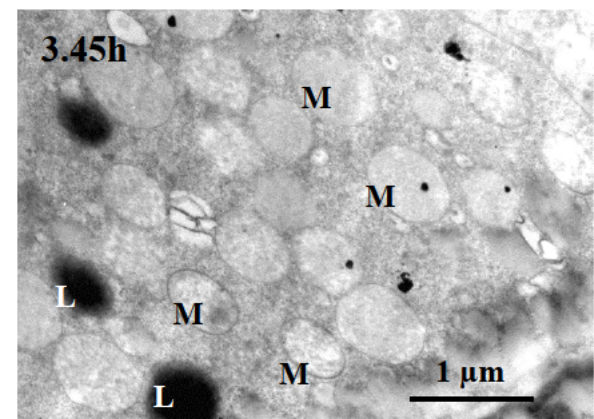
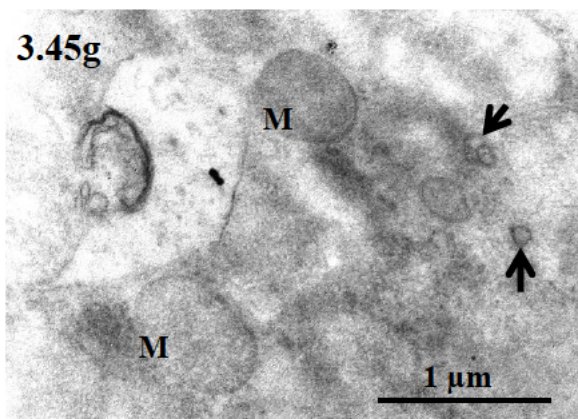
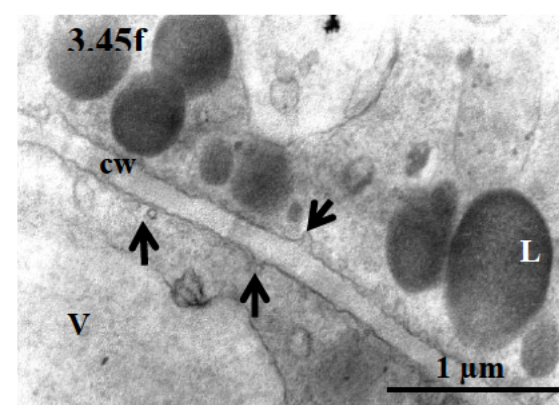
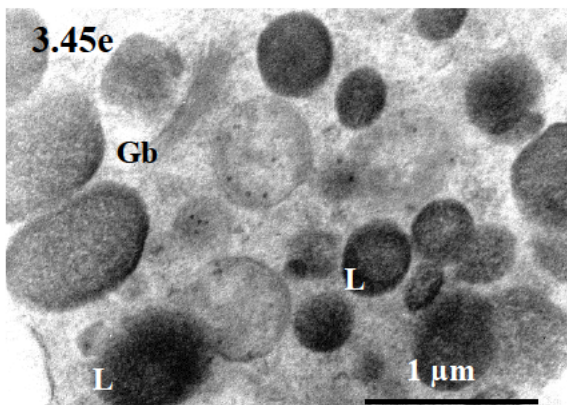
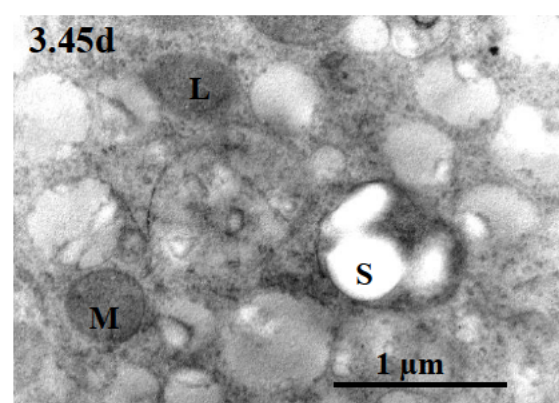
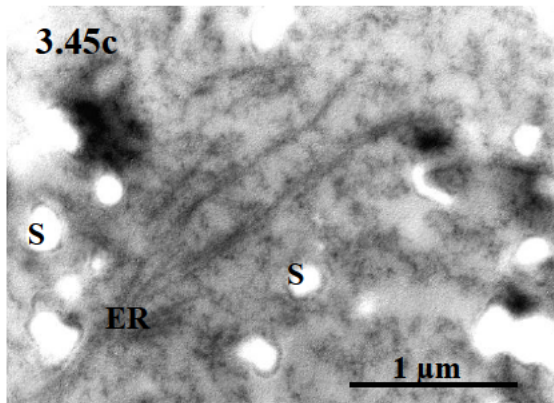
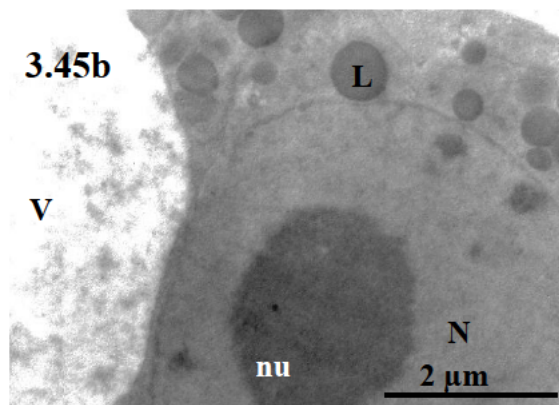
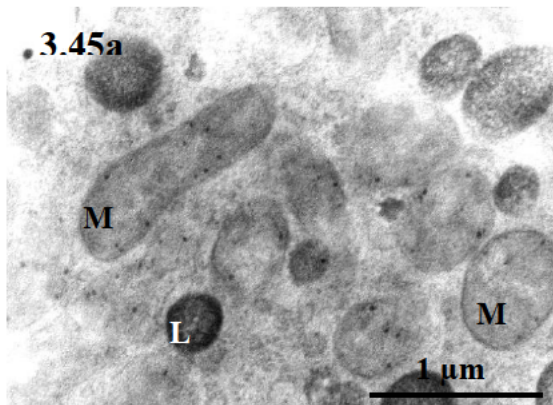
3.11.1.2 Ultrastructure of excised explants cryoprotected in cryoprotectants made up in either distilled or cathodic water

Explants were immersed in cryoprotectants that were made up in either distilled or cathodic water. The ultrastructure of the roots and shoots from these treatments are presented in Figs. 3.45a-h. The ultrastructure of explants immersed in distilled water prepared cryoprotectants did not show any abnormalities. The meristematic cells of the root had numerous mitochondria (Fig. 3.45a) and well developed nuclei with prominent nucleoli (Fig. 3.45b). These cells usually contained single large vacuoles (Fig. 3.45b). The shoot meristem showed signs of active metabolism with numerous lipid bodies and starch grains (Figs. 3.45c&d). Long profiles of endoplasmic reticulum (Fig. 3.45c) were also observed indicating active protein synthesis.

The root meristematic tissue of explants treated with cryoprotectants made up in cathodic water showed a well preserved ultrastructure. Numerous long ER profiles and Golgi bodies were noted within the cytomatrix (Fig. 3.45e) indicating active protein synthesis. As a sign of active metabolism, vesicles were associated with the plasma membrane (indicated by arrows) as well as importation of storage reserves illustrated by numerous dense lipid bodies present throughout the cytomatrix (Fig. 3.45f). The ultrastructure of the shoot meristem was well preserved displaying a metabolically active meristem. The cytomatrix was comprised of numerous vesicles (indicated by arrows) (Fig. 3.45g) as well as poorly developed mitochondria (Fig. 3.45h). Dense lipid bodies were scattered throughout the cytomatrix (Fig. 3.45h).

The ultrastructure of explants exposed to cryoprotectants did not show any signs of stress or abnormality irrespective of diluent (distilled or cathodic water). These were well preserved with well developed organelles indicating an active metabolic state.

Figures 3.45a&b Ultrastructure of the root meristem and Figs. 3.45c&d shows the ultrastructure of the shoot meristem of *E. capensis* immersed in cryoprotectants made up in distilled water. Figs. 3.45e&f Ultrastructure of the root meristem of explants cryoprotected in cryoprotectants made up in cathodic water, while Figs. 3.45g&h shows the ultrastructure of their shoot meristems. Fig. 3.45a shows well developed mitochondria (M) and dense lipid bodies (L). Well-developed nuclei (N), nucleoli (nu) and dense lipid bodies (L) are shown in Fig. 3.45b. Fig. 3.45c shows long profiles of endoplasmic reticulum (ER) and starch grains (S) while mitochondria (M), starch (S) and dense lipid bodies (L) are shown in Fig. 3.45d. Fig. 3.45e shows numerous dense lipid bodies (L) and Golgi body indicating active protein synthesis. Fig. 3.45f shows numerous dense lipid bodies (L) and numerous vesicles (ve) (indicated by arrows) associated with the cell wall (cw). Fig. 3.45g shows poorly developed mitochondria (lack of cristae) (M) and vesicles (indicated by arrows) scattered in the cytomatrix while Fig. 3.45h shows dense lipid bodies (L) and poorly developed mitochondria (M) in the cytoplasm of the shoot meristem.



3.11.1.3 Ultrastructure of excised explants after flash drying and rehydration in either CaMg or cathodic water

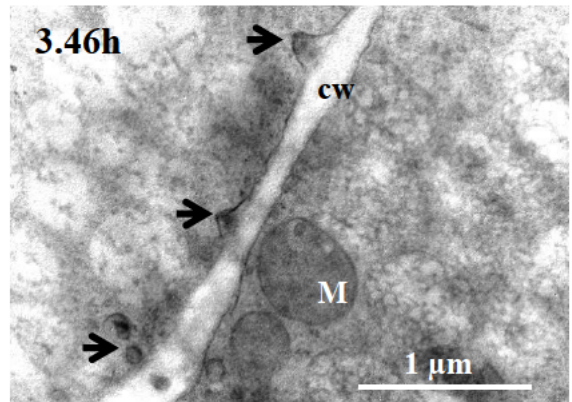
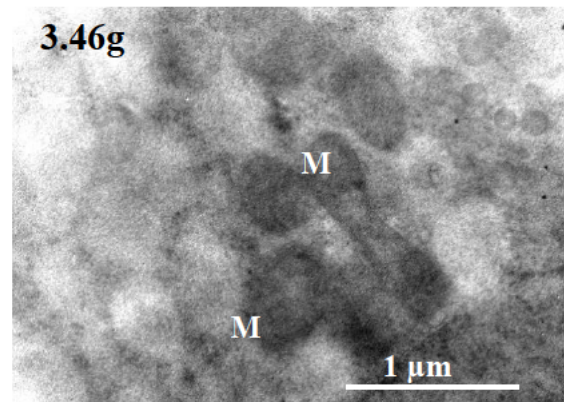
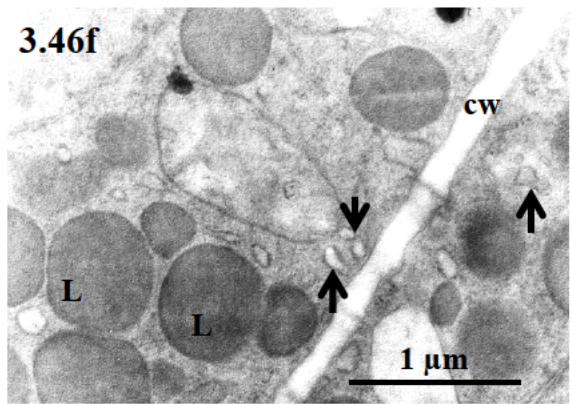
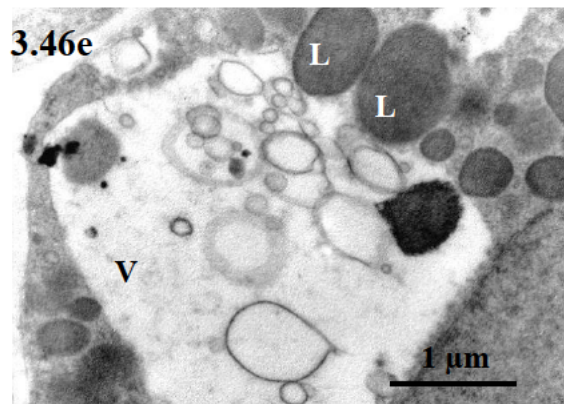
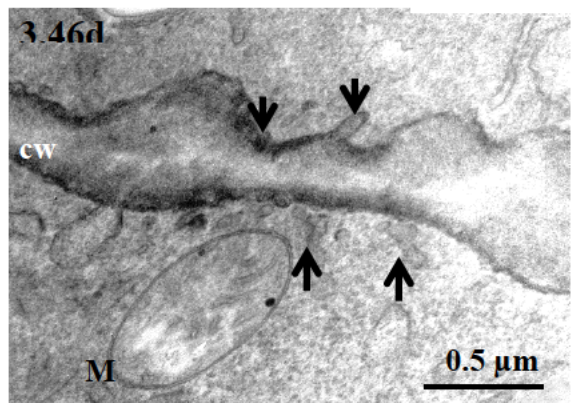
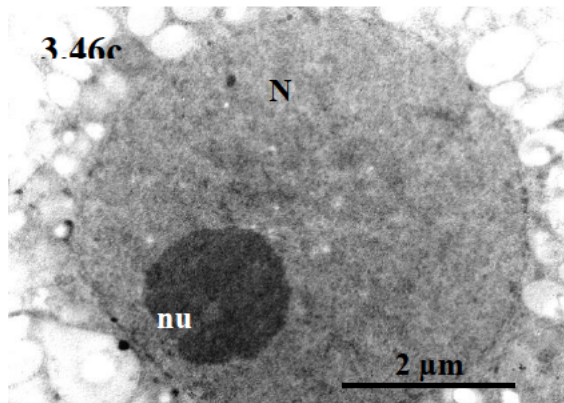
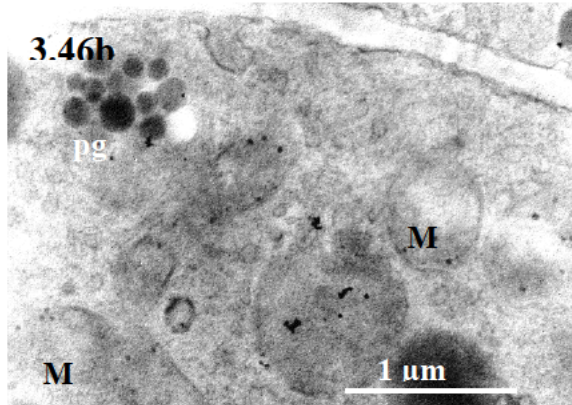
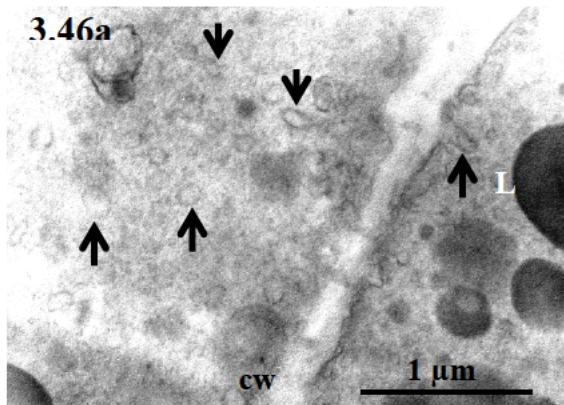
The effect of flash drying and rehydration on root and shoot ultrastructure was assessed (Figs. 3.46a-h). The explants were rehydrated in either CaMg or cathodic water.

After 10 min of conventional flash drying and 30 min rehydration in CaMg, the ultrastructure of the root meristem was comprised of cells that showed well defined organelles. Dense lipid bodies (Figs. 3.46a) as well as plastoglobuli within well developed plastids were observed (Fig. 3.46b). The accumulation of these reserves points toward active metabolism. A feature of the meristem was numerous poorly developed mitochondria (Fig. 3.46b). Numerous vesicles were scattered throughout the cytomatrix and were closely associated with the plasma membrane (indicated by arrows) (Fig. 3.46a). The ultrastructure of the shoot meristem was generally comprised of well developed nuclei, nucleoli (Fig. 3.46c) and mitochondria showing well developed cristae (Fig. 3.46d). Compared with the root meristem, the lipid bodies were less dense in the shoot meristem. Increased vesicle activity was observed in the vicinity of the plasma membrane (Fig. 3.46d).

After rehydration in cathodic water, assessment of the root meristem showed vacuoles that contained numerous vacuolar inclusions (Fig. 3.46e) indicating that cellular components were being recycled by the lytic enzymes contained therein (Lamb and Berjak, 1981). Numerous dense lipid bodies (Figs. 3.46f) present throughout the cytomatrix. Generally, the degree of mitochondrial development seemed to have advanced, displaying well developed cristae. Well developed, elongated mitochondria (Fig. 3.46g) were observed. Numerous well developed vesicles (indicated by arrows) which were closely associated with the plasma membrane were noted throughout the cytomatrix (Fig. 3.46f). The ultrastructure of the shoot meristem was characterised by a dense, granulated cytomatrix (Figs. 3.46g&h). Numerous lipid bodies were noted throughout the cytomatrix, however, these were less dense than those observed in the root meristem (Figs. 3.46g&h). Vesicles were prominent (indicated by arrows) (Fig. 3.46h) throughout the cytomatrix and in the vicinity of the plasmalemma.

Generally, the ultrastructure of both the root and shoot meristems were well preserved after flash drying and rehydration (in CaMg or cathodic water).

Figures 3.46a&b Ultrastructure of the root meristem of explants that were flash dried and rehydrated in CaMg and Figs. 3.46c&d shows that of the shoot meristem. Figs. 3.46e&f shows the ultrastructure of the root meristem of explants that were flash dried and rehydrated in cathodic water and Figs. 3.46g&h shows that of the shoot meristem. Fig. 3.46a shows numerous vesicles (indicated by arrows) in the cytoplasm and in the vicinity of the cell wall (cw) as well as dense lipid bodies (L). Fig. 3.46b shows numerous poorly developed mitochondria (M) in the cytoplasm and dense plastoglobuli (pg). Figs. 3.46c shows a well developed nucleus (N) with a prominent nucleolus (nu). Fig. 3.46d shows a well developed mitochondrion (M) showing prominent cristae. Vesicles (indicated by arrows) were closely associated with the cell wall (cw). Fig. 3.46e shows numerous dense lipid bodies (L) within the cytomatrix. The vacuole (V) is littered with vacuolar inclusions. Fig. 3.46f shows numerous well developed vesicles (indicated by arrows) closely associated with the plasma membrane of the cell wall (cw). The cytoplasm was characterised by dense lipid bodies (L). Fig. 3.46g from the shoot meristem shows elongated, dense mitochondria (M). Fig. 3.46h shows dynamic vesicle activity which is closely associated with the plasma membrane of the cell wall (cw). Dense mitochondria (M) were visible.



3.11.1.4 Ultrastructure of excised explants after cryoprotection, flash drying and rehydration in either CaMg or cathodic water

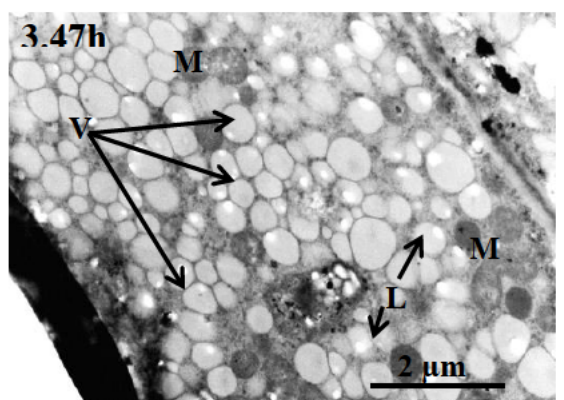
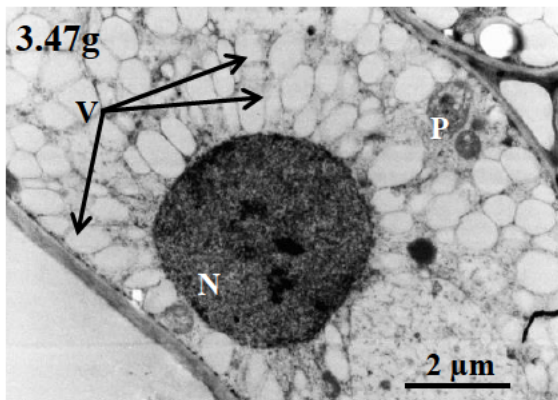
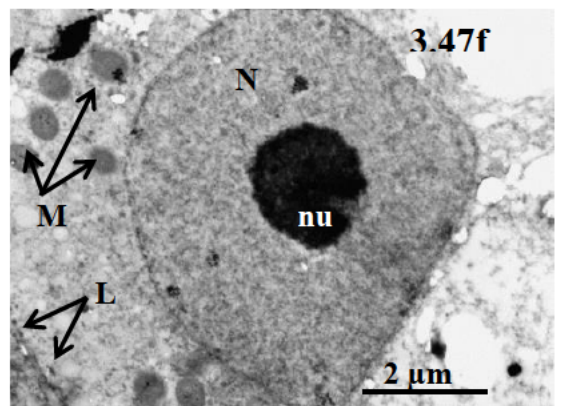
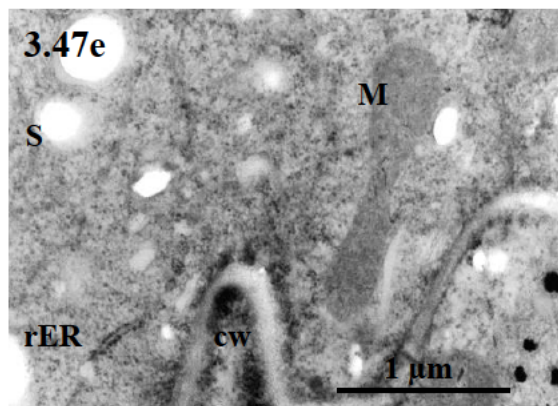
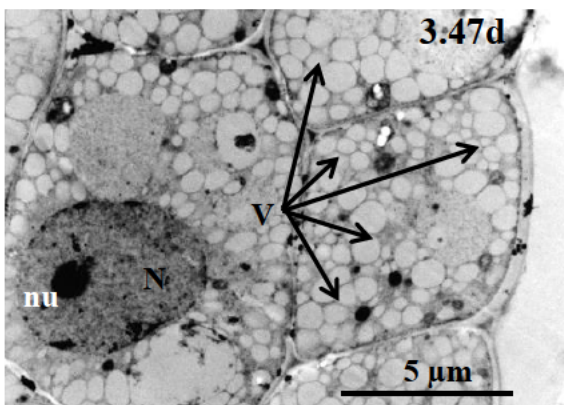
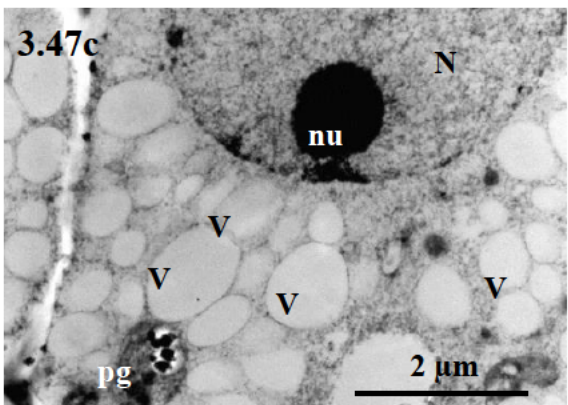
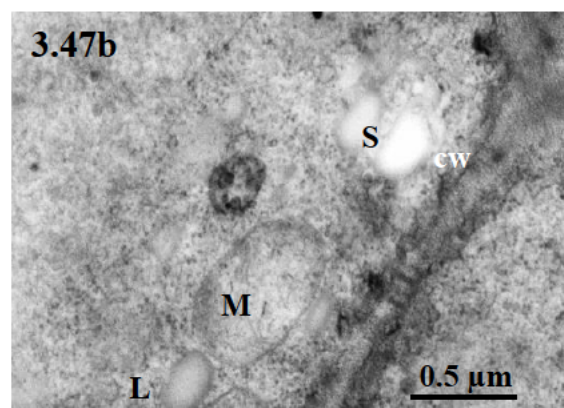
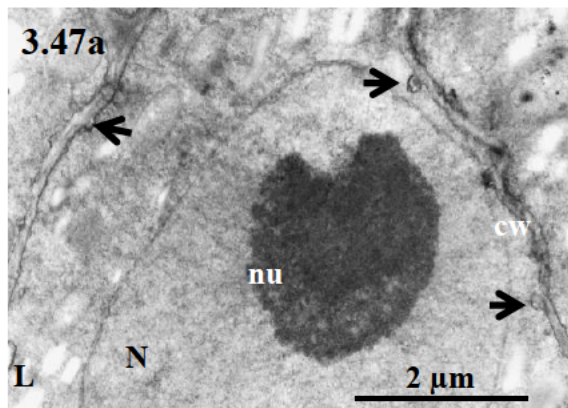
The explants were immersed in cryoprotectants as described in section 2.7, flash dried for 10 min then rehydrated in either CaMg or cathodic water. The ultrastructure of the roots and shoots are presented in Figs. 3.47a-h. When the explants were rehydrated in CaMg, the root ultrastructure did not show any signs of stress or deterioration. Well developed nuclei and nucleoli (Fig. 3.47a) were evident within the meristematic cells. Vesicles were closely associated with the plasma membrane (indicated by arrows) (Fig. 3.47a) and within the cytoplasm. Compared with the dense lipid bodies observed when the axes were flash dried and rehydrated, these appeared less dense and in some cases electron translucent implying active metabolism of reserve material (Figs. 3.47a&b). Mitochondria were scattered throughout the cytomatrix, however, these were poorly developed with poorly developed cristae (Fig. 3.47b). Well developed plastids with plastoglobuli were also observed (Fig. 3.47c). The ultrastructure of the tunica layer of the shoot meristem was largely preserved with well developed cell walls and organelles (Figs. 3.48c&d). However, there were indications that the tissue was experiencing stress as a high degree of vacuolation was observed (Fig. 3.47c&d). Despite this, well developed nuclei with numerous patches of heterochromatin (indicated by arrows) within the nucleoplasm and very dense nucleoli were noted (Figs. 3.47c&d).

After rehydration in cathodic water, the root ultrastructure showed short profiles of rough endoplasmic reticulum (Fig. 3.47e) suggesting active but subdued protein synthesis. Well developed nuclei and nucleoli (Fig. 3.48f) were observed. Numerous mitochondria (Figs. 3.47e&f) were observed throughout the cytoplasm. Numerous small, electron translucent lipid bodies were observed within the cytoplasm (Fig. 3.47f). The shoot meristem showed a high degree of vacuolation implying that the tissue was experiencing stress (Figs. 3.47g&h), although intact organelles were noted. These include well developed nuclei (Fig. 3.47g), plastids (Fig. 3.47g), mitochondria (Fig. 3.47h) and dense lipid bodies (Fig. 3.47h) indicating ongoing metabolism.

Taken together, ultrastructural observations of the explants from this treatment showed that the root meristematic cells were well preserved with dense cytomatrices, while the shoot

meristems displayed a high degree of vacuolation, suggesting that they were experiencing stress.

Figures 3.47a&b Ultrastructure of root meristem from explants that were cryoprotected, flash dried and rehydrated in CaMg. Figs. 3.47c&d shows the ultrastructure of the shoot meristem from this treatment. Figs. 3.47e&f shows the ultrastructure of root meristem from explants that were cryoprotected, flash dried and rehydrated in cathodic water. Figs. 3.47g&h shows the ultrastructure of their shoot meristems. Fig. 3.47a shows a well developed nucleus (N) with a prominent nucleolus (nu) and vesicles (indicated by arrows) closely associated with the plasmalemma. Electron translucent lipid bodies (L) suggest reserve utilisation. Fig. 3.47b shows a poorly developed mitochondrion (M). Less dense lipid bodies (L) and starch grains (S) were present in the cytomatrix. Fig. 3.47c shows a well developed nucleus (N) and nucleolus (nu). Dense plastoglobuli (pg) were noted in the cytomatrix. Numerous vacuoles (V) were evident. Fig. 3.47d shows a well developed nucleus (N) with numerous vacuoles (V) present throughout the cytomatrix of the tunica cell layer. Fig. 3.47e shows elongated mitochondria (M) and short profiles of rough endoplasmic reticulum (rER). Fig. 3.47f shows dense lipid bodies (L) in the cytomatrix. The nucleus (N) and nucleolus (nu) were well developed. Fig. 3.47g shows a high degree of vacuolation (V) in the cytoplasm of the shoot meristem, however, the nucleus (N) does appear dense and well developed. Fig. 3.47h shows highly vacuolated (V) cells in response to stress. Some organelles in the form of mitochondria (M) and dense lipid bodies are visible.



3.11.1.5 Ultrastructure of excised explants after cryoprotection, flash drying, cooling and retrieval in either CaMg or cathodic water

As described in chapter 2, explants were immersed in cryoprotectants, flash dried, cooled in LN slush and retrieved in either warm (40°C) CaMg or cathodic water. The ultrastructure of the root meristem of explants retrieved in CaMg was well preserved and typical of metabolically active tissue. Well developed nuclei with patches of heterochromatin (indicated by arrows) and nucleoli (Fig. 3.48a) were observed in the cytoplasm. Numerous mitochondria were observed throughout the cytomatrix (Figs. 3.48a&b). The cell walls were well developed with numerous associated vesicles (indicated by arrows) (Fig. 3.48a). Lipid bodies appeared electron translucent indicating an uptake of storage reserves presumably due to active metabolism (Fig. 3.48a). In contrast, the shoot meristem appeared to be highly vacuolated with no discernible organelles (Fig. 3.48c&d). In some cases, there appears to be large empty cavities within the cells, presumably where the vacuole once was (Fig. 3.48d).

When the explants were rehydrated in cathodic water, the root meristem cells showed well preserved, metabolically active ultrastructure (Figs. 3.48e&f). Well developed nuclei with heterochromatin patches (indicated by arrows) and nucleoli were noted throughout the cytomatrix (Fig. 3.48f). Numerous dense mitochondria were scattered throughout the dense cytoplasm (Figs. 3.48e&f). The lipid bodies appeared devoid of contents as indicated by their electron translucent appearance implying uptake of reserves during metabolism (Fig. 3.48e). The shoot meristem on the other hand appeared to be highly vacuolated. The cells were comprised of a few large vacuoles and numerous lipid bodies that filled the entire cytoplasm (Figs. 3.48g&h). There were no discernible organelles within the cytoplasm. The large vacuoles contained dense vacuolar inclusions, presumably another stress response i.e. accumulation of cellular contents in the vacuole (Figs. 3.48g&h).

After cooling and recovery, the ultrastructure of the root meristem was well preserved and was typical of metabolically active tissue, however, the shoot meristem showed a high degree of vacuolation and had disintegrated with no discernible organelles. Generally, the shoot meristem of the cathodic water retrieved explants was better preserved than their CaMg retrieved counterparts (Table 3.18). The improved preservation of shoot ultrastructure in the cathodic water retrieved explants resulted in 30% of these explants producing shoots.

Figures 3.48a&b Ultrastructure of root meristem from explants that were cryoprotected, flash dried, cooled in LN and rehydrated in CaMg. Figs. 3.48c&d shows the ultrastructure of the shoot meristem from this treatment. Figs. 3.48e&f shows the ultrastructure of root meristem from explants that were cryoprotected, flash dried, cooled in LN and rehydrated in cathodic water. Figs. 3.48g&h shows the ultrastructure of their shoot meristems. Fig. 3.48a shows well developed nuclei (N) with patches of heterochromatin (indicated by arrow) and dense nucleoli (nu). Dense mitochondria (M) were visible as well as numerous vesicles (indicated by arrows). Fig. 3.48b shows dense mitochondria (M) in a granulated cytoplasm (C) with well developed cell walls (cw). Fig. 3.48c&d shows highly vacuolated (V) shoot meristem cells with no discernible organelles. Fig. 3.48d shows meristem cells with large vacuoles and numerous lipid bodies present throughout the cytomatrix. Fig. 3.48e shows dense, well developed mitochondria (M) and electron translucent lipid bodies (L). Fig. 3.48f shows a well developed nucleus (N) with a prominent nucleolus (nu) and heterochromatin patches (indicated by arrows) within the nucleoplasm. Dense mitochondria (M) were distributed within the cytomatrix. Figs. 3.48g&h shows a high degree of vacuolation (V) with dense vacuolar inclusions and numerous lipid bodies (L).

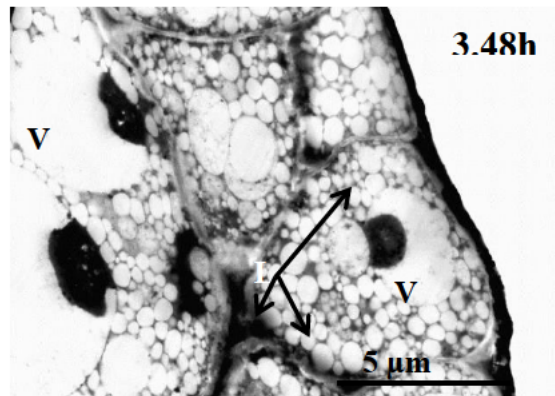
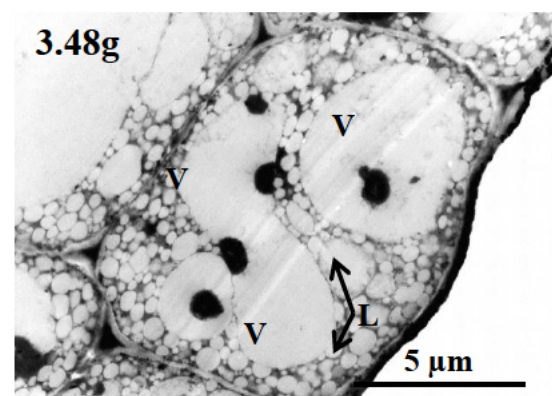
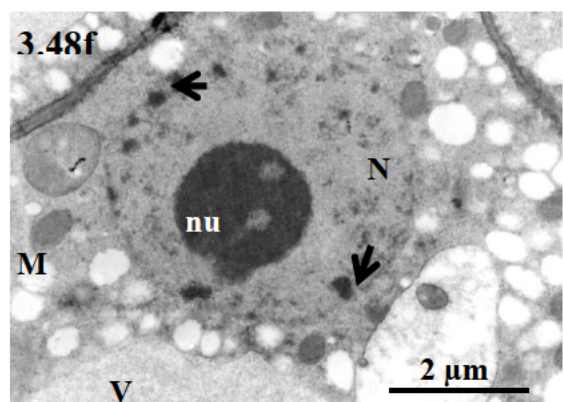
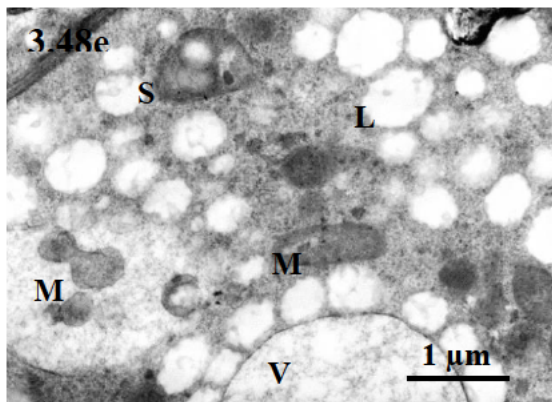
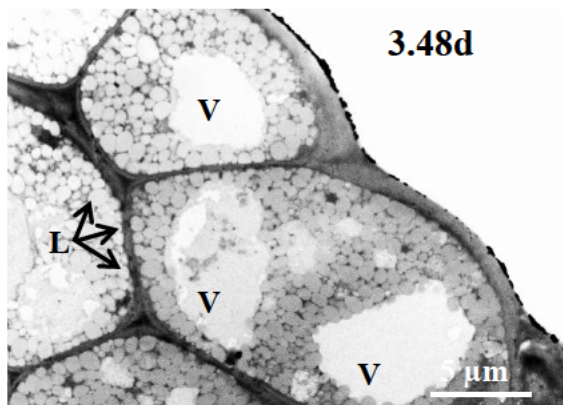
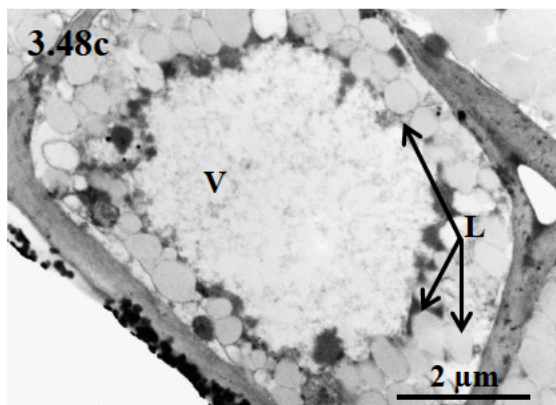
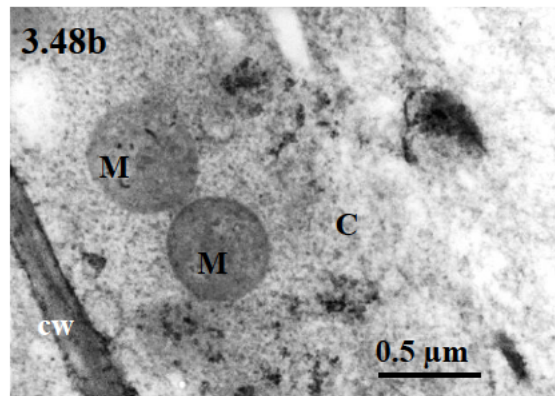
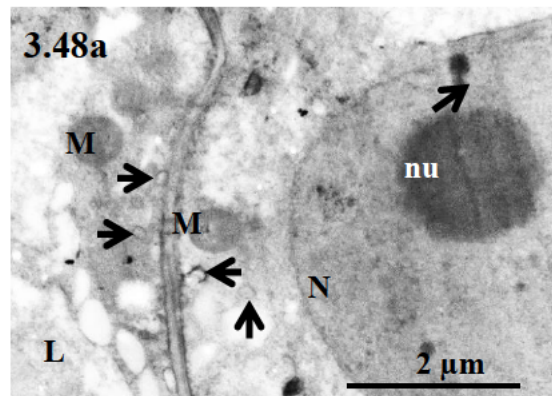


Table 3.18 The effect of cathodic water on root and shoot ultrastructure in explants of Port Elizabeth (PE) provenance after procedural steps of cryopreservation (E: excision; CP: cryoprotection; FD: flash drying; R: rehydration; C: cooling; Ret.: retrieval).

	E+CP		E+FD+R		E+CP+FD+R		E+CP+FD+C+Ret	
	d.H ₂ O	c.H ₂ O	CaMg	c.H ₂ O	CaMg	c.H ₂ O	CaMg	c.H ₂ O
Roots	Normal with numerous mitochondria and well developed organelles.	Normal with well-developed organelles.	Well defined organelles with dense lipid bodies and poorly developed mitochondria.	Advanced mitochondrial development. Increased vacuolar inclusions.	Well-developed nuclei present. Active metabolism of reserve material. Poorly developed mitochondria.	Well-developed nuclei. Short profiles of ER indicating subdued protein synthesis.	Ultrastructure was well preserved and metabolically active.	Organelles were well developed. Tissue indicated active metabolism.
Shoots	Normal with numerous lipid bodies long profiles of ER.	Normal with well preserved ultrastructure containing poorly developed mitochondria.	Well-developed nuclei, and mitochondria. Increased vesicle activity.	Dense cytomatrix containing well developed mitochondria. Enhanced vesicle activity.	Ultrastructure and organelles preserved but increased vacuolation. On average, there were 1.5X more vacuoles per cell compared with the cathodic water rehydrated explants. Average vacuole size: 3µm. Approximately 4 lipid bodies per cell.	Intact organelles observed but vacuolation increased. Average vacuole size: 5µm. Approximately 10 lipid bodies per cell.	Highly vacuolated. No discernible organelles. Large empty cavities in the cell. On average, there were twice as many vacuoles per cell compared with the cathodic water rehydrated explants. Average vacuole size: 2µm. Approximately 6 lipid bodies per cell.	Highly vacuolated with dense inclusions. No discernible organelles but the ultrastructure was generally better preserved than CaMg rehydrated explants. Average vacuole size: 4µm. Approximately 12 lipid bodies per cell.
Viability (%)	90	90	50	60	30	50	0	30

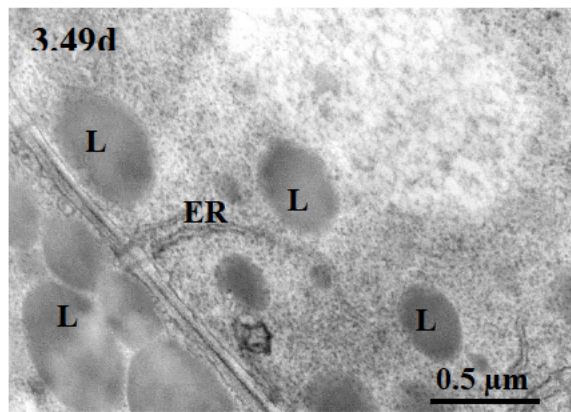
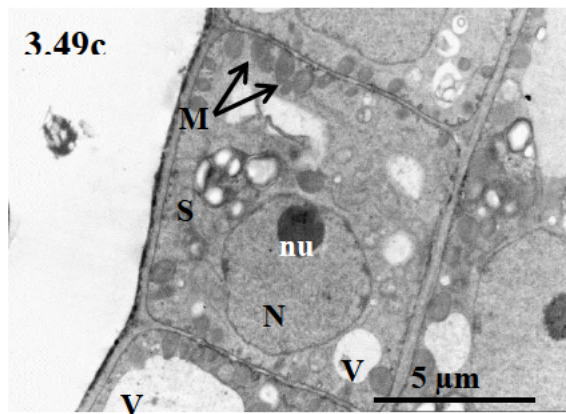
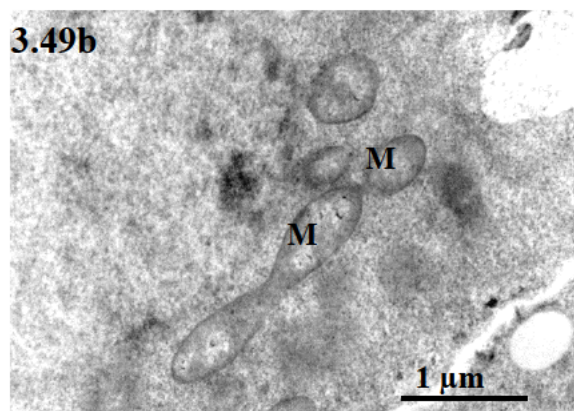
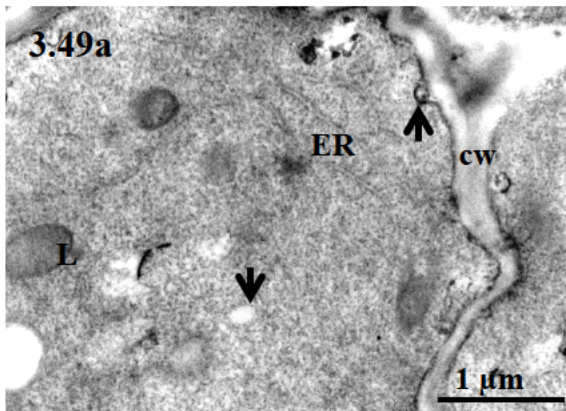
3.11.2 St Lucia

3.11.2.1 Ultrastructure of untreated, fresh (excised) explants

The ultrastructural integrity of the root and shoot meristem from freshly excised explants were assessed. The ultrastructure of the root meristem was congruent with active metabolic processes. Numerous short profiles of endoplasmic reticulum (Fig. 3.49a) scattered throughout the cytomatrix imply active protein synthesis and translation. Vesicles associated with the plasma membrane were clearly visible throughout the cytoplasm indicating active signal transduction and very likely, membrane turnover (Fig. 3.49a). Well developed, actively dividing mitochondria were observed regularly in the cytomatrix (Fig. 3.49b).

The ultrastructure of the shoot meristem was characterised by well developed nuclei and nucleoli with dense patches of heterochromatin within the nucleoplasm (Fig. 3.49c). Starch granules (Fig. 3.49c) were also observed (although less frequently) in the cytoplasm. The cells were usually comprised of a single large vacuole (Fig. 3.49c). Well-developed rough endoplasmic reticulum was observed in the cytomatrix (Fig. 3.49d). Dense lipid bodies (Fig. 3.49d) were arranged uniformly along the plasma membrane. Generally, the ultrastructure of both root and shoot meristem was well preserved with well-developed organelles indicating metabolically active tissue.

Figures 3.49a&b Ultrastructure of root meristem while Figs. 3.49c&d shows the ultrastructure of shoot meristem from freshly excised axes. The root ultrastructure shows well-developed mitochondria (M) and a prominent endoplasmic reticulum (ER). Vesicle activity (indicated by arrows) was evident. The shoot ultrastructure showed signs of active metabolism with well-developed nuclei (N) and nucleoli (nu). Dense lipid bodies (L) were present throughout the cytomatrix and along the periphery of the plasma membrane. Cells were typically comprised of single large vacuoles (V). Long profiles of endoplasmic reticulum (ER) were clearly visible.



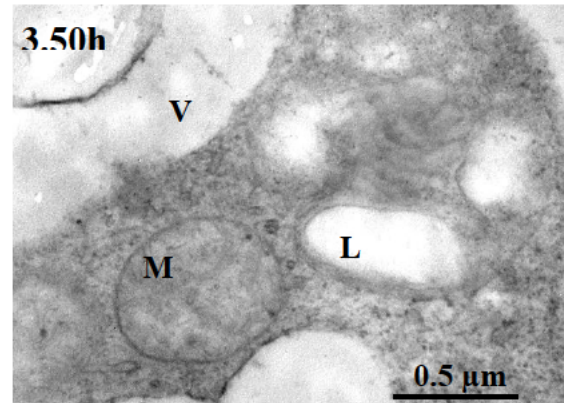
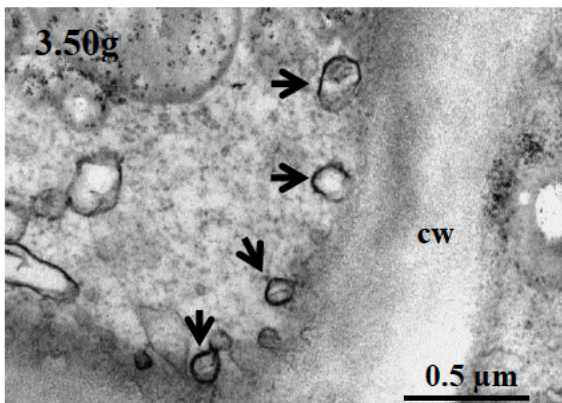
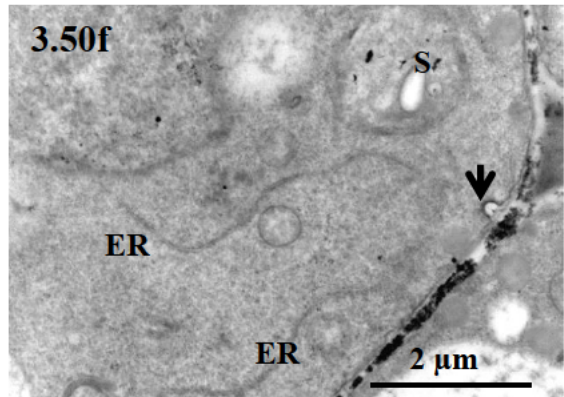
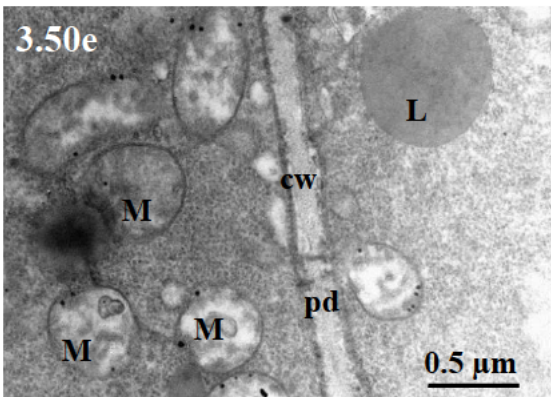
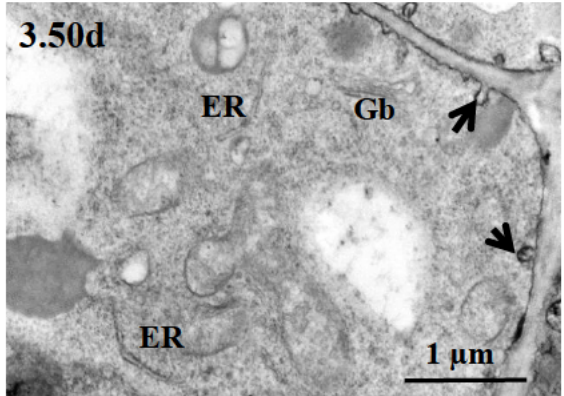
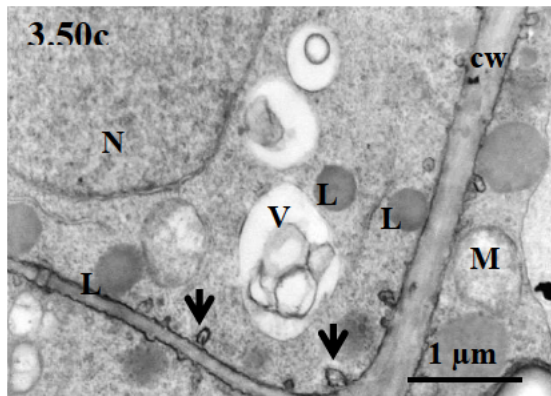
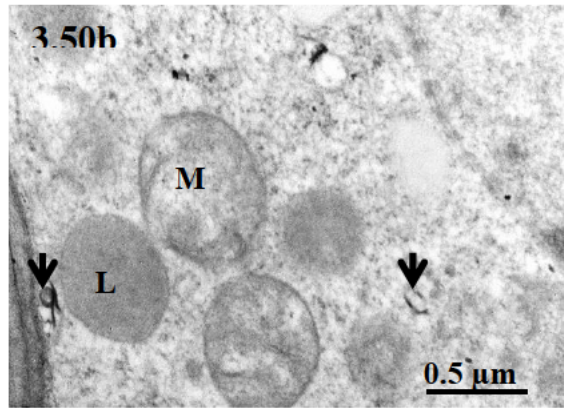
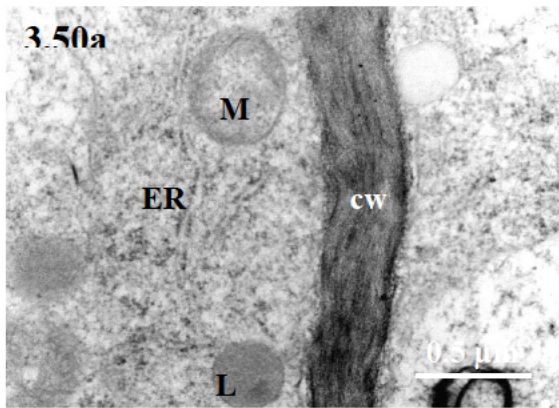
3.11.2.2 Ultrastructure of excised explants cryoprotected in cryoprotectants made up in either distilled or cathodic water

The explants were treated with cryoprotectants which were made up in either distilled or cathodic water. After immersion in distilled water prepared cryoprotectants, the ultrastructure of root meristematic cells was well preserved and showed well developed organelles. Although poorly developed, numerous mitochondria were visible throughout the cytomatrix (Figs. 3.50a&b). Short profiles of rER (Fig. 3.50a) were observed in the meristem pointing towards active protein synthesis in these cells. Vesicles (Fig. 3.50b) were associated with the plasma membrane and within the cytoplasm. Dense lipid bodies (Fig. 3.50a&b) occurred throughout the cytoplasm indicating an import of storage reserves presumably to fuel ongoing metabolism. The cells of the shoot meristem cells were metabolically active as indicated by prominent nuclei (Fig. 3.50c) with well-developed nuclear membranes. Extensive vesicle activity was noted (Figs. 3.50c&d). These were closely associated with the plasma membrane (Figs. 3.50c&d). Similar to the mitochondria observed in the root meristem, those in the shoot meristem were also poorly developed (Fig. 3.51c). Numerous short profiles of ER (Fig. 3.50d) and well developed Golgi bodies (Fig. 3.50d) indicating active protein synthesis and membrane turnover. Dense lipid bodies (Fig. 3.50d) were scattered throughout the cytoplasm.

When the explants were treated with cathodic water prepared cryoprotectants, the ultrastructure of the root meristem did not show any signs of stress. These cells had well-developed cell walls with well-defined plasmodesmata (Fig. 3.50e). Numerous poorly developed and electron translucent mitochondria (Fig. 3.50e) were scattered throughout the cytoplasm. Long ER profiles (Fig. 3.50f) were observed, indicating active protein synthesis. Storage reserve compounds in the form of dense lipid bodies (Fig. 3.51e) and starch (Fig. 3.50f) were present in the meristem cells. Vesicles were observed in the cytoplasm (Fig. 3.50f) and also in close association with the plasma membrane (Fig. 3.50f). The ultrastructure of the shoot meristem showed signs of typical metabolically active tissue. Numerous well-developed vesicles were closely associated with the plasma membrane (Fig. 3.50g) and in the cytoplasm (Fig. 3.50g). The cells were comprised of large, single vacuoles (Fig. 3.50h). Mitochondria were observed throughout the cytomatrix, however, these were well developed as defined by prominent cristae (Fig. 3.50h). Compared with those observed in the root meristem, lipid bodies in the shoot meristem appeared less dense, presumably due to reserve utilisation during metabolic processes (Fig. 3.50h). Generally, the ultrastructure from root

and shoot meristem of the explants that were treated with both distilled and cathodic water prepared cryoprotectants did not show any sign of stress or degradation. They instead displayed tissue that was typical of high metabolic activity with well-developed organelles.

Figures 3.50a&b Ultrastructure of the root meristem from explants that were immersed in cryoprotectants dissolved in distilled water, while Figs. 3.50c&d shows the ultrastructure of the shoots from this treatment. The root meristem showed a well preserved ultrastructure with short profiles of endoplasmic reticulum (ER) and numerous electron translucent mitochondria (M). Dense lipid bodies (L) and vesicles (indicated by arrows) were present throughout the cytomatrix. The shoot meristem displayed an ultrastructure typical of metabolically active tissue with well developed nuclei (N), long profiles of endoplasmic reticulum (ER) and prominent Golgi bodies (Gb). Numerous vesicles (indicated by arrows) were associated with the plasma membrane indicating either endocytotic or exocytotic activity. Dense lipid bodies (L) were present throughout the cytomatrix. Figs. 3.50e&f shows the ultrastructure of root meristem from explants that were treated with cryoprotectants made up in cathodic water. The ultrastructure was well preserved with long profiles of endoplasmic reticulum (ER) and poorly developed mitochondria (M). Large, well-developed plastids with starch grains (S) were present in the cytoplasm. Vesicle activity (indicated by arrows) was evident. Figs. 3.50g&h shows the ultrastructure of the shoot meristem from this treatment. Profuse vesicle activity (indicated by arrows) was noted. Mitochondria appeared to be at an advanced developmental stage showing prominent cristae. These cells were comprised of single, large vacuoles (V) and electron translucent lipid bodies (L).



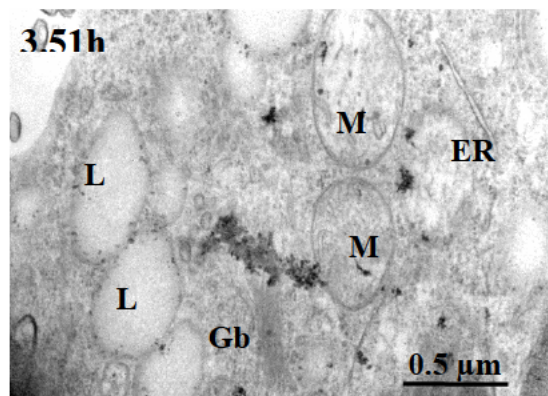
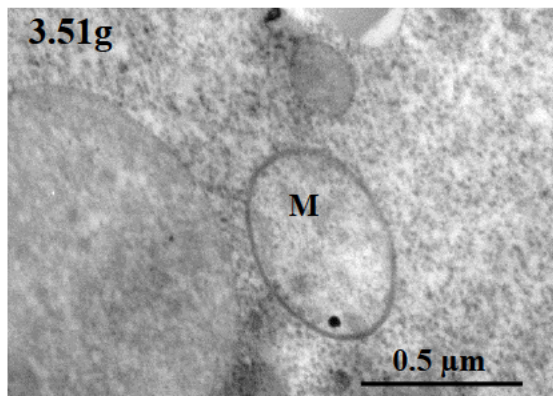
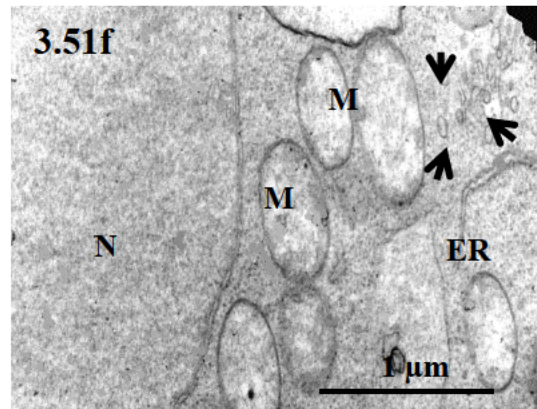
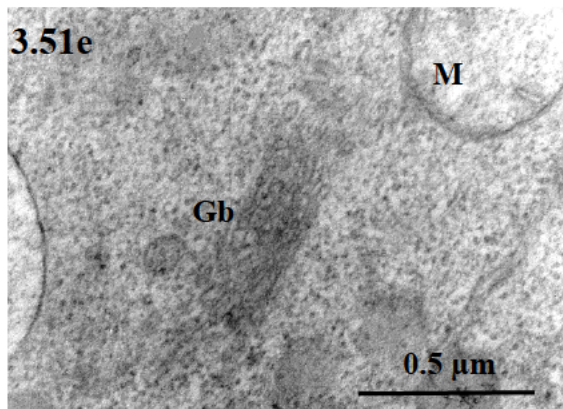
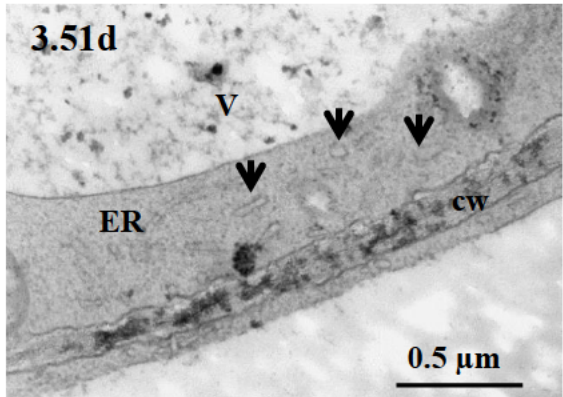
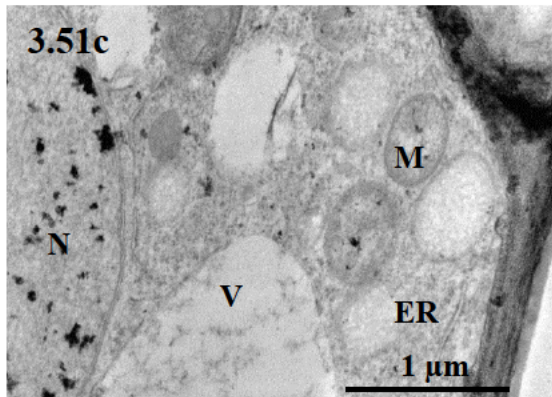
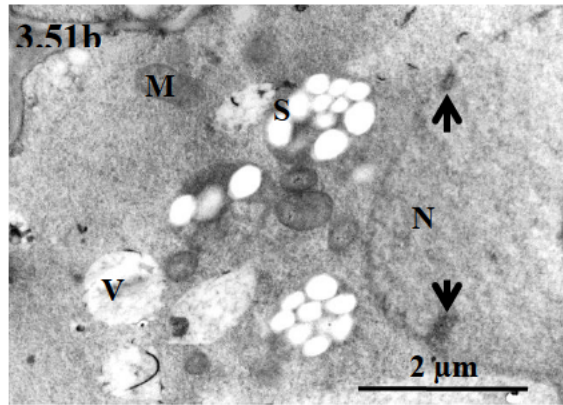
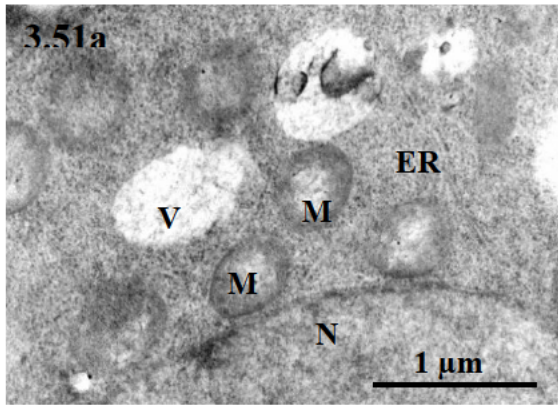
3.11.2.3 Ultrastructure of excised explants after flash drying and rehydration in either CaMg or cathodic water

The explants were flash dried and subsequently rehydrated in either CaMg or cathodic water. The ultrastructure of the root and shoot meristem from each of these treatments was assessed. The ultrastructure of the root meristem from CaMg rehydrated explants was dense and typical of metabolically active tissue, indicated by long profiles of ER (Fig. 3.51a) and numerous mitochondria (Fig. 3.51a). The mitochondria were poorly developed (no discernible cristae) and electron translucent (Fig. 3.51a). Numerous well-developed plastids with starch grains were present within the cytomatrix (Fig. 3.51b). Nuclei (Figs. 3.51a&b) were prominent throughout the cytomatrix with dense patches of heterochromatin in the nucleoplasm (Fig. 3.51b). The tunica layer of cells in the shoot meristem had well preserved ultrastructure. Numerous mitochondria, although electron translucent, were observed throughout the cytomatrix (Fig. 3.51c). Short profiles of ER were scattered within the cytomatrix (Fig. 3.51d) indicating protein synthesis activity. Cell walls were well developed with numerous closely associated vesicles (Fig. 3.51d).

After rehydration in cathodic water, the meristem of the root ultrastructure showed enhanced activity of the endomembrane system evidenced by the enhanced activity of the Golgi apparatus (Fig. 3.51e) and numerous vesicles (Fig. 3.51f) in the cytoplasm. Numerous electron translucent mitochondria (Figs. 3.51f), with the odd one (Fig. 3.51e) displaying well developed cristae implying an advanced developmental state. Short ER profiles (Fig. 3.51f) were observed in the cytomatrix implying protein synthesis and/or translation. The nuclei (Fig. 3.51f) were well-developed displaying the typical intact double membrane structure of the nuclear envelope. The ultrastructure of the shoot meristem was comprised of numerous electron translucent mitochondria within the cytomatrix (Fig. 3.51g&h). Metabolically active tissue with clearly visible Golgi bodies and its associated vesicles (Fig. 3.51h) were observed. It seems that storage reserves in the form of lipid (Fig. 3.51h) were imported into the meristem, presumably to fuel ongoing metabolism.

Overall, the ultrastructure of both root and shoot meristem displayed well preserved, metabolically active cells, irrespective of the rehydration medium.

Figures 3.51a&b Ultrastructure of the root meristem after it was flash dried and rehydrated in CaMg. Cells appeared metabolically active with numerous long profiles of endoplasmic reticulum (ER) and well-developed nuclei (N) that contained patches of heterochromatin (indicated by arrows). Large, well-developed plastids with numerous starch grains (S) were observed throughout the cytomatrix. Mitochondria (M) were poorly developed and electron translucent. Figs. 3.51c&d shows the ultrastructure of the shoot meristem after this treatment. The cytoplasm was comprised of single, large vacuoles (V) and numerous mitochondria (M) that did not show any significant cristae development. Short endoplasmic reticulum (ER) profiles and vesicles (indicated by arrows) were typical of metabolically active tissue. Figs. 3.51e&f shows the ultrastructure of the root meristem after the explants were flash dried and rehydrated in cathodic water. Although some mitochondria (M) appeared electron translucent, others showed well defined cristae. The presence of Golgi bodies (Gb) and vesicles (indicated by arrows) indicates enhanced activity of the endomembrane system. Short profiles of endoplasmic reticulum (ER) were present. Figs. 3.51g&h shows the ultrastructure of the shoot meristem. Mitochondria (M) appeared to be at different developmental stages with some appearing electron translucent and others showing well developed cristae. Although poorly developed, Golgi bodies (Gb) were present in the cytoplasm. Lipid bodies (L) appeared electron translucent implying reserve utilisation.



3.11.2.4 Ultrastructure of excised explants after cryoprotection, flash drying and rehydration in either CaMg or cathodic water

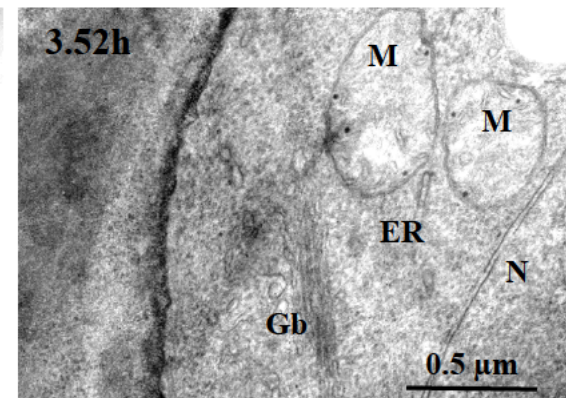
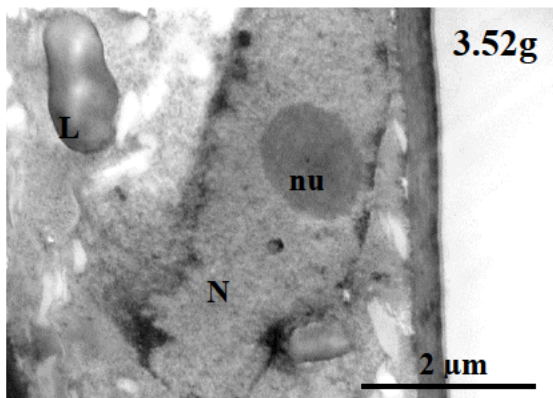
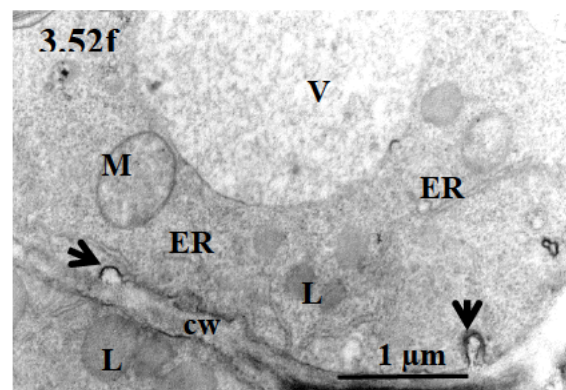
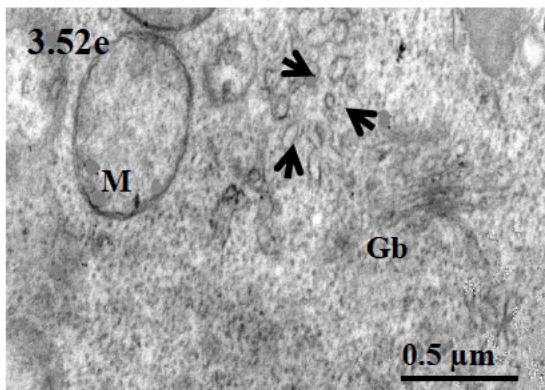
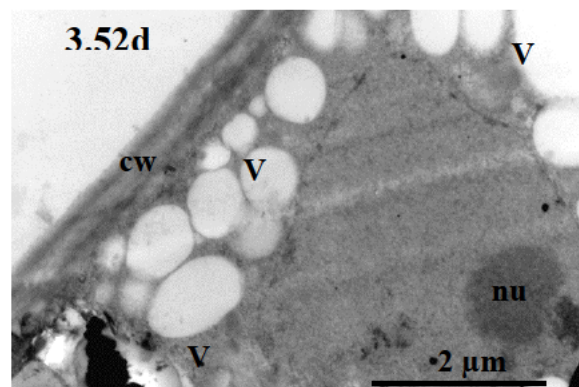
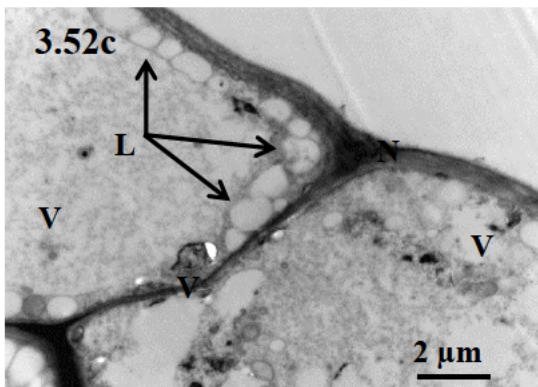
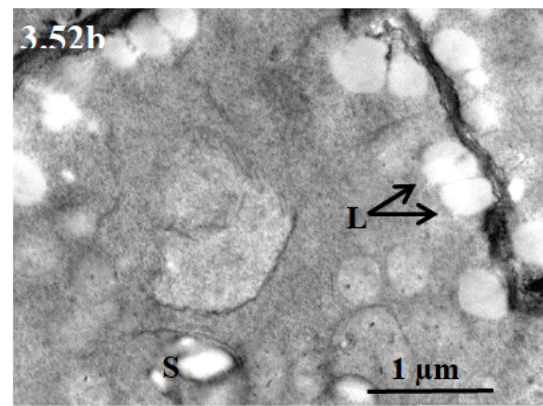
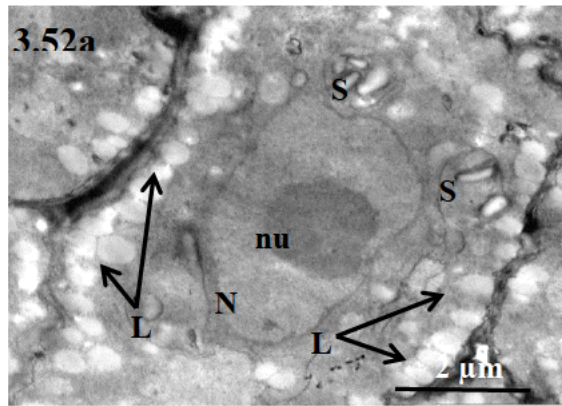
The explants were immersed in cryoprotectants, then flash dried for 20 min and rehydrated in either CaMg or cathodic water (in the dark for 30 min). After rehydration in CaMg, the root meristematic cells were well developed with prominent nuclei and nucleoli (Fig. 3.52a). A striking feature of these cells was the presence of many of lipid bodies that lined the periphery of the cytoplasm along the plasma membranes (Figs. 3.52a&b). This arrangement of lipid was not observed in the previous treatments and maybe linked to an enhanced metabolic state, presumably in response to stress (Pammenter *et al.*, 1998). In addition to lipid, well-developed plastids that contained starch grains were prominent in the cytoplasm (Fig. 3.52a). The cells of the shoot meristem also showed the accumulation of lipid in the peripheral regions of the cells along the plasma membrane (Fig. 3.52c). Vacuolation, a typical stress response was observed in the cytoplasm (Fig. 3.52d), although well developed nuclei and nucleoli were still prominent in the cytoplasm (Fig. 3.52d).

Numerous mitochondria, although poorly developed (Figs. 3.52e&f), were scattered throughout the cytomatrix in root meristematic cells from explants that were rehydrated in cathodic water. Well-developed Golgi bodies, vesicles and long profiles of ER indicating active protein synthesis and/or translation (Figs. 3.52e&f) were typical of an active endomembrane system in metabolically active cells. Dense lipid bodies (Fig. 3.52f) were present throughout the cytoplasm which was comprised of single, large vacuoles (Fig. 3.52f). The ultrastructure of the shoot meristem was well preserved displaying well developed nuclei and nucleoli (Fig. 3.52g). Dense lipid bodies (Fig. 3.52g) were present in the cytoplasm. Well-developed mitochondria with prominent cristae (Fig. 3.52h) indicated an advanced stage of development as required by metabolically active cells. Well-developed Golgi bodies and numerous vesicles indicate enhanced membrane activity. Short profiles of ER were observed in the cytoplasm (Fig. 3.52h) indicating active protein synthesis.

The shoot ultrastructure of the axes that were rehydrated in CaMg appeared to have shown signs of stress by increased vacuolation (Fig. 3.52c), however, this type of response was not observed in the shoots that were rehydrated in cathodic water. Using cathodic water effectively conserved the ultrastructure of the shoot meristem. The root meristem (from both

treatments) on the other hand were largely unaffected by this treatment displaying a well-preserved ultrastructure with well-developed organelles typical of metabolically active tissue.

Figures 3.52 a&b Ultrastructure of the root meristem after cryoprotection, flash drying and rehydration in CaMg. The root meristematic cells showed well developed nuclei (N) and nucleoli (nu). Numerous lipid bodies (L) were observed around the periphery of the cell. Plastids with starch grains (S) were also noted. Figs. 3.52c&d show the ultrastructure of the shoot meristem cells from this treatment. Although the nucleus (N) and nucleolus (nu) appeared well-developed, there was an accumulation of numerous small vacuoles (V) in the cytoplasm (vacuolation) suggestive of stress. Lipid bodies (L) lined the periphery of the cells. Figs. 3.52e&f shows the ultrastructure of root meristematic cells after the explants were immersed in cryoprotectants, flash dried and rehydrated in cathodic water. These cells appeared to be at an advanced development and enhanced metabolic state as shown by the long profiles of endoplasmic reticulum (ER), well-developed Golgi bodies (Gb) and numerous vesicles (indicated by arrows). Dense lipid bodies (L) were present throughout the cytomatrix. Figs. 3.52g&h show the ultrastructure of the shoot meristem from this treatment. The nuclei (N) and nucleoli (nu) were prominent in the cytoplasm. Mitochondria (M) were well-developed with prominent cristae. Golgi bodies (Gb), endoplasmic reticulum (ER) and dense lipid bodies (L) were featured in the cytoplasm.



3.11.2.5 Ultrastructure of excised explants after cryoprotection, flash drying, cooling and retrieval in either CaMg or cathodic water

Explants were immersed in cryoprotectants, flash dried and then tumble cooled in LN slush. They were subsequently retrieved in either warm (40°C) CaMg or cathodic water.

After retrieval in CaMg, the ultrastructure of the root meristem was well preserved and representative of metabolically active tissue. Although poorly developed, numerous mitochondria were observed in the cytoplasm (Fig. 3.53a). The meristem cells were characterised by single, large vacuoles which in some cases contained numerous inclusions (Fig. 3.53b). These inclusions imply that the cellular components were being broken down and recycled back into the cytoplasm (Lamb and Berjak, 1981). Long profiles of ER were noted in the cytomatrix as a consequence of heightened protein synthesis (Fig. 3.53a). Well-developed plastids that contained starch grains and plastoglobuli were observed (Fig. 3.53b). The shoot ultrastructure on the other hand had totally disintegrated with no discernible organelles (Figs. 3.53c&d). The cytoplasm was characterised by increased vacuolation and a destroyed cytoplasm (Figs. 3.53c&d).

After retrieval in cathodic water, the root ultrastructure displayed cell walls that were well preserved with prominent plasmodesmata (Fig. 3.53e). Long profiles of ER were observed in the cytoplasm (Fig. 3.53e). Dense lipid bodies were scattered throughout the cytomatrix (Figs. 3.53e&f). The cells were characterised by well-developed nuclei and nucleoli (Fig. 3.53f). The ultrastructure of the shoot meristem was typical of severely stressed tissue (Figs. 3.53g&h). The cells were highly vacuolated with no discernible organelles.

The ultrastructure of the root meristem after cooling and retrieval did not show any signs of stress or deterioration, irrespective of the recovery medium (i.e. CaMg or cathodic water). However, the shoot ultrastructure was totally disintegrated. Although shoots were not produced after recovery in both CaMg and cathodic water, the ultrastructure of the shoots that were recovered in cathodic water appeared more organised and less disintegrated than those recovered in CaMg (Table 3.19). It is probable that cathodic water had afforded more protection during the recovery step than CaMg.

Figures 3.53a&b Ultrastructure of the root meristem after the explants were immersed in cryoprotectants, flash dried, cooled in LN and recovered in CaMg. These cells appeared to metabolically active as indicated by the numerous mitochondria (M) scattered in the cytoplasm, long profiles of endoplasmic reticulum (ER) and single, large vacuoles (V) that contained some inclusions. Large plastids with starch (S) grains were also observed. The ultrastructure of the shoot meristem from this treatment (Figs. 3.53c&d) showed total destruction with no discernible organelles apart from numerous vacuoles (V) and a very granulated cytoplasm (C) – presumably remnants of cellular components. Figs. 3.53e&f shows the ultrastructure of root meristematic cells that were cryoprotected, flash dried, cooled in LN and recovered in cathodic water. These cells were typical of metabolically active cells with long profiles of endoplasmic reticulum (ER) and dense lipid bodies (L). Cells were comprised of single, large vacuoles (V) and well defined nuclei (N). Vesicles (indicated by arrows) were present in the cytoplasm. Cell walls (cw) were well-developed with distinct plasmodesmata (pd). The cells from the shoot meristem of this treatment (Figs. 3.53g&h) revealed highly vacuolated cells with no discernible organelles.

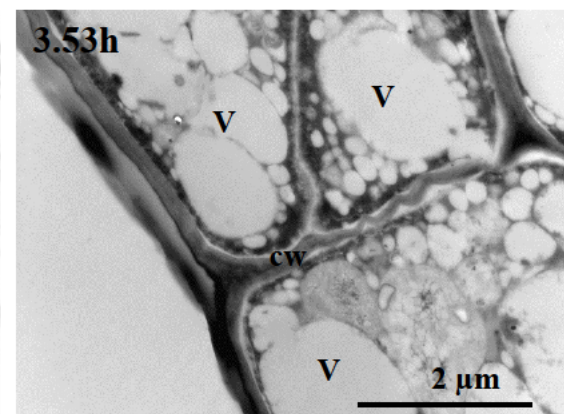
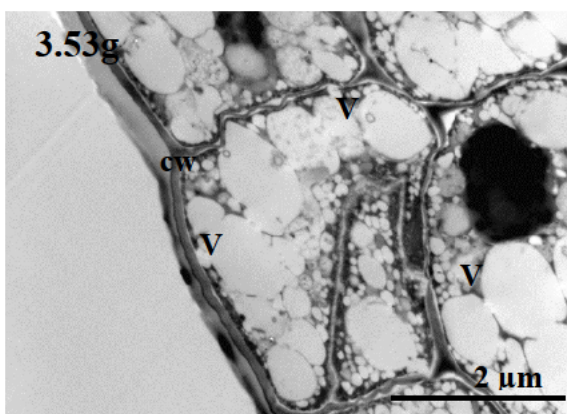
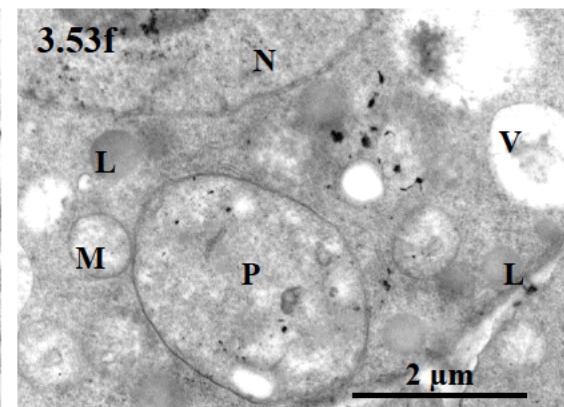
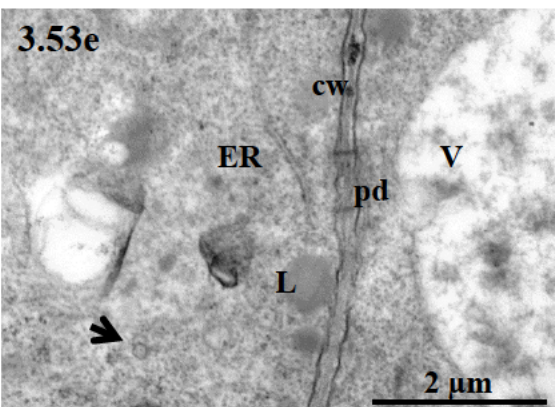
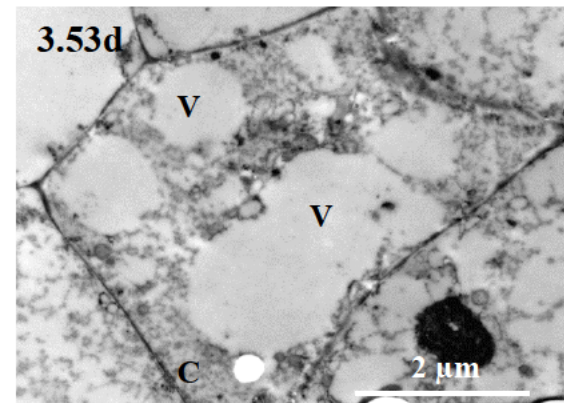
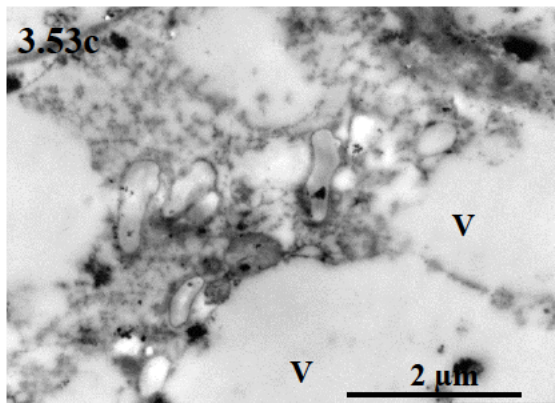
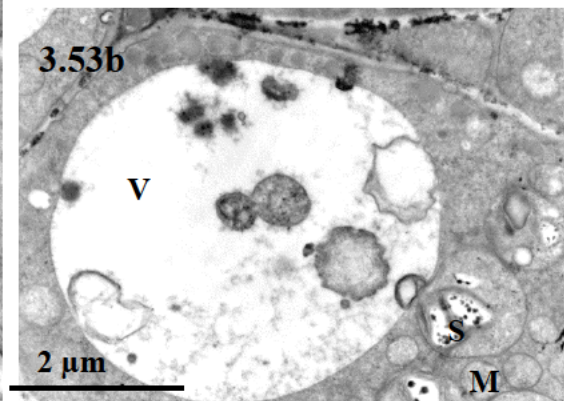
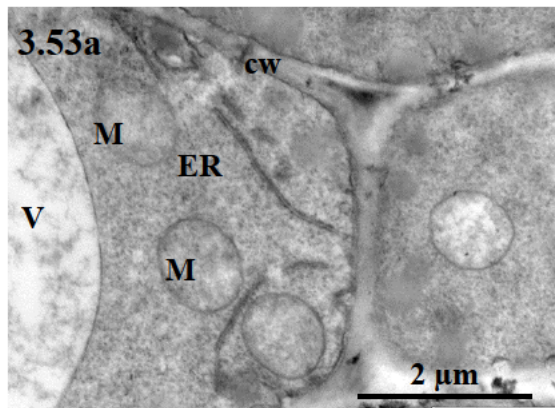


Table 3.19 The effect of cathodic water on root and shoot ultrastructure in explants of *St Lucia* provenance after procedural steps of cryopreservation (E: excision; CP: cryoprotection; FD: flash drying; R: rehydration; C: cooling; Ret.: retrieval).

	E+CP		E+FD+R		E+CP+FD+R		E+CP+FD+C+Ret	
	d.H ₂ O	c.H ₂ O	CaMg	c.H ₂ O	CaMg	c.H ₂ O	CaMg	c.H ₂ O
Roots	Well preserved ultrastructure. Numerous poorly developed mitochondria.	Well-developed plasma membranes with well-defined plasmodesmata. Long profiles of ER.	Long profiles of ER with poorly developed mitochondria.	Advanced mitochondrial development noted. Well-developed Golgi bodies and enhanced vesicle activity.	Cells contained well-developed nuclei and nucleoli. Numerous lipid bodies indicative of an enhanced metabolic state.	Long profiles of ER observed. Organelles were well-developed with numerous dense lipid bodies.	Well-developed ultrastructure with single large vacuoles that contained dense inclusions. Heightened protein synthesis.	Ultrastructure was well preserved with long profiles of ER.
Shoots	Well-developed organelles especially Golgi bodies. Extensive vesicle activity.	Metabolically active meristem. Cells comprised of large, single vacuoles.	Well preserved ultrastructure containing numerous poorly developed mitochondria and short profiles of ER.	Well-developed Golgi apparatus observed. Lipid bodies imported into the cytoplasm.	Well-developed Nuclei and nucleoli were prominent. Cytoplasm became vacuolated.	Ultrastructure was typical of metabolically active tissue. Short profiles of ER were observed.	Ultrastructure had totally disintegrated. No discernible organelles.	Highly vacuolated with no discernible organelles.
Viability (%)	90	90	60	70	40	50	0 (20% root production, 0% shoot production)	0 (30% root production, 0% shoot production)

CHAPTER 4: DISCUSSION

The simplest and most cost-effective approach to storing orthodox seeds requires low relative humidity and temperature. However, this is not possible for recalcitrant seeds because they cannot tolerate desiccation, and some may be chilling sensitive. The findings of this study are discussed below within the context of storage of chilled whole seeds (section A) and cryopreservation of germplasm (section B) of *E. capensis* from different provenances.

A) Chilling storage of whole seeds

4.1 Physiological responses

Pammenter and Berjak (1999) suggest that the storage of intact recalcitrant seeds is impossible for anything but the short-to medium-term. Moderate temperatures (e.g. ~16°C) were recommended, because of the need to maintain seeds hydrated and because some species cannot tolerate low temperatures. Additionally, after harvest, recalcitrant seeds remain metabolically active, and often start germinating during storage (Pammenter *et al.*, 1984) although additional water, which is unavailable to the stored seeds, is necessary for the completion of germination (Farrant *et al.*, 1986; Berjak *et al.*, 1989). Therefore, it seems likely that recalcitrant seeds in storage will undergo an initially mild, but with time an increasingly severe, water stress (Pammenter *et al.* 1994). It is known that mild dehydration stress of *E. capensis* seeds, which initially stimulates metabolic activity (Pammenter *et al.*, 1998) may well accelerate their deterioration, as the initiation of germinative metabolism is accelerated (Eggers *et al.*, 2007). Non-lethal, short-term chilling stress may act in a similar way.

In the seeds in the present study, water content remained more or less constant throughout storage. Also, there were no indications of radicle protrusion (i.e. completion of germination) at any of the storage temperatures, thus minimising (or eliminating) stress imposed by the requirement for an extraneous water source (Pammenter *et al.*, 1994) as a factor in seed deterioration. Therefore, the seed responses recorded were predominantly as a consequence of chilling. Results clearly showed that chilling tolerance of the seeds of *Ekebergia capensis* varies according to where they were collected, with tolerance being directly related to distance from the equator.

The fruit of *E. capensis* reached maturity at different times of the year at the different sites (provenances). The fruits were fully mature during the summer months in Tanzania and St Lucia, while those from PE were mature during the winter months. Mature fruit were collected during late December to early January from Tanzania; fruits were harvested during late February and early March at St Lucia and collections from PE were made during late May to June. At the time of fruit collection, mean minimum temperatures in these regions differed (South African data source - Surgeo <http://146.230.235.122/surgeosite/main.aspx>). The average minimum temperatures recorded in PE during May and June were 9° and 7°C, respectively. An average minimum temperature of 18°C was recorded in St Lucia during the months of January and February, while that for Dar-es-Salaam (Tanzania) was 25°C in December and January (Augustino and Gillah, 2005). Extrapolating from this, the seeds within the fruits were exposed to considerably different average minimum temperatures, PE<St Lucia<Tanzania. Recalcitrant seeds may be chilling sensitive and those of some species are deleteriously affected at temperatures lower than 15°C (Chin and Roberts, 1980). However, rather than on a generalised species basis, results from the present study indicate clear provenance- related chilling responses, with 80% of seeds from PE being able to tolerate temperatures as low as 1°C for 12 weeks (Fig. 3.7).

Seeds from St Lucia responded differently (compared with those from PE) when they were exposed to chilling. Viability decreased rapidly, reaching zero when seeds were stored at 3°C for 38 d (Fig. 3.8). These seeds clearly were not amenable to sustained low temperature, conditions to which they would not normally be accustomed in their natural environment. Slightly warmer temperatures (of 6° and 16°C) did not appear to affect the seeds adversely over the storage period (Fig. 3.8).

Seeds of Tanzanian origin were considerably more chilling sensitive than those from South Africa, all losing viability after 9 d when stored at 3°C (Fig. 3.9), and were clearly adversely affected at 6 and 16°C (Fig. 3.9). Under normal tropical environmental conditions, these seeds would not be exposed to temperatures much lower than 25°C, and certainly not for any sustained period.

Survival of the seeds from the coolest provenance, PE, suggests the possession and operation of mechanisms conferring at least some degree of chilling-tolerance, which are absent in seeds from the sub-tropical and tropical provenances. This has also been reported in other species, for example neem (Varghese and Naithani, 2000).

The ambient temperature during seed development can even influence whether a seed is considered genuinely recalcitrant. For example, Daws *et al.* (2004) showed that recalcitrant seeds of *Aesculus hippocastanum* collected from across Europe are significantly influenced by cumulative heat sum during development. In warmer environments, seed development progresses further before shedding. In a later study, similar trends were documented for *Acer pseudoplatanus* seeds (Daws *et al.*, 2006). In the latter study, differences in seed responses were sufficiently marked to indicate that while those from northerly provenances clearly showed recalcitrant behaviour, those from warmer temperature habitats might be categorised as being ‘intermediate’.

While similar considerations could have influenced the results obtained from the *E. capensis* seeds used in the present study, this is unlikely. Here, irrespective of provenance, 90-100% of the fresh seeds germinated within 2-3 d. Had under-development (as a result of lower cumulative heat sum) been a factor in the PE seeds, then this rapid germination response would have been unlikely. By contrast, Daws *et al.* (2004, 2006) showed marked lags in, and final percentage, of germination in seeds of both *A. hippocastanum* and *A. pseudoplatanus* from the more northerly, cooler provenances.

In terms of comparative mean water contents, those of cotyledons (a reflection of whole seed water contents) were remarkably similar ($0.60 - 0.68 \text{ g g}^{-1}$) irrespective of provenance. This was also the case for axes of seeds from PE and St Lucia, with mean water content of axes excised from seeds of Tanzania provenance being somewhat lower ($\sim 1.1 \text{ g g}^{-1}$ compared with $1.6 - 1.7 \text{ g g}^{-1}$ for the former two provenances). As whole fruits were harvested and transported to Durban, this is more likely to reflect some seed maturation rather than any water loss. Nevertheless, even if better developed, the seeds from Tanzania were the most chilling sensitive.

The studies on both *A. hippocastanum* and *A. pseudoplatanus* (Daws *et al.*, 2004; 2006) focused on desiccation sensitivity of seeds, paying particular attention to the effect of heat

sum on seed and fruit development as well as on reserve accumulation, with more dry mass and reserve accumulation in seeds that developed in warmer climates. In the present study, because the seeds were endocarp-enclosed, really rapid dehydration which has been shown to favour viability retention to considerably lower (axis) water contents in *E. capensis* (Pammenter *et al.*, 1998), was precluded. Nevertheless, the PE-provenance seeds lost water least rapidly, but retained higher viability to lower water contents as compared to those from St Lucia or Tanzania (Figs. 3.16 – 3.18). In contrast to the findings of Daws *et al.* (2004; 2006), the present data for *E. capensis* seeds indicate that desiccation sensitivity (Tanzania>St Lucia>PE) was inversely related to latitude (distance from the equator). Thus, for *E. capensis* seeds, it is unlikely that differences in desiccation tolerance are related to the degree of development resulting from cumulative heat sum as found for *A. hippocastanum* and *A. pseudoplatanus* (Daws *et al.*, 2004; 2006). The present observations on the relative desiccation sensitivities of *E. capensis* seeds from the three provenances are the same as those on chilling sensitivity, with both being Tanzania>St Lucia>PE.

4.2 Biochemical responses

The biochemistry of living organisms needs to be in a fine balance that ensures survival, with the numerous processes maintaining the organism in a state of homeostasis. Recalcitrant seeds are metabolically active when shed, and are variably desiccation sensitive (reviewed by Berjak and Pammenter, 2008), but the status of individual biochemical pathways (e.g. ROS and AOS levels) is likely to be indicative of metabolic deterioration.

Active protein synthesis has been suggested to be a good indicator of metabolic status (Salmen Espindola *et al.*, 1994). In the present study, the rate of protein synthesis declined irrespective of the provenance or temperature during storage of seeds of *E. capensis* (Figs. 3.10 – 3.12). As axis water content remained essentially constant under all storage conditions, decline in the rate of protein synthesis could not be attributed to water loss. Protein synthesis was probably reduced at all storage temperatures as a result of the stress imposed when recalcitrant seeds do not have an extraneous source of water to support ongoing metabolism, especially if germinative events have been initiated (Berjak *et al.*, 1989, Berjak and Pammenter, 2008). Despite this, however, during storage the seeds from PE retained full viability (Fig. 3.7) at 6°C as did those from St Lucia at 16°C. The reduction in the rate of

protein synthesis of the seeds from Tanzania corresponded to a reduction in viability (Fig. 3.12). Similarly, a study on the seeds of *Avicennia marina* showed that protein synthesis is correlated with viability; when synthesis reached its lowest level during storage, germination had dropped to 20% (Motete *et al.*, 1997). Results from the current study have shown that protein synthesis may be a useful parameter to predict longevity of seeds of *E. capensis* during chilled storage.

Maintenance of membrane integrity is important for the survival of seeds (Steponkus, 1984; Pammenter *et al.*, 1991, 1994; Bryant *et al.*, 2001), and various stresses can cause deterioration of membrane structure, for example, by altering the hydrophobic and hydrophilic domains (Leprince *et al.*, 1993; Somerville, 1995). Also, slow drying whole seeds of *E. capensis* has previously been shown to result in a large increase in electrolyte leakage, indicative of membrane damage (Pammenter *et al.*, 1998). In the present study, electrolyte leakage from the axes of seeds from PE (Fig. 3.13) appeared to peak after 2 weeks in storage and then decreased. The decrease in electrolyte leakage suggests that membrane damage associated with the initial temperature stress could have been repaired, based on the assumption of unimpaired repair mechanisms, (as suggested by e.g. Farrant and Kruger [2001] and Campos *et al.*, [2003]), for dehydration- and chilling- stressed vegetative tissue, respectively). The seeds from the St Lucia (Fig. 3.14) and Tanzanian (Fig. 3.15) provenances are assumed to have been unable to repair membranes, as solute leakage increased with time at all storage temperatures. However, increasing solute leakage in the material from St Lucia, could have at least in part resulted from the proliferation of fungi that occurred in some seeds after 38 d at 3°C.

This study showed that the most rapid leakage of electrolytes occurred in axes of seeds from Tanzania, suggesting that all storage temperatures were severely detrimental to membrane integrity. This was correlated with a rapid decline in seed viability (Fig. 3.9). After 9 d, viability dropped to 40, 10 and 0% in seeds when stored at 16°, 6° and 3°C, respectively. The seeds of the St Lucia provenance were less susceptible to chilling, with solute leakage from the axes increasing over 24 to 38 d of storage (Fig. 3.8). Leakage levels may well have increased further had the storage period been extended beyond 38 d. What is difficult to explain is that solute leakage after 38 d was essentially similar for seeds at all the storage temperatures, yet viability was zero for those stored at 3°C, and 100% in the case of the seeds stored at both 6 and 16°C. However, the correlation of leakage data with viability in the seeds

from St Lucia may have been confounded by the presence of fungi. Note also that solute leakage should have been recorded at 3 d intervals, but due to unpredictable fruiting and a shortage of seeds, this was not possible, accounting for the lack of data points between the 9 and 25 d sampling intervals.

4.3 Phylogeny

Analysis of ITS region sequences has been very popular in establishing the relatedness of populations and species of plants (Youngbae *et al.*, 1993; Schlötter *et al.*, 1994; Álvarez and Wendel, 2003; Muellner *et al.*, 2009). In this study, a phylogenetic analysis of the ITS 1 region of *Ekebergia* specimens from populations in the Cape and KwaZulu-Natal (South Africa) and from Tanzania was carried out. Results showed that the three groups of *Ekebergia capensis* were distinctly different, forming three well- to strongly-supported monophyletic clades separated by genetic distances of 3.1% to 6.0%. For comparison, analyses of sequence data from the National Centre for Biotechnology Information (NCBI) genbank show that the mean genetic distance between 40 species of *Aglaia* (Meliaceae) is 10.6% (minimum 0.4%, maximum 17.6%). Thus distances of 3.1% to 6.0% between the *Ekebergia* groups are not inconsistent with their being separate species, although a larger-scale phylogenetic study needs to be carried out, including the analysis of samples from more intermediate locations, and more DNA regions. However, their allopatric geographical distribution, and consequent growth in different climatic and environmental conditions, appears to have resulted in genetic differences between the groups presently studied.

Genetic differences appear to be reflected phenotypically. The shape of the fruits of *E. capensis* from Tanzania was distinctly different from those of PE and St Lucia (Figs. 1.1a and b). Furthermore, the chilling tolerance of the seeds from the different provenances differed. The seeds from the PE (Fig. 3.7) provenance were most tolerant to chilling while those from Tanzania (Fig. 3.9) were least so. The chilling tolerance of the different genetic groups appears to reflect the differing temperatures found under the prevailing climatic conditions, as mean, maximum and minimum temperatures in Tanzania are greater than those in the Cape. Thus, growth in different climatic regimes may have resulted in selection and adaptation of the trees and their seeds to growth in the prevailing environmental conditions of the different provenances (de Kroon *et al.*, 2005).

Other physiological, biochemical and ultrastructural differences may also be linked to the different genotypes. The genotypic composition of the seeds may have been altered as they were exposed to different environmental conditions over generations, as suggested by Quinn (1977). Provenance-related differences in chilling-sensitivity presently demonstrated for seeds of *E. capensis* argue either for natural selection facilitating phenology in line with latitudinal differences, and/or for marked genetic variability. Genetic variation was shown to have an impact on the ability of chilled roots of *Lycopersicon esculentum* and *L. hirsutum* to absorb ammonium (Bloom *et al.*, 1998), with *L. hirsutum* showing higher chilling tolerance and greater ability for ammonium uptake. However, analysis of the ITS 1 region of members of the Winteraceae showed that although this family is over 110 million years old, the molecular phylogeny is well resolved with very low sequence divergence (Youngbae *et al.*, 1993). Low sequence divergence was also found between populations of the alga, *Heterosigma akashiwo*, as analysis of the ITS region revealed that the samples from different geographic regions did not display genetic differences (Connell, 2000).

Although the genetic differences presently demonstrated may provide an explanation of the differences observed, due to difficulties experienced in sourcing material for analysis, only two representative samples could be obtained from Tanzania. Therefore, for the results to be more reliable, it is necessary to analyse more samples from Tanzania. Also, the KZN samples must be extended to include material from St Lucia itself, despite the fact that Mtunzini is relatively closely situated. However, it must be noted that this is a preliminary study and analysis of the ITS region alone cannot confirm that *E. capensis* from the different provenances are different enough to be classified as a sub-species.

4.4 Ultrastructure

The maintenance of the integrity of the ultrastructure is of paramount importance to viability retention. Broad surveys (Berjak and Pammenter, 2000; Berjak, 2001) suggest that ultrastructural studies can provide important information about metabolic status of axes from recalcitrant seeds, and help to elucidate the effects of dehydration. For example, Kioko *et al.* (2006) showed the importance of analysing the ultrastructure to explain recalcitrant seed behaviour and responses under a wide variety of conditions in axes from seeds of the meliaceous species, *Trichilia emetica*. Analysis of the ultrastructure has been found to reveal

the various changes that occur within cells as a result of exposure to stress or during progression toward germination (Berjak and Pammenter, 2000). Some studies have shown that subcellular damage as a result of exposure to chilling becomes more extensive and ultimately irreversible the longer a plant is exposed to chilling (Kratsch and Wise, 2000).

In addition to the analyses conducted in this study to elucidate the behavioural response of *E. capensis* to chilling, ultrastructural changes were also documented. The organelles of root meristem cells and adjacent cells of the PE batch showed that ultrastructural integrity was maintained throughout the 12-week storage period, irrespective of the storage temperature (see section 3.7). This observation supports the viability data (Fig. 3.7). The presence of well-developed organelles and ordered cytomatrices implies that the chilling treatment did not have an impact on cellular organisation, which was in line with retention of viability by the seeds. However, there was a marked difference in the chilling tolerance of the seed batches from St Lucia and Tanzania. The gradual loss in viability of the St Lucia seeds stored at 3°C (Fig. 3.8) was associated with the steady deterioration of the axis ultrastructure. Those seeds stored at 6° and 16°C showed a generally well-maintained ultrastructure when compared with the batch stored at 3°C. It is interesting to note that reducing the temperature by just a few degrees (i.e. 6 to 3° C) had a major impact on seed viability. A similar pattern emerged from analysis of the seeds from Tanzania. None of the seed lots were able to tolerate the cold, and deterioration of the axis ultrastructure was striking (Fig. 3.9).

The visualisation of numerous polysomes in the cytomatrix of axis cells of fresh (not stored) *E. capensis* seeds from all provenances was associated with the high rates of protein synthesis initially recorded, while the declines observed in the rate of protein synthesis could have been associated with the apparent reduction of polysomes that occurred. However, the correlation needs to be tested more rigorously in future studies.

Nevertheless, numerous cytomatrical polysomes appeared to persist in axis cells from the PE seeds, under all storage regimes. A possible explanation for the decrease in the rate of protein synthesis, in the case of the material from PE, is that due to the prolonged exposure to mild stress, the association of the sub-units required for translation of the mRNA took longer (Chinnusamy *et al.*, 2007) to occur. Alternatively – or perhaps additionally – the rate of mRNA transcription could well have been slowed when seeds were stored at reduced temperatures (Schaffer and Fischer, 1988), which was perhaps exacerbated by mild water

stress. These are aspects requiring further study for clarification.

The higher electrolyte leakage in the axes from the PE seeds compared to those from St Lucia and Tanzania suggests that damage to the plasma membranes had occurred. During the earlier stages of storage, the presence of numerous vesicles was considered to be a good indicator of membrane synthesis. However, with increasing duration in storage, a gradual decrease of the incidence of ER, Golgi bodies and vesicles became evident. This observation is consistent with the idea that membrane synthesis and turnover had become less efficient – and ultimately had ceased. A lack of membrane synthesis and repair in axes was probably responsible for the higher leakage compared with fresh seeds. In contrast, for the axes from the PE seeds, leakage decreased gradually from the peak observed after 2 weeks in storage at all temperatures (Fig. 3.13) and vesicles within the axis root tip cells were apparent throughout the experiment. This indicated that during storage the seeds from PE did not behave in the same way physiologically as those from St Lucia and Tanzania, with the axes of those from PE being able to repair membranes that may have been damaged as a result of the chilling stress [cf. observations made on leaves of species of *Coffea* by Campos *et al.* (2003)].

Fresh seeds from all the provenances had abundant lipid storage reserves and those from St Lucia also had reserves of starch. Storage tended to deplete reserves in the axes of seeds from all provenances. For example, storage reduced the starch deposits in axes of seeds from St Lucia. Furthermore, with only one exception (see below), in all samples irrespective of provenance, storage reduced the density of lipid bodies, implying that the contents were being utilised. The one exception to this observation, was that the lipid bodies in axis cells from the 12 week 6°C treatment of the PE seeds appeared essentially similar to those in the control material. For the PE seeds, maintenance at 16°C would have been associated with the most metabolically-active axes and thus they would have been expected to have utilised reserves more rapidly than those in seeds stored at the lower temperatures. Considering that utilisation and re-accumulation of reserves must be a dynamic process, it is suggested that reserves could have been imported from the cotyledons (since whole seeds were placed in storage) and re-assimilated in lipid bodies in the embryonic axis cells. However, the inferences about lipid metabolism based on the ultrastructural appearance need further studies.

Stressing seeds often induces vacuolation (Berjak and Pammenter, 2000). Within lytic

vacuoles, organelles and other cytoplasmic components are broken down into their basic molecular components, which is a normal event in turnover (Lamb and Berjak, 1981). However, stress applied to recalcitrant seeds or axes has consistently been shown to result in enhanced vacuolation and autophagic activity by vacuoles, apparently serving to remove damaged or redundant organelles and cytomatrical material (Berjak and Pammenter, 2000). In this study, the distribution of vacuoles in the fresh material appeared to be uniform, with cells containing one or two vacuoles per cell section (at similar magnifications) in material from PE and St Lucia. In comparison, axes from the Tanzanian seeds had 3-4 smaller vacuoles per cell section. With the application of chilling stress of increasing duration, *de novo* vacuole formation and autophagic activity became evident in all cases. This suggests that in stressed recalcitrant seeds damage occurs progressively, and autophagosome-like vacuoles are needed to recycle cytosolic components (Berjak and Pammenter, 2000). However, as was particularly evident in axes from the St Lucia seeds stored at 1 and 3°C, and for those from the Tanzanian axes irrespective of the storage temperature applied, there comes a stage when these – and presumably any other repair mechanisms – fail, with consequent loss of cellular integrity and axis/seed viability.

The extent of differentiation of the mitochondria is proportional to the rate of respiration in stored, recalcitrant seeds (Berjak and Pammenter, 2000). Generally, mitochondria appeared well developed in the fresh material from PE and St Lucia. However, when compared with the PE and St Lucia provenances, those in axes from Tanzanian seeds appeared to be less differentiated. This implies that fresh axes from seeds from PE and St Lucia would have had higher cellular respiratory rates (which, unfortunately, were not measured in the current study). The initial state of their development notwithstanding, deterioration of mitochondrial structure was evident in axes of seeds from St Lucia stored at 1 and 3°C and those of Tanzanian provenance even when stored at 6°C, for longer than 3 d which would progressively reduce respiration. However, at the ultrastructural level, mitochondrial degeneration was not evident in axis cells of the seeds of PE origin after 12 weeks of storage at 6°C. These organelles became more developed in axes from seeds originating in St Lucia, after 38 d at both 6 and 16°C. Measurement of respiration would have provided additional indication of the mitochondrial activity inferred from the ultrastructure, but was not done due to shortage of seeds and inconsistent fruiting seasons.

The seed batches from Tanzania and St Lucia stored at 3°C were all susceptible to the chilling temperatures to which they were exposed. A decline in seed viability was associated with increased vacuolation, mitochondrial and nuclear degeneration and finally, complete disintegration of the ultrastructure with no identifiable organelles. However, when viability was maintained in the PE seed lots and St Lucia batches stored at 6 and 16°C, the ultrastructure revealed metabolically active cells with well-developed mitochondria, nuclei and other organelles. In a similar study on axes of recalcitrant fluted pumpkin seeds (*Telfairia occidentalis*), the ultrastructure showed abnormalities after storage at 6° and 16°C (Ajayi *et al.*, 2006). Clustering of organelles, lobed nuclei, distended plastids and abnormal nuclear inclusions were observed in the root meristems of those axes. Complete disintegration of the axis cells was observed after storage of *T. occidentalis* seeds at 6°C for 4 weeks (Ajayi *et al.*, 2006). Other ultrastructural studies on chilling-sensitive seeds of *Vigna radiata* showed that in chilled embryonic axes the cells displayed whorls of rough endoplasmic reticulum (rER) surrounding clear regions of cytoplasm, plastids and mitochondria with vacuoles and enlargement of Golgi vesicles (Ishikawa, 1996) and disintegration of ultrastructure and accumulation of lipid droplets with increased staining of the stroma (Wise *et al.*, 1987). Continued exposure to chilling resulted in unstacking of grana and marked dilation of thylakoid intraspaces (Taylor and Craig, 1971; van Hasselt, 1974; Wu *et al.*, 1997). Evidently, chilling injury in chilling sensitive plant tissue or seeds manifests itself in deterioration of various ultrastructural components.

Visualisation of the cellular framework in terms of the nucleoskeleton and cytoskeleton would have provided further insight into the degradation of the ultrastructure as seen at TEM level. However, this aspect of intracellular structure, which requires application of antibodies for tubulin and phalloidin for actin, were beyond the scope of the present study. Nevertheless, the ultrastructural survey conducted provided considerable insight into the effect of chilling stress on *E. capensis* seeds from the three provenances.

B) Cryopreservation of recalcitrant seed germplasm

4.5 Explant selection and cryopreservation

The inability of whole recalcitrant seeds to be stored for prolonged periods is well documented (Berjak *et al.*, 1990; Bonner, 1990; Barbedo and Bilia, 1998; Pence, 2011; Berjak and Pammenter, 2013b). However, it is generally accepted that recalcitrant seeds maybe stored for short periods at high RH and at temperatures around 16°C (Berjak and Pammenter, 2004). The range of variability in chilling tolerance may be influenced by provenance as shown in Section A above. Currently, the only way to successfully store recalcitrant germplasm is by cryopreservation (Engelmann, 2011). However, the precise protocols that need to be used vary with species, as recalcitrant seeded species have different biochemical and physiological properties (Turner *et al.*, 2001). Generally, a typical cryopreservation protocol for a recalcitrant seed involves the isolation of a suitable explant, normally the embryonic axis (Engelmann, 2011; Berjak and Pammenter, 2013b, 2014), followed by cryoprotection, dehydration, freezing (cooling) and re-warming. The aim is to produce a viable seedling from the explant so that genetic variability can be maintained. Some of the problems in cryopreserving recalcitrant germplasm were highlighted in Section 1.6.1. This section of the present study investigated ways of overcoming some of those problems by assessing various aspects of germplasm physiology, biochemistry and ultrastructure.

It is important to optimise the rates of dehydration and cooling when cryopreserving explants from recalcitrant seeds (Wesley-Smith *et al.*, 2014). Suitable explants were selected on the basis of having a large surface area:volume ratio to facilitate the most rapid drying and cooling rate. Three explant types were tested, the first being embryonic axes without cotyledons attached. The explant type displayed reduced viability (70% - Table 3.14), probably due to mechanical damage and/ or the production of ROS in an oxidative burst upon the excision of cotyledons (Goveia *et al.*, 2004). The other two types of explants were excised with either a 1 or 2 mm³ piece of cotyledon remaining. Irrespective of the size of the attached cotyledon, these explant types were viable (Table 3a). While in an earlier study explants of *E. capensis* were excised with a 2 mm³ piece of cotyledon (Hajari *et al.*, 2011), here it was found that the size could be reduced to 1 mm³ without adversely affecting germination (Table 3.14). The reduced size of the attached cotyledon increased the surface area:volume ratio,

thereby increasing the rate of drying and heat exchange (i.e. cooling).

The aim of many cryopreservation protocols for recalcitrant germplasm is to reduce the water content of the selected explant to reduce lethal ice crystal formation during cooling (Walters *et al.*, 2002; Steinmacher *et al.*, 2007; Zámečník and Šesták, 2011; Pammenter and Berjak, 2014). Some cryopreservation protocols dehydrate biological tissue with cryoprotectants that are highly concentrated salt or sugar solutions (Engelmann, 2004; Lynch *et al.*, 2011; Salama *et al.*, 2018). One recommended treatment uses plant vitrification solution 2 (PVS2) which has been shown to alter freezing behaviour of shoots (Volk and Walters, 2006; Gebashe 2015). However, these techniques are usually employed when using smaller explants consisting of uniform tissue such as meristems. In the case of embryonic axes (explants used in this study), the most common and successful method for tissue dehydration is flash drying (Pammenter *et al.*, 1991).

Although protecting explants during freezing, cryoprotectants have been shown to be toxic to recalcitrant embryonic axes (e.g. Kistnasamy *et al.*, 2011; Best 2015). Therefore, in this study, various combinations of cryoprotectants (Table 2.4) were tested to allow selection of the least toxic combination, indicated by subsequent *in vitro* viability of embryos. It was found that the least toxic combination was that of a penetrating (glycerol) and non-penetrating (sucrose) cryoprotectant. Interestingly, an earlier study by Hajari *et al.*, (2011), showed that a cryoprotectant combination with sucrose was lethal to explants of *E. capensis*. The non-lethal effect of sucrose observed in this study could be attributed to the use of cathodic water (an exogenous antioxidant) during cryopreservation. The variation of explant responses to cryoprotectants (Table 2.4) may have been caused by differences in the fruiting and seeding of recalcitrant seeds of the same species across seasons as a result of varying environmental conditions (Daws *et al.*, 2006; Sershen *et al.*, 2007).

While application of cryoprotectants resulted in mild dehydration, this was not sufficient to remove all non-freezable water from tissues, as found in other studies (Pritchard *et al.*, 1995; Sun, 2000). To improve the success of cryopreservation, the maximum amount of non-freezable water must be extracted. This can be achieved by using either a slow or fast drying technique. It has been shown in previous studies that a slow drying rate reduces the survival of recalcitrant explants (Pammenter *et al.*, 1998; Liang and Sun, 2002; Perán *et al.*, 2006; Varghese *et al.*, 2011) and therefore many cryopreservation protocols (this study included)

employ a flash (fast) drying technique. A stream of dry air is passed over the explants to dry the explants to an optimal water content (Pence, 1990).

It is generally accepted that the target water content for successful cryopreservation of recalcitrant seeded embryonic axes is one which is low enough to minimise ice crystallisation upon cooling but high enough to keep a significant proportion of tissue viable after flash drying. The target water content was therefore selected as the lowest water content achieving at least 50% germination after flash drying. Explants from both PE and St Lucia provenances were flash dried before and after the application of cryoprotectants. Prior to the application of cryoprotectants, flash drying explants from the PE provenance for 10 min resulted in a water content of $0.39 \pm 0.05 \text{ g g}^{-1}$ while viability remained at 70% (Fig. 3.42). Similarly, flash drying explants from seeds of St Lucia provenance for 20 min reduced their water content to $0.39 \pm 0.23 \text{ g g}^{-1}$ and 75% (Fig. 3.43) of the explants remained viable. After application of cryoprotectants, flash drying for the same time (i.e. 10 and 20 min for PE and St Lucia, respectively) resulted in lower water contents ($0.32 \pm 0.09 \text{ g g}^{-1}$ and $0.32 \pm 0.10 \text{ g g}^{-1}$ for PE and St Lucia, respectively), but survival was unchanged. This demonstrated the non-toxic dehydrating properties of a sucrose+glycerol cryoprotectant combination. A lower water content means that there is less available water for lethal ice crystal formation (Hor *et al.*, 1990).

Although Wesley-Smith *et al.* (2014) demonstrated that ice crystallisation during freezing of recalcitrant tissue is not necessarily lethal, other studies have shown that ice crystal formation in itself is the primary cause of cell and tissue death after freezing of recalcitrant germplasm (Hor *et al.*, 1990; Volk and Walters, 2006; Pammenter and Berjak, 2014). Therefore, in addition to reducing the available water (by rapid drying), rapid cooling is pivotal to limit ice crystal formation. It is important to note that these variables are co-dependant because faster cooling can be achieved if the tissue has a lower water content because of the accelerated heat transfer rate. This has implications for recalcitrant explants because excessive drying will be lethal, while high water contents will also be lethal because of ice crystal formation during freezing. Maximum cooling rates can be achieved by reducing the water content of the explant to the lowest possible level without adversely affecting its viability.

Liquid nitrogen (LN; -196°C), in its natural state may generate a cooling rate of hundreds of degrees per second (Vertucci, 1989; Perán *et al.*, 2006). However, by using frozen LN or

“nitrogen slush” the cooling rate maybe increased by up to thousands of degrees per second (Wesley-Smith *et al.*, 2004). Nitrogen slush is obtained by placing LN in a vacuum which permits it to freeze at -210°C, and was used in this study. After cryoprotection and rapid drying, all explants were plunged in nitrogen slush to potentially achieve maximum cooling rate and thereby limit ice crystal formation. Rates of cooling were not measured during this study because it has been previously established that a fast cooling rate is necessary for survival of *E. capensis* explants, and this can be achieved using nitrogen slush (Perán *et al.*, 2006; Hajari *et al.*, 2011). Explants were re-warmed in a 40°C medium (2 min) followed by a 25°C medium (30 min), increasing the rate of thawing and rehydration (re-warming) that is equally important to minimise regrowth of ice.

4.6 Biochemical markers during cryopreservation of recalcitrant germplasm

Successful cryopreservation of recalcitrant explants is dependent on many factors, some of which are discussed above. Explant manipulations in preparation for cooling may upset the biochemical balance that exists in these systems. The balance focused on here, and which has recently been the focal point of many stress response studies (Uchendu *et al.*, 2010; Whitaker *et al.*, 2010; Gebashe, 2015; Naidoo *et al.*, 2016; Naidoo, 2017), occurs between ROS and antioxidants. Severe abnormalities, even death may result from over production of ROS, diminished or non-existent inherent antioxidant activity or both (Whitaker *et al.*, 2010; Naidoo *et al.*, 2016; Huang *et al.*, 2019).

Enhanced antioxidant capacity and reduced levels of $\cdot\text{O}_2^-$ resulted in higher explant survival of *Trichilia dregeana*, *Quercus robur*, *Lychee sinensis* and *Strychnos gerrardii* (Naidoo, 2017). The relationship between ROS and antioxidants is very complex. They do not work in isolation and are intimately associated with numerous other cellular events (Bailly *et al.*, 2008; Baxter *et al.*, 2013; Bharuth and Naidoo, 2020). ROS and TAA were isolated and tracked throughout this study to understand the response of recalcitrant explants not only to freezing, but also to cryoprotection, drying, cooling and re-warming.

Viability retention of flash dried *Trichilia dregeana* explants was associated with their ability to maintain high levels of antioxidants (Varghese *et al.*, 2011). Their study showed that explants from slow dried seeds were unable to regulate ROS levels due to a collapse of the antioxidant mechanisms. The findings from the study on *T. dregeana* highlighted the

importance of rapid rather than slow drying of recalcitrant explants.

Mechanical damage during excision of explants has been shown to negatively affect viability (Goveia *et al.*, 2004). These authors showed that excised explants of *T. dregeana* with an attached piece of cotyledon showed better viability than those without attached cotyledons. It was postulated and later shown (Whitaker *et al.*, 2010) that a prolonged burst of ROS, specifically $\cdot\text{O}_2^-$, occurs during excision, and this was suggested that it was responsible for viability loss. Excising explants with a piece of cotyledon attached meant that the explant would be larger, which would result in lower drying and cooling rates thereby prolonging the time for deleterious processes to occur (Varghese *et al.*, 2011). Explants of *E. capensis* were excised with 2 mm³ pieces of cotyledon to curtail the damaging effects of ROS on the embryonic axis (Hajari *et al.*, 2011). However, in the interest of reducing explant size to improve drying and cooling rates, explants with a 1 mm³ piece of cotyledon were used in this study. Reducing the size of the attached cotyledon had no adverse effect on viability prior to manipulations for cryopreservation, and, by reducing the explant size, drying and cooling rate (not measured) would have been increased.

Application of exogenous antioxidants has been shown to positively influence survival of recalcitrant explants that otherwise did not survive cryoprotection (Reed *et al.*, 2012). A widely used antioxidant, ascorbic acid (Smirnoff, 1996; Conklin, 2001; de Tullio and Arrigoni, 2003; Caverzan *et al.*, 2012), has been shown to improve survival in cryopreserved explants of *T. dregeana* (Naidoo *et al.*, 2016). Improved survival of *E. capensis* explants was observed when they were treated with DMSO, a known antioxidant (Hajari *et al.*, 2011). In the present study, cathodic water, a lesser known antioxidant was shown to improve the cryopreservation of explants of *E. capensis*. Results obtained were consistent with those from other studies that showed successful cryopreservation of recalcitrant explants after exogenous treatment with cathodic water (Berjak *et al.*, 2011; Naidoo *et al.*, 2016).

Based on the recent literature, it seems likely that excessive ROS formation is an important cause of poor explant survival during cryopreservation of recalcitrant seeds. This study focussed on two major ROS, *viz.* $\cdot\text{O}_2^-$ and H_2O_2 as these are considered to be harmful to cells (Garg and Machanda, 2009). Their levels were assessed at each cryo-preparative step (Fig. 2.3). The balance between the levels of $\cdot\text{O}_2^-$, H_2O_2 and antioxidants can determine whether an explant will survive (Naidoo, 2017). In freshly excised explants from the PE provenance, the ratio of $\cdot\text{O}_2^-:\text{H}_2\text{O}_2$ that was concomitant with viability was 21:1. By contrast, freshly excised

explants from St Lucia had 77 times more $\cdot\text{O}_2^-$ than H_2O_2 which resulted in 100% viability (Table 3.15). After cooling, there was 3.6 and 4 times more $\cdot\text{O}_2^-$ than H_2O_2 in the PE and St Lucia explants, respectively. The difference in the levels of TAA in explants in the fresh condition and after cooling and retrieval was not significant (Table 3.15). As suggested by Naidoo (2017), the changes in ROS and TAA levels and ratios may be responsible for the survival (or not) of explants after freezing and thawing.

Cryopreservation of recalcitrant germplasm is a particularly difficult process and success is highly variable (as reviewed by Berjak and Pammenter, 2014). Previous studies showed that stresses imposed at each cryopreparative step were cumulative, resulting in impaired shoot production. It was suggested that ROS, especially $\cdot\text{O}_2^-$, was a major factor in explant death after cryopreservation (Whitaker *et al.*, 2010). In this study, the relationship between ROS and TAA was assessed after each cryopreparative step. This is a fairly new area of investigation in cryopreservation of recalcitrant embryos and will, at the very least, provide a data base for comparison in future assessments on oxidative stress in other species. In the material from PE, 30% of the explants survived cooling when cathodic water was used as an antioxidant at each step of cryopreservation. The levels of ROS ($\cdot\text{O}_2^-$ and H_2O_2) were significantly lower when explants were rewarmed with cathodic water compared to rewarming with CaMg. However, there was no significant difference in the levels of TAA (Table 3.12). Explants from St Lucia on the other hand did not survive cooling, despite treatment with cathodic water at each cryopreparative step. When these explants were treated with cathodic water, the $\cdot\text{O}_2^-$ levels at each cryopreparative step was significantly lower with enhanced survival compared with the control treatments (Table 3.16). Despite significantly lower levels of ROS and enhanced levels of TAA after rewarming with cathodic water, the explants from St Lucia did not survive cooling. This implied that either a biochemical imbalance or physical factors (eg. ice crystal formation) resulted in a lack of shoot production, most likely a combination of both. Interestingly, there was no significant difference in the levels of $\cdot\text{O}_2^-$ and TAA between the explants from each provenance after they were cooled and rehydrated in cathodic water (Table 3.16). However, after the same treatment, the level of H_2O_2 was considerably lower in the explants of St Lucia compared with those from PE (Table 3.16). This observation further supports the idea that changes in levels of ROS and TAA are partially responsible for a lack of shoot production of the St Lucia explants, and reduced shoot production of PE explants.

4.7. Ultrastructure

Studying ultrastructure can provide invaluable information on the effects of stress on organisms, because it allows documentation of the effect of stress at the cellular level. Ultrastructural analysis can give vital information on the metabolic state of cells, especially within meristematic tissues. If the meristematic regions of the root or shoot are compromised, then it is likely that the tissue will not survive. As discussed in Section 1.6, manipulating recalcitrant explants for cryopreservation results in cumulative stresses which often overwhelms the coping capacity of the tissue (Pammenter and Berjak, 2014), and therefore in the present study, ultrastructural changes were assessed at each cryopreparative step.

Seedlings developed from all the embryos from both PE (Figs. 3.44 a-d) and St Lucia (Figs. 3.49 a-d) provenances. The ultrastructure of both the root and shoot meristems were intact, and typical of metabolically active tissue with no abnormalities and well-developed organelles. Although it is widely accepted that explant death occurs as a result of ice crystal formation during cooling, this idea has been subsequently refined. Wesley-Smith *et al.* (2014) pointed out that the size and location of the ice crystals are important in determining whether an explant will survive cryopreservation. Their study showed that embryonic axes of *Acer saccharinum* can survive cooling provided that the ice crystals are small and confined to the cytoplasm. Wesley-Smith *et al.* (2014) used an ultrastructural approach to show that the relationship between ice crystallisation and survival in plant tissues is affected by cooling and warming rates. Although ice crystallisation is an important factor influencing survival during the cooling and rewarming stages of cryopreservation, it was not the focus of this study. Instead, ice crystal (and physical) damage was inferred from ultrastructural features (e.g. membrane damage) in the root and shoot meristem of *E. capensis* explants.

Many studies have shown that while the production of roots from the embryos of recalcitrant seeds is not affected by cooling, shoot production was lacking (Hajari *et al.*, 2011; Gebashe, 2015; Naidoo, 2017). A lack of shoot production may be linked to a ROS burst during excision, but it may also be due to the loss of ultrastructural integrity, especially after cooling as observed in both PE (Figs. 3.48 c, d, g&h) and St Lucia explants (Figs. 3.53 c, d, g&h) (Perán, *et al.*, 2006). Therefore, ultrastructural assessment of the shoot meristem is an important aspect by which inferences relating to cellular deterioration and shoot survival may be made. Earlier ultrastructural studies on the responses of recalcitrant embryo response to

cooling assessed only the root meristem (Kioko *et al.*, 2006; Kistnasamy *et al.*, 2011; Sershen *et al.*, 2012a, b). However, it soon became obvious that the ultrastructure of the shoot meristem should also be assessed. In more recent years, it has become apparent that shoot meristems of recalcitrant embryos are more sensitive to cryopreparative manipulations, explaining why many studies report that after cryopreservation only roots can develop (Gebashe, 2015; Naidoo *et al.*, 2016; Naidoo, 2017). The present study analysed both root and shoot meristems of the embryos after each stage of cryopreservation to elucidate ultrastructural events commensurate with each manipulation (Gebashe, 2015; Naidoo, 2017).

Cryoprotectant toxicity was not evident in the tissue as indicated by the high viability of embryos excised from both PE (Figs. 3.45 a-h) and St Lucia (Figs. 3.50 a-h) provenances. Furthermore, when they were immersed in cryoprotectants prepared in cathodic water, a reduction in the levels of $\cdot\text{O}_2^-$ (Table 3.15) and H_2O_2 was evident (Table 3.15). The use of cathodic water as a diluent for cryoprotectants did not cause any abnormalities in tissue ultrastructure, in fact, the ultrastructure appeared to be better preserved.

Compared with fresh explants, the ultrastructure of root and shoot meristem of embryos from seeds collected in PE (Figs. 3.46 a-h) and St Lucia (Figs. 3.51 a-h) showed signs of enhanced metabolic activity after they were flash dried and rehydrated in either CaMg or cathodic water. Among other features, enhanced vesicle activity and mitochondrial development were visible in the ultrastructure regardless of the rehydration medium (Figs. 3.46a, d, f and h). It is known that dehydrating recalcitrant seeds (and explants) enhances germination (Pammenter *et al.*, 1998), and so ultrastructural changes observed here were very likely due to dehydration. Rehydrating embryos in cathodic water reduced the amount of ROS produced, and increased antioxidant levels as compared to those embryos rehydrated in CaMg. However, there were no discernible differences in their ultrastructure. This was expected since the exogenous application of an antioxidant serves to balance the redox system, which probably does not have consequences on the tangible cellular ultrastructure, but may affect survival.

Embryos excised from seeds of both PE (Figs. 3.46 e-h) and St Lucia (Figs. 3.51 e-h) origin showed marginally better viability after rehydration in cathodic water as compared to rehydration in CaMg after the combined stages of cryoprotection and flash drying. After this treatment, the root meristematic cells of the PE (Figs. 3.47 a-h) and St Lucia (Figs. 3.52 a-h)

embryos were well preserved and indicative of metabolically active tissue. However, the shoot meristem of these explants (both PE [Figs. 3.47 c, d, g and h] and St Lucia [Figs. 3.52 c,d,g and h] provenance) had begun to show signs of stress. Cathodic water rehydration of embryos excised from seeds originating from PE appeared to have no effect on the degree of vacuolation compared with their CaMg rehydrated counterparts. Some organelles were visible throughout the cytomatrix indicating partial retention of ultrastructural integrity. The shoot meristem ultrastructure of the St Lucia explants appeared to be better preserved than those of seeds from PE. After rehydration with CaMg, the St Lucia embryos showed signs of stress. However, there was a marked improvement in these shoot meristematic cells after rehydration in cathodic water (Figs. 3.52 g and h). The ROS quenching ability of cathodic water appeared to have improved the preservation of organelles that would otherwise have been damaged by uncontrolled ROS production. Interestingly, although explants from both provenances showed improved germination when rehydrated with cathodic water as compared to CaMg, embryos from seeds originating from St Lucia showed better vigour compared with those from PE (observations; data not shown). This observation can be attributed to better ultrastructural integrity noted in the St Lucia explants, particularly the shoot meristem.

Further ultrastructural deterioration of the shoot meristematic cells was observed after they were immersed in nitrogen slush. Compared with the previous treatment (i.e. cryoprotection and flash drying), a massive increase in vacuolation occurred. This was observed in the shoot meristems of embryos from PE (Figs. 3.48 c, d, g and h) and St Lucia (Figs. 3.53 c, d, g and h) seeds. Rehydration in either cathodic water or CaMg did improve shoot meristem ultrastructure, although, the shoot meristem (especially from those seeds of PE provenance) did show slightly better ultrastructural retention than explants treated with CaMg (Figs. 3.48 g&h). The inability of embryos from St Lucia seeds to produce shoots after cooling was reflected by the ultrastructural derangement observed (Figs. 3.53 g&h). The well preserved, metabolically active ultrastructure of St Lucia shoots observed after they were cryoprotected, flash dried and rehydrated in cathodic water became degraded after cooling and rewarming. The failure of these embryos to produce shoots may be attributed to ultrastructural disintegration in addition to changes in the biochemical balance (ROS and TAA). Both oxidative stress and physical damage of the tissue are crucial factors limiting survival after cooling. Although the shoots of PE explants showed a high degree of vacuolation, their ultrastructure did appear better preserved than those of St Lucia. The ultrastructural

preservation of the cathodic water rehydrated explants resulted in 30% of these explants producing shoots after cooling and rewarming. However, it is unlikely that the slightly improved preservation of ultrastructural integrity of the shoot meristem was the only reason that the PE embryos survived. It is likely that a balance of the oxidative system promoted by the application of cathodic water contributed to survival. Lipid accumulation in shoot ultrastructure was evident in the PE embryos (Figs. 3.48 g&h), but was lacking in those from St Lucia (Figs. 3.53 g&h). It is possible that lipid accumulation may play a role in the ability of the PE explants to tolerate chilling temperatures (an observation noted in section A as well) and perhaps provide protection during freezing.

The ultrastructure of the root meristem was well preserved after cryopreparative steps and was not adversely affected by the various manipulations. Rehydrating with either cathodic water or CaMg did not influence ultrastructural integrity. These meristem cells were characterised by well-developed organelles and a dense, granulated cytoplasm. The deleterious effect of cooling noted in shoot meristem ultrastructure was not observed in root meristem ultrastructure resulting in root production after each cryopreparative step, even after cooling. Based on root meristem ultrastructural preservation, shoots do not possess the same mechanisms as roots to tolerate freezing and thawing.

With the application of cathodic water, ultrastructural preservation of both roots and shoots were observed in successfully cryopreserved recalcitrant germplasm of *Strychnos gerrardii* (Berjak *et al.*, 2011; Naidoo, 2017) and *Quercus robur* (Naidoo, 2017). However, shoot meristems showed ultrastructural deterioration and degradation in those that did not survive freezing (even after application of cathodic water as an antioxidant) e.g. *T. dreageana* (Gebashe, 2015; Naidoo, 2017), *T. emetica* (Gebashe, 2015) and *Lychee sinensis* (Naidoo, 2017). Similarly, unsuccessfully cryopreserved explants of *E. capensis* showed extensive damage to the shoot ultrastructure (Perán *et al.*, 2006). Shoot failure and ultrastructural deterioration after cryopreservation appeared to be characteristic of explants from the Meliaceae family.

CHAPTER 5: CONCLUDING REMARKS

This study has shown that recalcitrant seeds of *Ekebergia capensis* of tropical and sub-tropical origins cannot tolerate chilling. Furthermore, whole recalcitrant seeds of *E. capensis* cannot be maintained in hydrated storage for extended periods. Some loss of viability (after 12 weeks at 1°C) indicated that even those seeds from PE would not last indefinitely under hydrated storage conditions. Although it is well known that recalcitrant seeds of some tropical species cannot tolerate chilling (King and Roberts, 1980; Bedi and Basra, 1993), the exact reasons for this are unknown. Results from the present study showed that various aspects of metabolism were adversely affected by storage at unfavourably low temperatures, to the detriment of vigour and viability of the seeds. Loss of viability of seeds from St Lucia, and especially from Tanzania, was also accompanied by ultrastructural deterioration, in some cases to the point where no organelles were discernible.

This study showed that the sensitivity of *E. capensis* seeds to chilling was determined by where they were collected from; seeds of warm temperate origin are least susceptible to chilling temperatures. Preliminary analysis of nuclear ribosomal ITS 1 sequences has indicated that there is a distinct separation among the populations sampled. Clear genetic differences existed between the three populations (PE, St Lucia and Tanzania) sampled here, suggesting that the collections represent different sub-species or even different species. However, the number of samples and sampled areas needs to be increased, and also more genetic studies carried out before unequivocal conclusions can be drawn about the relatedness of the populations. In conjunction with molecular genetic analyses, in-depth investigations into some of the biochemical parameters (that could not presently be done) may assist in providing the explanation of the different, provenance-related responses of the seeds to chilling.

It was clear from this and other studies that ‘traditional’ cryopreservation methods were unsuccessful at cryopreserving explants of *E. capensis*. A novel approach of using cathodic water as an exogenous antioxidant during cryopreservation was successful in achieving explant survival (at least in explants of PE provenance). This study showed that application of cathodic water reduced ROS formation ($\cdot\text{O}_2^-$ and H_2O_2) after each cryopreparative step compared with treatments devoid of an exogenous antioxidant.

It was shown that the levels of ROS and total aqueous antioxidants exist in a very delicate balance, the alteration of which resulted in explant death. Ultrastructural evidence supports the idea that cell death occurred as a result of membrane damage due to ice crystal formation after freezing. Despite this observation, it is unlikely that a lack of shoot development can be attributed exclusively to a biochemical imbalance or ultrastructural deterioration. These two facets are probably intimately associated and inter-dependant, with cell death resulting from an impairment of either one or both mechanisms.

Importantly, the influence of provenance has been demonstrated in this study. Explants of PE origin survived cooling while those of St Lucia did not. It is likely that this may in part be due to the different genome, but also the high lipid content as observed in the ultrastructure. It is unfortunate that more seeds from Tanzania could not be sourced.

Ultrastructural evidence shows that the metabolic activity of root meristem cells were probably maintained after cooling. Presumably, this does not disrupt the antioxidant machinery within cells, thereby enabling them to counter the harmful effects of ROS. Shoot meristem cells contrariwise gradually undergo deleterious ultrastructural changes during cryopreparative steps. The stresses imposed by these manipulations were cumulative and perhaps started a chain reaction whereby a loss of ultrastructural integrity resulted in compromised antioxidant machinery, further exacerbating the inability of the shoot to recover from cooling stress.

This was the first study that reported successful cryopreservation of *E. capensis* with the application of cathodic water. This study also highlighted the importance of ROS and the retention of ultrastructural integrity to achieve successful cryopreservation of recalcitrant seeded explants.

The treatment of explants of *E. capensis* from PE with cathodic water resulted in successful cryopreservation. To achieve improved post cryopreservation success for *E. capensis*, future studies should focus on the application of other antioxidants during the cryopreservation process. Nevertheless, the outcomes of this study have made significant advances towards characterising and alleviating some of the physiological, biochemical and ultrastructural stresses associated with cryopreservation of recalcitrant explants.

CHAPTER 6: REFERENCES

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Appendix

Preparation of stock solutions: 10X TBE

53.89g Tris-HCl

24.96g Boric acid powder

1.86g Ethylenediamine tetraacetic Acid (EDTA) up to 500ml with distilled water, adjust pH to 8.3

0.5X TBE

1 in 19 dilution of 10X TBE

=> 5mls 10X TBE + 95 mls distilled water Ethidium bromide

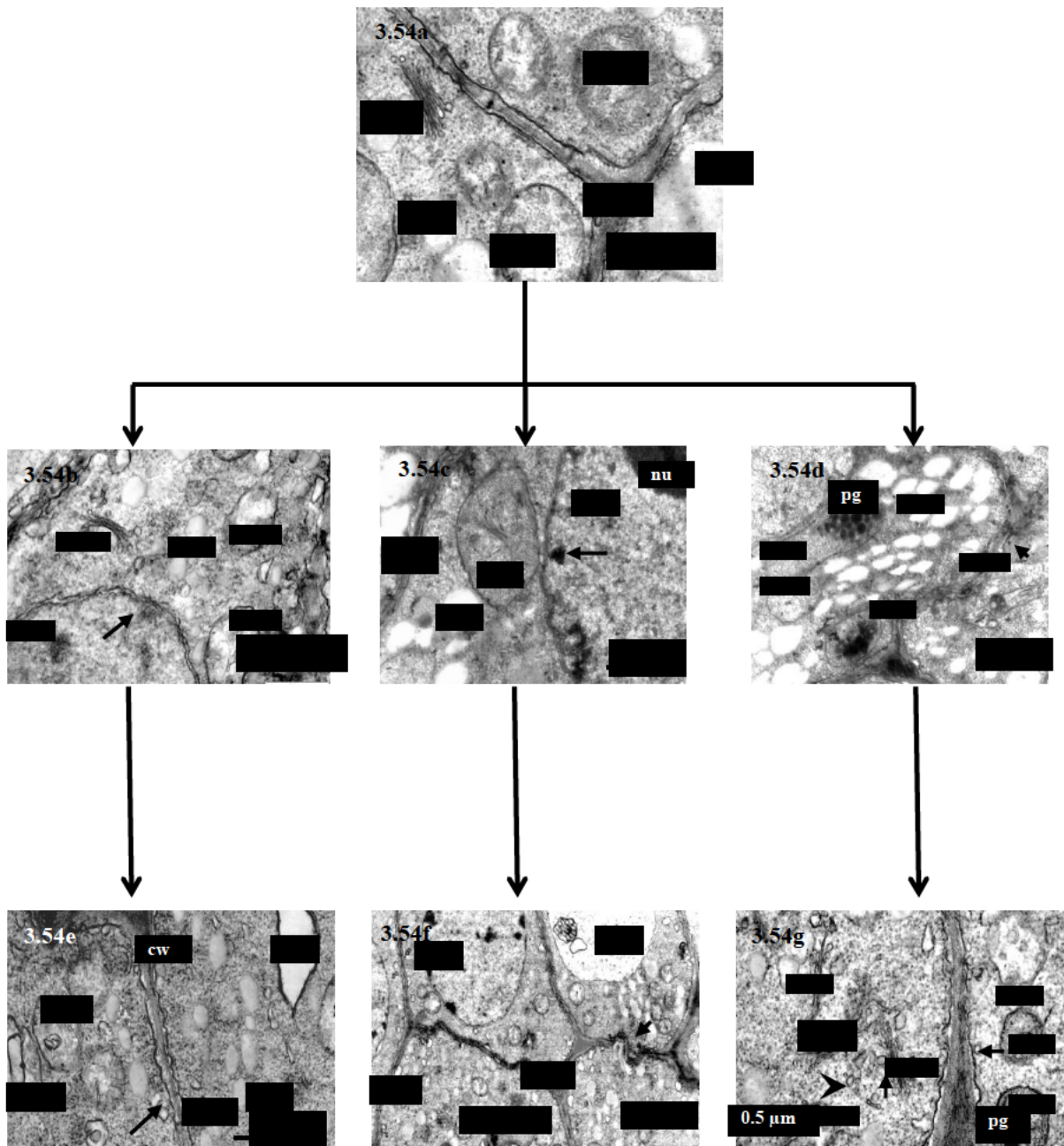
Stock: 10mg ml⁻¹

10mg of ethidium bromide + 1ml distilled water

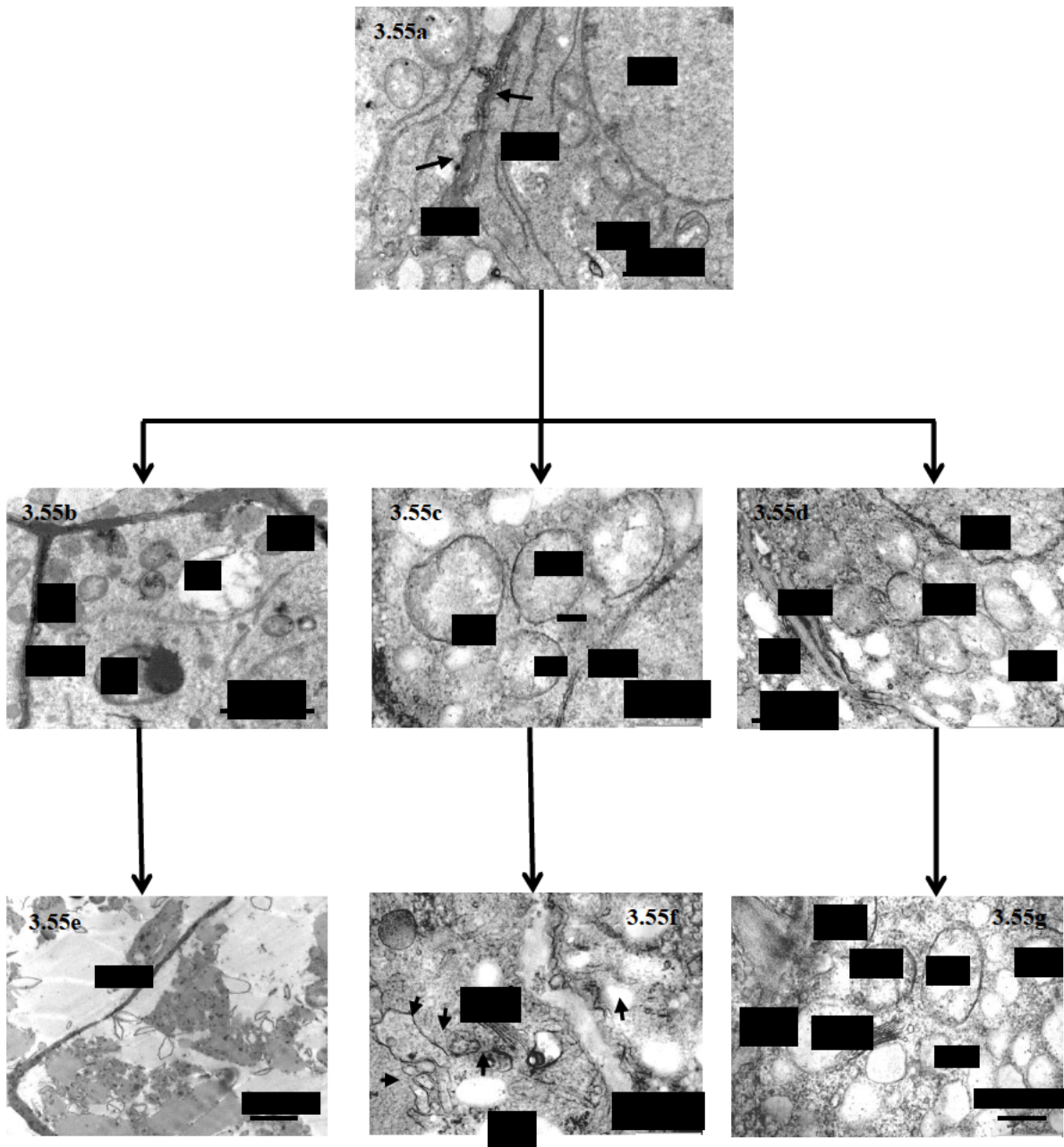
0.05mg ml⁻¹

1 in 200 dilution

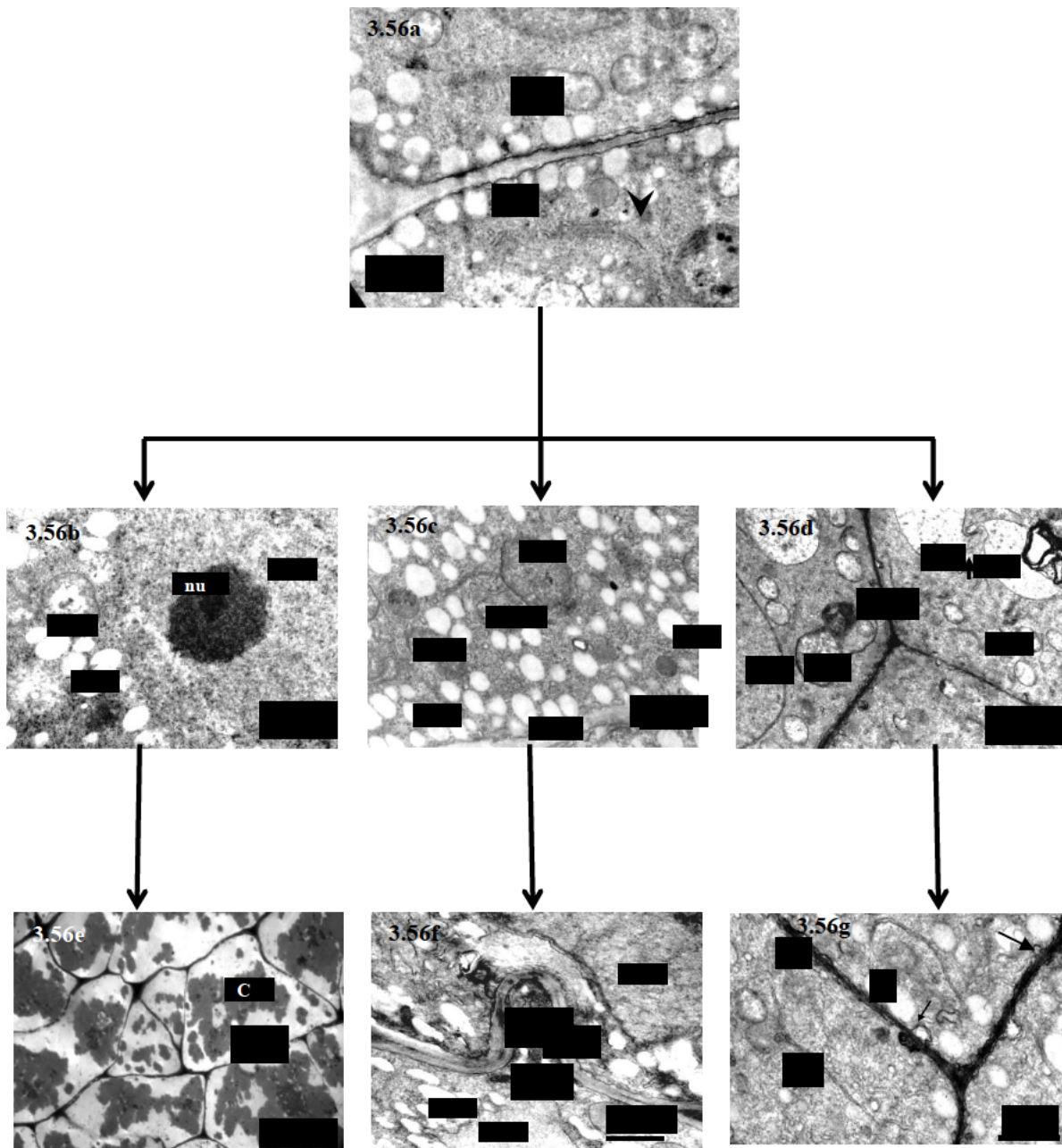
=> 0.1 ml of 10mg ml⁻¹ ethidium bromide in 19.9 ml distilled water



Figures 3.54a-g summarises the ultrastructural changes observed in the root meristematic cells of *E. capensis* from PE provenance when they were stored at chilling temperatures. The ultrastructure of fresh seeds was well preserved indicating active metabolic activity (Fig. 3.54a). After 2 weeks in storage at 1°C (Fig. 3.54b), ultrastructural integrity was maintained showing no abnormalities. The ultrastructure of the seeds stored for two weeks at 3°C (Fig. 3.54c) and 6°C (Fig. 3.54d) showed signs of heightened metabolic activity as indicated by organelle development. After 12 weeks, the ultrastructure of the axes stored at 1°C (Fig. 3.54e) was generally well maintained, however, there were indications of subtle abnormalities. The ultrastructure of the axes stored at 3°C (Fig. 3.54f) and 6°C (Fig. 3.54g) was typical of highly metabolically active tissue.

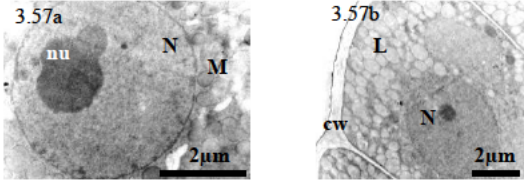
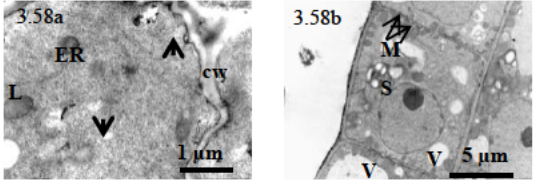
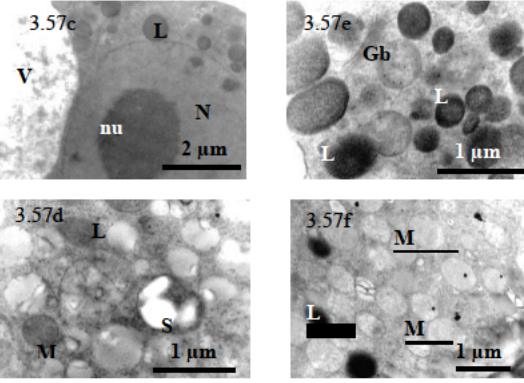
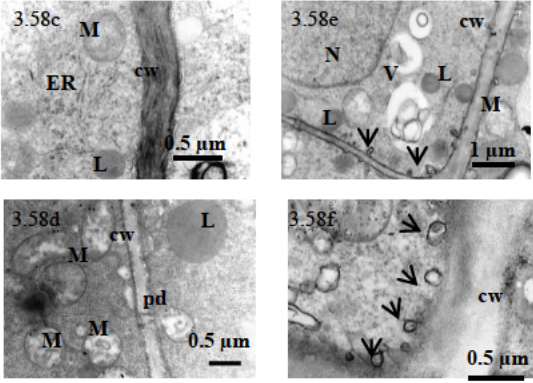
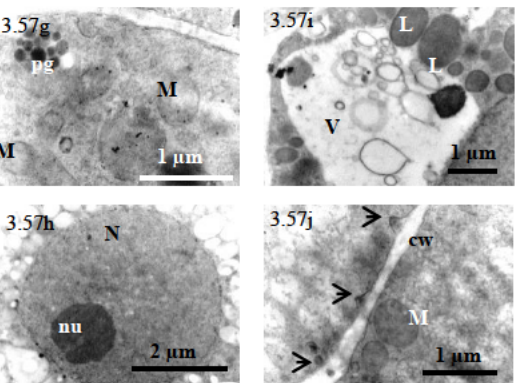
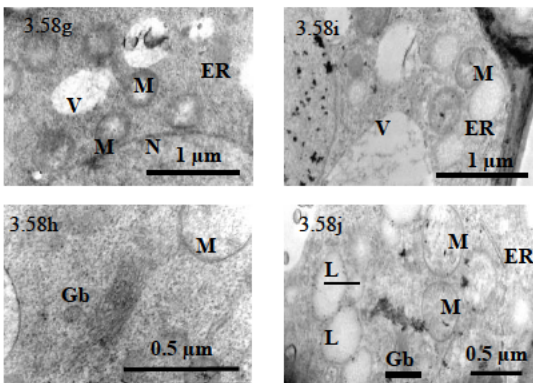


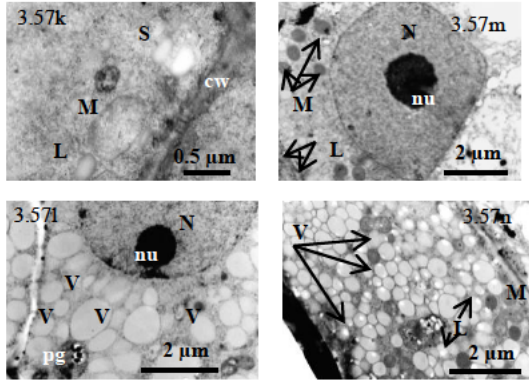
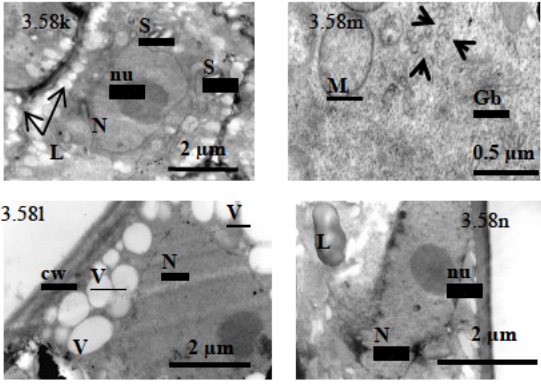
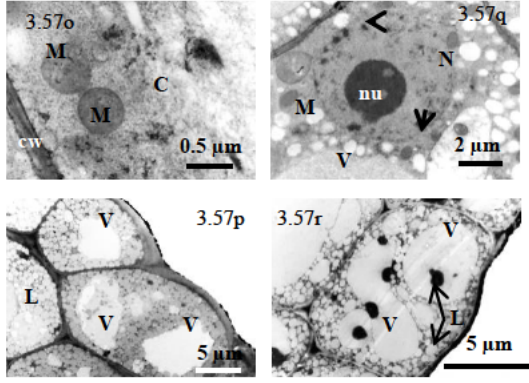
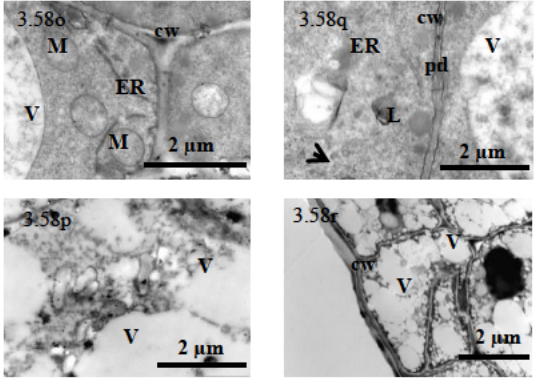
Figures 3.55a-g summarises the ultrastructural changes observed in the root meristem of *E. capensis* from St Lucia that were stored at chilling temperatures. The organelles in fresh were well preserved and metabolically active. After storage for 3 d at 3°C (Fig. 3.55b), highly metabolically active tissue was observed. Well preserved organelles were observed in the ultrastructure of axes that were stored at 6° (Fig. 3.55c) and 16°C (Fig. 3.55d) for 18 d. After 38 d in storage, the ultrastructure of the axes stored at 3°C (Fig. 3.55e) had completely disintegrated while highly metabolic tissue was typical of the ultrastructure of axes stored at 6° (Fig. 3.55f) and 16°C (Fig. 3.55g) for 38 d.



Figures 3.56 a-g shows the ultrastructural changes observed in the axes of *E. capensis* from Tanzania after they were stored at chilling temperatures. Fresh seeds (Fig. 3.56a) were metabolically active as indicated by the well preserved organelles in their ultrastructure. After 3 d storage at 3°C, ultrastructural deterioration was evident while this was not the case in the axes of the 6° (Fig. 3.56c) and 16°C (Fig. 3.56d) stored seeds. After 9 d in storage at 3°C (Fig. 3.56e), the ultrastructure of the axes had deteriorated with no identifiable organelles. Some ultrastructural deterioration was evident in the axes of the 6° (Fig. 3.56f) and 16°C (Fig. 3.56g) stored seeds.

Table 3.20 Summary of the ultrastructural differences in root and shoot meristem of *E. capensis* from PE and St Lucia

PE	SL
 <p>Figures 3.57a and b show the well preserved ultrastructure of root and shoot meristem, respectively, of <i>E. capensis</i> from PE.</p>	 <p>Figures 3.58a and b show the well preserved ultrastructure of root and shoot meristem, respectively, of <i>E. capensis</i> from SL.</p>
 <p>Explants of <i>E. capensis</i> from PE were immersed in cryoprotectants that were made up in either distilled or cathodic water. Figures 3.57 c&d shows the intact ultrastructure of the root and shoot meristem, respectively, they were immersed in cryoprotectants made up in distilled water. Figs. 3.57e shows the metabolically active ultrastructure of root meristem after immersion in cryoprotectants made up in cathodic water while Fig. 3.57f shows the shoot meristem after this treatment.</p>	 <p>Explants of <i>E. capensis</i> from SL were immersed in cryoprotectants that were made up in either distilled or cathodic water. Root (Fig. 3.58c) and shoot (Fig. 3.58d) ultrastructure of explants immersed in distilled water prepared cryoprotectants were preserved with no abnormalities. Similarly, well preserved ultrastructure was observed in the root (Fig. 3.58e) and shoot (Fig. 3.58f) when they were immersed in cathodic water prepared cryoprotectants.</p>
 <p>Explants of <i>E. capensis</i> from PE were flash dried and rehydrated in either CaMg or cathodic water. The root and shoot ultrastructure was well preserved after rehydration in CaMg (Figs. 3.57g&h, respectively) and cathodic water (Figs 3.57i&j, respectively).</p>	 <p>Explants of <i>E. capensis</i> from SL were flash dried and rehydrated in either CaMg or cathodic water. The root and shoot ultrastructure from the CaMg rehydration treatment was indicative of metabolically active tissue (Figs. 3.58g&h, respectively). Those</p>

 <p>Explants of <i>E. capensis</i> from PE were cryoprotected, flash dried and rehydrated in either CaMg or cathodic water. After rehydration in CaMg, the root ultrastructure (Fig. 3.57k) was well preserved while the shoot ultrastructure (Fig. 3.57l) showed signs of stress with increased vacuolation. Similarly, the root ultrastructure (Fig. 3.57m) of the explants rehydrated in cathodic water was well preserved but the shoot ultrastructure from this treatment was vacuolated (Fig. 3.57n).</p>	<p>explants that were rehydrated in cathodic water after flash drying had well preserved root (Fig. 3.58i) and shoot (Fig. 3.58j) ultrastructure.</p>  <p>Explants of <i>E. capensis</i> from SL were cryoprotected, flash dried and rehydrated in either CaMg or cathodic water. The root ultrastructure (Fig. 3.58k) was maintained after rehydration in CaMg, however, some vacuolation was observed in the shoot ultrastructure (Fig. 3.58l). When the explants were rehydrated in cathodic water, the root ultrastructure (Fig. 3.58m) was preserved and there appeared to be far fewer vacuoles in the shoots (Fig. 3.58n) compared with their CaMg rehydrated counterparts.</p>
 <p>Explants of <i>E. capensis</i> from PE were cryoprotected, flash dried, cooled in LN and rehydrated in either CaMg or cathodic water. The root ultrastructure of the CaMg retrieved explants was metabolically active (Fig. 3.57o) while the ultrastructure of the shoot was highly vacuolated (Fig. 3.57p). The root ultrastructure of the cathodic water retrieved explants was well maintained (Fig. 3.57q) while the shoot ultrastructure from this treatment was highly vacuolated (Fig. 3.57r) and better preserved than their CaMg rehydrated counterparts.</p>	 <p>Explants of <i>E. capensis</i> from SL were cryoprotected, flash dried, cooled in LN and rehydrated in either CaMg or cathodic water. After rehydration in CaMg, the root ultrastructure (Fig. 3.58o) was well preserved but the shoot ultrastructure (Fig. 3.58q) had completely disintegrated. When the explants were rehydrated in cathodic water, their root ultrastructure (Fig. 3.58p) was typical of metabolically active tissue. Although the shoot ultrastructure (Fig. 3.58r) was highly vacuolated and abnormal, it appeared better preserved than those shoots rehydrated with CaMg.</p>