Some investigations on the responses to desiccation and exposure to cryogenic temperatures of embryonic axes of *Landolphia kirkii*

by

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As the candidate's supervisors we have approved this dissertation for submission.

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Abstract

Landolphia kirkii is scrambling shrub forming an integral part of the flora along the coastal areas of north-eastern South Africa. The non-sustainable harvesting of fruit as food source, by monkeys and rural communities and the highly recalcitrant nature of their seeds threatens the continuation of the species. In addition, the ability of the plants to produce high quality rubber makes its long-term conservation highly desirable. Previously, attempts have been made to cryopreserve germplasm of *L. kirkii*, but no survival had been recorded at cryogenic temperatures of below -140ºC.

The present study reports on the effects of rapid dehydration, chemical cryoprotectants and various cooling rates, thawing and imbibition treatments on survival of embryonic axes excised with cotyledons completely removed, as well as with 3 mm portion of each cotyledon attached, from fresh, mature, recalcitrant seeds of *L. kirkii*. Survival was assessed by the ability for both root and shoot development in *in vitro* culture, the tetrazolium test and electrolyte leakage readings.

At seed shedding, embryonic axes were at the high mean water content of 2.24 g g^{-1} (dry mass basis). All axes (with and without attached cotyledonary segments) withstood rapid (flash) drying to a water content of *c*. 0.28 g g^{-1}; however, the use of chemical cryoprotectants, singly or in combination, before flash-drying was lethal. Rapid cooling rates were detrimental to axes flash-dried to 0.28 g g^{-1}, with no explants showing shoot production after exposure to -196ºC and -210ºC. Ultrastructural examination revealed that decompartmentation and loss of cellular integrity were associated with viability loss after rapid cooling to cryogenic temperatures, although lipid bodies retained their morphology regardless of the thawing temperature employed. Furthermore, analysis of the lipid composition within embryos of *L. kirkii* revealed negligible amounts of capric and lauric acids, suggested to be the medium-chained saturated fatty acids responsible for triacylglycerol crystallisation when lipid-rich seeds are subjected to cryogenic temperatures. Hence, lipid crystallisation was not implicated in cell death following dehydration, exposure to cryogenic temperatures and subsequent thawing and

rehydration. Rapid rehydration of embryonic axes of *L. kirkii* by direct immersion in a calcium-magnesium solution at 25ºC for 30 min (as apposed to slow rehydration on moistened filter paper or with rehydration in water) was associated with highest survival post-dehydration. Cooling at 1ºC min^{-1} and 2ºC min^{-1} facilitated survival of 70 and 75% respectively of axes with attached cotyledonary segments at 0.28 g g^{-1} after exposure to -70ºC. Viability retention of 40 and 45% were recorded when embryonic axes with attached cotyledonary segments were cooled at 14 and 15ºC min^{-1} to temperatures below -180ºC. However, no axes excised without attached cotyledonary segments produced shoots after cryogenic exposure. The use of slow cooling rates is promising for cryopreservation of mature axes of *L. kirkii*, but only when excised with a portion of each cotyledon left attached.

Preface

The experimental work described in this dissertation was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, under the supervision of Professors Patricia Berjak and Norman W. Pammenter.

This study represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

_____

Provain Kistnasamy

Declaration 1 - Plagiarism

I, Provain Kistnasamy declare that

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Declaration 2 - Publications

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1: Kistnasamy, P., Berjak, P. and Pammenter, N.W. 2011. The effects of desiccation and exposure to cryogenic temperatures on embryonic axes of *Landolphia kirkii*. *CryoLetters* **32** (1): 28 – 39.

I carried out all the experimental work and wrote this paper and my supervisors provided input on a series of draft copies.

Signed: .....

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Chapter 1: Introduction

1.1 Plant genetic resource conservation

Biodiversity can be defined as the total sum of biotic variation, ranging from the genetic level to ecosystems, and can be used to indicate the diversity of species, the diversity within species, and the diversity of ecosystems (Martens *et al*., 2003). Recent reports indicate that the loss of global biodiversity is accelerating at an alarming rate (Chapin III *et al*., 2000; Martens *et al*., 2003; Rao, 2004). The causes of this loss can be attributed to a variety of reasons including destruction of tropical rainforests (Ford-Lloyd and Jackson, 1991; Turner, 1996; Gullison *et al*., 2007), increases in development activities (Rao, 2004; Reidsma *et al*., 2006), changes in agricultural practices (McLaughlin and Mineau, 1995; Rao, 2004; Reidsma *et al*., 2006), invasion by alien species (Diamond, 1989; Sax and Gaines, 2003) and the much publicised climate change due to elevated atmospheric carbon dioxide concentrations (Kappelle *et al*., 1999; Malcolm *et al*., 2006; Botkin *et al*., 2007). To prevent extinction of species by the above-mentioned causes, plant germplasm is conserved by a variety of methods and techniques. Germplasm represents all the different genotypes i.e. the genetic diversity of a species. The main objective of its conservation is to ensure that useful germplasm is available at any given time (Nitzsche, 1983) to meet the needs of different users (Rao, 2004).

The loss of plant biodiversity is most worrying, with food sources and plants with medicinal properties being threatened with extinction. Rao (2004) highlighted this in stating that "plant genetic resources for food and agriculture are the basis of global food security." Franks (1999) also suggested that it is widely accepted that the genetic base of commercial cultivars has been narrowed down, particularly due to plant breeders no longer utilising primitive, related but unselected forms such as landraces, but rather choosing to use germplasm that is highly selected (referred to as elite material). The International Union for Conservation of Nature (IUCN) Red List of endangered species further emphasises this plight showing that 8724 out of the 18 351 species (i.e. 48%)

threatened with extinction are plants (IUCN, 2010). The conservation of germplasm through conventional, as well as novel techniques/methods is of the highest priority and can be broadly divided into two strategies of approach, *in situ* and *ex situ* conservation (Ford-Lloyd and Jackson, 1991; Nevo, 1998; Withers and Engelmann, 1998; Engelmann and Engels, 2002; Rao, 2004; Benson, 2008).

1.2 *In situ* conservation

In situ conservation, also referred to as 'on site' conservation, is the conservation of wild species (Gladis, 2000) and uncultivated plant communities (Rao, 2004) within their natural habitats (Park *et al*., 2005) and ecosystems, which include protected wilderness areas and reserves (Withers and Engelmann, 1998). However, Prance (1997) stated that *in situ* conservation is not restricted to protected areas of undisturbed natural ecosystems. That author and others further suggested that disturbed areas harbour a large amount of biodiversity, and disturbance is of vital importance for the maintenance of dynamic stability (White and Jentsch, 2001; Odion and Sarr, 2007), as well as for the increase of species diversity (Prance, 1997; Jentsch and White, 2002). For example, many wild crop species which are becoming vitally important because of the genetic erosion of much of the breeding material, occur in disturbed areas rather than in natural protected areas (Prance, 1997).

In situ conservation within farming areas, termed on-farm conservation, is relatively new (Hammer *et al*., 2003) but is being widely applied by farmers for the conservation of agricultural biodiversity (crop cultivars) in agro-ecosystems (Rao, 2004). This technique is employed to protect, maintain, develop and use local varieties of crop species (Hammer *et al*., 2003; Park *et al*., 2005), the agro-biodiversity of which, should be self-supporting and favour dynamic, evolutionary genetic processes and the complexity of genetic interactions in the agricultural ecosystem (Park *et al*., 2005). The genetic diversity provided by on-farm conservation provides farmers with more options to develop new and more productive crops that are adapted to environmental change, and are resistant to pests and diseases, through selection and breeding (Rao, 2004).

The major benefit of *in situ* conservation is that the genetic system is maintained, while allowing the ongoing processes of natural selection, evolution and adaptation (Kleinschmit, 1994) through the interaction between species and their physical environment (Kleinschmit, 1994; Prance, 1997; Franks, 1999). Conversely, it is not always possible to conserve species in their natural habitat due to total habitat destruction, including invasion by alien species (Prance, 1997). Furthermore, *in situ* conservation requires the permanent appropriation of land, constant management (Williams, 1997), and is vulnerable to technological innovation and diffusion, economic and political change, and potentially the most important factor, environmental changes (Brush, 2000). Therefore a viable alternative to *in situ* conservation, i.e. *ex situ* conservation, has to be developed. However, the two approaches are no longer seen as exclusive alternatives (Brush, 2000), where *in situ* conservation can be dismissed in favour of *ex situ* (Maxted *et al*., 1997); they are rather viewed as complementary approaches (Brush, 2000). It has been suggested that neither method alone, is sufficient to conserve the total range of genetic resources that exist and that each approach addresses different aspects of genetic resources (Maxted *et al*., 1997; Brush, 2000).

1.3 *Ex situ* conservation

Ex situ, in contrast to *in situ* conservation, involves the collection of plant germplasm from its natural environment and protecting it in an artificial environment such as field genebanks, *in vitro* storage, DNA storage, cryopreservation, pollen storage and seedbanks or genebanks (Engelmann and Engels, 2002). It is used to safeguard populations that are in danger of destruction, replacement, deterioration or even extinction (Rao, 2004). Field genebanks are generally the preferred method of storage for plant species such as banana, potato, yam, cassava, sugarcane and sweet potato that either do not produce seeds, or produce seeds that are sterile or highly heterozygous, in which case clonal propagation is utilised to conserve genotypes (Engelmann and Engels, 2002; Rao, 2004). Field genebanks, although providing easy access to and observation of conserved material (Engelmann and Engels, 2002; Rao, 2004), much like *in situ* conservation, have certain drawbacks and limitations that threaten their conservation security (Withers and Engels,

1990; Engelmann and Engels, 2002; van Slageren, 2003). These include the higher costs involved in the maintenance of genetic material, exposure of the plant species to diseases and pests, human error, vandalism and natural catastrophes such as gales, fire, drought, floods etc. (Engelmann and Engels, 2002; Rao, 2004).

Seed genebanks and biotechnology techniques for *ex situ* conservation of plant germplasm offer viable, complimentary alternatives. There are two main approaches to storing plant germplasm, either through active or base collections. Active collections are generally stored at 0°C and 8% moisture content for 10 – 15 years (e.g. Pita *et al*., 1998; Ruiz *et al*., 1999) and are generally made available to plant breeders and similar patrons for research (Acquaah, 2007), regeneration and evaluation (Sackville Hamilton and Chorlton, 1997). However, to make germplasm from active genebanks available, curators must increase the amounts of germplasm accessible to fulfil requirements expeditiously, and thus increase the chances of jeopardising accessions (Acquaah, 2007). While active collections are generally put into place for short- and medium-term conservation, base collections are aimed at long-term germplasm conservation and are seen as the most comprehensive collections of the genetic variability of species (Acquaah, 2007) and as back-up reserves and replenishment for active collections (Sackville Hamilton and Chorlton, 1997; Yadav *et al*., 2007). Germplasm for base collections is held at lower moisture contents than active collections and at temperatures ranging between -18 and -196°C (Engelmann and Engels, 2002; Acquaah, 2007).

Advances in biotechnology, particularly in the fields of *in vitro* culture techniques and molecular biology, are contributing to improved conservation and management of genetic resources (Rao, 2004). *In vitro* conservation also holds certain advantages over other *ex situ* methods in that genetic material is not subjected to environmental hazards, vandalism, theft etc. (Withers and Engelmann, 1990; Williams, 1997; Engelmann and Engels, 2002; Rao, 2004), while being conserved in a pathogen-free state which facilitates safe distribution (Rao, 2004). *In vitro* techniques, whether involving slow growth procedures used as a short- to medium-term option by keeping sterile plant tissue or plantlets on a nutrient gel-medium, or cryopreservation for long-term storage of plant

germplasm in liquid nitrogen (LN) or over in the vapour above LN (Rao, 2004), is especially vital for the preservation of vegetatively propagated material and recalcitrant-seeded species (Engelmann and Engels, 2002; Rao, 2004).

1.3.1 Seed storage as a means of *ex situ* germplasm conservation

It is widely suggested that the storage of plant genetic resources is economically feasible, safe and most achieved via the storage of naturally produced seeds (Francisco-Ortega *et al*., 1994; Hong and Ellis, 1996; Phartyal *et al*., 2002; Mycock *et al*., 2004; Walters, 2004). The effectiveness of seedbanks in conserving large amounts of intra-specific diversity is highlighted by the fact that 90% of the 6.1 million accessions stored in genebanks are maintained as seed (Linington and Pritchard, 2001; Engelmann and Engels, 2002) and due to developments and improvements in techniques over the years, can be stored in this manner for decades (Engelmann and Engels, 2002). Seed storage is imperative not only for planting material from one growing season to the next, intra-seasonal food reserves and feedstock for domesticated animals, but also for long-term conservation of genetic resources (Hong and Ellis, 1996; Berjak and Pammenter, 2008). Seeds are also easily and conveniently distributed to plant breeders, farmers, researchers and other users and are less likely to carry pathogens than other plant germplasm material (Engelmann and Engels, 2002). However, the major difficulties associated with seed storage is that not all plant species are amenable to conventional seedbanking as seeds exhibit a range of storage behaviour under routine storage conditions (Hong and Ellis, 1996; Pritchard, 2004). Some of these aspects are discussed below.

1.4 Seeds

Seeds hold the genetic information and diversity that is required for the continuation of a species and constitute the most easily and economically feasible storage option (Roberts, 1991; Wang *et al*., 1993; Theilade and Petri, 2003) for re-establishment of populations affected by environmental factors, over-population, pests and diseases etc. Thus their conservation, as well as handling and transportation, is of vital importance. Seeds can be

broadly categorised into two groups (although each may be further subdivided) based on their sensitivity or tolerance to desiccation, and thus their amenability to storage (Roberts, 1973; King and Roberts, 1980; Farrant *et al*., 1993). A seed that is tolerant to desiccation and that can be stored at low water content, under low relative humidity and low temperatures for predictably long periods with little effect on viability, is referred to as an orthodox seed (Roberts, 1973). A seed that is sensitive to desiccation and is killed by drying to water contents as high as 20 – 30% (Pritchard, 2004), is classified as non-orthodox being either intermediate (Ellis *et al*., 1990a) or recalcitrant (Roberts, 1973). Recalcitrant seeds progress towards germination when wet and therefore cannot be stored under hydrated conditions for anything other than the short-term (Pammenter and Berjak, 1999).

Non-orthodox, and in particular recalcitrant seed behaviour, is not merely a matter of desiccation sensitivity. Recalcitrant seeds are metabolically active when they are shed, in contrast to orthodox types which are quiescent (Berjak and Pammenter, 2004a). Ellis *et al*. (1990a) showed that coffee (*Coffea Arabica*) seeds survived both desiccation to a water content of approximately 10% (wet mass basis) and subsequent storage at 15°C for a year. However, seeds deteriorated more rapidly at lower temperatures or lower water contents. These seeds did not fully satisfy the definitions of either recalcitrant or orthodox seed behaviour, and therefore the term 'intermediate' was introduced (Ellis *et al*., 1990a). This term is used to describe seeds that demonstrate relatively desiccation-tolerant post-harvest behaviour, but will not withstand desiccation to levels as low as orthodox seeds (Pammenter and Berjak, 1999).

The original categorisation of seeds into orthodox and recalcitrant types was based on only a narrow range of species comprising mostly crop species that held economic importance (Pammenter and Berjak, 1999). As research has expanded to include a wider spectrum of species from diverse habitats, the range of post-harvest responses observed has also increased (Pammenter and Berjak, 1999). Due to the obvious variability in post-harvest behaviour of seeds across species, their behaviour could be considered as a

continuum, with extreme recalcitrance and orthodoxy on either end, and subtle gradations in between (Berjak and Pammenter, 2000; 2001).

1.4.1 Orthodox seeds

Many orthodox seeds are capable of naturally drying to water contents of 20% or less [presumably on a fresh mass basis (fmb)] on the mother plant and are harvested or shed in this state (Roberts and King, 1980). Developing orthodox seeds acquire desiccation tolerance early, prior to or during the final developmental phase on the parent plant (Vertucci and Farrant, 1995; Berjak, 2006a), and thereafter enter a phase of maturation drying (Bewley and Black, 1994). The acquisition, as well as the maintenance of desiccation tolerance, is based on an interplay of a suite of mechanisms and processes during early development (Pammenter and Berjak, 1999; Berjak and Pammenter, 2004b; 2008). Due to the ability of orthodox seeds to tolerate desiccation, they are also able to withstand further drying (Berjak and Pammenter, 2000) down to at least 5% (presumably on a fmb) without damage (Roberts and King, 1980), and can therefore be stored for many years under cold, dry conditions (Vertucci and Farrant, 1995).

Ellis and Roberts (1980) suggested that if orthodox seeds are of a high quality after harvest, and low water content is maintained through controlled conditions of low temperatures and relative humidity, then the period for which they can be stored without deterioration can be predicted. However, the absolute longevity of seed accessions differs greatly among species even in the same environment (Hong and Ellis, 1996). Equations predicting seed longevity in storage, that take into account variations within species and initial seed quality (Ellis and Roberts, 1980), have shown that the storage time of orthodox seeds increases logarithmically with a decrease in water content and temperature, as well as changes in oxygen levels dependent on the water content of the seeds (Ellis and Roberts, 1980; Roberts and Ellis, 1989; Pritchard and Dickie, 2003; Walters, 2004). However, ultra-drying of orthodox seeds below a certain point either does not increase (e.g. Ellis *et al*., 1989; 1990b; 1992) or may in fact slightly reduce seed storage longevity (e.g. Ellis *et al*., 1989; 1990b; c; Buitink *et al*., 2000), even when stored

at genebank temperatures (Vertucci and Roos, 1990; 1993; Pritchard and Dickie, 2003). Additionally, Vertucci and Roos (1993) suggested that the benefit of low temperatures in slowing some of the aging reactions of stored seeds may be lost if these seeds are overly dried. Moreover, those authors further suggested that seeds that are extremely dry and have therefore lost structurally important water, age more rapidly under very cold conditions compared with more moderate conditions. Studies based on thermodynamic considerations have suggested that the optimum water contents for storage of seeds should increase with decreasing temperatures (Vertucci and Roos, 1993; Vertucci *et al*., 1994a; Buitink *et al*., 1998).

Although the longevity of stored orthodox seeds can be greatly improved by manipulations of the above-mentioned parameters, these seeds will eventually die if conditions favourable for germination are not achieved (Smith and Berjak, 1995) or unless germination is inhibited by dormancy (Roberts and Ellis, 1989). Additionally, all orthodox seeds deteriorate under storage conditions (Harrington, 1973; Roberts, 1973; Smith and Berjak, 1995; Walters, 2003; Berjak and Pammenter, 2008), with the time dependent on the species (Walters and Towill, 2004; Walters *et al*., 2005; Probert *et al*., 2009).

Studies have also shown that rapid uptake of water by dry seeds can result in imbibition damage (Powell and Matthews, 1978; Ellis *et al*., 1982) and that the lower their moisture content, the more susceptible seeds are to damage (Pollock, 1969; Ellis *et al*., 1982).

1.4.2 Recalcitrant seeds

Recalcitrant seeds are generally large seeds that are most commonly produced by trees growing in tropical and sub-tropical regions (Chin, 1995; Dickie and Pritchard, 2002; Berjak and Pammenter, 2003a; Daws *et al*., 2005). Some examples include many perennial tropical species such as rubber [*Hevea brasiliensis* (Normah *et al*., 1986)], coconut [*Cocos nucifera* (Assy-Bah and Engelmann, 1992)], tea [*Camellia sinensis* (Wesley-Smith *et al*., 1992; Berjak *et al*., 1993)], cocoa [*Theobroma cacao* (Chandel *et*

al., 1995)], avocado [*Persea americana* (Raja *et al*., 2001)] and jackfruit [*Artocarpus heterophyllus* (Wesley-Smith *et al*., 2001a)], which are of great commercial and economical importance (Roberts and King, 1980). Many non-commercial plant species such as *Avicennia marina* (Farrant *et al*., 1993), *Barringtonia racemosa* (Farrant *et al*., 1996; Berjak, 2000; Naidoo, 2008), *Bruguiera gymnorrhiza* (Farrant *et al*., 1996), *Trichilia dregeana* (Kioko *et al*., 1998; Berjak *et al*., 2004), *Trichilia emetica* (Kioko *et al*., 2006; Varghese *et al*., 2009) and *Ekebergia capensis* (Walker, 2000; Perán *et al*., 2006) also produce recalcitrant seeds. Furthermore, many aquatic and temperate (usually forest tree species) species are also known to produce seeds that are recalcitrant in nature (Probert and Longley, 1989; Pritchard, 1991; Finch-Savage *et al*., 1994; Suzuki *et al*., 2007).

Recalcitrance is not limited to any single type of plant and studies have revealed that many herbaceous plants including geophytes [e.g. *Lilium longiflorum* (van der Leede-Plegt *et al*., 1992), *Scadoxus puniceus* (Sershen *et al*., 2007; 2008), *Amaryllis belladonna* (Sershen *et al*., 2007; 2008; 2010) and *Haemanthus montanus* (Naidoo *et al*., 2010; Sershen *et al*., 2011)] and graminoids [e.g. *Zizania palustris* (Probert and Longley, 1989; Kovach and Bradford, 1992; Vertucci *et al*., 1994b; 1995), *Zizania texana* (Vertucci *et al*., 1994b; Walters *et al*., 2002b) and *Spartina alterniflora* (Chappell Jr and Cohn, 2011)] as well as woody plant species [e.g. *Machilus thunbergii* (Lin and Chen, 1995), *Trichilia dregeana* (Drew *et al*., 2000; Berjak and Mycock, 2004; Goveia *et al*., 2004) and *Ekebergia capensis* (Walker, 2000; Perán *et al*., 2006)] from both dicotyledonous (e.g. von Teichman and van Wyk, 1991; 1994; Berjak *et al*., 1992; Farrant *et al*., 1993) and monocotyledonous plants (e.g. Farrant *et al*., 1989; Sershen *et al*., 2007) produce recalcitrant seeds (Berjak and Pammenter, 2000; 2003a).

1.4.2.1 Lipid composition

Lipid composition may contribute to desiccation tolerance in orthodox seeds and to the lack thereof in recalcitrant types (Berjak and Pammenter, 2008). Previous studies (Liu *et al*., 2006) have shown that membrane lipid composition, in particular, differs

significantly between recalcitrant and orthodox seeds. Nkang *et al*. (2003) revealed that total fatty acid composition may change within seeds, depending on the desiccation temperatures they are subjected to, significantly affecting viability. It has also been proposed that coalescence of oil-bodies during recalcitrant seed imbibition after dehydration may occur (Leprince *et al*., 1998). Triacylglycerols (TAGs) are an important storage reserve in seeds of some species, and may affect storage characteristics and germination (Crane *et al*., 2006). However, although the mechanisms involved are still not clear (Crane *et al*., 2006), it has been suggested that TAGs also serve as a reservoir for substrates and as protectants in ageing reactions (Walters, 1998). Work by Vertucci (1989b) showed that TAG composition is related to damage under certain conditions when seed tissue is exposed to cryogenic temperatures (below -140°C).

Studies have shown that seeds which exhibit intermediate behaviour, survive both low temperatures and low water contents independently, but do not survive when exposed to both stresses simultaneously (Ellis *et al*., 1990a; d; Crane *et al*., 2003; 2006; Volk *et al*., 2006a; 2007). Crane *et al*. (2003; 2006) and Volk *et al*. (2006a; 2007) showed that low temperatures crystallise lipids, predominantly the saturated long- and medium-chain storage lipids, at both high and very low water contents (reviewed by Berjak and Pammenter, 2008). Exposure of seeds to water when the TAGs are still crystallised is lethal, because intracellular structure is irreversibly perturbed when lipid bodies explode revealing sharp, translucent shards penetrating through the lipid bodies, reminiscent of extant crystalline structures, as demonstrated in studies on *Cuphea* species (Crane *et al*., 2003; 2006; Volk *et al*., 2006a; 2007).

Although the transition from the dry to the hydrated state, which occurs during early imbibition, is lethal to seeds if lipid reserves are crystalline, the reason for this is still not known (Volk *et al*., 2006a). Volk *et al*. (2007) revealed that even though lipid and water do not mix, there was a profound interaction between water and crystalline TAGs in *Cuphea* species. This interaction, which could be brought on by only brief imbibition, resulted in massive cellular disruption which was lethal to seeds containing crystallised TAGs. However, heat exposure prior to imbibition melted the TAGs and eliminated

damage on rehydration, and allowed the seeds to germinate normally (Crane *et al*., 2003; 2006; Volk *et al*., 2006a; 2007). Seeds that exhibit intermediate or recalcitrant storage behaviour may contain lipids that melt at relatively high temperatures (Volk *et al*., 2006a; 2007). Thawing temperatures of 25°C, used for most thawing protocols, may be lower than those required for lipid melting (e.g. Volk *et al*., 2006a; 2007), which may perhaps be a reason for the limited success of non-orthodox seed types with regards to *in vitro* regeneration post-thawing, following exposure to cryogenic temperatures. This was further advocated by Neya *et al*. (2004), who showed that imbibition of lipid-rich non-orthodox *Azadirachta indica* seeds with warm water (30°C), alleviated the effects of imbibition stress that resulted when cold water was used. The same dynamic may be true for embryonic axes of *Landolphia kirkii* (the material for the present investigation) which are known to have a high lipid content (Berjak *et al*., 1992).

1.5 Desiccation sensitivity

Recalcitrant seeds undergo little to no maturation drying during the final phase of development (Finch-Savage and Blake, 1994; Pammenter and Berjak, 1999), and are hydrated and metabolically active when shed (Pammenter and Berjak, 1999), with many species able to germinate without the addition of water (King and Roberts, 1980). The warm, moist tropic environments provide the perfect conditions for recalcitrant seeds to germinate and establish seedlings throughout the year (King and Roberts, 1980). Recalcitrant seeds cannot tolerate desiccation and Pammenter and Berjak (1999) suggested that the desiccation sensitivity of the seed is related to the rate of metabolic activity.

When attempting to store seeds of a particular species, it is imperative that one determines the characteristics of the seeds, i.e. whether they are orthodox or non-orthodox, and, if the seeds are recalcitrant, to determine the degree of recalcitrance. The range of shedding water contents of embryonic axes of recalcitrant seeds varies markedly among species (Berjak and Pammenter, 2008). Berjak and Pammenter (2004a; 2008) suggested that this range is generally between 0.4 and 4.0 g g^{-1} on a dmb, but can

possibly be even higher (Pammenter and Berjak, 1999). Additionally, water content at shedding appears to be intra-seasonally variable within the same species (Berjak *et al*., 1989), and this should also be taken into consideration. Consequently, shedding water content of a seed alone, cannot be the defining factor when assessing the degree of recalcitrance, but rather assessed complementarily with the seed's response to desiccation, as well as storage behaviour (Berjak and Pammenter, 2001).

Because they do not tolerate desiccation, recalcitrant seeds cannot be stored under dry, cold conditions (King and Roberts, 1980; Vertucci *et al*., 1991) as employed for orthodox types. Orthodox seeds undergo profound changes when desiccation tolerance is acquired during development, involving the presence and interplay of a suite of mechanisms (discussed in section 1.4.1), but in non-orthodox seeds, the expression and interaction of the factors concerned is incomplete (Pammenter and Berjak, 1999; Berjak and Pammenter, 2004c). It is therefore thought that recalcitrant seed behaviour is a consequence of events (or the lack of these events) during seed development, and the absence or ineffective expression of one or more of these could determine the relative degree of desiccation of seeds of a particular species (Pammenter and Berjak, 1999). The specific processes or mechanisms that have been suggested to confer and/or contribute to desiccation tolerance have been outlined and reviewed by Pammenter and Berjak (1999) and are as follows:

1) Physical characteristics of the intracellular environment, specifically:

- decreased levels of vacuolation;
- quantity and nature of the accumulated insoluble reserves;
- reaction of the cytoskeleton;
- arrangement of the DNA, chromatin and nuclear structure;

2) Intracellular de-differentiation leading to minimal exposure of membrane surface area and possibly that of the cytoskeleton;

3) 'Switching off' of metabolic activity;

4) Occurrence and efficiency of antioxidant systems;

5) Accumulation and functions of protective molecules, including late embryogenic abundant proteins (LEAs), sucrose and certain oligosaccharides or galactosyl cyclitols;

6) Deployment of certain amphipathic molecules [although since then it appears that there is no *in vivo* evidence for this (Golovina, pers. comm.[1])];

7) Presence and effective functioning of a peripheral oleosin layer around lipid bodies;

8) Presence and functioning of repair mechanism during rehydration.

It has also been suggested that the ability of seeds to tolerate desiccation is an ancestral trait, and that over generations this trait has been lost in species with recalcitrant seeds (Farnsworth, 2000; Oliver *et al*., 2000; Dickie and Pritchard, 2002). Pammenter and Berjak (2000) offered the opposite scenario, suggesting rather that seed recalcitrance is the ancestral condition and desiccation tolerance of seeds was acquired through evolution. Furthermore, to add to the desiccation sensitivity of recalcitrant seeds, many species also produce seeds that are chilling-sensitive and they cannot be stored at low temperatures (even above 0°C) (Roberts, 1973; King and Roberts, 1980).

Due to their inability to tolerate desiccation, all recalcitrant seeds lose viability at relatively high water content, but the degree of water loss tolerated varies with the species (King and Roberts, 1980; Roberts and King, 1980; Normah *et al*., 1986; Pritchard and Prendergast, 1986; Farrant *et al*., 1988; 1989; Pence, 1990; Berjak and Pammenter, 2000; 2004a; c; 2008; Wesley-Smith *et al*., 2001a). Thus, there are different degrees of recalcitrance, ranging from seeds that are minimally to those that are maximally sensitive to desiccation (Farrant *et al*., 1988). This implies that recalcitrant seeds of some species

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may survive longer at defined tissue water potentials than those of other species, possibly due to the semi-acquisition or semi-operation of mechanisms that permit orthodox seeds to tolerate desiccation (Berjak and Pammenter, 2008). Pammenter and Berjak (1999) suggested that the more metabolically active a seed is when it is shed; the less tolerant it will be to desiccation.

1.6 Dehydration of recalcitrant seeds

Dehydration of recalcitrant seeds is a necessary practice for long-term conservation of their genetic resources. When hydrated seeds are exposed to LN during cryopreservation (see section 1.7.1), lethal freezing damage occurs as a result of ice formation. On the other hand dehydration to water contents at which ice formation does not occur invariably results in desiccation damage (King and Roberts, 1980; Wesley-Smith *et al*., 2001a). Dehydration, when applied as a pretreatment to cryopreservation, reduces the amount of water available for ice crystal formation and growth, which can occur readily during cooling and storage at low temperatures (Meryman and Williams, 1985; Pritchard and Prendergast, 1986; Pammenter *et al*., 1991; 1998; Vertucci *et al*., 1991; Berjak *et al*., 1992; Pammenter and Berjak, 1999; Wesley-Smith *et al*., 2001a). Many recalcitrant seeds can be dehydrated to water contents that approximate the hydration level at which point all remaining water is non-freezable, and if this so called 'structural' water, which is associated with intracellular structures and macromolecules (Pammenter *et al*., 1998; Pammenter and Berjak, 1999), is removed, conformation changes and irreversible desiccation damage occurs (Pammenter *et al*., 1993; Wesley-Smith *et al*., 2001a). Furthermore, the desiccation damage that results from drying seeds below this hydration level, which includes changes in membrane phase behaviour (Pammenter *et al*., 1998; Hoekstra *et al*., 1999; Bryant *et al*., 2001), is greater at lower temperatures (Kovach and Bradford, 1992; Vertucci *et al*., 1995). These lower temperatures are said to intensify water stress (Walters *et al*., 2002a). In contrast to this, orthodox seeds are able to withstand removal of a considerable portion of this non-freezable water without decreasing viability (Pammenter *et al*., 1991; Vertucci and Farrant, 1995).

In order to establish successful protocols for cryopreservation of recalcitrant material, a balance between minimal freezing and desiccation damage needs to be achieved (King and Roberts, 1980; Pritchard and Prendergast, 1986; Chandel *et al*., 1995; Wesley-Smith *et al*., 1992; 2001a). Additionally, the variability in post-harvest behaviour of seeds among different recalcitrant-seeded species and even within species, depending on the developmental status (see section 1.6.1), must also be taken into consideration when attempting to establish guidelines for manipulation of seed water content (Farrant *et al*., 1989; Berjak *et al*., 1992; 1993; Pammenter *et al*., 1998; Pammenter and Berjak, 1999; Wesley-Smith *et al*., 2001a). Moreover, Pammenter *et al*. (1998) suggested that due to the differential response of recalcitrant seeds to dehydration at different drying rates, it is not possible to define a 'critical water content' at which viability is lost (see section 1.6.2).

Dehydration removes water that is critical for survival; it induces a number of stresses within cells, affecting chemical and metabolic processes, protective functions, and membrane and organellar integrity, collectively termed desiccation-induced damage (Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Walters *et al*., 2001; Wesley-Smith *et al*., 2001a). These different stresses can be attributed to the many roles that water plays in the functioning of cells (Walters *et al*., 2002a). Vertucci and Farrant (1995) suggested that if a seed is dehydrated and thus water removed, chemical and metabolic processes that can occur only at the original water content level, are no longer possible. Those authors further suggested that the protective function of water is obviously diminished by its removal and, particularly in the case of recalcitrant seeds, damaging effects will occur due to a lack of tolerance mechanisms. However, often it may be unclear whether a change in morphology, metabolic activity or cellular ultrastructure is a consequence of dehydration, i.e. a sign of damage, or a protective strategy (Walters *et al*., 2002a). Furthermore, desiccation damage is not indicated by differences between the hydrated and dry state, but by the resumption of normal activity upon rehydration (i.e. damage measured by irreversible change as opposed to those that are reversible) (Walters *et al*., 2002a).

1.6.1 Effects of developmental status on response to dehydration

There has been sufficient evidence from a number of studies (Berjak *et al*., 1992; 1993; Farrant *et al*., 1993; Daws *et al*., 2006; reviewed by Leprince *et al*., 1993; Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002) indicating that the developmental status of seeds has a marked impact on their response to desiccation. It is generally understood that orthodox seeds are desiccation tolerant; however these seeds are not able to tolerate drying at all stages of their development (Bewley and Black, 1994; Kermode and Finch-Savage, 2002). During the early stages of seed development, orthodox species are desiccation sensitive and only later undergo a transition to a desiccation-tolerant state (Kermode, 1990; Bewley and Black, 1994; Kermode and Finch-Savage, 2002). Orthodox seeds undergo three main developmental phases: histodifferentiation, increase in fresh mass and reserve deposition, and maturation drying (Bewley and Black, 1994; Kermode, 1997; Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002; Daws *et al*., 2006). It is thought that the acquisition of full desiccation tolerance happens at the transition between reserve deposition and maturation drying (Kermode and Bewley, 1985; Pammenter and Berjak, 1999; Daws *et al*., 2006). At the onset of maturation drying, the accumulation of dry mass stops and fresh mass decreases due to an overall loss of water from the orthodox seeds (Kermode, 1990; Bewley and Black, 1994; Pammenter and Berjak, 1999; Walters, 2000; Kermode and Finch-Savage, 2002; Daws *et al*., 2006).

Due to the limited number of studies on the developmental processes among the spectrum of desiccation-sensitive species (Pammenter and Berjak, 1999), it is difficult to unequivocally chart the developmental aspects of recalcitrant seeds. However, some generalities can be drawn (Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002). Recalcitrant seeds undergo initial histodifferentiation and reserve accumulation much in the same manner as those of orthodox types (Farrant *et al*., 1992; Finch-Savage and Blake, 1994; Finch-Savage, 1996; Pammenter and Berjak, 1999). The difference in development patterns between orthodox and recalcitrant seeds however, lies in the fact that, whereas orthodox seeds reach a point where dry mass accumulation ceases, in

recalcitrant seeds accumulation continues until they are shed (Farrant *et al*., 1992; Finch-Savage and Blake, 1994; Kermode and Finch-Savage, 2002). This increase in dry mass results in a net decrease in percentage water content at the final stage of development, as dry mass accumulates at a higher rate than water (Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002). However, this decrease in percentage water content is not comparable with the maturation drying phase in orthodox seeds as there is little or no net water loss (Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002).

Towards the end of seed development, the decrease in percentage water content of recalcitrant seeds coincides with a slight increase in desiccation tolerance (Finch-Savage, 1996; Berjak and Pammenter, 1997; Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002). Such increases in desiccation tolerance with increasing developmental status have been shown in a number of species including *Acer pseudoplatanus* (Hong and Ellis, 1990a), *Quercus robur* (Finch-Savage, 1992), *Camellia sinensis* (Berjak *et al*., 1993) and *Aesculus hippocastanum* (Farrant *et al*., 1997; Pammenter and Berjak, 2000). However, it must be remembered that this increased desiccation tolerance with decreased water content is relative, as it is being compared with the highly desiccation-sensitive, earlier stages of seed development. These seeds still remain metabolically active, desiccation sensitive and require no dehydration to stimulate germination. Consequently, these seeds are more inclined towards rapid germination and establishment (Kermode and Finch-Savage, 2002; Pritchard *et al*., 2004; Daws *et al*., 2005) rather than suited to long-term storage. Daws *et al*. (2004), in their study on 36 desiccation-sensitive species, proposed that this tendency towards rapid germination associated with the desiccation sensitivity of seeds at shedding is beneficial. The results of the fore-mentioned study showed that rapid germination minimised seed predation, and the low investment of resources in physical defense mechanisms (i.e. endocarp and/or testa) meant more efficient use of resources in seed provisioning. Therefore this relative increase in desiccation tolerance with increased development should not be taken as seeds exhibiting orthodox behaviour, but rather simply as a slight shift in position on the continuum scale of seed behaviour.

Furthermore, it must be noted that this pattern of increased desiccation tolerance (i.e. lower critical water content) with increased development is not consistent across all non-orthodox species. For example, Farrant *et al*. (1993) showed that seeds of *Avicennia marina* demonstrated no change in water content during seed development after they had become germinable, and thus exhibited no increase in desiccation tolerance. Some recalcitrant seeds may also exhibit increased desiccation sensitivity during the final stages of seed development (Pammenter and Berjak, 1999). This has been shown in studies by Fu *et al*. (1994) (*Clausena lansium* and *Litchi chinensis*), Ellis *et al*. (1990d) (*Coffea arabica*) and most notably, Berjak *et al*. (1992) (*Landolphia kirkii*). Investigations on seeds of *L. kirkii* suggested that the decrease in desiccation tolerance during development from immature to mature embryonic axes is as a consequence of an increase in percentage water content brought about by a decrease in lipid content during development (Berjak *et al*., 1992) (see section 1.4.2.1).

Despite the apparent metabolic changes during development of seeds, there appears to be no clear end point to development of recalcitrant seeds (Kermode and Finch-Savage, 2002). In fact it has also been put forward that recalcitrance is as a result of premature termination of seed development (Finch-Savage, 1992; Finch-Savage and Blake, 1994). Furthermore, the variability of developmental status, even at the point of seed shed, is illustrated by Finch-Savage and Blake (1994) who showed that recalcitrant seeds of *Quercus robur* from the same tree were shed at different water contents in different years, with those shed at the lower water contents being most desiccation tolerant. Studies performed on *Acer pseudoplatanus* by Daws *et al*. (2006) have shown that provenance and environmental conditions also have a marked impact on both desiccation and germination. The results of their investigations indicated that colder conditions induced *A. pseudoplatanus* seed shed at a higher water content and thus a less developed stage, resulting in seeds that were more desiccation sensitive. Therefore, it is imperative that when undertaking dehydration studies on recalcitrant seeds, the precise stage of development of the seeds, the provenance, climatic conditions and harvesting or collection protocols are known (Berjak *et al*., 1993). Additionally, the influence of pre-

shedding development is that, generally, a high level of metabolic activity is associated with high desiccation sensitivity (Pammenter and Berjak, 1999).

1.6.2 Rates of drying

Another major factor that contributes to the variability in the response of desiccation-sensitive seeds to dehydration is the rate of drying (Pammenter *et al*., 1991; 1998; Berjak *et al*., 1993; Pammenter and Berjak, 1999; Wesley-Smith *et al*., 2001a; Berjak and Pammenter, 2008). The rate of dehydration determines the time taken for seed or axis material to pass through a series of declining water contents (Pammenter and Berjak, 1999). However, evaluating the effects of dehydration rates is confounded by the fact that many studies compare slow drying rates of whole seeds with rapid dehydration of embryonic axes (e.g. Pammenter *et al*., 1991; Berjak *et al*., 1993; Pritchard *et al*., 1995; Kioko *et al*., 1998; Pritchard and Manger, 1998), while others evaluate the drying rates of whole seeds (e.g. Farrant *et al*., 1985; Pritchard, 1991; Bonner, 1996; Pammenter *et al*., 1998) or embryonic axes only (e.g. Liang and Sun, 2000; Wesley-Smith *et al*., 2001a; Liang and Sun, 2002; Ntuli *et al*., 2011). The general consensus for non-orthodox seeds though is that the faster the drying, the lower the water content that can be tolerated (Farrant *et al*., 1985; 1989; Normah *et al*., 1986; Pritchard and Prendergast, 1986; Berjak *et al*., 1989; 1993; Pritchard, 1991; Potts and Lumpkin, 1997; Pammenter *et al*., 1998; Pritchard and Manger, 1998; Pammenter and Berjak, 1999; 2000; Kundu and Kachari, 2000; Ajayi *et al*., 2006a; Berjak and Pammenter, 2008). The opposite has been observed for orthodox seeds in that the slower the dehydration rate, the higher the survival (Pammenter and Berjak, 1999). It has been suggested that a slower drying rate of orthodox seeds allows adequate time for the induction and operation of protective mechanisms within the seeds (only if they haven't been induced already) (Pammenter and Berjak, 1999). As the example most relevant to the present investigation, Pammenter *et al*. (1991), working on *Landolphia kirkii*, showed that slow drying of intact seeds to axis water contents of approximately 0.9 to 0.7 g g^{-1} (dmb) caused lethal damage, whereas very rapid (flash) drying of excised embryonic axes permitted removal of water to approximately 0.3 g g^{-1} (dmb). The same authors also suggested that the difference

between desiccation-tolerant and desiccation-sensitive seeds is that tolerant seeds tolerate loss of freezable water, whereas sensitive seeds can lose this water without obvious damage (at least in the short-term) only if it is removed very rapidly.

Although the effects of dehydration rates have been observed on whole recalcitrant seeds (Farrant *et al*., 1985; Pritchard, 1991; Finch-Savage, 1992; Pammenter *et al*., 1998), these effects are markedly less when compared to that of isolated embryos (Pammenter *et al*., 1998). It has been questioned whether the removal of embryonic axes from the storage tissue of seeds (i.e. cotyledon/s, and possibly the endosperm) (Berjak *et al*., 1993), could elicit different responses to dehydration when compared to intact seeds (Finch-Savage, 1992; Berjak *et al*., 1993; Leprince *et al*., 1999). However, a study on *Ekebergia capensis* showed that whole seeds that were rapidly dried retained full viability at substantially lower embryonic axis water contents than seeds that were slowly dried (Pammenter *et al*., 1998). Similarly, studies on excised embryonic axes of *Artocarpus heterophyllus* (Wesley-Smith *et al*., 2001a) and *Quercus robur* (Ntuli *et al*., 2011) showed that when axes were rapidly (flash) dried, viability was reduced only at water contents <0.3 g g^{-1} (dmb) compared with 0.8 g g^{-1} (dmb) when excised axes were slowly dehydrated. These investigations suggest a response to dehydration rate rather than responses to removal of cotyledons or endosperm tissue (Pammenter and Berjak, 1999).

Another argument is that the large size of most recalcitrant seeds may simply not facilitate rapid drying rates (Pammenter *et al*., 1991; Chandel *et al*., 1995; Pence, 1995; Pammenter and Berjak, 1999; Berjak, 2000; Wesley-Smith *et al*., 2001a; Berjak and Pammenter, 2004a; 2008; Daws *et al*., 2005; Sershen *et al*., 2007) and this may explain the reason for the lower impact of different dehydration rates on whole seeds than when compared with that of isolated embryos. Additionally, Pence (1995) suggested that tissues within seeds may lose water at different rates, and therefore the overall seed water content may in essence, be very different from that of the embryonic axis itself. This was shown to be the case for seeds of *Elaeis guineensis*, which exhibited an overall seed water content that was 10% less than the actual axis water content (Grout *et al*., 1983). Additionally, in some species including *Quercus rubra* (Pritchard, 1991), *Quercus robur*

(Finch-Savage, 1992) and *Castanea sativa* (Leprince *et al*., 1999), tissues within recalcitrant seeds have demonstrated differences in their sensitivity and thus responses to desiccation. Therefore, slower and more rapid dehydration rates may in essence have a very similar impact on embryonic axes held within whole seeds. On the other hand, isolated embryonic axes are not surrounded by additional tissues that lose water at different rates, and the effects of dehydration rates therefore may be markedly greater.

However, even root and shoot tissue within isolated embryos of *Araucaria hunsteinii* (Pritchard and Prendergast, 1986; Pritchard *et al*., 1995), *Quercus rubra* (Pritchard, 1991), *Castanea sativa* (Pence, 1992) and *Quercus robur* (Poulsen, 1992) exhibited different responses and tolerances to dehydration. Even so, when embryonic axes, somatic embryos or meristems are isolated from the seeds and are dehydrated by flash-drying (Berjak *et al*., 1990; Wesley-Smith *et al*., 2001a; Pammenter *et al*., 2002), rapid dehydration rates can be achieved in many species [e.g. *Hevea Brasiliensis* (Normah *et al*., 1986), *Landolphia kirkii* (Pammenter *et al*., 1991), *Ekerbergia Capensis* (Pammenter *et al*., 1998; Perán *et al*., 2006) and *Quercus robur* (Berjak *et al*., 1999)].

It is suggested that the effectiveness of rapid drying at lowering the water content before viability reduction in recalcitrant material is not through the elicitation of any form of desiccation tolerance (Pammenter *et al*., 1998; Wesley-Smith *et al*., 2001a; Berjak and Pammenter, 2008); rather, rapid drying allows less time for deleterious water-based reactions to progress, and damage to accumulate (Berjak *et al*., 1989; 1993; Pammenter *et al*., 1991; 1998; Pritchard, 1991; Berjak and Pammenter, 1997; 2008; Wesley-Smith *et al*., 2001a). These deleterious reactions that cause a loss in viability occur when desiccation-sensitive material is slowly dehydrated (Pammenter *et al*., 1998; Wesley-Smith *et al*., 2001a). Pammenter *et al*. (1991) suggested that viability loss associated with recalcitrant seeds, when they are dehydrated fast enough to pass through the intermediate water contents associated with the deleterious aqueous-based reactions, is as a result of the removal of the structural-associated or non-freezable water (reviewed by Pammenter and Berjak, 1999). This may also be the case for whole seeds as suggested by Pritchard (1991) working on *Quercus rubra*, who showed that rapid dehydration provided less time

for desiccation-induced deteriorative changes to take place. A separate study on whole seeds of *Avicennia marina* (Farrant *et al*., 1985) offers an alternative to this reasoning, suggesting that as a consequence of the increased time afforded by slow drying, germination-associated development events are allowed to occur during the initial stages of drying, thereby increasing desiccation sensitivity (Berjak *et al*., 1989).

It is important to remember that recalcitrant seed tissue cannot be viewed as potentially desiccation tolerant, regardless of how rapidly the tissue can be dehydrated (Berjak and Pammenter, 2003a; 2008). In fact, embryonic axes of recalcitrant seeds will lose viability at ambient or refrigerated temperatures rapidly if left, even for a few hours, at the low water contents attained through flash-drying (Walters *et al*., 2001; Berjak and Pammenter, 2008). There is also a point below which they cannot survive, and this lower limit is always a higher water content than to that which intermediate and orthodox seeds can be dried (Pammenter and Berjak, 1999; Berjak, 2006a; Berjak and Pammenter, 2008). Therefore, in simple terms, the more rapidly dehydration is achieved with recalcitrant material, the shorter is the exposure to the water range that permits damaging aqueous-based reactions (Berjak and Pammenter, 2003a; Ntuli *et al*., 2011). Furthermore, even when recalcitrant explants are rapidly dehydrated to lower water contents without any adverse impact on initial viability, they do not remain viable for long periods (possibly hours to a few days) (Berjak and Pammenter, 2003b) in this partially dehydrated state (Vertucci and Farrant, 1995; Pammenter *et al*., 1998; Walters *et al*., 2001; 2002a).

1.7 Storage of recalcitrant seeds

In recent years, the loss of plant genetic resources and the need for environmental protection has come to the foreground (Chin, 1995; van Slageren, 2003). National and international programs have been implemented for the conservation of crop plants or their wild relatives by a variety of techniques and methods (Chin, 1995) (discussed in sections 1.2 and 1.3). Genebanks in the form of seedbanks for the storage of orthodox seeds, and *in vitro* banks for other explants, have been established worldwide for short-, medium-

and long-term storage (Levin, 1990; Roberts, 1991; Chin, 1995). Genebanking essentially keeps the genetic material of a plant, be it in the form of seeds or alternate explants, in a state of suspended animation (van Slageren, 2003). Large numbers of seeds of a particular species are collected, dried and stored in airtight aluminium and glass containers at sub-zero temperatures, usually in large deep-freezers kept at -20°C (van Slageren, 2003). The longevity of orthodox seeds in storage is said to increase logarithmically with decreasing water contents (Ellis and Roberts, 1980; Chin, 1995). However, the storage lifespan of recalcitrant seeds is curtailed by the fact that they are desiccation sensitive and so cannot be stored in the dry state, and if maintained hydrated they will initiate germination in storage (reviewed by Pammenter and Berjak, 1999). Additionally, recalcitrant seeds are variable in size and viability (Chin, 1995), and many, particularly those of tropical origin, may be chilling sensitive (Roberts, 1973; King and Roberts, 1980; Chin, 1995; Pammenter and Berjak, 1999) (see section 1.4.2). Therefore recalcitrant seeds cannot be stored under the conventional conditions designed for orthodox seeds and short- to medium-term storage remains a challenge.

Short- and medium-term storage techniques currently employed for recalcitrant seeds are generally limited to imbibed or hydrated storage. It has been suggested for some species a technique of partial desiccation of seeds to water contents slightly below their shedding water content may be beneficial to prevent germination in storage (Chin, 1995; Pritchard *et al*., 1995). However, the work of Drew *et al*. (2000) and Eggers *et al*. (2007) have demonstrated that partial dehydration of some recalcitrant seeds actually decreases storage lifespan. Seeds are generally stored at ambient temperatures or slightly below (Berjak *et al*., 1989; Chin, 1995) in the range of 7 to 20°C (Chin, 1995) in order to minimise early germination by decreasing metabolic rates (Pammenter and Berjak, 1999). However, these methods are accompanied by their own set of problems because hydrated seeds are more susceptible to attack by pests, infection by diseases (Chin, 1995) and microbial proliferation (King and Roberts, 1980; Mycock and Berjak, 1990; Berjak, 1996; Berjak and Pammenter, 2004a; 2008). Fungal contamination has been shown to accelerate the intrinsic deterioration of recalcitrant seeds in storage (Bilia *et al*., 1999). Moreover, results from studies conducted on seeds of *Hopea parviflora* (Sunilkumar and

Sudhakara, 1998) and *Avicennia marina* (Calistru *et al*., 2000) demonstrate that the application of fungicidal treatments prior to hydrated storage extends the hydrated-storage lifespan supporting the conclusion that mycofloral contamination decreases storage lifespan. However, Berjak and Pammenter (2004b) stated that "as most fungicides target particular fungal species or groups, it is necessary to assess the efficacy of systemic fungicides in curtailing proliferation of the specific fungi involved, as well as ensuring that the treatment itself does not damage the seeds." Furthermore, even under minimal, or the absence of these contaminants, recalcitrant seeds ultimately lose viability under these conditions as stored, hydrated recalcitrant seeds are metabolically active and so undergo germination-associated changes (Pammenter *et al*., 1994; Pammenter and Berjak, 1999; Berjak and Pammenter, 2004a; 2008). It has been suggested that the hydrated storage period of recalcitrant seeds is dependant on the rate of germinative development in storage (Berjak *et al*., 1989). This variability amongst recalcitrant seeds means that optimum storage conditions are generally species-specific and often only elucidated by the process of trial and error (Berjak, 2006a; Sershen *et al*., 2007). Recalcitrant seeds therefore generally have a storage lifespan under hydrated conditions ranging from a few days to 1 – 2 years (King and Roberts, 1980; Berjak *et al*., 1989; Farrant *et al*., 1989; Pammenter *et al*., 1994; Chin, 1995; Pammenter and Berjak, 1999), which proves inadequate for the long-term storage needed for germplasm preservation (Pammenter and Berjak, 1999).

Over the past few decades cryopreservation has become the leading method for the potential long-term preservation of recalcitrant-seeded germplasm in the form of whole seeds, embryonic axes and somatic meristems and embryos (e.g. Pritchard and Prendergast, 1986; Chaudhury *et al*., 1991; Vertucci *et al*., 1991; Assy-Bah and Engelmann, 1992; Wesley-Smith *et al*., 1992; Pammenter and Berjak, 1999; Shimonishi *et al*., 2000; Sershen *et al*., 2007; reviewed by Engelmann, 2000; Berjak *et al*., 2000a; Walters *et al*., 2008). This technique and the attendant problems are discussed in the sections that follow.

1.7.1 Cryopreservation

Cryopreservation involves exposure to ultra-low temperatures, storage and subsequent recovery of viable cells, tissues, organs and organisms so they resume normal metabolic functioning [generally in LN or its vapour phase at temperatures between -140 and -196°C (Benson, 2008; Day *et al*., 2008)]. These low temperatures curtail biochemical activity and substantial molecular movements necessary for reactions without killing the cells (Benson and Bremner, 2004; Benson, 2008), and it has been suggested that under the appropriate conditions, stability and hence longevity, is enhanced in cells (Day *et al*., 2008). Furthermore, cryopreservation imparts security of biological materials as the risk of genetic selection, loss of totipotency and contamination is minimised (Benson and Lynch, 1999; Benson, 2008; Day *et al*., 2008). Cryopreservation requires very little maintenance and once protocols have been established and samples stored in LN, all that is required is that the levels of the cryogen are regularly checked and replenished (Harding, 2004; Benson, 2008). Thus, the technique is not only more cost-effective than other storage methods such as culture collections, but also saves valuable personnel hours spent on subculturing (Engelmann, 2004; Benson, 2008). The avoidance of routine subcultures also importantly decreases the risk of contamination (Benson, 2008). Therefore, cryopreservation has been widely utilised in a variety of sectors including horticultural, medical, healthcare, biotechnology, forestry, aquaculture and veterinary science (Benson *et al*., 2002; 2006; Engelmann, 2004; Day *et al*., 2008) since germplasm can, theoretically, survive for indefinite periods (Tessereau *et al*., 1994; Engelmann, 1997; Menges and Murray, 2004; Berjak and Pammenter, 2004a; Leunufna and Keller, 2005).

This technique, if it can be successfully achieved, is the most robust and efficient long-term storage option for non-orthodox seed germplasm which cannot be stored using conventional seed storage methods for any meaningful period (Normah *et al*., 1986; Pritchard and Prendergast, 1986; Kartha and Engelmann, 1994; Berjak *et al*., 1996; 1999; 2000a; Engelmann, 2000; 2004; Berjak and Pammenter, 2004a; b; 2008; Perán *et al*., 2006; Sershen *et al*., 2007; Benson, 2008; Walters *et al*., 2008). Cryopreservation

protocols exist for more than 100 plant species (Harding, 2004) in a variety of explant forms (Kartha and Engelmann, 1994). However, the development of cryopreservation protocols for recalcitrant-seeded species are vastly less advanced when compared with vegetatively propagated species (Engelmann, 2004). As is the case with dehydration and short- to medium-term storage of non-orthodox material, the responses of germplasm to cryopreservation has been highly variable among species as well as among explants within the same species, necessitating the empirical determination of protocols for each species and explant employed (Pence, 1995; Wesley-Smith *et al*., 1995; Kioko *et al*., 1998; Sershen *et al*., 2007). Due to this, some of the cost saving incurred by the use of cryopreservation is sometimes offset by the unavoidable use of protocols for recalcitrant seeds that are suboptimal, resulting in impaired recovery responses (Benson, 2008) and therefore larger numbers of explants need to be cryopreserved in order to ensure at least some survival post-retrieval (Reed, 2008).

Day *et al*. (2008) suggested that despite the diversity of the biological materials that have been successfully cryopreserved, all storage protocols developed and employed are based upon the knowledge of three factors *viz*. water behaviour, cryo-injury and cryoprotection. Broadly based on these factors, the general approach to cryopreservation of non-orthodox seed material involves the manipulation, optimisation and collective operation of a number of factors including the size, physiological state and selection of an appropriate explant type, decontamination protocols, tissue water content and dehydration methods and rates, characteristics and concentration of chemical cryoprotectants, cooling rates and methodology, thawing and rehydration temperatures and rates, and development of *in vitro* technology for establishment of vigorous seedlings (Withers, 1979; Benson, 1993; Harding, 2004; Berjak and Pammenter, 2008). These factors are discussed below.

1.7.1.1 Explant type

Berjak (2006b) suggested that in order to achieve successful cryostorage, the smallest explant that will subsequently give rise to a plant needs to be used. As discussed in section 1.4.2, recalcitrant seeds generally are large (Chin, 1995; Berjak *et al*., 2000a;

Wesley-Smith *et al*., 2001a; Berjak and Pammenter, 2003a; b; 2004a; 2008; Pammenter *et al*., 2011) with the embryo/embryonic axis comprising only a small percentage of the overall dry weight of the seed (Chin *et al*., 1989; Berjak and Pammenter, 2004a; 2008). Additionally, tissues within these recalcitrant seeds have also been shown to differ in their responses and sensitivity to desiccation (e.g. Pritchard and Prendergast, 1986; Pritchard, 1991; Finch-Savage, 1992; Pence, 1992; Poulsen, 1992; Pritchard *et al*., 1995; Leprince *et al*., 1999). Therefore, it is very difficult to achieve rapid and uniform dehydration and cooling of the embryos in whole seeds (Pence, 1995; Pammenter and Berjak, 1999; Berjak, 2000; Wesley-Smith *et al*., 2001a; Berjak and Pammenter, 2004a; 2008; Daws *et al*., 2006; Sershen *et al*., 2007), and in many cases attempts to freeze these large seeds have failed (e.g. Becwar *et al*., 1983; Jörgensen, 1990; Ahuja, 1991; González-Benito and Pérez-Ruiz, 1992; Chandel *et al*., 1995). However, although very seldom (Berjak *et al*., 1989; Wesley-Smith *et al*., 1992; 2001a), some non-orthodox seeded species have been successfully cryopreserved using whole seeds [e.g. *Carica papaya* (Chin and Krishnapillay, 1989), *Coffea liberica* (Normah and Vengadasalam, 1992), *Azadirachta indica* (Berjak and Dumet, 1996), *Wasabia japonica* (Potts and Lumpkin, 1997) and *Warburgia salutaris* (Kioko *et al*., 2003)].

With regards to non-orthodox seeds, and recalcitrant seeds in particular, the removal of the embryos or embryonic axes from its nutrient supply (i.e. the cotyledon(s) and/or endosperm) within the seed, and its subsequent use in cryopreservation and *in vitro* culture, has been the most popular approach for their long-term storage in recent years (Berjak and Pammenter, 2004a; 2008; Berjak, 2006a). In most cases, this approach generally achieves the objective of size reduction for cryopreservation as recalcitrant embryonic axes are usually in the range of 1 – 3 mm (Berjak and Pammenter, 2004a; Berjak, 2006a), and amenability to flash (rapid) drying (Pammenter *et al*., 2002). The use of embryonic axes as germplasm for cryopreservation has achieved success with many non-orthodox species including *Fagus grandiflora* (Pence, 1990), *Quercus faginea* (González-Benito and Pérez-Ruiz, 1992), *Camellia sinensis* (Wesley-Smith *et al*., 1992; Chandel *et al*., 1995), *Artocarpus heterophyllus* (Chandel *et al*., 1995), *Quercus robur*

(Berjak *et al*., 1999), *Aesculus hippocastanum* (Wesley-Smith *et al*., 2001b), *Aesculus glabra*, *Juglans nigra* (Pence, 2003) and *Poncirus trifoliata* (Wesley-Smith *et al*., 2004a).

With many tropical and sub-tropical species, axes need to be excised from their cotyledons in order to attain explants that are suitably small to process for cryopreservation (Pammenter *et al*., 2011). However, excision of embryonic axes from cotyledons is known to cause considerable damage to the shoot apex (Goveia *et al*., 2004; Berjak and Pammenter, 2004a; 2008; Pammenter *et al*., 2011) and following cryopreservation, shoot production often does not occur, but rather axes survive to form callus and/or roots (Pammenter *et al*., 2011). Pammenter *et al*. (2011) suggested that this is particularly the case for dicotyledonous species that have fleshly cotyledons. Species that have demonstrated this are axes of *Trichilia dregeana* (Kioko *et al*., 1998; Goveia *et al*., 2004), *Ekebergia capensis* (Perán *et al*., 2006), *Trichilia emetica* and *Protorhus longifolia* (Pammenter *et al*., 2011). It has been suggested that the excision of embryonic axes from the seed potentially exposes them to an oxidative burst [i.e. the production of reactive oxygen species (ROS)], which is a natural primary response to wounding (Minibayeva *et al*., 1998; Goveia *et al*., 2004; Roach *et al*., 2008; Whitaker *et al*., 2010).

In some instances embryos/embryonic axes are still too large for cryopreservation and alternative explants need to be used (Berjak and Pammenter, 2004a; Berjak, 2006a). Modern techniques are successfully implementing cryopreservation of all types of explants including embryonic callus, apices, somatic embryos, meristems, pollen, nodal buds and cell suspensions of temperate and tropical species (Engelmann, 2004; Berjak and Pammenter, 2004a; Berjak, 2006b).

1.7.1.2 Dehydration as a pretreatment for cryopreservation

Whether seeds, embryonic axes or alternative explants, tissues in the hydrated state do not tolerate sub-zero temperatures (Meryman and Williams, 1985; Normah *et al*., 1986; Vertucci *et al*., 1991) and therefore, long-term storage efforts through cryopreservation are adversely affected. Thus, for successful cryopreservation, water content of explants,

amongst other parameters, need to be optimised so as to minimise damage by desiccation at low water contents, or by freezing at higher water contents (Normah *et al*., 1986; Pence, 1990; Chaudhury *et al*., 1991; Vertucci *et al*., 1991; Wesley-Smith *et al*., 1992; 2001a; Berjak and Pammenter, 2004b).

Dehydration of explants not only removes water in the tissue, but also promotes transition of the aqueous solution to a viscous amorphous glassy phase through the process of vitrification (Dumet and Benson, 2000; Engelmann, 2000; Berjak and Pammenter, 2004a). Controlling the state of water by stabilising glasses is essential if successful cryopreservation strategies are to be developed (Benson, 2008; Buitink and Leprince, 2008). Dehydration increases the concentration of cellular solutes (Leprince *et al*., 1999; Benson, 2007). As the solutions within the cells of the explant material become concentrated during drying, it may become supersaturated, accompanied by an increase in viscosity (Koster, 1991). If the viscosity reaches the point at which diffusion of water is prevented (Koster, 1991; Leprince *et al*., 1999), the solution is regarded as a glass. A glass is defined as an amorphous metastable state that resembles a plastic solid brittle material, but retains the disorder of a liquid (Franks, 1985; Walters *et al*., 2005). For example, a study by Leprince *et al*. (1999), using electron spin resonance (ESR) spectroscopy on recalcitrant *Castanea sativa* seeds, showed that when isolated embryonic axes were dried, the cytoplasmic viscosity increased steadily from a water content of 1.6 down to 0.4 g g^{-1}. Furthermore, below a water content of 0.35 g g^{-1} the ESR spectra reading became distorted, which is indicative of a spin probe that had become immobilised in a semi-solid environment, suggesting that a glass had formed within the cytoplasm (Leprince *et al*., 1999). Those authors also showed that drying rate did not influence the rise in viscosity during water loss.

Koster (1991) also suggested that sugar mixes similar to those found in axes that are desiccation sensitive, form glasses only at sub-zero temperatures and their properties may also play a role in desiccation tolerance. Furthermore, Buitink *et al*. (2000) and Buitink and Leprince (2008) suggested that storage stability of seeds is related to the molecular mobility as well as the packing density of the intracellular glass, and therefore, the

physico-chemical properties of intracellular glasses provide stability for long-term survival. Therefore the benefits of glass formation and vitrification to explants undergoing desiccation include the highly viscous glass filling space during dehydration, the sheer bulk of which may therefore prevent the collapsing of tissue (Burke, 1986; Koster, 1991; Sakai, 2004; Buitink and Leprince, 2008). Additionally, glass formation may suspend chemical reactions that require molecular diffusion thereby ensuring stability (Burke, 1986; Koster, 1991; Sun and Leopold, 1993; 1997; Buitink *et al*., 1998; Sakai, 2004; Buitink and Leprince, 2008). This phase change also stabilises membranes as glasses may permit the continuance of hydrogen bonding at the interface between the glass and hydrophilic surfaces in the cell (Burke, 1986) and may reduce the possible lethal ice crystal formation during explant cooling (Stanwood, 1985; Reed, 1996; Benson *et al*., 2006). Furthermore, glasses do not drastically change fluid structure thereby making them less damaging (Benson, 2007).

In order to establish successful cryopreservation procedures, achieving rapid dehydration of non-orthodox germplasm (discussed in section 1.6.2) whilst maintaining viability is vital (Wesley-Smith *et al*., 1992; 2001a; Pammenter *et al*., 1998; 2002; Berjak and Pammenter, 2008). Wesley-Smith *et al*. (2001a) suggested that drying recalcitrant axes to water contents facilitating cryopreservation should ensure survival of a critical number of cells in order to ensure that normal seedlings are produced. Flash-drying to sub-lethal water contents has been shown to achieve this by reducing the time of dehydration stress to which germinative cells are exposed (Wesley-Smith *et al*., 2001a). As discussed above, dehydration increases cytoplasmic viscosity (Leprince *et al*., 1998; 1999; Wesley-Smith *et al*., 2004a), which hinders ice crystal growth on immersion of explants in the cryogen (Luyet *et al*., 1962; Wesley-Smith *et al*., 2004a). Furthermore, lower water contents are associated with lower freezing damage (Meryman and Williams, 1980; Vertucci, 1990; Wesley-Smith *et al*., 1992; 2004a; Farrant and Walters, 1998) and higher glass transition temperatures (Meryman and Williams, 1980; Leprince and Walters-Vertucci, 1995). However, for successful cryopreservation, there needs to be a balance between avoiding ice crystal damage (rapid cooling rates and lower water contents) and desiccation damage (higher water contents) (Berjak and Pammenter, 2004a).

1.7.1.3 Cryoprotection

Cryoprotectant solutions are believed to prevent or reduce the physical injuries that result in cellular and tissue damage of germplasm exposed to cryogenic temperatures (Suzuki *et al*., 2005; Volk and Walters, 2006; Benson, 2008). It has been suggested that successful cryopreservation protocols are dependent on cryoprotective strategies in combination with optimising cooling rates preceding the final immersion of explants in LN (Benson, 2004; 2008; Fuller, 2004; Day *et al*., 2008). Success has been achieved with cryopreservation by combining rapid dehydration and chemical cryoprotection (Sakai, 2000; Shimonishi *et al*., 2000; Benson, 2008), followed by cooling, either relatively slowly (Chaudhury *et al*., 1991; Harding *et al*., 2004; Benson *et al*., 2006) or ultra-rapidly (Wesley-Smith *et al*., 1992; Pammenter and Berjak, 1999; Volk *et al*., 2006b). Cryoprotection involves the pretreatment of explants by direct exposure to concentrated cryoprotective solutions (Meryman and Williams, 1985; Sakai, 1985; Chaudhury *et al*., 1991; Mycock *et al*., 1995; Sershen *et al*., 2007), or by preculturing them on a medium containing the cryoprotectant (Pritchard *et al*., 1982; Dumet *et al*., 1994; Cho *et al*., 2001; Varghese *et al*., 2009). These chemical cryoprotectants are a heterogeneous group of compounds that can be broadly categorised as penetrating/colligative and non-penetrating/osmotic types that are often used in combination (Fahy *et al*., 1984; Meryman and Williams, 1985; Benson, 2004; 2008; Volk *et al*., 2006b; Day *et al*., 2008).

During dehydration and controlled cooling, water is removed from cells resulting in a more concentrated cytoplasm. However, excessive concentration of solutes, known as colligative injury (Benson *et al*., 2005; Benson, 2007; Day *et al*., 2008), is detrimental to cell functioning, and was shown to be the primary cause of freezing injury (Lovelock, 1953). Therefore, a balance needs to be achieved between dehydration and solute concentration and this is where penetrating cryoprotectants are effective (Day *et al*., 2008).

Colligative cryoprotectants are small molecules that penetrate the plasma membrane, although their permeability often varies between cell types (Volk *et al*., 2006b; Benson,

2008; Day *et al*., 2008). Glycerol and dimethyl sulphoxide (DMSO) are the most common penetrating cryoprotectants applied to plant material (Suzuki *et al*., 2005; Benson, 2007; 2008; Day *et al*., 2008). They operate by entering the cytoplasm and depressing the freezing point of the intracellular solution and acting as a cellular solvent, ameliorating the damaging effects of increased solute concentrations that occur when water is lost from the cell (Benson, 1990; 2004; 2007; 2008; Santarius and Franks, 1998; Benson *et al*., 2005; Day *et al*., 2008). This is achieved by minimising the toxic electrolyte concentration proportionally to the amount of water maintained in the liquid state below 0°C (Kartha and Engelmann, 1994). Penetrating cryoprotectants are believed to obviate the potentially lethal reduction in cell volume from water loss (Benson, 2007; 2008; Day *et al*., 2008) and, due to their equal distribution across the germplasm, a more uniform cooling rate can be achieved, thus improving recovery (Benson, 2007; 2008).

Non-penetrating or osmotic cryoprotectants such as sucrose are generally larger molecules and have multiple protective roles (Farrant *et al*., 1977; Day *et al*., 2008). They are believed to function by causing osmotic dehydration, thus reducing the amount of water available for freezing, as well as lowering the freezing point and impairing ice crystal nucleation by restricting the molecular mobility of water molecules (Storey and Storey, 1996; Benson, 2004; 2007; 2008; Fahy *et al*., 2004; Fuller, 2004; Benson *et al*., 2005; Volk and Walters, 2006; Day *et al*., 2008). It is generally recommended that penetrating additives be used in combination with non-penetrating cryoprotectants as the latter are said to lower the necessary but potentially damaging high concentrations of colligative cryoprotectants required to attain a stable glass (Fahy *et al*., 1984; Benson, 2007; Day *et al*., 2008).

The application of chemical cryoprotectants promotes the formation of glass (Dumet and Benson, 2000; Engelmann, 2000; Benson, 2007) because the intracellular solution is so concentrated that the cytoplasm vitrifies and ice crystal growth is inhibited (see section 1.7.1.2). Even if ice crystals do form from the remaining intracellular water, they are generally so small that they are innocuous and non-injurious (Meryman and Williams, 1985; Benson *et al*., 2005; Benson, 2007; 2008; Day *et al*., 2008).

The application of single cryoprotectant solutions has been shown to be generally less effective than when used in combinations of two or more, which significantly increases survival post-cooling (Finkle and Ulrich, 1979; Chen *et al*., 1984; Withers, 1985; Fahy *et al*., 1986; Tao and Li, 1986; Towill, 1995). However, the penetration of each cryoprotectant component and thus their effectiveness, may change with the temperature of their application (Fahy *et al*., 1984; Finkle *et al*., 1985; Benson, 2007), cell type (Fahy *et al*., 1984; Finkle *et al*., 1985; Benson, 2007; Volk and Caspersen, 2007), culture age (Harding *et al*., 1996), totipotency (Benson *et al*., 1992), capacity to overcome stress (Benson *et al*., 1992; 1995) and other components in the cryoprotection solution (Fahy *et al*., 1984; Finkle *et al*., 1985). Furthermore the effect of cryoprotectants may also be species-specific (Fahy *et al*., 1984; Finkle *et al*., 1985; Benson, 2008). Additionally, apart from the general physical protective properties mentioned above, it has been suggested that chemical cryoprotectants provide additional defenses against freezing injury (Benson and Bremner, 2004; Fuller, 2004; Volk *et al*., 2006b; Day *et al*., 2008). Chemical cryoprotectant solutions can stabilise both proteins (Arakawa and Timasheff, 1982; Arakawa *et al*., 1990; Volk *et al*., 2006b; Day *et al*., 2008) and membranes (Rudolph and Crowe, 1985; Kartha and Engelmann, 1994; Fuller, 2004; Volk *et al*., 2006b; Day *et al*., 2008), as well as acts as antioxidants (Orthen *et al*., 1994; Shen *et al*., 1999; Yancey, 2005; Day *et al*., 2008). However, the chemical toxicity of cryoprotectant solutions, especially at high concentrations can damage cell structures, impair physiological function and interfere with microfilament and microtubule structure and configuration (Bhandal *et al*., 1985; Fahy, 1986; Tao and Li, 1986; Withers, 1988; Steponkus *et al*., 1992; Fahy *et al*., 2004; Volk *et al*., 2006b). Although the exposure time and concentration of cryoprotectant solutions differ with the species, concentrations between 5 – 15% are commonly applied and appear tolerable prior to immersion into LN (Withers, 1985; Kartha *et al*., 1988; Mycock *et al*., 1995; Cyr, 2000). Cryoprotectants are generally applied at 0°C or room temperature, in a one-dose concentration, or stepwise. The latter is suggested in order to avoid osmotic shock (Benson, 2008). However, a balance between the effectiveness of chemical cryoprotectants and their toxicity to cells within plant germplasm needs to be empirically determined and optimised as toxicity can be germplasm- and species-dependent (Dumet and Benson, 2000; Benson, 2008).

1.7.1.4 Controlled rate and slow cooling

The first techniques employed for the routine cryopreservation of plant germplasm involved slow, controlled rate cooling (Withers and King (1980; Benson, 2004; 2007; 2008; Benson *et al*., 2005) and still form the basis of many current plant germplasm cryopreservation protocols (Benson, 2004; 2008; Panis and Lambardi, 2006). The basic methodology has been modified, adapted, and optimised for a number of plant systems (Benson, 2004). Slow cooling rates of 0.1 – 10°C min^{-1} (Touchell *et al*., 2002; Benson, 2004; 2007; 2008) down to sufficiently low temperatures in the range of -30 to -80°C, within a programmable freezer, are generally adopted for most cryopreservation protocols (Benson, 2004; 2008). These slow cooling rates minimises the risk of damaging intracellular ice formation (Mazur, 1984; Kartha, 1985; Meryman and Williams, 1985; Kartha and Engelmann, 1994; Benson, 2004; Benson *et al*., 2005; Day *et al*., 2008). Explants are subsequently immersed in LN (Kartha, 1985; Benson, 2004; 2007; 2008; Benson *et al*., 2005; Day *et al*., 2008) or continued stepwise reduction in temperature to -196°C is adopted (Kartha, 1985; Sershen *et al*., 2007). The former is commonly referred to as two-step cooling (Morris, 1978; Engelmann, 1993; Day *et al*., 2000; Benson *et al*., 2005; Benson, 2007).

Slow cooling rates are believed to encourage the migration of water to extracellular spaces and therefore the formation of a few large extracellular ice crystals, and alleviates the formation of potentially-lethal intracellular ice that generally occurs together with extracellular freezing events, when explants are rapidly cooled (Fahy *et al*., 1984; Franks, 1985; Kartha, 1985; Mazur, 2004; Benson *et al*., 2005; Day *et al*., 2008). Slow, progressive temperature reduction will initiate the nucleation of ice extracellularly, restricting the subsequent ice formation to the external *milieu* (Kartha and Engelmann, 1994; Reed and Uchendu, 2008) and leaving the interior of the cell super-cooled rather than frozen (Benson *et al*., 2005). A water vapour difference is established between the frozen exterior and the interior of the cell resulting in quasi-equilibration through the movement of intracellular water to the exterior of the cell whereupon it freezes, a process referred to as cryodehydration (Benson *et al*., 2005). Mazur (1963; 2004) explained that

the cooling rate of cells controls the water movement between cell compartments, which invariably affects the cell solute concentration. This phenomenon (i.e. increase in cytoplasmic solute concentration) is believed to bring about the transition of the remaining intracellular water to a glass (Kartha, 1985; Mazur, 1990; Wesley-Smith *et al*., 1995; Dumet and Benson, 2000; Engelmann, 2000; Day *et al*., 2008).

An optimised protocol for controlled rate cooling (using a programmable freezer) has a major advantage over other approaches in that hundreds of vials of germplasm can be cryopreserved at one time (Benson, 2004; 2008; Reed and Uchendu, 2008). This technique is highly efficient for large-scale processing of high volumes of germplasm such as culture collections holding many accessions (Benson, 2004; 2008). Most model programmable freezers are computerised (Benson *et al*., 2005; Benson, 2008; Day *et al*., 2008) allowing the user to manipulate multiple parameters, resulting in more robust and reliable methods in addition to many different optimisation options (Benson, 2008; Day *et al*., 2008). Programmable freezers are used extensively in the commercial sector (Benson, 2008) and controlled slow cooling has proven a successful cryopreservation method when applied to many types of germplasm including meristems [e.g. *Pyrus communis* (Reed, 1990), *Cichorium intybus* (Demeulemeester *et al*., 1993), *Mentha spicata* (Hirai and Sakai, 1999) and *Melia azedarach* (Scocchi *et al*., 2004), reviewed by Kartha, 1985; Benson, 1995; Reed and Uchendu, 2008], dedifferentiated cultures [e.g. *Bromus inermis* (Ishikawa *et al*., 1996), *Gossypium hirsutum* (Rajasekaran, 1996), *Helianthus tuberosus* (Swan *et al*., 1999) and *Nicotiana tabacum* (Kobayashi *et al*., 2005), reviewed by Withers, 1985], embryogenic cultures [e.g. *Oryza sativa* (Lynch and Benson, 1991), *Ipomoea batatas* (Blakesley *et al*., 1995; Bhatti *et al*., 1997) and several *Citrus* species (Pérez *et al*., 1997)], somatic embryos [e.g. *Larix Eurolepsis*, *Picea mariana* (Kobayashi *et al*., 1990), *Citrus sinensis* (Marin and Duran-Vila, 1988) and *Castanea sativa* (Corredoira *et al*., 2004)] and whole seeds [e.g. *Pisum sativum*, *Glycine max*, *Helianthus annuus* (Vertucci, 1989a), 10 native Western Australian species (Touchell and Dixon, 1993), *Wasabia japonica* (Potts and Lumpkin, 1997) and *Coffea arabica* (Dussert *et al*., 1998; 2000)].

In contrast to the numerous advantages of programmable freezers, the drawbacks associated with the use of this equipment include, the initial monetary outlay for the equipment (Benson, 1999; 2004; 2008), its regulatory servicing and routine checks, safety, and the cost associated with supplying the large amounts of LN required to cool the freezing chamber, all of which can prove prohibitive for smaller, non-profit laboratories (Benson, 2008). Low-budget alternatives such as Mr Frosty (Nalgene^{TM}, New York, U.S.A.) are therefore employed by these laboratories for small batches of germplasm samples (Percy *et al*., 2000; Benson, 2008; Day *et al*., 2008; Amaral *et al*., 2009). This low maintenance system uses isopropanol as the coolant to passively freeze polypropylene cryovials at a constant rate within an ultra freezer (-80°C) (Harding *et al*., 2004; Menges and Murray, 2004; Benson, 2007; 2008; Day *et al*., 2008). However, Mr Frosty too has its limitations in that cooling rates cannot be manipulated as the cryovials held within the systems chamber can be cooled only at a fixed rate of 1°C min^{-1} down to between -20 and -80°C (dependent on the solvent's cooling characteristics).

Successful cryopreservation protocols have also been developed for partially dehydrated plant germplasm by plunging cryovials directly into LN, with many explant types including embryos/embryonic axes [e.g. *Hevea brasiliensis* (Normah *et al*., 1986), *Aruacaria hunsteinii* (Pritchard and Prendergast, 1986), *Cocos nucifera* (Assy-Bah and Engelmann, 1992), *Aesculus hippocastanum* (Pence, 1992), *Poncirus trifoliata* (Radhamani and Chandel, 1992), *Prunus amygdalus* (Chaudhury and Chandel, 1995), *Trichilia dregeana* (Kioko *et al*., 1998), *Sechium edule* (Abdelnour-Esquivel and Engelmann, 2002), *Arachis hypogaea* (Gagliardi *et al*., 2002) and various Amaryllid species (Sershen *et al*., 2007)], embryonic cultures [e.g. *Ipomoea batatas* (Blakesley *et al*., 1995; Bhatti *et al*., 1997) and *Picea mariana* (Touchell *et al*., 2002)], meristems [e.g. *Sechium edule* (Abdelnour-Esquivel and Engelmann, 2002)], whole seeds [e.g. *Coffea liberica*, *Vigna sesquipedalis* (Normah and Vengadasalam, 1992) and *Citrus suhuiensis* (Makeen *et al*., 2005)] and somatic embryos [e.g. *Castanea sativa* (Corredoira *et al*., 2004)] reporting at least some survival. Cryopreservation protocols have also been developed in which dehydrated germplasm is immersed in LN vapour [e.g. whole seeds of *Helianthus annuus*, *Glycine max* and *Pisum sativum* (Vertucci, 1989a) and embryonic

axes with attached cotyledons of *Camellia sinensis* (Kim *et al*., 2002; 2005)]. Although slightly faster cooling rates are achieved by plunging cryovials directly into LN compared with programmable freezers and the use of Mr Frosty, it is still classified as slow cooling (e.g. Vertucci, 1989a; Wesley-Smith *et al*., 2001b; Sershen *et al*., 2007). Documented rates achieved using this technique however, varies significantly between experiments [e.g. *c*. 10°C min^{-1} (Vertucci, 1989a); 3 – 5°C s^{-1} (Sershen *et al*., 2007)]. It has been suggested though that the cooling rate achieved within cryovials plunged into LN is species-specific and influenced by the moisture content and the size of the germplasm (Vertucci, 1989a).

1.7.1.5 Rapid cooling

Rapid dehydration and cooling rates have been employed for successful cryopreservation and post-thaw survival of isolated zygotic axes of a variety of species, for example *Camellia sinensis* (Wesley-Smith *et al*., 1992), *Trichilia dregeana* (Kioko *et al*., 1998), *Quercus robur* (Berjak *et al*., 1999), *Poncirus trifoliata* (Wesley-Smith *et al*., 2004a) and various amaryllid species (Sershen *et al*., 2007). However, one danger associated with flash-drying is that it may result in excessive water loss from the explant, thus killing germplasm before any cooling trials are performed (Berjak and Pammenter, 2004a). An ultra-rapid cooling technique has been developed to overcome this problem, especially for the cryopreservation of embryonic axes of recalcitrant-seeded species that cannot tolerate desiccation (Wesley-Smith *et al*., 1992; 1999; 2001b; 2004a; Berjak and Pammenter, 2004b).

The most common method of ultra-rapid cooling, of the order of several hundred of degrees per second (Berjak and Pammenter, 2000b; Wesley-Smith *et al*., 2004b), is achieved by tumble mixing naked explants in a polystyrene container with nitrogen slush [LN supercooled to -210°C (Echlin, 1992)] (e.g. Wesley-Smith *et al*., 1992; Sershen *et al*., 2007; Ngobese *et al*., 2010). Alternatively, rapid cooling rates are also attained by use of a plunging device (spring-loaded) to directly immerse individually-mounted axes into nitrogen slush or isopentane, at an average velocity of 1.2 m s^{-1} to a depth of 160 mm

(e.g. Wesley-Smith *et al*., 1995; 1999; 2001b; 2004a; b). Although cooling rates as high as *c*. 1300°C s^{-1} (Wesley-Smith *et al*., 2004a) and *c*. 1700°C s^{-1} (Wesley-Smith *et al*., 2004b) have been attained using the above-mentioned technique, ultra-rapid cooling rates and survival following this cooling are dependent on good thermal contact between the sample and the cryogen (Wesley-Smith *et al*., 1992), and the complex interaction among the physical attributes such as mass, water content and geometry of the explant (Wesley-Smith *et al*., 2001b).

Water is said to undergo a transition into cubic or hexagonal ice configuration between temperatures of -40 and -138°C (Robards and Sleytr, 1985). Wesley-Smith *et al*. (2004b) stated that the cooling rate down to these temperatures influences the location, size and number of ice crystals formed within cells and tissues. It was further suggested that higher cooling rates prevent ice crystal formation and growth by rapidly traversing through this critical temperature range (Robards and Sleytr, 1985; Wesley-Smith *et al*., 1995; 2001b). This invariably permits cooling of explants at higher water contents that are still tolerable during cryopreservation, thereby facilitating better post-thaw survival (Luyet, 1960; 1965; Vertucci *et al*., 1994b; Berjak *et al*., 1996; Wesley-Smith *et al*., 2001b; Berjak and Pammenter, 2004a; Benson *et al*., 2005). Due to the relatively large size of most explants though, the total prevention of intracellular ice formation within relatively hydrated tissues is unachievable (Luyet, 1960; Ryan and Purse, 1985). However, rapid cooling is effective in limiting the amount of ice that is formed rather than completely avoiding it (Wesley-Smith *et al*., 2001b; 2004b).

1.7.1.6 Thawing

The survival and subsequent recovery of explants to produce normal, functional seedlings following cryopreservation is dependent on a number of carefully defined factors and manipulations (Berjak and Mycock, 2004) including the optimisation of rewarming parameters after cryostorage (Kioko, 2003; Berjak and Mycock, 2004; Wesley-Smith *et al*., 2004a; Day *et al*., 2008). Day *et al*. (2008) suggested that relaxation of glasses that may occur on rewarming can fracture fragile and particularly rigid structures within cells.

Additionally, much like cooling, thawing/warming of germplasm can result in the formation of ice crystals due to the devitrification of the metastable glasses within cells, which invariably poses a major threat to cellular integrity (Bowers, 1990; George, 1993; Benson, 2007; 2008; Day *et al*., 2008). Furthermore, innocuous ice crystals are capable of growing to a size that may cause cellular injury via a process of recrystallisation (Sakai and Yoshida, 1967; Leibo *et al*., 1970; Bowers, 1990; Mazur, 1990; George, 1993; Benson, 2007; Day *et al*., 2008). It has been suggested that devitrification and glass relaxation during thawing can be related, at least in part, to the warming rates (Benson *et al*., 2006; Benson, 2007; Day *et al*., 2008). Moreover, dependent upon the rates of cooling, the cryoprotectants employed and the type of tissue, thawing rates can also affect ice stability (Benson 2007). Day *et al*., (2008) proposed that if warming rates are too slow, glasses can devitrify and form ice. In addition to ice formation (and glass relaxation), slow thawing may also result in unbalanced metabolism (Bowers, 1990; George, 1993; Kioko, 2003).

As discussed in section 1.7.1.5, ice crystal formation generally occurs in the temperature range between -40 and -138°C (Robards and Sleytr, 1985) and is a function of the mobility of water (Luyet *et al*., 1962; Rall and Fahy, 1985), which is reduced below -40°C (MacKenzie, 1977), and severely restricted below -100°C (Wikefeldt, 1971). Therefore, much as is the case for cooling, faster thawing rates through this temperature range are thought to limit recrystallisation, ice crystal formation and growth and their associated cellular damage (Mazur, 1984; Kartha, 1985; Robards and Sleytr, 1985; George, 1993; Kioko, 2003; Wesley-Smith *et al*., 2004a; Benson, 2007). However, Benson (2007; 2008) cautions that if warming rates are too rapid, stress-cracks and fractures can occur. Furthermore, studies have suggested that the warming rate required to avoid cellular damage following cryostorage is largely determined by the time spent in the temperature range that supports ice crystal formation during cooling (Mazur, 1990; Wesley-Smith *et al*., 2004a). Moreover, the damage incurred during thawing can be avoided/limited only through the effective balance between water content and cooling rates (Wesley-Smith *et al*., 2001b; 2004a), as well as the use of cryoprotectants (Wesley-Smith *et al*., 2001b; Benson, 2007).

Rapid thawing is generally achieved by immersing explants for 1 to 2 min directly in distilled water (e.g. Wesley-Smith *et al*., 1992; 1999; 2001b; 2004b; Kartha and Engelmann, 1994; Berjak *et al*., 1999; 2000b; Sershen *et al*., 2007), or in liquid growth medium, held at a temperature between 35 and 45°C (e.g. Wesley-Smith *et al*., 1992; Kartha and Engelmann, 1994; Grout, 1995; Kioko *et al*., 1998; Mix-Wagner *et al*., 2002). Alternatively, germplasm cryostored within cryovials can be thawed by plunging cryovials into a water bath held at 25 – 45°C (e.g. Withers and King, 1980; Find *et al*., 1993; Kioko *et al*., 1998; Burch and Wilkinson, 2002; Nadarajan *et al*., 2006; Benson, 2008; Varghese *et al*., 2009). Recent studies have also involved the use of a solution of 1µM $CaCl_2.2H_2O$ and 1mM $MgCl_2.6H_2O$ [calcium-magnesium (CaMg)] as a thawing and rehydration medium which was shown to be more effective than distilled water (Berjak *et al*., 1999; Mycock, 1999; Berjak and Mycock, 2004; Varghese *et al*., 2009). This is further discussed below.

1.7.1.7 Rehydration

Rehydration of explants that have been either partially dehydrated and cryopreserved, or dehydrated only, is an important step in their survival and formation of normal seedlings. Similar to the thawing process, rehydration generally involves directly submerging the experimental material in distilled water (e.g. Wesley-Smith *et al*., 2001a; 2004b; Perán *et al*., 2004) or liquid culture medium (e.g. Quain *et al*., 2009) for *c*. 20 – 40 min. Simultaneous thawing/rehydration of explants either in distilled water (e.g. Berjak *et al*., 1999; 2000a) or liquid medium (e.g. Wesley-Smith *et al*., 1992; Mycock *et al*., 1995) has also been successfully applied to a number of cryopreservation protocols at either ambient temperature (25°C) or at 35 – 40°C. Alternatively, slow rehydration can be carried out using filter paper moistened with liquid growth medium or distilled water (e.g. Leprince *et al*., 1998; Wesley-Smith *et al*., 2001b; 2004a; Perán *et al*., 2004). A study conducted by Perán *et al*. (2004) showed that rapid rehydration by direct immersion resulted in higher germination when compared to slow rehydration. Much in the same manner as slow dehydration (see section 1.7.1.2), slow rehydration is suggested to expose recalcitrant tissue to the intermediate water contents that bring about aqueous-based

degradation processes (Vertucci and Farrant, 1995; Pammenter *et al*., 1998; Walters *et al*., 2001).

The rehydration of dry biological material is known to result in the leakage of low molecular weight substances (Hoekstra *et al*., 1999), although this leakage is understood to subside as rehydration progresses in desiccation-tolerant tissue, provided that initially the material is not very dry (Pammenter *et al*., 2002). Ultra dry material (Hong and Ellis, 1990b; Pammenter *et al*., 2002) and/or low temperatures (Hobbs and Obendorf, 1972; Bramalage *et al*., 1978) can result in extensive imbibition damage. Furthermore, recalcitrant seeds and axes will be damaged at even higher water contents (Pammenter *et al*., 2002; Perán *et al*., 2004), and imbibition damage is exacerbated at low temperatures and with increased storage time (Sacandé *et al*., 1998; Perán *et al*., 2004). To avoid damage of orthodox seeds due to rapid imbibitional leakages at low temperatures, seeds or axes undergo a pre-humidifying step (Pammenter *et al*., 2002; Perán *et al*., 2004), which was a common practice for recalcitrant germplasm (e.g. Berjak *et al*., 1992; 1993; Leprince *et al*., 1998; Pammenter *et al*., 1998). However, in our laboratory, this step has now been superseded by immersion of recalcitrant material in a CaMg solution.

Studies performed with recalcitrant axes of *Quercus robur* (Berjak *et al*., 1999; 2000a) revealed that when explants exposed to drying-freezing treatments were rapidly rehydrated in distilled water at ambient temperature, the roots produced showed no gravitropic response. This abnormality was due to failed reassembly of the cytoskeleton and so statolith deposition (Berjak *et al*., 1999; 2000a; Berjak and Mycock, 2004) as a result of improper rehydration procedures (Berjak and Mycock, 2004; Berjak and Pammenter, 2004b). However, rehydration of recalcitrant germplasm in a CaMg solution at ambient temperature achieved promising results (Berjak *et al*., 1999; Berjak and Mycock, 2004; Perán *et al*., 2006; Varghese *et al*., 2009), with this rehydration protocol being associated with the reassembly of the actin cytoskeleton (Berjak and Pammenter, 2004b) and normal statolith deposition in root cap columella cells, which is correlated with the downward curvature of the roots (i.e. gravitropism) (Berjak and Mycock, 2004; reviewed by Berjak and Pammenter, 2004b).

1.7.1.8 Viability assessment and *in vitro* regeneration

The ultimate goal of plant cryopreservation is the establishment and normal development of seedlings from cryostored germplasm. There are numerous techniques to evaluate the vigour, viability and cellular structure of plant tissue, including the tetrazolium test (ISTA, 1999) (e.g. Bittencourt *et al*., 1997; de Camargo *et al*., 1997), electrolyte leakage (e.g. Vertucci, 1989b; c; Pammenter *et al*., 1991; 1998; Berjak *et al*., 1992; Wesley-Smith *et al*., 2001a; b), and electron microscopy (e.g. Berjak *et al*., 1992; 1999; Wesley-Smith *et al*., 1992). However, these methods are not considered as rigorous at assessing the success of a cryopreservation protocol as the regeneration and establishment of normal, fully-functional, callus-free seedlings (Pammenter *et al*., 2002; Benson, 2007).

Many problems can arise at this re-establishment step of cryopreservation (Berjak and Pammenter, 2004b). Since recalcitrant seeds are generally too large to be cryopreserved (discussed in section 1.7.1.1), embryonic axes are usually separated from the endosperm or cotyledons of the seed and, in so doing, its nutrient source is lost. This necessitates the formulation of a tissue culture germination medium for embryonic axes post-thawing. The selection of the correct medium is an essential step in the development of a successful cryopreservation protocol (Berjak and Pammenter, 2004b). Ideally, the medium should contain the ingredients found in the seed endosperm such as vitamins, minerals, sucrose and may also include plant growth regulators (PGRs) such as kinetin, benzylaminopurine (BAP), gibberellic acid (GA), naphthaleneacetic acid (NAA) and cytokinin (George, 1993). A formulation developed by Murashige and Skoog (1962), or a modification thereof, is usually employed as a starting point for recalcitrant germplasm. Additives such as PGRs are species-dependent and Kane (2004) suggested that the complexity of the culture medium is inversely proportional to the size of the explant. In addition to the culture medium, decontamination protocols, photoperiod conditions and temperature at which *in vitro* cultures are kept all need to be empirically determined for explants of each species.

1.8 *Landolphia kirkii* and rationale for this study

The seeds of *L. kirkii* T.-Dyer, a member of the Apocyanaceae, are characterised as being recalcitrant (Berjak *et al*., 1989; 1992; Pammenter *et al*., 1991; Vertucci *et al*., 1991). The plant, which is native to southern Africa, is abundantly distributed along coastal and sandforest areas, mainly in northern KwaZulu-Natal. *Landolphia kirkii* is a scrambling shrub, or woody climber that can reach up to 8 metres, and produces fruit biannually. In the ripened state the fruit of *L. kirkii* constitutes a prominent food source for monkeys and birds while remaining popular among rural communities. This species also has potential for the rubber industry as the fruit and stems exude large amounts of milky latex, which can be used to produce high quality rubber (Munro, 1981). The plant produces relatively large (approximately 40 – 100 mm in diameter), pale green fruit with white dots, turning yellowish-orange upon ripening (Pooley, 1993). *Landolphia kirkii* produces narrowly oval, large endospermous seeds (approximately 1.5 g and 10 – 15 mm long) (Berjak *et al*., 1992). The embryonic axis is fully developed when the fruit is shed, and there are no tendencies towards dormancy (Pammenter *et al*., 1991). The embryonic axis constitutes less than 0.1% of the dry mass of the mature seed (Berjak *et al*., 1990) and is situated apically, which facilitates its excision from the extremely hard endospermic tissue (Berjak *et al*., 1992). If maintained at their original water content, the seed will lose viability in approximately one month (Pammenter *et al*., 1991), as metabolic demands of the embryo exceed the endogenous seed tissue water. However, wet storage of seeds of *L. kirkii* has been shown to increase the desiccation sensitivity of embryonic axes (Berjak *et al*., 1992). Therefore, cryostorage of the embryonic axes excised from *L. kirkii* axes should offer a means of germplasm preservation.

Previous studies have demonstrated that the extent of desiccation tolerated by recalcitrant seeds of a variety of species could be altered by the rate of drying (Farrant *et al*., 1988; Berjak *et al*., 1990; 1992; Pammenter *et al*., 1991; Berjak and Pammenter, 1994). Embryonic axes of seeds of *L. kirkii* have been shown to tolerate a lower water content when dried very rapidly (flash-dried), than when slowly dehydrated (Pammenter *et al*., 1991). However, the 'critical water content' for survival is dependent on the

developmental status of the seeds: immature axes could be flash-dried to lower water levels without apparent damage compared with mature and germinating seeds (Vertucci *et al*., 1991; Berjak *et al*., 1992). Previous work carried out on mature axes of *L. kirkii* has shown that the original water content of about 1.5 g g^{-1} (dmb) can be reduced to approximately 0.32 g g^{-1} when flash-dried for 30 min, while the viability was maintained at 80% (Pammenter *et al*., 1991). Vertucci *et al*. (1991), recorded success in terms of mature axis survival of *L. kirkii* at a temperature of -70°C using a slow cooling rate of 10°C min^{-1}, but no survival was recorded when axes were exposed to -150°C (Wesley-Smith *et al*., 1992; Berjak, pers. comm.[2]).

The present study was aimed at successful preservation of zygotic germplasm of *L. kirkii* at temperatures below -140°C, widely regarded as the optimum for cryogenic storage (Benson, 2008). In the previous research on *L. kirkii* the axes alone (i.e. without attached cotyledonary tissue) were removed from the seeds (Pammenter *et al*., 1991; Vertucci *et al*., 1991; Berjak *et al*., 1992). This may have resulted in excision injury (Pammenter *et al*., 2011), which was possibly exacerbated at temperatures below -70°C. For the purposes of the current study, therefore, axes were also removed with a portion of each 'paper-thin' cotyledon attached (see Figure 2.1) in order to observe the possible effects of excision injury and their responses, compared with those from which the cotyledons had been severed flush with the axis surface. Prior to cooling trials, the desiccation sensitivity of axes was assessed through flash-drying in order to establish the optimum water content amenable to cooling. Other studies have shown that chemical cryoprotectants increased the probability of explant survival following cryogenic cooling (Shimonishi *et al*., 2000; Benson, 2008). Although this may not generally apply across recalcitrant zygotic axes of all species (Kioko, 2003; Sershen *et al*., 2007), the effects of selected cryoprotectants were tested as part of the present investigation. Thereafter, the effects of various cooling rates and techniques on the survival of embryonic axes only and embryonic axes with attached cotyledonary segments were assessed.

[2] Berjak, P. University of KwaZulu-Natal, Durban, South Africa

As discussed in section 1.4.2.1, exposure of seeds to water when the TAGs are crystallised is lethal. Therefore, before any cooling trials were performed, a pilot experiment was conducted to determine if this was, in fact, the case for embryonic axes of *L. kirkii*, the cells of which have previously been demonstrated to contain considerable lipid (TAG) reserves (Berjak *et al*., 1992). Fatty acid composition of total and polar fractions of the axes and cotyledons of *L. kirkii* were determined. Protocols employing ultra-rapid and/or rapid cooling methods have shown to be the most promising for long-term cryopreservation of recalcitrant germplasm (e.g. Wesley-Smith *et al*., 1992; 2004a; Kioko *et al*., 1998; Berjak *et al*., 1999; Sershen *et al*., 2007). Therefore, for the initial pilot study embryonic axes of *L. kirkii* were also exposed to both ultra-rapid and rapid cooling and various thawing combinations (after Volk *et al*., 2006a; 2007) to examine for possible lipid body melting prior to imbibition. The effects of these treatments on cellular structure, and the possible occurrence of lipid crystallisation within embryonic axes post exposure to cryogenic temperatures was ultrastructurally examined using transmission electron microscopy (TEM).

Chapter 2: Materials and methods

2.1 Harvesting and seed handling

Recalcitrant seeds are physiologically active upon shedding and some lose water relatively readily (Berjak *et al*., 1989). It is thus imperative that their provenance and mode of harvest are known, and that their post-harvest handling is strictly controlled (Berjak *et al*., 1989). Fully mature fruits of *Landolphia kirkii* were hand-harvested in October of 2008 and February and October of 2009 from a wild population in False Bay (27° 58' 32.84"S, 32° 19' 42.38"E), South Africa, enclosed in heavy plastic bags that were periodically aerated, and were conveyed by road to the laboratory at the University of KwaZulu-Natal (UKZN), Durban, within 48 h. The fruits are hard coated and contain between 15 and 40 mature endospermous seeds, to which the sticky pulp adheres. Wet storage of recalcitrant seeds has been shown to increase desiccation sensitivity of embryonic axes of some species [e.g. *Avicennia marina* (Farrant *et al*., 1985) and *Camellia sinensis* (Berjak *et al*., 1993)] including *L. kirkii* (Berjak *et al*., 1992). Additionally, the seeds of *L. kirkii* lose little to no water in the intact fruit (Pammenter *et al*., 1991), hence for storage for up to two weeks at 16°C, fruits were left intact, surface decontaminated by rinsing in a 1% (m/v) sodium hypochlorite (NaOCl) solution, dusted with the fungicide Benomyl (active ingredient benzimidazole, Villa Protection, South Africa) and stored in sterilised plastic trays. Seeds were removed from the fruits and cleaned of the sticky lactiferous pulp by gently rubbing inside a piece of thin nylon mesh under a stream of water and removing excess pulp using a blunt scalpel. After cleaning the seeds, the embryonic axes (or axes with attached cotyledonary segments) were immediately excised from the hard endosperm. This was done by using a blunt scalpel to transversely lift one side of the endosperm of the seed and detaching it from the attached cotyledon and axis (careful attention was paid not to split the axes during this process) (see Figure 2.1) after which axes were easily excisable.

2.2 Water content determination

To determine the shedding water content as well as the range of water contents post-flash-drying, twenty axes with attached cotyledonary segments when present, were individually weighed on a Mettler MT5 six place balance. Weights of embryonic axes were recorded before and after drying in an oven at 80°C for 48 h, and expressed on a dmb (g H_2O g^{-1} dry mass; g g^{-1}).

2.3 Decontamination protocol

Various surface decontaminants were tested in an attempt at curtailing bacterial and fungal growth associated with embryonic axes of *L. kirkii*. These included 1% (m/v) sodium hypochlorite (NaOCl) for 5 and 10 min, 0.2% (m/v) mercuric chloride ($HgCl_2$) for 30 seconds and 1 min, and 1% (m/v) calcium hypochlorite [$Ca(OCl)_2$] for 5 and 10 min. All decontaminants tested were prepared with sterile deionised water and poured under aseptic conditions within a laminar flow cabinet into sterile 90 mm Petri dishes containing the excised embryonic axes. Closed Petri dishes were gently agitated by hand throughout the duration of decontamination. After decontamination for the chosen times, decontaminating solutions were decanted and axes rinsed three times with a sterile CaMg solution [containing 1µM $CaCl_2.2H_2O$ and 1mM $MgCl_2.6H_2O$ (Mycock, 1999)]. In order to ascertain the effectiveness of decontaminants at preventing mycofloral proliferation, axes were subsequently blotted dry with sterile filter paper and cultured in 90 mm Petri dishes (5 axes per Petri dish) containing 35 ml full strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium comprising 4.42 g l^{-1} MS salts, 30 g l^{-1} sucrose, 8.0 g l^{-1} bacteriological agar, pH 5.6 – 5.8. Petri dishes were sealed with Parafilm® and placed in a growth room with a photosynthetic photon flux density (PPFD) of 52 µmol m^{-2} s^{-1}, 16 h light/8 h dark photoperiod and 24°C day/21°C night temperatures.

2.4 Germination medium

After an appropriate decontamination protocol was established, nine different germination media were tested to identify the most successful medium for germination of *L. kirkii* embryonic axes (with and without attached cotyledonary segments) (see section 2.5 and Figure 2.1). All media tested comprised of 30 g l^{-1} sucrose and 8.0 g l^{-1} bacteriological agar, pH 5.6 – 5.8. The constituents of each medium were as follows:

1. a) 4.42 g l^{-1} (full-strength) MS medium
   b) 2.21 g l^{-1} (half-strength) MS medium
   c) 1.105 g l^{-1} (quarter-strength) MS medium

2. a) 4 g l^{-1} activated charcoal was added to medium 1. a)
   b) 4 g l^{-1} activated charcoal was added to medium 1. b)
   c) 4 g l^{-1} activated charcoal was added to medium 1. c)

A medium formulated for *Hevea brasiliensis* (Normah *et al*., 1986) (after Pammenter *et al*., 1991; Vertucci *et al*., 1991) containing growth regulators was modified as follows:

3. a) 0.7 µM kinetin, 1.0 µM NAA and 1.4 µM GA was added to 2. a)
   b) 0.7 µM kinetin, 1.0 µM NAA and 1.4 µM GA was added to 2. b)
   c) 0.7 µM kinetin, 1.0 µM NAA and 1.4 µM GA was added to 2. c)

Axes were decontaminated with 1% $Ca(OCl)_2$ for 10 min (decided from the results obtained; see section 3.1 later) and cultured in 90 mm Petri dishes (five axes per Petri dish) containing 35 ml of each of the germination media described above, sealed with Parafilm® and placed in the dark for the first week. Thereafter Petri dishes were transferred to growth room conditions described in section 2.3.

2.5 Viability assays

Seeds were removed from the fruits, cleaned (as described in 2.1), the embryonic axes excised, in one case with cotyledons completely removed and in the other, with a segment of approximately 3 mm of each cotyledon attached (after Goveia *et al*., 2004) (see Figure 2.1) and placed in Petri dishes on filter paper moistened with a CaMg solution. Axes were thereafter surface decontaminated in a laminar air flow cabinet with a 1% $Ca(OCl)_2$ solution for 10 min and rinsed three times with a sterile CaMg solution (see section 3.1). The 2,3,5-triphenyl-tetrazolium (TTZ) test (ISTA, 1999) was used to test initial viability of axes immediately after excision. A transverse section was made through excised axes and immediately treated with a 1% (m/v) aqueous TTZ solution and incubated in the dark at 25±1°C. Colour development was observed in embryos after 8 h. The indications of the TTZ test were confirmed when a separate cohort of axes were decontaminated with 1% $Ca(OCl)_2$ solution for 10 min, rinsed three times with a sterile CaMg solution and cultured in 90 mm Petri dishes (five axes per Petri dish) containing medium 3. b) described in section 2.4 (decided from the results obtained; see section 3.2 later). Petri dishes were sealed with Parafilm® and placed under growth room conditions described in section 2.3. Embryonic axes were subcultured onto fresh germination medium at three-week intervals. Axes (both with cotyledons completely removed and those with attached cotyledonary segments) were scored as surviving upon greening and when both radicles and shoots had each extended at least 2 mm. Axes that turned necrotic or showed no development after two months were scored as non-surviving, which was also confirmed by the TTZ test and electrolyte measurements.

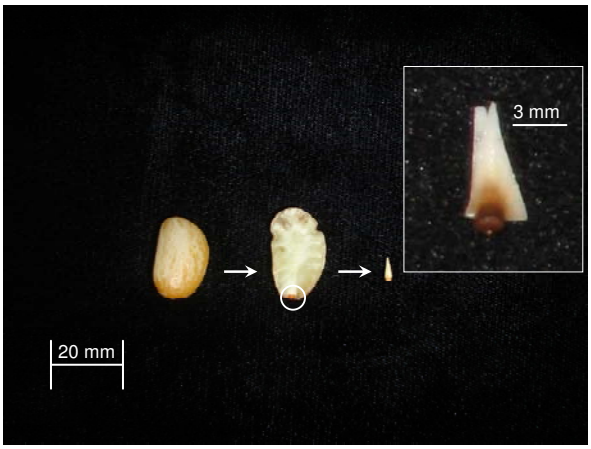

Figure 2.1: Endospermous seed (left) revealing 'paper-thin' cotyledons and embryonic axis (circled, centre) of *Landolphia kirkii*. Insert: excised axis with ~3 mm of each cotyledon attached

2.6 Dehydration

Before any cooling treatments were applied to axes, the optimum axis water content avoiding desiccation damage but sufficiently low for non- or minimally-injurious cooling, was determined. Embryonic axes were excised from fresh seeds (as described in section 2.1) in two batches (one batch with cotyledons completely removed and one with a 3 mm segment of each cotyledon attached) and accumulated in Petri dishes on filter paper moistened with a sterile CaMg solution before cryoprotection (when applied). Axes were then flash-dried at 5 min intervals up to 75 min, and the water contents of ten axes were gravimetrically determined individually at each flash-drying interval (described in section 2.2). Flash-drying involved dehydrating axes on a fine-mesh nylon beneath which a computer fan circulated an air stream through 250 ml activated silica gel, and then over the embryonic axes within a 500 ml glass jar.

2.7 Rehydration

Axes dried for various time intervals to different water contents were rehydrated by direct immersion in a sterile CaMg solution for 30 min at 25±1°C in the dark. Rehydrated axes were decontaminated with 1% $Ca(OCl)_2$ for 10 min (see section 3.1), rinsed three times with a sterile CaMg solution and assessed for germination totality and rate by embryo culture on germination medium (five axes per Petri dish). Petri dishes were kept in the dark for the first week and thereafter transferred to growth room conditions described in section 2.3. The drying-rehydration experiment was repeated three times and only small differences between replicates were recorded. Therefore the data presented in the results section are the bulked data from the three replicates.

2.8 Lipid composition

2.8.1 Electron microscopic morphometry

The total lipid content (dmb) of fresh, mature embryonic axes of *L. kirkii* was estimated using electron microscopic morphometry. Ten transmission electron micrographs from different axes (refer to sections 2.12 and 3.10) at magnifications ranging between 10 000 – 15 000 X, were randomly selected and enlarged onto prints of 210 X 290 mm. A transparent grid consisting of 20 X 28 parallel lines (10 mm^2 squares) and 560 test points was placed over the electron micrographs. The volume of cells occupied by lipid bodies were estimated by dividing the number of points falling on lipid bodies by the total number of points falling within the cells (after Loud, 1962; Weibel and Bolender, 1973).

2.8.2 Determination of total lipid content (dmb)

To determine the estimated total lipid dry mass of the embryonic axes using the estimated volume of cells occupied by lipid bodies, the following calculation was used:

Area of cells occupied by non-lipid: (100-x)%
Area of cells occupied by lipid: x% (calculated using electron microscopic morphology)

Volume of cells occupied by non-lipid: (1-x)
Volume of cells occupied by lipid: x

Fresh mass of non-lipid: (1-x) g g^{-1} (assuming a density of non-lipid cellular material of 1.0 g cm^{-3})
Fresh mass of lipid: (x * 0.8) (assuming density of 0.8 g cm^{-3} of lipid)

Water associated with non-lipid portion of cells:
Initial water content of embryonic axes: 2.24 g g^{-1}
Removing water from non-lipid portion of cells reduces mass by 1/2.24 g g^{-1}
Dry mass of non-lipid portion of cells: [(1-x)/2.24 g g^{-1}] = p
Dry mass of lipid portion of cells: (x * 0.8) = q

Therefore lipid portion (%) of cells (dmb): q/(p + q) X 100

2.8.3 Determination of fatty acid composition

Fatty acid composition was determined for the non-polar lipid fractions in embryos of *L. kirkii*. Freeze-dried embryos were homogenised with a mortar and pestle and lipid was extracted from powdered embryos using a 2:1 solution of chloroform:methanol (Bligh and Dyer, 1959). After centrifugation, the supernatant held in a test tube was dried down using nitrogen gas (N_2) and was thereafter dissolved in chloroform. The polar and non-polar fractions were then separated by elution over solid-phase extraction cartridges (Biotage-Isolute®, Germany) containing silica. Fatty acid methyl esters were prepared using diethyl ether and 20% tetramethylammonium hydroxide (TMAH) (Metcalfe and Wang, 1981) and characterised using an Agilent Technologies 6890N network gas chromatograph system.

2.9 Cryoprotection

Embryonic axes were excised from seeds with ~3 mm of each cotyledon attached and accumulated in Petri dishes moistened with a sterile CaMg solution before cryoprotection. Sucrose, glycerol, DMSO, a combination of sucrose and glycerol, a combination of sucrose and DMSO, a combination of glycerol and DMSO, and sucrose, glycerol and DMSO in combination were the cryoprotectants tested in this study. Axes were exposed to 5% (v/v) cryoprotectant solutions [(m/v) for sucrose] in 90 mm Petri dishes for 1 h under dark conditions at 25±1°C before being transferred to 10% solutions of the same cryoprotectant (or cryoprotectant combinations) for an additional 1 h (after Sershen *et al*., 2007). All axes were then blotted free of cryoprotectant with filter paper. A batch of cryoprotected axes (45 axes per cryoprotectant/combination of cryoprotectants) were thereafter flash-dried at 5 min intervals from 10 min up to 25 min towards a target water contents in the range of *c*. 0.35 – 0.25 g g^{-1} (as identified from the results obtained; see section 3.5). Axes flash-dried after cryoprotection were rehydrated in a CaMg solution for 30 min in the dark at 25±1°C (as described in section 2.7). Axes were thereafter decontaminated with 1% $Ca(OCl)_2$ (see section 3.1) and rinsed three times with a sterile CaMg solution. To assess the effects of cryoprotectant treatments, axes were cultured on germination medium (30 axes per treatment, five axes per Petri dish) and placed under growth room conditions described in section 2.3. Axes not treated with any cryoprotectants were also cultured at the same time to serve as a control for comparison under the same growth room conditions. Axes were scored as surviving when they greened and radicles and shoots had each extended at least 2 mm (see section 2.5). Fifteen axes from each treatment (cryoprotected only and cryoprotected+flash-dried) were used to gravimetrically determine axis water content (prior to rehydration) as described in section 2.2.

2.10 Cooling

2.10.1 Rapid cooling

A pilot experiment ascertained whether crystallisation in the intracellular TAGs (within lipid bodies) occurred, and then, whether warming to various temperatures would control possible lipid melting before imbibition (after Volk *et al*., 2006a). Fresh embryonic axes of *L. kirkii* excised with attached cotyledonary segments (2.24±0.05 g g^{-1}) and axes flash-dried to *c*. 0.28±0.04 and 0.11±0.05 g g^{-1} (see results later) were exposed to rapid cooling by LN (cooling rate of 100°C min^{-1}) and ultra-rapid cooling by nitrogen slush (cooling rate of 200°C min^{-1}) (Wesley-Smith, pers. comm.[3]). To attain rapid cooling, LN (200 ml) was held in a polystyrene cup (250 ml) and tumble mixed into a second polystyrene cup (250 ml) containing the naked, fresh or flash-dried embryonic axes. For ultra-rapid cooling, nitrogen slush, which is LN supercooled to -210°C (Echlin, 1992), was formed by placing LN (200 ml) in a polystyrene cup into a desiccator and using a vacuum pump to lower the pressure and thus form slush. Nitrogen slush was then poured into a second polystyrene cup (as above) containing the naked embryonic axes. Embryonic axes were maintained in the cryogen for 30 min before being removed and placed into Petri dishes and warmed to 5°C (in a refrigerator), 25°C (on the laboratory bench) or 45°C (in an incubator) for at least 1 h before imbibition treatments were initiated. Axes were imbibed in the dark on filter paper moistened with water (after Volk *et al*., 2006a) or a CaMg solution within Petri dishes at 25°C for 12 h. Thereafter, axes were decontaminated and cultured (five axes per Petri dish) for assessment of viability (as described in section 2.5) under growth room conditions described in section 2.3. To further assess viability, axes were subjected to the TTZ test (as described in section 2.5) and measurements of electrical conductivity of leakage were taken (see section 2.11). Axes exposed to each of the above mentioned treatments were also prepared for TEM.

For all rapid cooling trials, the above-mentioned protocol was repeated by tumble mixing LN or nitrogen slush into a polystyrene cup holding embryonic axes, either with

[3] Wesley-Smith, J. University of KwaZulu-Natal, Durban, South Africa

cotyledons completely removed and or with cotyledonary segments attached, flash-dried to *c*. 0.28±0.04 g g^{-1} (see results later). However, after 30 min in the cryogen, LN was poured out and axes were immediately thawed by immersion in a CaMg solution at 40°C for 2 min. Axes were then transferred to CaMg solution at 25±1°C for 30 min rehydration in the dark.

Following rehydration, axes were decontaminated and cultured (five axes per Petri dish) for assessment of viability (as described in section 2.5) under growth room conditions described in section 2.3.

2.10.2 Slow cooling

2.10.2.1 Cooling in cryovials

Axes flash-dried to *c*. 0.28±0.04 g g^{-1} (see results later), either with cotyledons completely removed or with attached cotyledonary segments, were enclosed in sterile 2 ml polypropylene cryovials (Greiner bio-one™; five axes per cryovial), further sealed with Parafilm®, mounted on aluminium cryocanes and plunged into LN within a Dewar cryovat for a minimum of 72 h (after Sershen *et al*., 2007; Varghese *et al*., 2009). The cooling rate of these axes was determined using a thermocouple (Newport Electronics inc., model i800). The 300 µm thermocouple tip was inserted into individual axes of *L. kirkii* and the cooling rates recorded to ascertain the actual cooling rate of the axes, rather than the cooling rate within the cryovial. After four days, cryovials containing the dehydrated embryonic axes were removed from the Dewar, cryovials immediately opened and naked axes immersed in a CaMg solution at 40°C for 2 min to thaw. Axes were thereafter transferred to a CaMg solution at 25±1°C for 30 min to rehydrate in the dark. Post-rehydration, axes were decontaminated and cultured (five axes per Petri dish) to assess germination (as described in section 2.5) under growth room conditions described in section 2.3.

2.10.2.2 Cooling in Mr Frosty

Batches of embryonic axes (either with cotyledons completely removed or with attached cotyledonary segments) were slow-cooled in a cryo-freezing container, Mr Frosty. The apparatus is designed to hold cryovials within wells of a plastic insert located inside the container and surrounded by isopropanol in the outer chamber that cools at 1°C min^{-1} to -70°C when the apparatus is placed in a -80°C freezer. Embryonic axes were dehydrated to *c*. 0.28±0.04 g g^{-1} by flash-drying (see results later), enclosed within cryovials (five axes per cryovial), further sealed with Parafilm®, inserted into Mr Frosty and placed within a -80°C ultra-freezer (after Quain *et al*., 2009; Varghese *et al*., 2009). After 95 min (the time taken to drop the temperature of Mr Frosty from room temperature to -70°C), cryovials were maintained at -70°C for a further 4 h within the freezer. For two-step cooling, cryovials were removed from Mr Frosty after 95 min within the ultra-freezer and either immediately transferred to LN by immersing the naked embryonic axes in the LN, or sealed cryovials were mounted on aluminium cryocanes and plunged into LN within a Dewar cryovat. These axes were held in LN for at least 24 h. For all treatments (Mr Frosty only and two-step cooling), axes were retrieved from the cryogen and thawed by immersion in a CaMg solution at 40°C for 2 min, prior to rehydration in CaMg at 25±1°C for 30 min in the dark. Embryonic axes were decontaminated and processed for viability assessment as described in section 2.5 under growth room conditions (see section 2.3).

2.10.2.3 Programmable freezer

A range of cooling rates was also tested using the Kryo 360-1.7 programmable freezer (Planer Plc. Middlesex, U.K.). Cooling rates of 2, 5, 10, 15, 20 and 50°C min^{-1} were used to cool embryonic axes with attached cotyledonary segments from 25°C to -180°C. Axes flash-dried to a water content of *c*. 0.28±0.04 g g^{-1} (see results later) were enclosed in cryovials (five axes per cryovial). Cryovials were mounted onto aluminium straws and inserted into the ampoules that were radially positioned within the freezer chamber. In an attempt to replicate the freezing rate of Mr Frosty using the programmable freezer, axes were also cooled to -70°C using a cooling rate of 2°C min^{-1} [as a cooling rate of 1°C

min^{-1} was impractical when using this programmable freezer (see section 3.9.2)]. Cooling rates were controlled and monitored using computer software Delta T™. Once the required temperature was reached (i.e. -70 or -180°C) and maintained for at least 5 min, axes were retrieved from the programmable freezer and were immediately immersed in a CaMg solution at 40°C for 2 min for thawing. Rehydration was performed in the dark at 25±1°C for 30 min in a CaMg solution (as discussed earlier). Axes were decontaminated and processed for viability assessment (described in section 2.5) under growth room conditions described in section 2.3.

2.11 Electrolyte leakage

Electrolyte measurements were recorded in addition to performing the TTZ test and germination by tissue culture described in section 2.5. The rate and the quantity of electrolytes that leaked out from embryonic axes is suggested to give a reliable assessment of the damage in the tissue, and is thought to be linked to plasmalemma abnormalities and/or cell death and is said to correlate well with viability characteristics (e.g. Vertucci, 1989c; Pammenter *et al*., 1991; Berjak *et al*., 1992; Wesley-Smith *et al*., 2001a; b). The levels of electrolyte leakage was measured for five replicates of fresh axes, as well as axes exposed to dehydration and combinations of dehydration, cooling and thawing treatments. These measurements were taken on an individual-axis basis. Axes were immersed directly into 2 ml of distilled water within the wells of a CM 100 multi-cell conductivity meter (Reid and Associates cc., Durban, South Africa). The conductivity of their leachate was monitored at 30 min intervals for a period of 18 h. Levels of electrolyte leakage were normalised by the axis dry weight. The conductivity values for the control (distilled water), if any, was subtracted from all sample leachate conductivity values. The rate of electrolyte leakage was calculated after 13 h (see results later) and data were expressed as $\mu S\ cm^{-1}\ min^{-1}\ g^{-1}$ dry mass.

2.12 Electron microscopy

After the imbibition period mentioned in section 2.10.1, radicles were excised, fixed overnight at 5°C in a 2.5% glutaraldehyde solution buffered to pH 7.2 with 0.1 M phosphate buffer and containing 0.5% caffeine. The tissue samples were rinsed three times in a 0.1 M phosphate buffer and post-fixed with 0.5% aqueous osmium tetroxide for 1 h at 25±1°C. After three washes in phosphate buffer, the samples were gradually dehydrated in a gradient ethanol series (10, 30, 50, 75 and 100%) and ultimately in 100% propylene oxide. Note that to achieve good ultrastructural preservation of material, dehydration had to be carried out with increasing concentrations of ethanol solutions rather than increasing concentrations of acetone solutions, as acetone solution had been shown to cause severe plasmolysis of meristematic cells of *L. kirkii*. Samples were thereafter infiltrated with equal parts propylene oxide and epoxy resin for 4 h and subsequently infiltrated with epoxy resin for 24 h (Spurr, 1969). The specimens were then orientated in moulds in the same low viscosity epoxy resin and polymerised at 70°C for 8 h. Thick sections were cut with glass knives on a Reichert Ultracut E microtome, stained with a 1% alkaline aqueous toluidine blue solution and viewed using a Nikon Biophot light microscope. Ultra-thin sections were cut with glass knives, collected on copper grids and post-stained with a saturated aqueous uranyl acetate solution for 10 min followed by lead citrate for 10 min (Reynolds, 1963). Thin sections were viewed with a Jeol 1010 transmission electron microscope at 100kV accelerating voltage and images digitally captured with an iTEM image analysis software package.

2.13 Statistical analysis

The Kolmogorov-Smirnov test was used to test if data were normally distributed. All data exhibited normal distribution and therefore no transformation was required. Each experiment was repeated three times. Data were analysed using one-way analysis of variance (ANOVA) and either Scheffe's or Duncan's post-hoc tests.

Chapter 3: Results and discussion

3.1 Decontamination

In vitro cultures of axes of *Landolphia kirkii*, like most plant material, are susceptible to both bacterial and fungal contamination. This susceptibility is further increased when embryonic axes are excised for use as explants for germplasm conservation (Mycock and Berjak, 1990; Calistru *et al*., 2000; Berjak *et al*., 2000a; Sutherland *et al*., 2002). Micro-organisms associated with recalcitrant embryonic axes are not destroyed even by exposure to LN and will continue to proliferate upon retrieval of the explant from the cryogen and regeneration on germination medium (e.g. Kioko, 2003; Naidoo, 2006). Therefore an effective decontamination protocol needs to be in place before any cooling trials can be performed.

Berjak *et al*. (2000b) and Berjak and Pammenter (2003a; 2004a) advocated the use of NaOCl, $HgCl_2$ and $Ca(OCl)_2$ to curtail fungal and bacterial micro-organisms associated with the surface of recalcitrant embryonic axes. These decontaminants were tested in the present study at various concentrations and durations (Table 3.1). A 1% solution of NaOCl for 5 (e.g. Sershen *et al*., 2007) and 10 min (e.g. Sershen *et al*., 2008) as well as a 0.2% solution of $HgCl_2$ for 0.5 and 1 min (e.g. Ahmad *et al*., 2010), followed by rinsing (three times) with a sterile CaMg solution (see section 2.3) were toxic, killing all embryonic axes of *L. kirkii* (Table 3.1). Although decontamination with a solution of 1% $Ca(OCl)_2$ for 5 min (e.g. Naidoo *et al*., 2010; Ngobese *et al*., 2010) was not toxic to axes, it proved ineffective in curtailing bacterial and fungal growth (Figure 3.1). However, increasing the duration of decontamination to 10 min with the same decontaminant and subsequent rinsing of axes with a sterile CaMg solution was effective in preventing mycofloral proliferation (Table 3.1). Several other studies involving plant material have reported effective decontamination using the same protocol or modifications thereof, for example *Psathyrostachys juncea* seeds (Wang *et al*., 2002), chickpea seeds (Dawar *et al*., 2007), *Trithuria austinensis* seeds (Tuckett *et al*., 2010), floral explants of *Theobroma cacao* (Li *et al*., 1998), zygotic embryos of *Haemanthus montanus* (Naidoo *et al*., 2010)

and embryonic axes of *Trichilia dregeana* (Berjak and Mycock, 2004), *Telfairia occidentalis* (Ajayi *et al*., 2006b) and *Phoenix reclinata* (Ngobese *et al*., 2010). This decontamination protocol was therefore employed for all subsequent experiments in the current investigation. Regardless of the viability of embryonic axes after various cryoprotection, dehydration and cooling manipulations, none of the explants revealed any signs of contamination. It is suggested that the milky latex exuded by the seeds of *L. kirkii* may in fact possess anti-fungal and anti-bacterial properties that possibly work in conjunction with the decontamination solution (Berjak, pers. comm.[3]).

Table 3.1: Effects of chemical decontaminants tested at various concentrations and durations on embryonic axes with attached cotyledonary segments of *Landolphia kirkii*

Decontaminant	**Duration (minutes)**	**Efficacy**
1% NaOCl	5	Toxic
	10	Toxic
0.2% $HgCl_2$	0.5	Toxic
	1	Toxic
1% $Ca(OCl)_2$	5	Non-toxic Ineffective
	10	Non-toxic Effective

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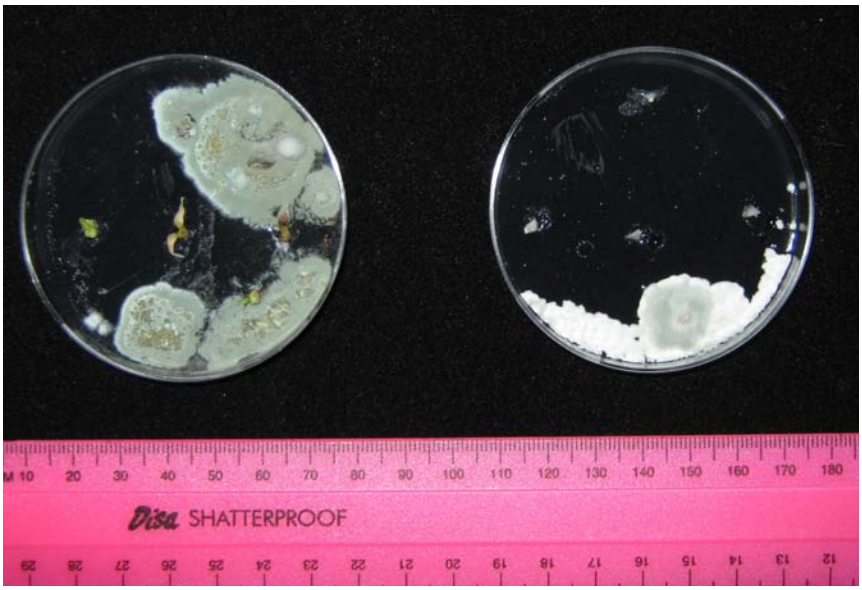


Figure 3.1: Bacterial and fungal contamination of embryonic axes with attached cotyledonary segments of *Landolphia kirkii* on *in vitro* culture medium after treatment with 1% $Ca(OCl)_2$ for 5 minutes (scale in mm)

3.2 Germination medium

Kioko *et al*. (1998) and Berjak and Pammenter (2004b) pointed out that the selection of the appropriate regeneration procedure, including tissue culture medium, is of vital importance in the recovery of cryopreserved plant material. Furthermore, Normah and Makeen (2008) suggested that factors arising in the recovery medium adversely influence the rate of recovery of frozen embryonic axes that are already suffering desiccation and freezing stresses. The use of MS, which is a universal plant nutrient supplement, in germination medium for recalcitrant embryonic axes has been reported for several studies (e.g. Normah *et al*., 1986; Pammenter *et al*., 1991; Liang and Sun, 2000; Wesley-Smith *et al*., 2001a; Goveia *et al*., 2004; Sershen *et al*., 2007; Varghese *et al*., 2009; Whitaker *et al*., 2010). Various culture media containing MS were assessed for germination of embryonic axes of *L. kirkii* (Table 3.2). Embryonic axes with attached cotyledonary

segments cultured on media containing 30 g l^{-1} sucrose and either full or half-strength MS produced roots (80 and 90% respectively). Shoot elongation was negligible (0 and 15% respectively) on these media; however, cotyledon greening was observed (40 and 15% respectively) (Table 3.2). The supplementation of germination medium with PGRs to increase shoot production has been employed in a number of studies on recalcitrant explants (e.g. Normah *et al*., 1986; Hor *et al*., 1990; Ashburner *et al*., 1993; Martínez *et al*., 2003; Ajayi *et al*., 2006a; b). When a cocktail of PGRs [0.7 µM kinetin, 1.0 µM NAA and 1.4 µM GA (final concentrations in the medium)] was added to the above mentioned germination media, while root production increased minimally, shoot production increased substantially to 60 and 70% respectively with 30% cotyledon greening observed on both media (Table 3.2).

The inclusion of the adsorbent activated charcoal in the germination medium was shown to improve shoot production in embryonic axes of *Trichilia dregeana* (Goveia *et al*., 2004). The lack of shoot production is a problem associated with a burst of ROS which is a response to the wounding associated with the excision of the cotyledons (Goveia *et al*., 2004; Berjak and Pammenter, 2004b; Pammenter *et al*., 2011) (refer to section 1.7.1.1). Furthermore, the exudation of inhibitory substances (suspected to be polyphenolics) into the medium from cut cotyledonary surfaces has been thought to retard root growth of *T. dregeana* axes *in vitro* (Goveia *et al*., 2004; Berjak and Pammenter, 2004b). Activated charcoal has been shown to promote and enhance the normal development of embryos of 11 recalcitrant-seeded species and it is believed that it functions to remove the toxic substances released in the medium (polyphenolics), owing to injury during excision of explants (Chin *et al*., 1988). If these substances are not removed, normal development is hindered as they promote unorganised growth and inhibit embryogenesis, root formation and elongation (Fridborg and Erickson, 1975; Chin *et al*., 1988). In the present study, the addition of 4 g l^{-1} activated charcoal further increased shoot production to 80 and 100% for full and half-strength MS (with added PGRs) respectively (Table 3.2). Therefore, the medium containing sucrose, half-strength MS, PGRs and activated charcoal proved most effective in promoting seedling production from embryonic axes of *L. kirkii* excised with attached cotyledonary segments, producing 100% root and shoot formation (Table 3.2). It

should be noted that when axes alone (i.e. with no attached cotyledonary segments) were cultured on the same medium, root and shoot production was essentially the same as that of axes with attached 3 mm cotyledonary segments (data not shown). This medium was therefore used for all subsequent experiments in the current study.

Table 3.2: Germination responses of embryonic axes of *Landolphia kirkii* excised with attached cotyledonary segments cultured on six different growth media (n=30)

Medium	Root Protrusion (%)	Shoot Elongation (%)	Cotyledon Greening (%)
Full MS	80	0	40
Half MS	90	15	15
Full MS + PGRs	90	60	30
Half MS + PGRs	90	70	30
Full MS + PGRs + activated charcoal	100	80	20
Half MS + PGRs + activated charcoal	100	100	0

3.3 Shedding water content

Embryonic axes with attached cotyledonary segments that were excised from fresh seeds of *L. kirkii* had the relatively high mean water content of 2.24±0.05 g g^{-1} (embryonic axes alone were shed at 2.13±0.06 g g^{-1}). This is in keeping with other species that produce recalcitrant seeds that are usually shed at high water contents of >0.4 g g^{-1} and are generally a reliable predictor of their desiccation intolerance (Pammenter *et al*., 1991; 1998; Hong and Ellis, 1998; Berjak and Pammenter, 1999). Although inter-annual variability of axis shedding water content has been observed and is believed to be a

common feature of embryonic axes of recalcitrant seeds (e.g. Tompsett and Pritchard, 1993; Finch-Savage and Blake, 1994; Berjak *et al*., 1996; 2004; Finch-Savage, 1996; Daws *et al*., 2004; Sershen *et al*., 2008; reviewed by Berjak and Pammenter, 2004a), this was not the case over the two years of the current investigation. A possible explanation for the lack of inter-seasonal variability of axis shedding water content of *L. kirkii* is that fruit were hand-harvested at the same stage of maturity and from the same trees in both years of collection. As discussed in section 1.6.1, studies have reported that developmental status plays a major role in the shedding water contents and desiccation tolerance of recalcitrant seeds. Moreover, previous work on *L. kirkii* embryonic axes by Berjak *et al*. (1992) has shown that the maturity status of seeds affect the overall lipid content and thus the overall water content of the embryonic axes (see section 3.5). Those authors reported immature embryonic axes to have a water content that was higher [by 170% or 2.6 g g^{-1} (dmb)] than those of mature embryonic axes.

However, studies on recalcitrant seeds of *Aesculus hippocastanum* (Tompsett and Pritchard, 1993) and of *Quercus robur* (Finch-Savage and Blake, 1994) have shown that seeds from the same tree were shed at different water contents in different years, and therefore varied in their desiccation tolerance. However, the exact factors that cause these inter-annual variations are unknown (Daws *et al*., 2004). Work by Daws and colleagues (2004; 2006) showed that provenance and environmental conditions impacted on the shedding water content of recalcitrant seeds. Results of a study conducted on *A. hippocastanum* by Daws *et al*. (2004) have shown that environmental conditions (e.g. air temperature) during seed development have an effect on the desiccation sensitivity of these recalcitrant seeds. Those authors attributed the inter-annual variation in desiccation tolerance of seeds, recorded in their investigation, to be as a consequence of a change in the development period, with air temperature significantly influencing maturity status at dispersal (Daws *et al*., 2004). Differences in shedding water contents of embryonic axes of *L. kirkii* can be seen when results from the present study are compared with previous investigations on the same species. Pammenter *et al*. (1991) and Berjak *et al*. (1992) recorded an axis shedding water content of *c*. 1.5 g g^{-1} (dmb) when fruit of *L. kirkii* were handed-harvested in 1991. Fruit hand-harvested for the current study, from an alternate

location to that of Pammenter *et al*. (1991) and Berjak *et al*. (1992), showed a shedding water content 50% or 0.74 g g^{-1} (dmb) higher. It can therefore be assumed that this difference in water content can be attributed to differences in provenance, environmental conditions, or a combination of the two as well as the development stage when harvested.

3.4 Assessment of initial viability

Initial viability was determined using a TTZ test, with all newly excised embryonic axes staining red (Figure 3.2). Viability was confirmed by seedling development *in vitro*. Axes from fresh, newly harvested seeds of *L. kirkii* were readily germinable, greening after three days in culture, and after two weeks, showed both root and shoot production forming functional seedlings (Figure 3.3). By week six, leaves started to expand. The seedlings retained 100% viability after two months in culture. Such vigour does indicate that these seeds were fully mature when harvested [full maturity in recalcitrant seeds can be difficult to identify (Berjak and Pammenter, 2004a) as in some species seed development continues into germination (Berjak *et al*., 1984; Kermode and Finch-Savage, 2002; Pritchard *et al*., 2004)]. A study on recalcitrant *Telfairia occidentalis* embryonic axes showed that when axes were cultured with a portion of cotyledon attached, vigour (assessed by *in vitro* culture) was increased (i.e. rate of development) but root growth was retarded when compared to shoot growth (Ajayi *et al*., 2007). When the cotyledon was completely removed from embryonic axes of *T. occidentalis*, although vigour decreased, root growth was normal and proportional to shoot growth (Ajayi *et al*., 2007). Conversely, other research on dicotyledonous recalcitrant-seeded species (Goveia *et al*., 2004; Whitaker *et al*., 2010; reviewed by Pammenter *et al*., 2011), showed that excising axes at the axis/cotyledon node or even with 1 mm basal cotyledonous segment attached (Goveia *et al*., 2004), was associated with failure of shoot development. In the case of *Trichilia dregeana* axes, leaving an attached 2 mm segment of cotyledon facilitated normal shoot development (Goveia *et al*., 2004) (see sections 3.9.3 and 3.9.4). With regard to the present investigation however, essentially identical initial viability results were obtained for axes excised with the cotyledons completely removed and those excised with a portion of both cotyledons attached. However, subsequent experiments

were still carried out both on axes with cotyledonary segments attached, and axes with cotyledons completely removed [as it has been suggested that excision damage may manifest itself only during the later stages of cryopreservation protocols (discussed in section 3.9.4)].

Figure 3.2: TTZ test results for *Landolphia kirkii* (a) fresh axes (2.24±0.05 g g^{-1}) (dmb) and axes flash-dried to (b) 0.28±0.04 g g^{-1} (dmb) and (c) 0.11±0.05 g g^{-1} (dmb). (major grid scale in cm, minor gridlines in mm).

Row a: Red to orange staining of embryonic axes and cotyledonary segments (refer to bars for comparison) – fully viable axes;
Row b: Red staining of axes but not cotyledonary segments – all axes viable, cotyledonary segments having apparently lost respiratory capacity;
Row c: No reaction with TTZ – axes non-viable

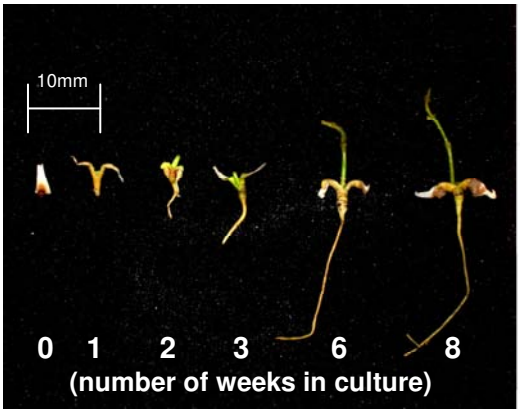


Figure 3.3: Growth and development of fresh, mature axes of *Landolphia kirkii* excised with cotyledonary segments, in culture over a period of eight weeks

3.5 Responses to dehydration

The embryonic axes from mature seeds of *L. kirkii* could be flash-dried to water contents between 1.60±0.07 and 0.28±0.04 g g^{-1}, showing maintenance of 100% viability and no apparent adverse effect on vigour after immediate rehydration in a CaMg solution and culture on germination medium (Figures 3.4 and 3.5). However, dehydration of the axes to water contents of *c*. 0.21±0.07 g g^{-1} (after 55 min flash-drying) and lower resulted in a considerable decrease in vigour (assessed by germination proportion) and viability when assessed by *in vitro* culture (Figures 3.4 and 3.5). Additionally, when assessed by the TTZ test, fresh embryonic axes and axes dehydrated to a water content of 0.28±0.04 g g^{-1} (after 25 min flash-drying) revealed metabolically active meristems, confirming their viability (Figure 3.2). Axes with water contents of 0.11±0.05 g g^{-1} (i.e. below 0.21±0.07 g g^{-1}) stained minimally, showing little respiratory activity within the axial tissue (Figure 3.2). These results were further verified by electrolyte leakage measurements (see section 3.8.3).

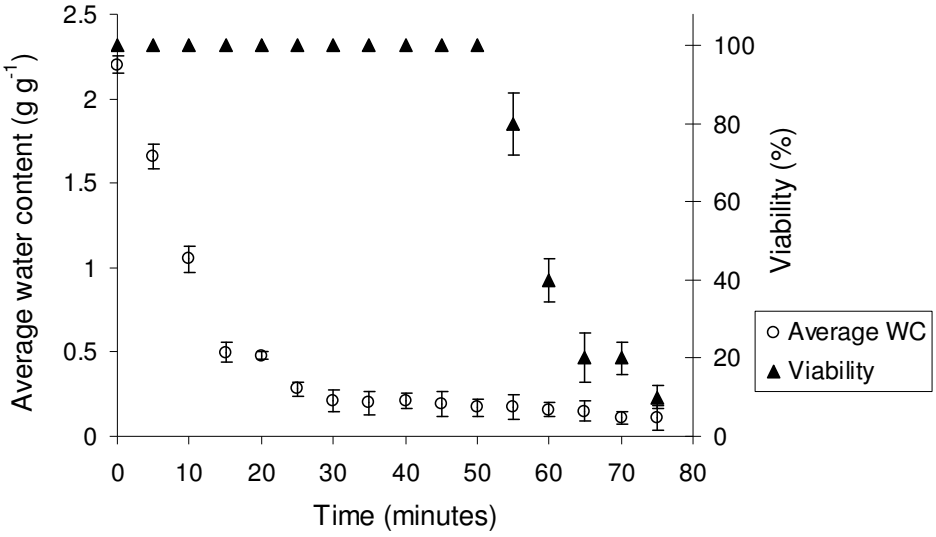


Figure 3.4: Drying time course and corresponding viability (root plus shoot protrusion) of axes excised with attached cotyledonary segments of *Landolphia kirkii*. Bars indicate one standard deviation about the mean

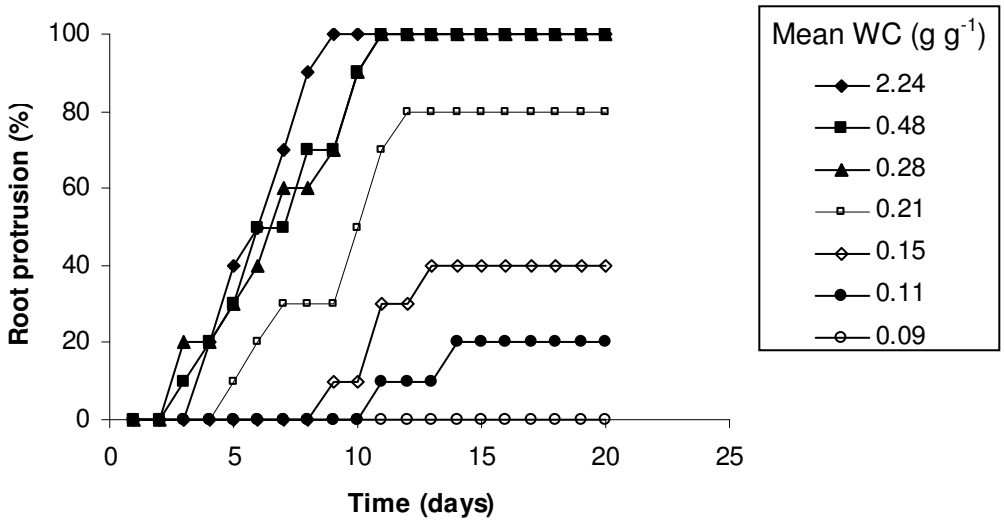


Figure 3.5: The relationship between water content (WC) of axes excised with attached cotyledonary segments after flash-drying, and rate and germination proportion (WC and viability data corresponds to Figure 3.4). Statistical analysis shows significant differences among treatments (Duncan post-hoc test; $p \leq 0.01$)

Initial dehydration of fresh embryonic axes was rapid with a water loss of 1.7 g g^{-1} within the first 15 min of flash-drying (Figure 3.4). However, dehydration below 0.28±0.04 g g^{-1} was relatively slow (little water loss subsequent to drying for 30 min). This suggests that the loss of vigour and viability to be the outcome of both water content *per se*, and the duration of sustained drying stress, ≥ 50 min to 0.21±0.07 g g^{-1} as opposed to 25 min to reach 0.28±0.04 g g^{-1} (this demonstrates the concept of duration versus intensity of a stress). The findings of this investigation are in agreement with those of Pammenter *et al*. (1991) and Vertucci *et al*. (1991), who reported that mature axes of *L. kirkii* could be flash-dried rapidly to water contents of approximately 0.3 g g^{-1} (after 30 min flash-drying), before any discernable decline in viability. However, further dehydration was reported as being slow. This behaviour is analogous to that observed by Wesley-Smith *et al*. (2001b) for embryonic axes excised from recalcitrant seeds of *Aesculus hippocastanum*. Those authors reported that flash-drying reduced water content of axes by 70% during the first 30 min, but only by 17% further over the subsequent 110 min. The same research group also recorded similar findings when working on recalcitrant embryonic axes of *Poncirus triofoliata* (Wesley-Smith *et al*., 2004a). Furthermore, Berjak *et al*. (1992) showed that immature embryonic axes of *L. kirkii* dried to a water content lower than 0.2 g g^{-1} after only 20 min of flash-drying, but recorded results similar to those of Figure 3.4 for mature axes. It is also reported that the seeds of *L. kirkii* reach their desiccation tolerance peak just prior to seed shed (Berjak *et al*., 1992; reviewed by Pammenter and Berjak 1999). Therefore results in Figure 3.4 further support the initial viability data in indicating that the embryonic axes used for the current study were at full maturity, and hence at their most amenable to cryopreservation.

Water in seed tissue above a water content of 0.3 g g^{-1} generally freezes using routine cooling rates (Vertucci, 1990). Water in tissue below this concentration is associated with macromolecular structures and surfaces within cells, is sometimes referred to as 'bound' water, and requires slower cooling or prolonged exposure to low temperatures to freeze (Vertucci, 1990). Pammenter *et al*. (1991) showed that whereas the removal of freezable water by flash-drying was achievable without short-term deleterious effects on viability, removing non-freezable water was not readily accomplished and was associated with

damage. Those authors reported the amount of non-freezable water in embryonic axes of *L. kirkii* to be 0.28 g g^{-1} (dmb) regardless of drying rate. From the results of the current investigation, it is suggested that the water removed after 25 min of flash-drying was freezable, due to the high desiccation rate achieved and lack of deleterious effects on vigour and viability (Figures 3.4 and 3.5). Therefore, most of the water removed after >25 min flash-drying was non-freezable, evident by the slower drying rate and decreases in vigour and viability, suggesting desiccation damage (Figures 3.4 and 3.5).

Pammenter *et al*. (1991) showed further that the desiccation response of the axes was dependent on the rate of drying; if seeds of *L. kirkii* were dried slowly over silica gel, viability drastically declined at axis water contents of ~1.2 g g^{-1}. Therefore, it was suggested that the desiccation-sensitive seeds of *L. kirkii* can lose only freezable water from the tissue, without obvious damage, if dehydration is rapid (Pammenter *et al*., 1991). Similar results have been found in other studies on recalcitrant explants [e.g. *Camellia sinensis* (Berjak *et al*., 1993), *Ekebergia capensis* (Pammenter *et al*., 1998), *Trichilia dregeana* (Kioko *et al*., 1998), *Quercus robur* (Pritchard and Manger, 1998), *Artocarpus heterophyllus* (Wesley-Smith *et al*., 2001a), *Theobroma cacao* and *Ginkgo biloba* (Liang and Sun, 2002)]. Those studies reported that the damage incurred by seed tissue through dehydration to a particular water content is inversely related to the rate at which the explants were dehydrated (i.e. the faster the drying rate achieved, the less damage sustained). Furthermore, a study on recalcitrant seeds of *Ekebergia capensis* has shown short-term, rapid dehydration to non-injurious water contents, increases germination rate (Pammenter *et al*., 1998).

Due to the differential responses to dehydration at different drying rates of recalcitrant seeds, Pammenter *et al*. (1998) suggested that it is not possible to unambiguously define a 'critical water content' below which viability is lost. This necessitates the empirical determination of a water content and corresponding drying time that is amenable to cryogenic cooling for specific species of recalcitrant germplasm. Additionally, although rapid dehydration is suggested to minimise or even avoid metabolic-based damage of recalcitrant tissue, drying to/or approximating to the level of non-freezable water is

associated with lethal, irreversible desiccation damage *sensu stricto* (Pammenter *et al*., 1998; Walters *et al*., 2001). This damage occurs as a result of desiccation-sensitive tissue losing a fraction of the non-freezable water associated with macromolecular structures (Walters *et al*., 2001). Moreover, the objective of flash-drying is to dehydrate axes rapidly to water contents that are amenable to non- or minimally-injurious cooling, while subjecting them to the least amount of desiccation stress possible (King and Roberts, 1980; Pritchard and Prendergast, 1986; Wesley-Smith *et al*., 1992; 2001a; Chandel *et al*., 1995). Based on these factors, although embryonic axes of *L. kirkii* could be flash-dried for a longer period and to a slightly lower water content without decreasing viability (e.g. 45 min flash-drying to a water content of 0.25±0.08 g g^{-1}) (see Figure 3.4), an average 'optimum' water content of 0.28±0.04 g g^{-1} after 25 min of flash-drying was chosen as the most appropriate for all subsequent cooling trials.

Finch-Savage (1992), working on recalcitrant embryonic axes of *Quercus robur*, suggested that removal of the embryonic axis from the cotyledons may possibly influence the responses of the isolated axis to dehydration. With regard to the present investigation however, although data in Figures 3.4 and 3.5 are for axes with cotyledonary segments attached, results showed no differences in response to desiccation of embryonic axes of *L. kirkii* with cotyledons completely removed and those excised with a 3 mm portion of both cotyledons attached (data not shown). Pammenter *et al*. (1998) supported this conclusion when they observed that the responses to desiccation at different drying rates of *Ekebergia capensis* seeds was not an artefact of removing the axis from the influence of the cotyledons.

As discussed in section 1.4.2.1, seed material of *L. kirkii* is known to have a high lipid content and lipid content and composition may contribute to the temperature response in recalcitrant seeds. The area of cells occupied by lipid was estimated at 26% (by electron microscopic morphometry). This figure was substituted into the calculation shown in section 2.8.2, revealing fresh, mature embryos to contain ~39% total lipid content (dmb). When the values were adjusted for the high lipid content within the cells, the water contents are observed as much higher (Figure 3.6). Thus, the optimum water content

amenable for cryopreservation appears to be closer to 0.39±0.06 g g^{-1} rather than 0.28±0.04 g g^{-1}. However, this water content still falls within the range of 0.15 and 0.4 g g^{-1}, suggested by other studies to be the optimum at which recalcitrant embryonic axes can be successfully cryopreserved (e.g. Vertucci *et al*., 1991; Berjak and Dumet, 1996; Kioko *et al*., 1998; Wesley-Smith *et al*., 2004a; b; Perán *et al*., 2006; Sershen *et al*., 2007). Nevertheless, the water content values quoted in this contribution will be based on the values prior to storage lipid corrections in order to correlate with previous investigations on *L. kirkii*, which did not adjust for the high non-polar lipid fraction within the embryos (refer to sections 3.6 and 3.10).

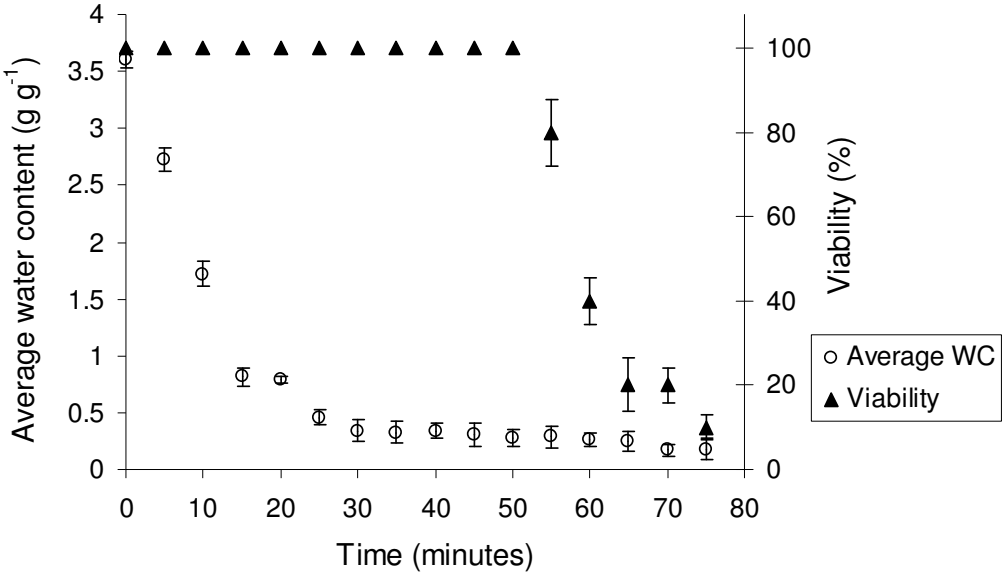


Figure 3.6: Drying time course (after adjustment for storage lipid) and corresponding viability (root plus shoot protrusion) of axes excised with attached cotyledonary segments of *Landolphia kirkii.* Bars indicate one standard deviation about the mean

Total lipid content (dmb) =~39%.

Therefore WC (after adjustment for storage lipid) = WC values from Figure 3.4/0.61

e.g. initial WC (from Figure 3.4) 2.24g g^{-1}/0.61 = 3.67g g^{-1}

3.6 Lipid Composition

Wesley-Smith *et al*. (1992) suggested the existence of stresses (in addition to those induced by ice damage alone) during rapid cooling to cryogenic temperatures. Studies have shown that the damage inflicted on seed material by a combination of low water contents and low temperatures can be explained by phase changes of water and water-soluble components of the cells (e.g. Sun and Leopold, 1994; Koster *et al*., 2000). Crane *et al*. (2006) showed that the water contents and temperatures that induce damage in seeds with intermediate storage physiology do not correspond to those that result in phase changes of water or water-soluble components and, changes may be attributed to TAGs in seeds. To add to this, Volk *et al*. (2007) suggested that understanding the behaviour of lipids in seeds during their storage and imbibition is essential in the development of long-term storage of species that are generally difficult to conserve. Moreover, in the present study, it was initially suspected that TAG crystallisation within lipid bodies found in embryonic axes of *L. kirkii*, might have resulted in their rapid disintegration upon rehydration, as has been demonstrated for embryos of the other species (e.g. Vertucci, 1989a; Crane *et al*., 2003; 2006; Volk *et al*., 2006a; 2007). It has been proposed that rehydration of crystallised TAGS results in cellular disruption that cannot be reversed and consequent reduced germination (Crane *et al*., 2003; 2006; Volk *et al*., 2007). Therefore, lipid transitions may play an important role in the viability of recalcitrant tissue (Crowe and Crowe, 1986).

Previous work on embryonic axes of *L. kirkii* by Berjak *et al*. (1992) revealed that lipid deposits form a substantial intracellular component of immature axes. Although a significant decline in lipid content as axes matured was noted, possibly due to autophagy of these reserves during development (Berjak *et al*., 1992), lipid deposits are characteristic of mature *L. kirkii* embryonic axes (see section 3.10). These observations are supported by the lipid analysis from the current study, showing that the fresh, mature embryos of *L. kirkii* are lipid rich, containing ~39% total lipid content (dmb). A large proportion (44%) of this total lipid constitutes the non-polar fractions. The fresh embryos contained high proportions of long-chain saturated fatty acids, 47% palmitic (C16) and

17% stearic (C18) (Table 3.3). Unsaturated fatty acids comprised 25% of the total storage lipid and was made up entirely of linoleic (C18:2) acid as no traces of either oleic (C18:1) or linolenic (C18:3) acids were detected (Table 3.3). Previous investigations have reported primarily saturated, medium-chain fatty acids (C8 – C14) to be the main cause of TAG crystallisation within seeds (Crane *et al*., 2003; 2006; Volk *et al*., 2006a; 2007). However, results indicated that collectively, only 11% of the storage lipid was made up of medium-chain saturated fatty acids capric (C10), lauric (C12) and myristic (C14), with no trace of caprylic acid (C8) (Table 3.3). Nevertheless, Vertucci (1989c) speculated that TAG interaction with water can trigger sensitivity to freezing damage. That author proposed that phospholipids are believed to contain freezable water, and even minute quantities may cause devastating effects on seed viability during lipid transitions. Furthermore, it has been suggested that the optimisation of water contents will minimise the negative effects associated with crystalline TAGs during storage (Volk *et al*., 2007).

Table 3.3: Fatty acid composition of the non-polar lipid fractions within fresh embryos of *Landolphia kirkii*

Fatty Acid	Chain Length	Lipid Composition (%)
Caprylic	C8:0	0
Capric	C10:0	1
Lauric	C12:0	3
Myristic	C14:0	7
Palmitic	C16:0	47
Stearic	C18:0	17
Oleic	C18:1	0
Linoleic	C18:2	25
Linolenic	C18:3	0
Total		100

From the data presented in Table 3.3 it can be noted that 75% of the non-polar fatty acid composition of fresh embryos of *L. kirkii* were saturated. Nkang *et al*. (2003), working on recalcitrant *Telfairia occidentalis* seeds, showed that the high saturated fatty acid content of the seeds was decreased, resulting in an increase in both mono and polyunsaturated fatty acids, when seeds were desiccated to 42% of their shedding water content at 28°C. The increase in unsaturated fatty acid content was accompanied by viability loss (Nkang *et al*., 2003). This change in lipid composition may also occur in and have an effect on mature *L. kirkii* axes that lose viability at a water content of approximately 0.39±0.06 g g^{-1} (after adjustment for lipid composition) after dehydration at 25°C (Figure 3.6). Nkang *et al*. (2003) did show that when seeds were dehydrated at 5°C, the high saturated fatty acid composition was retained and the loss of viability was delayed. This suggested that lipid composition has a marked impact on desiccation tolerance and sensitivity of seeds. However, due to restraints with regard to available seed numbers, dehydration of embryonic axes of *L. kirkii* at different temperatures could not be assessed.

The results of this investigation showed that capric and lauric acids constitute approximately 1 and 3% respectively of the total non-polar fraction of lipid within embryos of *L. kirkii* (Table 3.3). These values are low when compared to the 28% capric and 56% lauric acids found within seeds of *Cuphea wrightii* and the 75% capric and 2% lauric acids within seeds of *Cuphea lanceolata* reported by Volk *et al*. (2006a). Those authors suggested that the high concentration of these two medium-chain saturated fatty acids may be responsible for the TAG crystallisation when seeds are subjected to cryogenic temperatures. They also reported that the proportions of capric and lauric acids confer a difference in melting temperatures of the crystallised TAGs. Further research by Volk *et al*. (2007), on seeds of six species of *Cuphea*, showed that in addition to the proportions of capric and lauric acids, other medium-chain saturated fatty acids, *viz*. caprylic and myristic acids, not only affected their sensitivity to hydration, but also impacted greatly upon the TAG melting temperatures of α, β' and β crystals. They showed that where seeds from some *Cuphea* species could tolerate cold exposure (-80°C), since they possessed TAGs that melted at standard warming (22°C for 1 h), imbibition (22°C for 4 h) and germination (seeds placed on damp filter paper in Petri

dishes and incubated at 25°C with 16 h light/8 h dark photoperiod) conditions, other species contained TAGs with higher melting temperatures that germinated only when seeds were warmed to above 35°C, following low temperature (-80°C) exposure. However, the generally low levels of these fatty acids within the embryos of *L. kirkii* [11% collectively (Table 3.3)] suggest that lipid body crystallisation within the embryos is unlikely. Nevertheless, to investigate possible TAG crystallisation and whether warming at higher temperatures prevented possible subsequent fatal lipid body explosions, fresh and flash-dried embryonic axes of *L. kirkii* were subjected to rapid cooling and various thawing and rehydration treatments (see section 3.8.1). These results were corroborated by TEM images (see section 3.10).

3.7 Cryoprotection

It should be noted that in order to test the effects of cryoprotection solutions in combination with flash-drying on the viability of embryonic axes (as shown in Figure 3.8), high seed numbers are required. Therefore the plant material available allowed only the testing of embryonic axes with attached cotyledonary segments and not axes that were excised with cotyledons completely removed. The effect of cryoprotection was ascertained by comparing the viability of embryonic axes subjected to specific cryoprotection treatments with that of the control (i.e. fresh axes not subjected to cryoprotection, dehydration or cooling trials). Fresh axes subjected to exposure to all cryoprotectants, singly and in combination, retained 100% viability when assessed by *in vitro* culture immediately after treatment (Table 3.4 and Figure 3.8). However, the aim of this study was to freeze axes that had not only been cryoprotected but also dehydrated to a water content amenable to cooling. Dehydration will gradually increase the concentration of penetrating cryoprotectants (glycerol and/or DMSO in the present study) in the cells (Kioko, 2003). However, when dehydration to mean water contents of *c*. 0.28±0.04 g g^{-1}, chosen as the most amenable to cooling (see section 3.5), was preceded by cryoprotection, no axis survival was recorded in culture (Table 3.4 and Figure 3.8). While axes treated with cryoprotectants alone also gave uniformly positive results on TTZ testing and *in vitro* culture, those treated with cryoprotectants and subsequently

flash-dried and rehydrated did not stain at all, indicating a lack of any respiratory activity (Figure 3.7).

Table 3.4: The effect of chemical cryoprotection on the viability of fresh and flash-dried embryonic axes of *Landolphia kirkii* excised with attached cotyledonary segments (n=30)

Cryoprotectant (5% for 1 h followed by 10% for another 1 h)	**Germination following cryoprotection treatment only (%)**	**Germination following cryoprotection and dehydration to *c.* 0.28±0.044 g g^{-1} (%)**
Sucrose	100	0
Glycerol	100	0
DMSO	100	0
Sucrose + Glycerol	100	0
Sucrose + DMSO	100	0
Glycerol + DMSO	100	0
Sucrose + Glycerol + DMSO	100	0

Figure 3.7: TTZ test results for *Landolphia kirkii* (a) fresh axes (2.24±0.05 g g^{-1}) (dmb), (b) axes cryoprotected with sucrose and flash-dried to 0.28±0.04 g g^{-1} (dmb), (c) axes cryoprotected with glycerol and flash-dried to 0.28±0.04 g g^{-1} (dmb) and (d) axes cryoprotected with DMSO and flash-dried to 0.28±0.04 g g^{-1} (dmb). (major grid scale in cm, minor gridlines in mm)

Row a: Red to orange staining of embryonic axes and cotyledonary segments (refer to bars for comparison) – fully viable axes;
Rows b, c and d: No reaction with TTZ – axes non-viable

Despite the successful use of chemical cryoprotectants for cryopreservation in studies conducted on embryos/axes of recalcitrant seeds of other species (e.g. de Boucaud *et al*., 1991; Assy-Bah and Engelmann, 1992; Kioko *et al*., 1998; Walters *et al*., 2002b; Martínez *et al*., 2003; Sershen *et al*., 2007), their use was precluded for embryonic axes of *L. kirkii*, proving lethal to all when cryoprotection was followed by rapid dehydration. Mycock *et al*. (1995) suggested that although cryopreservation pretreatments such as dehydration and chemical cryoprotection have proved successful for zygotic embryos, this is not always the case, and optimum pretreatments need to be determined on a species-specific basis. Furthermore, Fuller (2004) conceded that cryoprotectants are chemicals that are not normally encountered by living organisms, and although cryoprotectant concentrations between 5 and 10% have been shown to be tolerable, most

chemical cryoprotectants are toxic to some degree (Mycock *et al*., 1995; Gui *et al*., 2004; Fuller, 2004). Berjak and Pammenter (2004b) also observed that although the use of cryoprotectants has been effective when applied to somatic embryos, they appear highly injurious to excised axes. Therefore, it has been suggested that the major limitation of cryoprotectants is imposed by their phytotoxicity (Bajaj, 1985; Finkle *et al*., 1985; Canavate and Lubain, 1994), which may cause irreversible cell structure injury, both before and after exposure to cryogenic temperatures (Farrant *et al*., 1977; Mycock *et al*., 1991). Furthermore, chemical cryoprotectants may induce reversible structural alterations to cell organelles including lipid components of the membranes (Costello and Gulik-Krzywicki, 1976) and membrane particles (Kirk and Tosteson, 1973; Farrant *et al*., 1977; Stolinski and Breathnach, 1977), or retard explant growth processes (Mycock *et al*., 1991). The cytotoxicity of chemical cryoprotectants varies with the type and concentration of the cryoprotectant and most importantly, could be species- and even cell-type-specific (Kartha, 1985; Mycock *et al*., 1991; Fuller, 2004; Benson, 2008; Walters *et al*., 2008). Additionally, it has been reported that the cytotoxic effects of certain individual cryoprotectants may be alleviated only by combining them with another cryoprotectant (Bajaj, 1985; Withers, 1985).

Farrant *et al*. (1977) and Fuller (2004) further proposed that the damage caused to tissue by chemical cryoprotection is a direct function of concentration, time and temperature of exposure, as well as the rate of addition. Numerous studies support this, generally showing that higher concentrations of, and prolonged exposure to chemical cryoprotectants adversely affect viability of various biological tissues (e.g. Nowshari *et al*., 1995; Gui *et al*., 2004; Volk *et al*., 2006b; reviewed by Fahy, 1986; Fuller, 2004). A review on cryoprotectant toxicity by Fahy (1986) also showed that the use of high concentrations of cryoprotectants resulted in more damage that could be accounted for on the basis of the calculated increase in solute concentrations, thus suggesting cryoprotectant toxicity. Studies have also shown that chemical cryoprotectants may cause the disassembly of microtubules and microfilaments that could be reversed only if the cryoprotectant exposure times and temperatures were reduced (Fuller, 2004) (although specific details were not provided by the author). Furthermore Volk *et al*. (2006b)

showed that when mint shoot tips were treated with glycerol, damage was significantly less when the exposure temperature was 0°C rather than at room temperature (22°C). This is possibly due to lower temperatures reducing the permeability of cryoprotectants (McGann, 1978). However, Fuller (2004) argued that lowering exposure temperatures, and thus the passive permeation of cryoprotectants, in itself may cause problems as it takes longer to achieve the sufficient concentrations required to facilitate cryoprotection.

Ashwood-Smith (1987) warned that in addition to chemical, osmotic toxicity will also be detected if cryoprotectant exposure is not optimised. When chemical cryoprotectants are added to biological explants, they traverse cellular membranes relatively slowly compared with water. This results in a rapid efflux of water from the cells, with associated volume collapse, and is referred to as osmotic toxicity (Leibo *et al*., 1978). With regard to the present investigation, embryonic axes survived exposure to cryoprotectants but viability decreased drastically when explants were flash-dried (Figure 3.8). Prior to culture on germination medium or treatment with TTZ, axes were rehydrated in a CaMg solution and it is therefore possible that the opposite of osmotic toxicity may have occurred. Rehydration of embryonic axes, whilst they were still loaded with cryoprotectants, may have led to rapid over-swelling of the cells (Fuller, 2004). It has been stated that cells can tolerate only moderate repeated changes in cell volume without significant damage (Pegg, 2002) and therefore over-swelling of cells within axes of *L. kirkii* may have led to their death.

In the present study, four of the seven cryoprotectant treatments tested (alone or in combination), contained the penetrating cryoprotectant DMSO. This cryoprotectant has been shown to be highly phytotoxic (Canavate and Lubain, 1994), although this effect appears to be species-specific with regard to exposure time, as well as being dependent on the size of the explant (Sakai, 2004). Fahy (1986) suggested that the toxicity of cryoprotectants may in fact manifest itself in the form of extra freezing-injury (termed 'cryoprotectant-associated freezing-injury') over and beyond the freezing-injury due to classic, well-known causes. Simply stated, cryoprotectants may cause added injury that are not as a result of the cryobiological properties of the cryoprotectant, but rather are due

to direct cytotoxic effects (Fahy, 1986; Fuller, 2004). Fahy (1986) also reported an increase in cryoprotectant-associated freezing-injury with increasing concentrations of DMSO. Furthermore, Kim *et al*. (2009) showed a decrease in growth recovery when concentrations of DMSO were increased. Dimethyl sulphoxide is also known to bind to monomers of actin thus reducing the extent of the actin filaments within cells (Morriset *et al*., 1993) and therefore disrupting the cytoskeleton. Kioko (2003), working on embryonic axes of *Trichilia dregeana*, recorded similar reductions in viability when cryoprotected axes were dehydrated, and suggested that this cytoskeleton disruption may have further reduced the percentage of axes developing into plantlets.

One possible approach to combat the toxicity of cryoprotectant solutions would be to decrease their concentration at application. As mentioned earlier, dehydration of explants will increase the concentration of penetrating cryoprotectants intracellularly. With regard to the present study, although 5 and 10% concentrations of cryoprotectant/cryoprotectant combinations were applied to embryonic axes, the intracellular concentration following flash-drying may in fact have been significantly higher. Therefore, if the concentrations of cryoprotectants/cryoprotectant combinations during application are reduced, after dehydration (which will cause an increase in intracellular cryoprotectant concentration) the final intracellular concentration may not be much higher than if there was initially no dehydration step. Although cryoprotectant concentrations of 5 – 15% are commonly employed for cryopreservation studies (discussed in section 1.7.1.3), it is possible that reducing concentrations to 1 and 2% may result in lower final concentrations after dehydration. As viability was maintained at 100% (when there was no dehydration step) (Table 3.4), it is therefore likely that such intracellular concentrations (5 and 10%) was not damaging to cells in embryonic axes of *L. kirkii*. Although studies using such low concentrations of cryoprotectant solutions are few, some success has been reported [e.g. *Chlamydomonas reinhardtii* (Crutchfield *et al*., 1999)]. Nevertheless, with respect to the current investigation, testing of different concentrations of cryoprotectant solutions, exposure times and exposure temperatures were not feasible because of the high seed numbers required to carry out adequate drying curves such as those in Figure 3.8 (as discussed above).

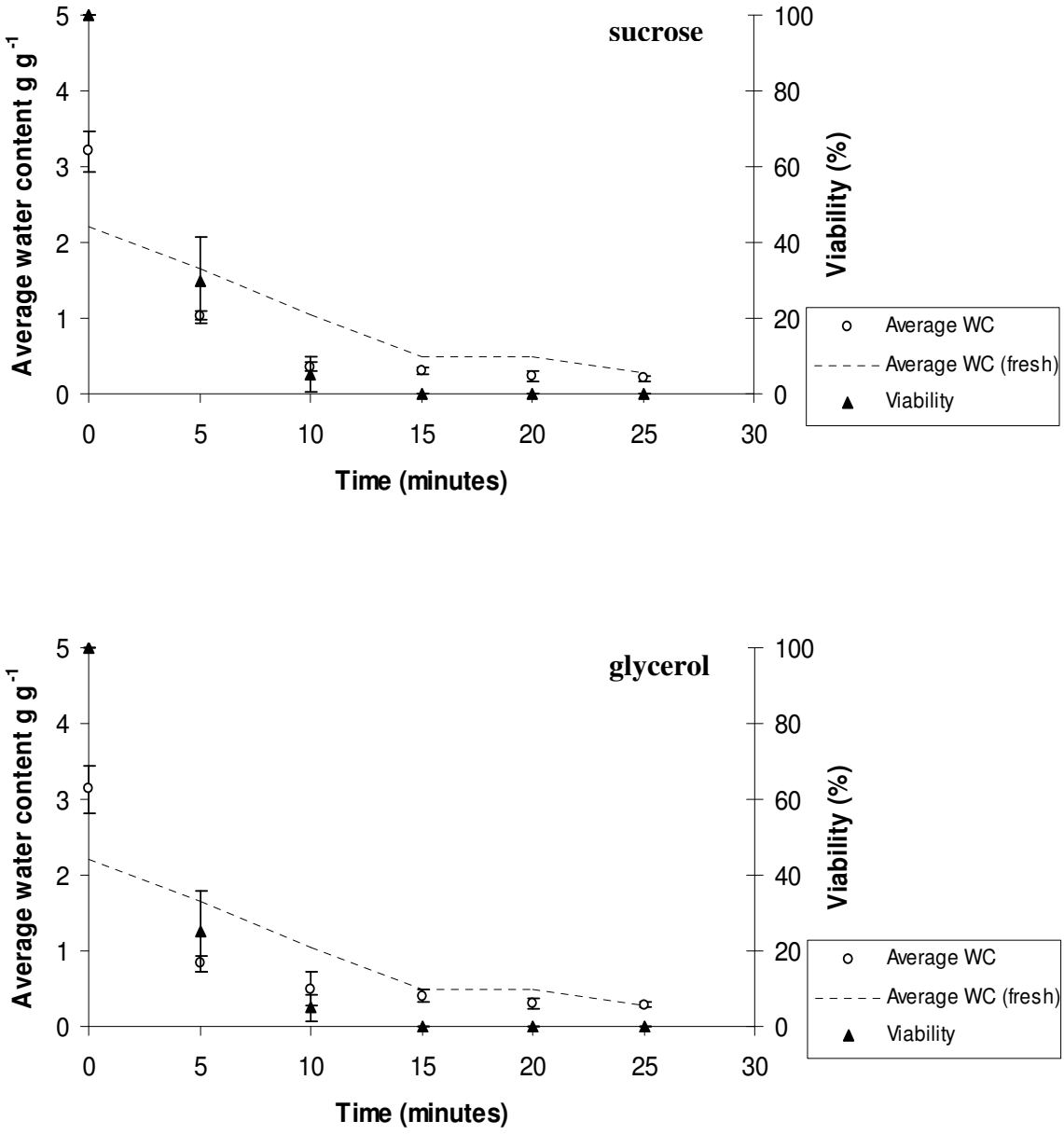


Figure 3.8: Drying time course and corresponding viability (root plus shoot protrusion) of axes excised with attached cotyledonary segments of *Landolphia kirkii* treated with cryoprotectants (singly and in combinations). Dashed line indicates drying time course for fresh, non-cryoprotected axes excised with attached cotyledonary segments. Bars indicate one standard deviation about the mean

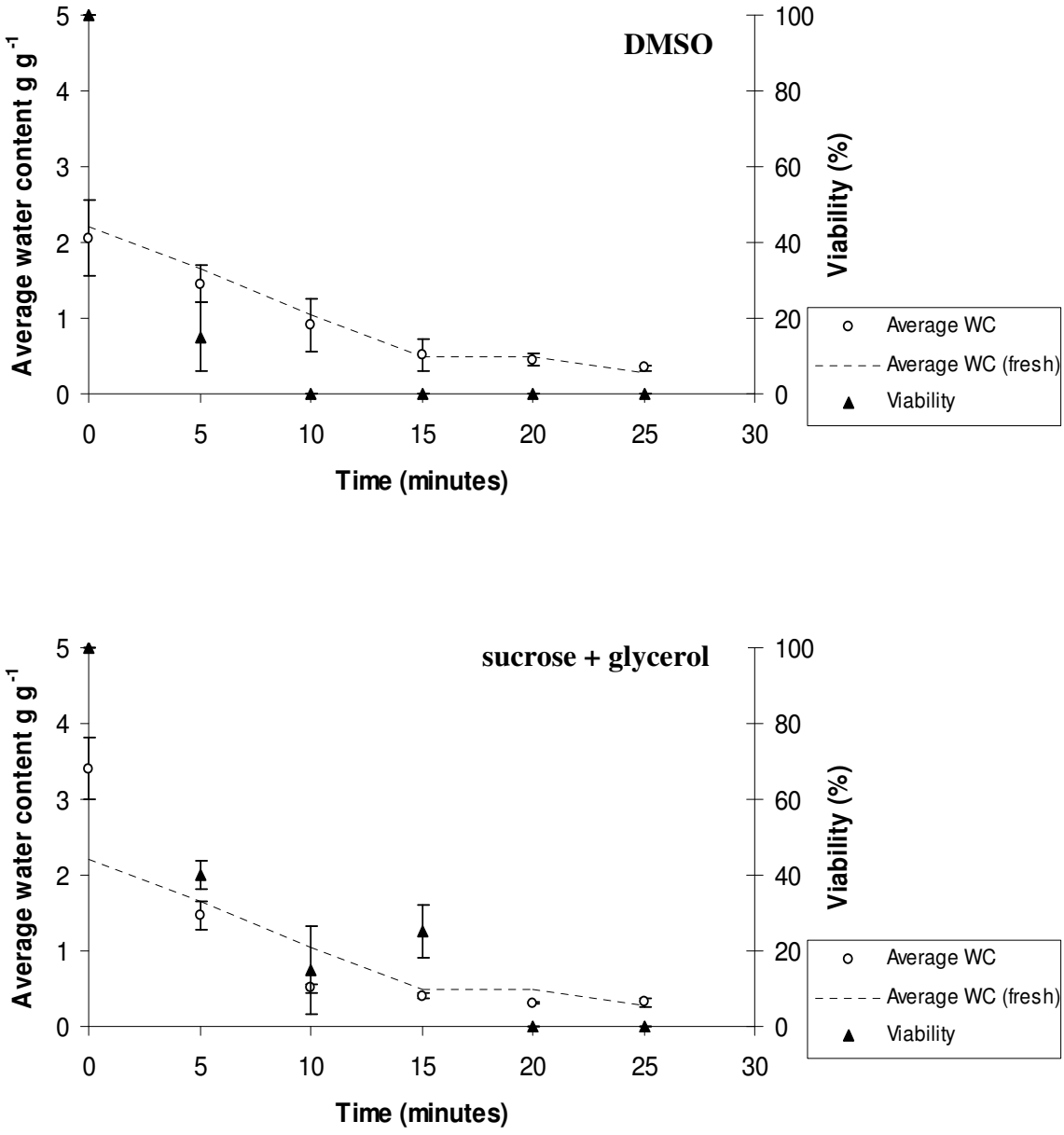
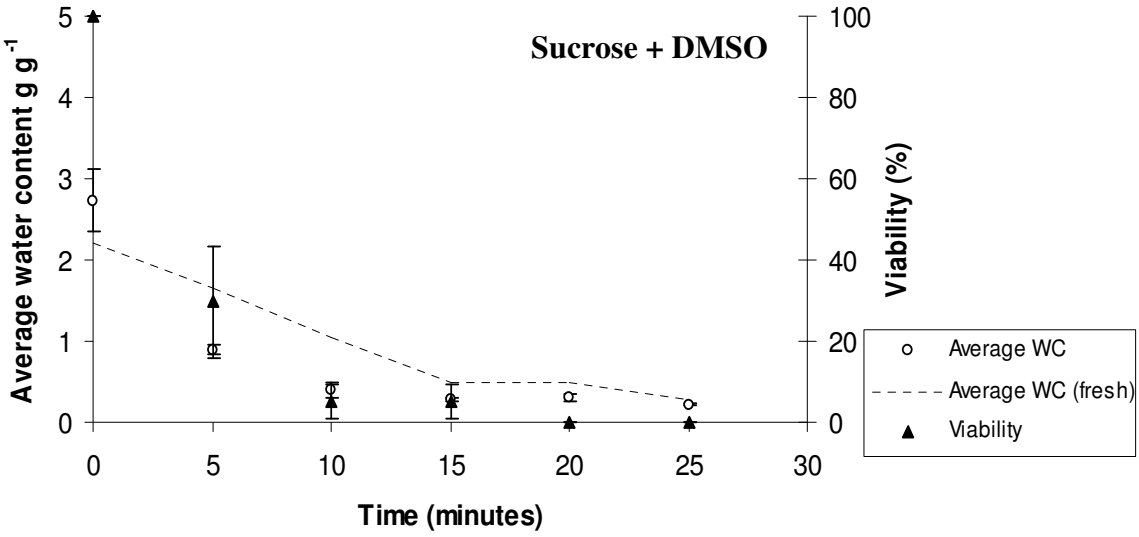


Figure 3.8 ***continued…***: Drying time course and corresponding viability (root and shoot protrusion) of axes excised with attached cotyledonary segments of *Landolphia kirkii* treated with cryoprotectants (singly and in combinations). Dashed line indicates drying time course for fresh, non-cryoprotected axes excised with attached cotyledonary segments. Bars indicate one standard deviation about the mean



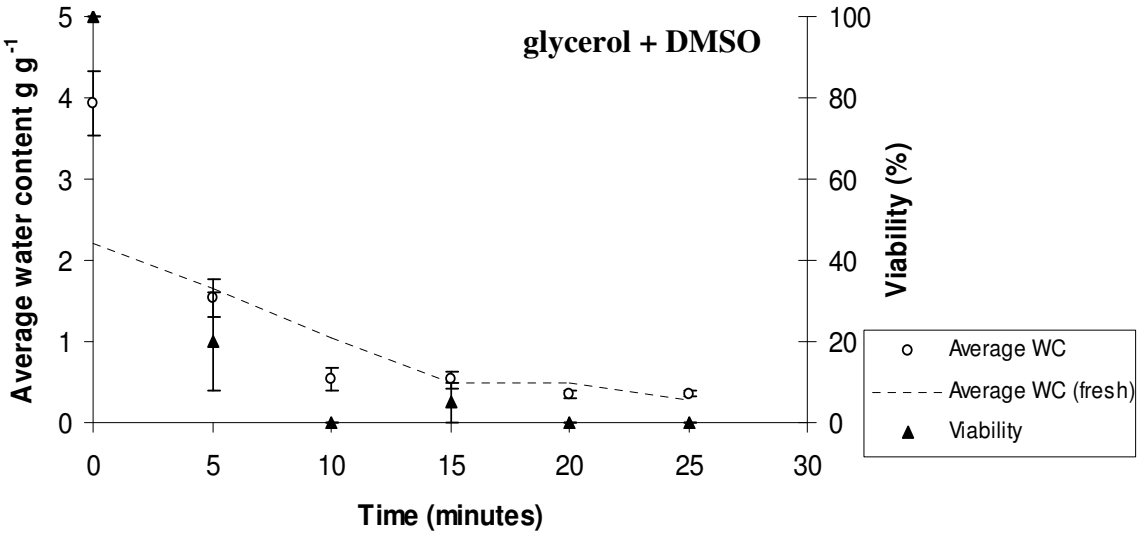


Figure 3.8 ***continued…***: Drying time course and corresponding viability (root and shoot protrusion) of axes excised with attached cotyledonary segments of *Landolphia kirkii* treated with cryoprotectants (singly and in combinations). Dashed line indicates drying time course for fresh, non-cryoprotected axes excised with attached cotyledonary segments. Bars indicate one standard deviation about the mean

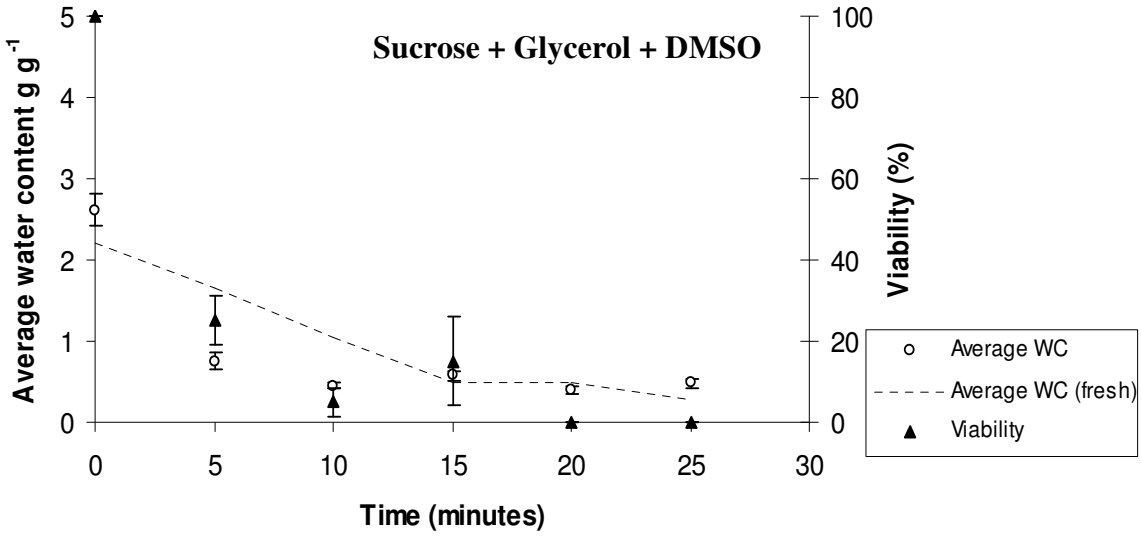


Figure 3.8 *continued…*: Drying time course and corresponding viability (root and shoot protrusion) of axes excised with attached cotyledonary segments of *Landolphia kirkii* treated with cryoprotectants (singly and in combinations). Dashed line indicates drying time course for fresh, non-cryoprotected axes excised with attached cotyledonary segments. Bars indicate one standard deviation about the mean

By exposing embryonic axes to penetrating cryoprotectants, the amount of solutes within the cells will increase (glycerol and DMSO are less volatile than water) and therefore the water content of axes should be lower after exposure to cryoprotectants. Surprisingly however, six of the seven cryoprotectant treatments tested resulted in an increase in the initial water content of embryonic axes of *L. kirkii* (Figure 3.8). Combinations of sucrose and glycerol, and glycerol and DMSO, showed the highest increases, with water contents of 3.41±0.34 and 3.98±0.28 g g^{-1} respectively. These values are significantly higher than that of non-cryoprotected axes that were shed at a water content of 2.24±0.04 g g^{-1}. Unusually, only DMSO treatments resulted in a lowered water content of the embryonic axes. Upon flash-drying of axes that had been cryoprotected (singly and in combination), water contents were consistently lower than that of non-cryoprotected axes dried for

similar periods (Figure 3.8). Although the general increase in initial water contents after immersion in cryoprotectant solutions compared to non-cryoprotected axes is surprising, reduction in water contents following sucrose cryoprotection of embryonic axes have been reported for other species including *Trichilia dregeana* (Dumet and Berjak, 1996; Kioko, 2003), *Trichilia emetica* (Kioko, 2003) and fifteen species of Amaryllidaceae (Sershen *et al*., 2007). Tissue dehydration has been achieved through the use of sucrose and other non-penetrating cryoprotectants which are suggested to promote vitrification during freezing (Vanoss *et al*., 1991; Storey and Storey, 1996; Benson, 2004; 2008; Fuller, 2004; Volk and Walters, 2006; Day *et al*., 2008). However, penetrating cryoprotectants such as glycerol do not function by tissue dehydration (Thierry *et al*., 1997; Benson, 2008). Therefore, the lower water contents recorded for axes cryoprotected with glycerol and flash-dried, when compared to non-cryoprotected axes flash-dried for the same time, were unexpected. Although the reason for this is unknown, Sershen *et al*. (2007) proposed a possible explanation in that glycerol may change the physical characteristics of the axial tissue which permits more rapid transfer of water from the interior of the tissue to the surface. This water is then lost to the air stream provided by the flash-drier. Furthermore, adding glycerol is like adding dry matter to the explant, which will decrease measured water content. Whatever the direct effects of the cryoprotectants on axis cells of *L. kirkii* were, the net result was that their application was lethal to all axes if followed by flash-drying and therefore their use was excluded from all subsequent cooling trials.

3.8 Cooling

3.8.1 Initial trials

As discussed earlier, initial cooling trials were undertaken with the purpose of testing for possible TAG crystallisation within embryonic axes of *L. kirkii*. Previous studies on mature axes of *L. kirkii* have shown that cryopreservation was possible when axes were dried to the 'optimal' water content between 0.30 and 0.45g g^{-1} (Vertucci *et al*., 1991). Their data showed that exposure of axes to sub-zero temperatures at water contents lower

and higher than their 'optimal' water contents resulted in reduced survival post-cooling (43% and 50% respectively), compared with axes with 'optimal' water contents exposed to -70°C, which yielded greater than 90% survival (Vertucci *et al*., 1991). However, no survival was recorded for cryopreservation of immature and germinating axes (Vertucci, *et al*., 1991). Therefore, those authors suggested that successful cryopreservation of *L. kirkii*, like other recalcitrant species, is dependent on the developmental stage of the tissues, although rate of freezing may play a role as well. For the current contribution, three water contents based on the desiccation trials (section 3.5) were selected for initial cryopreservation studies. These were fresh (2.24±0.05 g g^{-1}), 'optimum' (0.28±0.04 g g^{-1}) and 'detrimental' (0.11±0.05 g g^{-1}), chosen to determine their effect on viability and also to assess, by TEM, the damage incurred by desiccation, freezing and combinations of both. At these three water contents, embryonic axes were exposed to rapid and ultra-rapid cooling by tumble-mixing in LN and nitrogen slush respectively (see section 2.10.1).

Due to limited seed material, this initial experiment was conducted only on axes excised with a 3 mm segment of each cotyledon still attached. No embryonic axes of *L. kirkii* exposed to sub-zero temperatures and subsequent thawing and imbibition treatments showed survival when viability was assessed by *in vitro* culture and the TTZ test (Table 3.5). Fresh axes imbibed on filter paper moistened with water or a CaMg solution for 12 h only (i.e. without temperature and thawing treatments), showed viability of 80 and 95% respectively when assessed in the same manner. However, viability of 15 and 65% resulted when axes flash-dried to a 'optimal' water content (0.28±0.04 g g^{-1}), were imbibed on filter paper for 12 h (without temperature and thawing treatments) moistened with water and a CaMg solution respectively (Table 3.5). Axes at 'detrimental' water contents (0.11±0.05 g g^{-1}) that underwent the same imbibition treatments revealed 0% viability for both treatments. From these results it is clear that neither cooling rates, thawing procedures nor imbibition methods adopted for this initial trial, were amenable to successful cryopreservation of embryonic axes of *L. kirkii*. However, in all cases whether axes had been subjected to dehydration or not, irrespective of the temperature of thawing, lipid bodies within meristematic and other cells of *L. kirkii* remained intact, with no evidence of disintegration having occurred (see section 3.10).

Table 3.5: Viability of embryonic axes of *Landolphia kirkii* excised with cotyledonary segments following dehydration, cooling, thawing and imbibition treatments (n=30)

Water content ($g\ g^{-1}$)	Cooling treatment (LN/Slush)	Thawing temperature (°C)	Imbibition treatment*	Viability (%)
2.24	None	None	Water	80
2.24	None	None	CaMg	95
2.24	LN	5	CaMg	0
2.24	LN	25	CaMg	0
2.24	LN	45	CaMg	0
2.24	Slush	5	CaMg	0
2.24	Slush	25	CaMg	0
2.24	Slush	45	CaMg	0
0.28	None	None	Water	15
0.28	None	None	CaMg	65
0.28	LN	5	CaMg	0
0.28	LN	25	CaMg	0
0.28	LN	45	CaMg	0
0.28	Slush	5	CaMg	0
0.28	Slush	25	CaMg	0
0.28	Slush	45	CaMg	0
0.11	None	None	Water	0
0.11	None	None	CaMg	0
0.11	LN	5	CaMg	0
0.11	LN	25	CaMg	0
0.11	LN	45	CaMg	0
0.11	Slush	5	CaMg	0
0.11	Slush	25	CaMg	0
0.11	Slush	45	CaMg	0

* Imbibition temperature of 25°C for all treatments

3.8.2 Rehydration

It is not clear from the results of these initial cooling trials whether it was as a consequence of drying, cooling, thawing or imbibition rates, or a combination of the four that resulted in the loss of viability. However, Table 3.5 does indicate that the imbibition method employed on axes of *L. kirkii* is pertinent to their survival. These results suggested that CaMg should be the preferred solution for rehydration of embryonic axes of *L. kirkii*. As discussed in section 1.7.1.7, Berjak *et al*. (1999; 2000a), working on *Quercus robur*, showed that when partially dehydrated recalcitrant axes were rehydrated in distilled water, roots lacked a gravitropic response. Furthermore, those authors reported that rehydration in distilled water resulted in shoot production that was abnormal and eventually became necrotic. However, these abnormalities appeared to be rectified when a CaMg solution rather than distilled water was used for rehydration (Berjak *et al*., 1999; 2000a). Similarly, recalcitrant embryonic axes of *Trichilia dregeana* rehydrated with distilled water, lacked roots with gravitropic responses (Berjak and Mycock, 2004). However, when these axes were rehydrated with a CaMg solution or when axes were not dehydrated and therefore did not require rehydration, roots developed with strong gravitropic responses (Berjak and Mycock, 2004). Furthermore, those authors reported that axes rehydrated in a CaMg solution showed higher vigour and had greened to a marked extent in comparison to axes rehydrated in distilled water after seven days of *in vitro* culture. It was suggested that the lack of root graviperception after rehydration is as a result of ineffective cation uptake from the growth medium, resulting in disruption of starch synthesis and deposition (Berjak and Mycock, 2004). It was also proposed that CaMg plays a vital role in maintenance of the actin component of the cytoskeleton, which appears to be involved with intracellular organisation, more specifically with the proximal positioning of the nucleus (Berjak and Mycock, 2004). Therefore, with regard to imbibition after cooling and thawing treatments in this investigation, a CaMg solution was used for all further experiments as opposed to distilled water.

It must be noted that when fresh axes (2.24±0.05 g g^{-1}) and axes flash-dried to 'optimum' water contents (0.28±0.04 g g^{-1}) were rehydrated by plunging into a CaMg solution, higher viability (100% for both) (Figure 3.4) was achieved compared with axes that were rehydrated by slow imbibition on filter paper dampened with a CaMg solution (95% for fresh and 65% for flash-dried) (Table 3.5). Leprince *et al*. (1998), working on desiccation-sensitive cocoa seeds, established that when these seeds were slowly rehydrated on moistened filter paper, coalescence of oil bodies in cotyledons occurred. Coalescence of lipid bodies is a common abnormality associated with deterioration of cells from seeds (Smith and Berjak, 1995). Pammenter and Berjak (1999) suggested that the role of oleosins in desiccation tolerance is of importance in seeds that are particularly lipid-rich such as *Azadirachta indica* (Berjak *et al*., 1995) and therefore, possibly seeds of *L. kirkii* (see section 3.6). The findings of Leprince *et al*. (1998) implied that the rate of rehydration is similar in its effect as the rate of dehydration. Previous studies (Pammenter and Berjak, 1999; Perán *et al*., 2004) have also suggested that slow rehydration of zygotic axes led to greater accumulation of damage, as tissue is exposed to intermediate hydrated levels for a longer time period, thus allowing more time for aqueous-based deleterious processes to occur (Vertucci and Farrant, 1995; Walters *et al*., 2001; 2002a; Perán *et al*., 2004). This effect is similar to that of slow dehydration (Pammenter and Berjak, 1999; Perán *et al*., 2004). Furthermore, Perán *et al*. (2004) suggested that slow rehydration on moistened filter paper (as opposed to direct immersion by plunging), permits free oxygen access to the tissue, thereby possibly enhancing the rate of production of damaging ROS. Moreover, those authors advise that slow imbibition of dehydrated orthodox plant tissue permits re-establishment of functional membranes (Perán *et al*., 2004). Similar techniques have been used on recalcitrant tissue (Berjak *et al*., 1992; 1993; Leprince *et al*., 1998; Pammenter *et al*., 1998) with the assumption that, if slow rehydration of partially dehydrated material is not beneficial, at least it is not damaging (Perán *et al*., 2004). However, this may not always be the case for desiccation-sensitive material (Perán *et al*., 2004). Perán *et al*. (2004), working on embryos or axes from three desiccation-sensitive species *viz. Artocarpus heterophyllus*, *Podocarpus henkelii* and *Ekebergia capensis*, demonstrated that rapid rehydration by direct immersion may prevent the accumulation of damage associated with rehydration by reducing the time spent at

intermediate hydration levels. Those authors showed that generally, all material that was rapidly rehydrated by direct immersion resulted in higher survival than when slowly rehydrated on moistened filter paper.

3.8.3 Electrolyte Leakage

Due to the difficulties associated with assessing post-cryogenic temperature exposure and the damage incurred by recalcitrant axes during dehydration, cooling and rehydration, as well as time constraints, measurements of electrical conductivity of leakage were also made to assess viability after the pilot experiment that was conducted to ascertain whether crystallisation in the intracellular TAGs occurred. Electrolyte leakage from seed tissue can give a reliable assessment of damage within seeds and is directly linked to plasmalemma abnormalities, providing a good indication of viability (Ching and Schoolcraft, 1968; Villers, 1973; Parrish and Leopold, 1978; Bewley, 1979; Becwar *et al*., 1982; Vertucci, 1989; Pammenter *et al*., 1991; 1993; Berjak *et al*., 1992; 1993; Wesley-Smith *et al*., 2001a). Furthermore, Berjak *et al*. (1993) stated that the effect of various stresses on seed survival and on electrolyte leakage is inversely related. Readings were taken for axes subjected to the various cooling, thawing and imbibition manipulations of the initial cryopreservation trials.

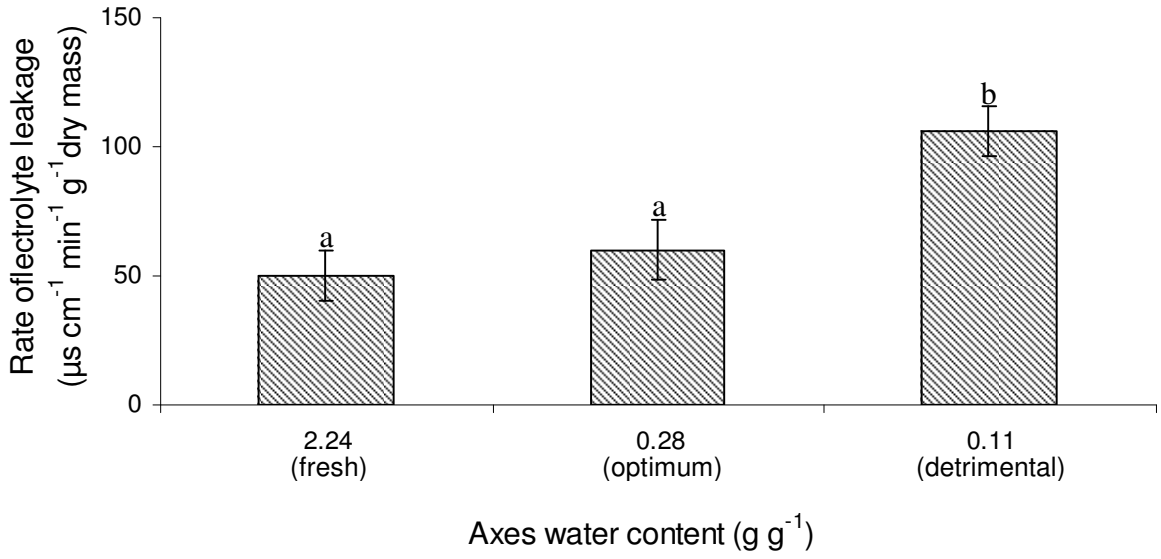


Figure 3.9: Rates of electrolyte leakage from embryonic axes excised with attached cotyledonary segments of *Landolphia kirkii* rapidly dehydrated to different water contents. Error bars show standard error means. A Kolmogorov-Smirnov test was carried out which showed normal distribution of data. A one-way ANOVA was performed showing significant difference between treatments ($p<0.05$). This was followed by a Scheffe's post-hoc test

Average conductivity measurements were plotted as a function of soaking time. This showed that electrolyte leakage stabilised after 13 h (data not shown). Average conductivity readings over the 13 h were used to assess the damage within the axes. Rates of leakage of electrolytes from isolated axes with attached cotyledondary segments corroborated the viability results of both dehydration, and to some degree cooling and rehydration treatments (Figures 3.9 and 3.10). Although the rate of electrolyte leakage increased only slightly between fresh axes (2.24±0.05 g g^{-1}) and axes at 'optimal' water contents (0.28±0.04 g g^{-1}), there was a marked increase in leakage rate when axes were flash-dried to 'detrimental' levels (0.11±0.05 g g^{-1}) (Figure 3.9). Pammenter *et al*. (1998) suggested that the relationship between electrolyte leakage and water content generally follows a typical pattern of constant leakage to a 'critical water content,' at which point a

steep escalation occurs. Similar findings have been reported for embryonic axes of *Artocarpus heterophyllus* (Pammenter *et al*., 1993; Wesley-Smith *et al*., 2001a), *Camellia sinensis* (Berjak *et al*., 1993), *Glycine max* (Sun and Leopold, 1993), *Theobroma cacao* (Li and Sun, 1999; Liang and Sun, 2000), embryos of *Chrysalidocarpus lutescens* and whole seeds of *Acer saccharium* (Becwar *et al*., 1982) and *Shorea robusta* (Chaitanya and Naithani, 1994). The leakage results from the present contributions coincided well with those studies as well as that of Pammenter *et al*. (1991) and Berjak *et al*. (1992) who found that the levels to which mature embryonic axes of *L. kirkii* can be flash-dried before marked increase in leakage occurred, was between 0.25 and 0.3 g g^{-1} and, as was the case with the current contribution, this steep increase in leakage coincided with a drastic decline in viability. Further analogy with those studies was that leakage remained essentially unchanged until a water content of 0.25 – 0.3 g g^{-1} (Figure 3.9). Metabolic imbalance seems to begin relatively early in the desiccation process and Leprince *et al*. (1999) suggested that toxic products of this imbalance will eventually lead to irreparable damage to cellular membranes and subsequent leaking of electrolytes (Leprince *et al*., 2000).

Although it is difficult to identify a clear pattern for the present study with regard to leakage, Figure 3.10 does show a definite increase in rates of electrolyte leakage from axes subjected to various combinations of cooling and thawing treatments as compared with fresh axes that were not exposed to severe temperature or rehydration treatments but where imbibed only. It would have been expected that rates of electrolyte leakage would have been significantly higher for axes dehydrated to 'detrimental' water contents and cooled in either LN or nitrogen slush compared with axes dehydrated to 'optimum' levels and subjected to the same cooling treatments. However, Figure 3.10 shows axes cooled at 'optimum' water contents to have higher rates of leakage than those that at were cooled at 'detrimental' levels. Furthermore, all cooling treatments for which electrolyte leakage readings were taken had a viability of 0% (Table 3.5) making determining any relationship between viability and rates of electrolyte leakage of axes impossible. Therefore, leakage readings from Figure 3.10, in this case, are rather not informative.

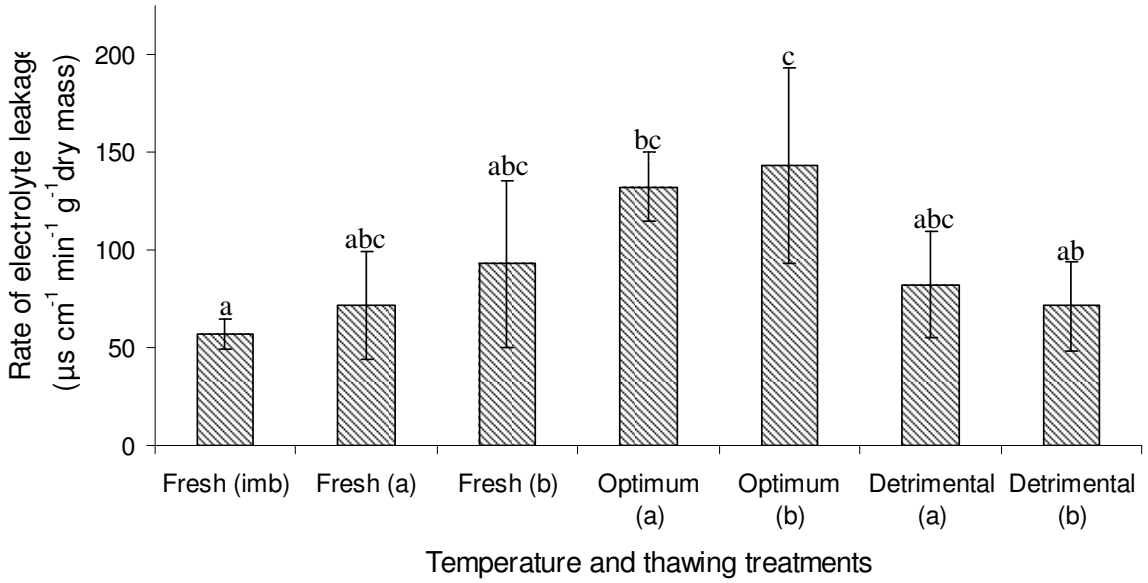


Figure 3.10: Rates of electrolyte leakage from embryonic axes excised with attached cotyledonary segments of *Landolphia kirkii* subjected to various drying, cooling and thawing treatments. Fresh WC= 2.24±0.05 g g^{-1}; optimum WC= 0.28±0.04 g g^{-1} and detrimental WC= 0.11±0.05 g g^{-1}. imb = imbibed only; (a) = cooled in LN, thawed at 25°C; (b) = cooled in nitrogen slush, thawed at 25°C. Error bars show standard error of the means. A Kolmogorov-Smirnov test was carried out which showed normal distribution of data. A one-way ANOVA was performed showing significant difference between treatments ($p<0.05$). This was followed by a Scheffe's post-hoc test

3.9 Cryopreservation trials

It should be noted, that with regard to germination after cooling, axes which had been excised with the cotyledons completely removed performed similarly to axes that were excised with ~3 mm of both cotyledons attached when assessed by root protrusion (data not shown). However none of the axes excised with the cotyledons completely removed developed shoots after exposure to cryogenic temperatures (data not shown). Since it was

decided that survival would be scored as production of both roots and shoots, all data presented hereafter are based to results obtained from cryopreservation trials on embryonic axes excised with cotyledonary segments attached only.

3.9.1 Rapid cooling

Rapid and ultra-rapid cooling is generally considered the most promising method for cryopreservation of recalcitrant embryonic/zygotic axes (Berjak, pers. comm.[3]). Leprince and Walters-Vertucci (1995) suggested that the partial dehydration of axes can reduce the heat capacity of tissue as well as encouraging the formation of glasses. This partial dehydration of embryonic axes of *L. kirkii* was achieved by flash-drying only (see section 3.5) as osmotic dehydration, through the use of non-penetrating chemical cryoprotectants, resulted in the death of all the axes (see section 3.7). Although cryoprotection and partial dehydration of recalcitrant embryonic axes has shown to optimise rapid and ultra-rapid cooling rates of larger explants (e.g. Wesley-Smith *et al*., 1992; 2004a; Kioko *et al*., 1998; Sershen *et al*., 2007), the size of explants is a major limiting factor for rapid cooling and avoiding freezing damage (Ryan and Purse, 1985; Wesley-Smith *et al*., 1992; Wesley-Smith, 2002; Walters *et al*., 2008). Ryan and Purse (1985) and Wesley-Smith *et al.* (1992) suggested that specimens smaller than 100 µm are most amenable to rapid cooling so as to ensure adequate freezing rates throughout the specimen. However, the size of embryonic axes with attached cotyledonary segments of *L. kirkii*, greatly exceed this recommended size limit. For the current contribution, although rapid (direct immersion in LN) and ultra-rapid (direct immersion in nitrogen slush) cooling showed high root protrusion post-thawing (70 and 100% respectively), no shoot development was observed for either treatment (Table 3.6). The larger size of explants may have contributed to this post-thaw viability loss. In contrast, many rapid cooling techniques such as direct emersion in LN (e.g. Normah *et al*., 1986; Janeiro *et al*., 1996; González-Benito, 1998; Kioko, 2003), direct emersion in nitrogen slush (e.g. Wesley-Smith *et al*., 1992; 2001b; Kioko *et al*., 1998; Wesley-Smith, 2002; Sershen *et al*., 2007) and freezing

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in melting isopentane (e.g. Berjak *et al*., 1999; Walker, 2000; Wesley-Smith *et al*., 2001b) have been successfully employed for the cryopreservation of recalcitrant embryonic axes. The success of these protocols can be attributed to the cooling rates (Kioko, 2003) as these cooling rates are suggested to move embryonic tissue through the ice nucleation temperatures faster than ice crystal growth can occur, and the intracellular solution is said to form microcrystalline ice or vitrify (Dubochet *et al*., 1982; James, 1983; Robards and Sleytr, 1985; Wesley-Smith *et al*., 1992; 2004b; Engelmann, 1993; Day *et al*., 2008). Both of these outcomes are considered to be non-injurious to cellular membranes (Kioko, 2003; Benson *et al*., 2006; Day *et al*., 2008).

Table 3.6: The effect of different cooling treatments on root protrusion and shoot development from embryonic axes of *Landolphia kirkii* excised with attached cotyledon segments and subjected to rapid drying to a water content of 0.28±0.04 g g^{-1} (n=30)

Treatment	**Cooling rate (°C min^{-1}) to -196°C**	**Root protrusion (%)**	**Shoot development (%)**
Nitrogen slush†	12000[2]	100±0[c]	0±0[a]
LN†	6000[2]	70±5[a]	0±0[a]
Mr Frosty	1 (to -70°C)	80±8.7[b]	70±5[c]
Mr Frosty + LN†	1 (to -70°C) + 6000[2]	70±5[a]	10±8.7[a]
Mr Frosty + LN	1 (to -70°C) + 14	75±5[ab]	10±8.7[a]
Cryovials	14	80±0[b]	40±10[b]

A Kolmogorov-Smirnov test was carried out which showed normal distribution of data. A one-way ANOVA was performed showing significant difference between treatments ($p<0.05$). Numbers followed by the same letter in the same columns are not significantly different according to the Duncan post-hoc test

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† Direct immersion of naked embryonic axes

In addition to the cryogen used, the thermal mass of the explant also plays a major role in the cooling rates attained (Meryman, 1956; Bald, 1987; Walters *et al*., 2008). To achieve cooling rates of 6000 – 12000°C min^{-1}, as attempted in the present investigation (see Table 3.6), it has been suggested that the total mass of the explant plus water needs to be in the range of 1 – 2 mg (Wesley-Smith, 2002; Walters *et al*., 2008). At high water contents, smaller explants are required; this ultimately becomes a limiting factor and at high water contents these high cooling rates are not achievable. As discussed previously, the axes of *L. kirkii* used for this study were excised with approximately 3 mm of each cotyledon attached. Walters *et al*. (2008) stated that the tissue size affects the thermal mass as well as the surface area to volume ratios, which in turn will affect the cooling rate of the tissue. After dehydration of *L. kirkii* embryonic axes to the water content chosen to be the most amenable to cryopreservation (i.e. 0.28±0.04 g g^{-1}), the mass of the single explants were between 3 and 4.5 mg, more than double the recommended mass. Therefore, due to the mass, in addition to the size of explants employed in the current study, it is unlikely that the extremely high cooling rates necessary to prevent ice crystallisation and to bring about vitrification of partly-dehydrated axes could be attained (Kioko, 2003). Walters *et al*. (2008) reiterate this, stating that "as the sample dry mass increases, the allowable range of water contents narrows and it becomes physically impossible to cool hydrated tissues fast enough to prevent lethal freezing injury." Those authors however, differed from Wesley-Smith (2002) with regard to the range of thermal mass required to achieve cooling rates higher than 6000°C min^{-1}. Wesley-Smith (2002) suggested that if recalcitrant explants have a dry mass of ≤6 mg, a broad range of water contents can be cooled at rates between 6000 and 30000°C min^{-1} and, if tissue has a dry mass of <1 mg, cooling rates faster than 60000°C min^{-1} are attainable for tissue of a suitable range of water contents.

The lack of survival of embryonic axes of *L. kirkii* after exposure to rapid and ultra-rapid cooling methods may also be a function of the intracellular water within the tissue. Although the water content at which these axes were exposed to cryogenic temperatures is quoted as 0.28±0.04 g g^{-1}, after correction for the high lipid content of the tissue, the water content is closer to 0.39±0.06 g g^{-1}(see section 3.5). This figure is at the higher

limits of the range of 0.2 – 0.4 g g^{-1} suggested by Vertucci and Leopold (1987) to be the amount of non-freezable water within recalcitrant seeds. Therefore, it is very possible that some of the water remaining within the embryonic axes of *L .kirkii* after flash-drying to 0.39±0.06 g g^{-1} (after correction for lipid content) is constituted by the freezable component. Kioko (2003) suggested that even a small proportion of freezable water could provide the basis for lethal ice crystallisation during exposure of axes to rapid cooling protocols, which is dictated by the relatively large thermal mass of the axis/cotyledonary explants.

3.9.2 Controlled rate and slow cooling

The effects of slow cooling (1 and 14°C min^{-1}) on survival (i.e. root and shoot protrusion) of embryonic axes excised with attached cotyledonary segments of *L. kirkii* dehydrated to 0.28±0.04 g g^{-1} are presented in Table 3.6. Similarly to flash-dried axes subjected to rapid and ultra-rapid cooling, slow cooling trials also recorded high root protrusion (≥70%) by axes of *L. kirkii* (Table 3.6). However, in contrast to rapid and ultra-rapid cooling, axes flash-dried to 0.28±0.04 g g^{-1} and subjected to slow cooling recorded at least some shoot production (≥10%), with axes cooled at 1°C min^{-1} to -70°C (Mr Frosty) revealing the highest survival (assessed by root and shoot production) (Table 3.6). These results mirrored those of Vertucci *et al*. (1991) on *L. kirkii*, in that slow cooling down to -70°C was associated with the highest survival of embryonic axes. Axes cooled to -70°C in Mr Frosty produced 80% root protrusion with 70% shoot development (Table 3.6). Similar success with the use of Mr Frosty for cryopreservation of other plant tissue has also been reported (e.g. Christianson, 1998; Day and Harding, 2008).

The water content at which embryonic axes of *L. kirkii* [0.28±0.04 g g^{-1} (0.39±0.06 g g^{-1} after correction for lipid content)] were cooled fell within the narrow range of 0.3 – 0.4 g g^{-1} suggested for slow cooling (Vertucci, 1989b). Previous success, in terms of survival of embryonic axes of *L. kirkii*, cooled to -70°C by Vertucci *et al*. (1991), was achieved by employing a slow cooling rate of 10°C min^{-1}. However, this cooling rate (10°C min^{-1}), falls at the upper limits of the range considered to be regarded as slow cooling

[0.1 – 10°C min^{-1} (Touchell *et al*., 2002; Benson, 2004; 2007; 2008)]. It was suspected that post-thaw survival of embryonic axes of *L. kirkii* could be improved if cooling rates slower than 10°C min^{-1} were employed, as has been shown for recalcitrant embryonic axes of *Quercus robur* (Poulsen, 1992), *Quercus suber* and *Quercus ilex* (González-Benito *et al*., 2002). Mazur (1963; 2004) explained that the rate of change of temperature influences the rate at which water is transported out of cells during freezing and therefore, indirectly, the probability of intracellular freezing. If water can leave the cells rapidly enough to maintain near thermodynamic equilibrium across the cell membrane, the cytoplasm within the cell will not cool below its freezing point (i.e. super-cool), and the ice formed will all be situated externally of the cells (Pegg, 2007). However, if cooling rates are too rapid, cytoplasm becomes super-cooled, and it becomes more likely that the cell will lethally freeze internally (Pegg, 2007). For example, Rubinsky and Pegg (1988) working on hepatocytes, showed that intracellular freezing is unlikely to occur at 1°C min^{-1} but is possible at cooling rates higher than 10°C min^{-1}. Thus, a cooling rate of 1°C min^{-1} essentially eliminated the risk of intracellular ice damage during freezing of hepatocytes. Therefore, a cooling rate of 1°C min^{-1} (within Mr Frosty) down to -70°C as well as further two-step cooling down to -196°C, either at 14°C min^{-1} (ascertained for axes of *L. kirkii* using a thermocouple) in polypropylene cryovials or by directly plunging naked axes in LN, were adopted for some treatments in this study.

Although dehydrated axes cooled to -70°C in Mr Frosty performed best regarding shoot production, the aim of the present study was to cryopreserve embryonic axes of *L. kirkii* at cryogenic temperatures of -140°C and lower (Benson, 2008). The successful use of controlled rate two-step cooling for the cryopreservation of plant germplasm is well documented (Morris, 1978; Morris and Canning, 1978; Withers, 1978; Chen *et al*., 1985; Reed and Lagerstedt, 1987; Towill, 1988; Heszky *et al*., 1990; Reed, 1990; Reed *et al*., 2003; Uchendu and Reed, 2008). This technique aims to moderate two injurious components, the effects of damaging colligative solutions, and ice formation (Mazur, 2004; Benson, 2007). The initial slow programmed cooling step of two-step cooling is thought to safely facilitate the movement of intracellular water to the exterior of the cell, allowing for vitrification of the cytoplasm when explants are subsequently plunged into

LN (dependent on the water content of explants) (Pearce, 2004; Wesley-Smith *et al*., 2004a; Benson *et al*., 2005; Reed and Uchendu, 2008). However, it should be noted that most successful cryopreservation protocols employing this technique, pretreat explants with cryoprotectant solutions (Schrijnemakers and Van Iren, 1995; Benson, 2007; Reed and Uchendu, 2008). It is thought for plant tissue that, at the freezing point of the cryoprotectant solution, ice nucleation is initiated resulting in ice forming in the cryoprotectant solution and the intercellular spaces (Reed and Uchendu, 2008). Cell membranes are protected from these damaging ice crystals by the cell wall (Reed and Uchendu, 2008). Although the use of Mr Frosty to cool plant tissue to -70°C (at a cooling rate of 1°C min^{-1}) before plunging cryovials into LN has shown to be successful in other studies on plant material (e.g. Vendrame *et al*., 2001; Cho *et al*., 2007; Varghese *et al*., 2009; Ngobese *et al*., 2010; Ozudogru *et al*., 2010; reviewed by Day and Harding, 2008), when such an approach was employed for the current study on embryonic axes of *L. kirkii*, although root protrusion was 80%, shoot production achieved was only 10% (Table 3.6). Additionally, when a separate batch of axes were cooled to -70°C using Mr Frosty before naked axes were plunged directly into LN (at *c*. 6000°C min^{-1}) the root and shoot protrusion recorded was only 70 and 10% respectively (Table 3.6). It is possible that because the use of cryoprotectant solutions on embryonic axes of *L. kirkii* had to be precluded from current cryopreservation trials (explained in section 3.7); this may have played a role in the poor post-thaw survival recorded for two-step cooling.

Therefore, the best survival at cryogenic temperatures, assessed by both root and shoot production, was when embryonic axes within cryovials were plunged in LN at a cooling rate of *c*. 14°C min^{-1} (Table 3.6). These embryonic axes showed 80% root production with 40% shoot development (all axes that did develop shoots had also eventually produced roots). This slow rate achieved by cooling explants within polypropylene cryovials, has been successfully utilised in a number of studies on recalcitrant embryonic axes/embryos including *Corylus avellana* (Normah *et al*., 1994; Reed *et al*., 1994), *Azadirachta indica* (Chaudhury and Chandel, 1996), *Sechium edule* (Abdelnour-Esquivel and Engelmann, 2002), *Aesculus glabra* (Pence, 2003), *Castanea sativa* (Corredoira *et al*., 2004) and various amaryllid species (Sershen *et al*., 2007). However, it should be

noted, as discussed previously (see section 1.7.1.4), that the rates of cooling achieved using this technique vary markedly between investigations and is dependent on the species as well as the water content of the explants.

In an attempt to optimise survival by more accurately controlling cooling rate, further cooling trials were carried out using a computerised programmable freezer. Due to the lack of shoot production achieved when axes excised with cotyledons completely removed were subjected to the same cooling treatments as shown in Table 3.6 (data not shown) and because of a lack of large quantities of seed material, only embryonic axes excised with portions of the cotyledons attached were used for cooling in the programmable freezer. A range of cooling rates (2, 5, 10, 15, 20 and 50°C min^{-1}) were tested and the viability results are presented in Table 3.7. The cooling courses achieved by use of the controlled rate freezer at different cooling rates are shown in Figure 3.11 (graphs were re-drawn due to the poor quality of the original graphs produced by the programmable freezer). Figure 3.11 shows that although programmable freezers are suggested to accurately and precisely control freezing rates over a broad range of temperatures (Benson, 2004; 2007; 2008; Benson *et al*., 2005; Day *et al*., 2008), the cooling rates actually achieved differed from those that are quoted, especially when faster cooling rates were employed (e.g. Figure 3.11f, g). In order to replicate a similar cooling rate to that achieved by the use of Mr Frosty, a cooling rate of 2°C min^{-1} down to -70°C was employed (Figure 3.11a). Cooling rates of 1°C min^{-1} (as previously achieved by use of Mr Frosty) or lower were not feasible using this particular model of programmable freezer. The controlled rate instruments use large amounts of LN to simply cool the freezing chamber (Benson *et al*., 2005; Benson, 2007; 2008). For example, to achieve a cooling rate of 2°C min^{-1} down to -180°C in the present study (Figure 3.11b), 32 litres of LN was required. Therefore, in order to attain cooling rates as low as 1°C min^{-1} even higher volumes of LN are consumed, making such slow cooling rates using this particular programmable freezer impractical.

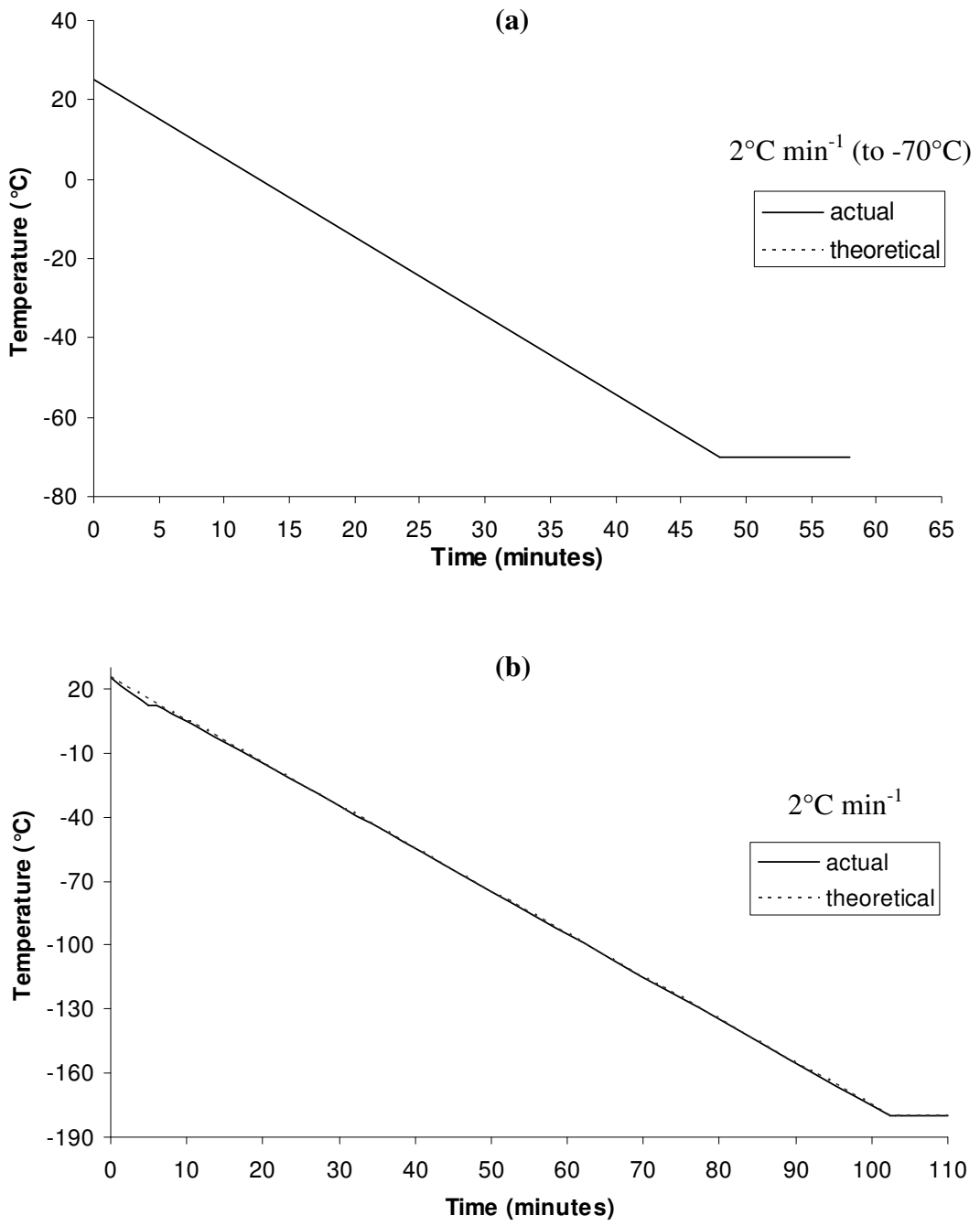
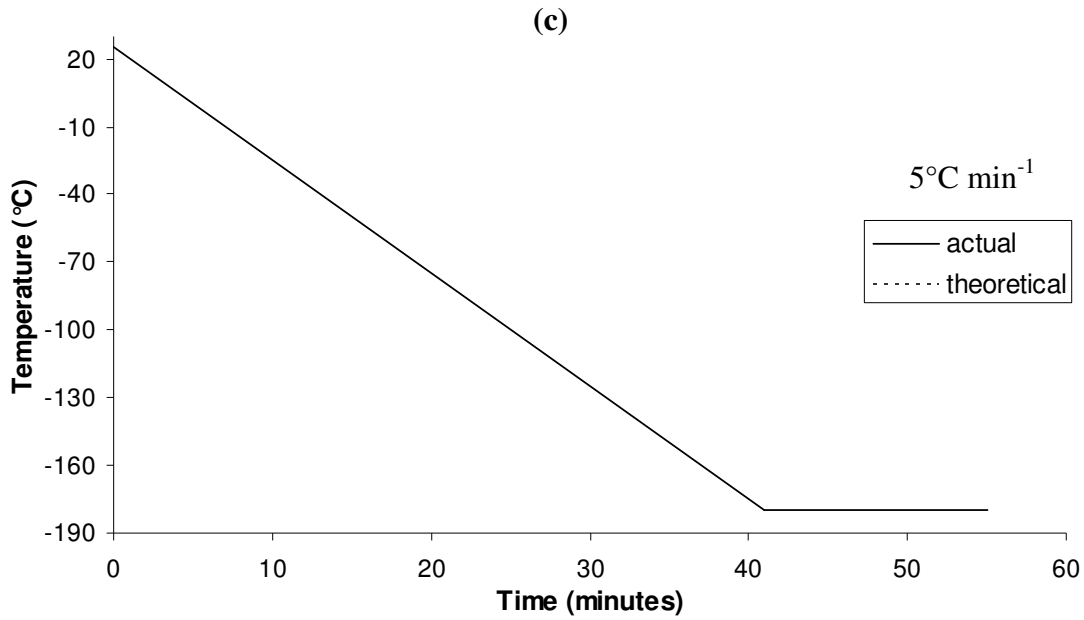


Figure 3.11a – b: Cooling time course (theoretical and actual course achieved) of axes of *Landolphia kirkii* excised with attached cotyledonary segments, flash-dried to 0.28±0.04 g g^{-1} (dmb) and cooled within a programmable freezer to -180°C (all graphs were reproduced from original print-outs generated from the Kryo 360-1.7 programmable freezer using computer software Delta T™). (a) Cooling rate of 2°C min^{-1} (to -70°C). (b) Cooling rate of 2°C min^{-1}



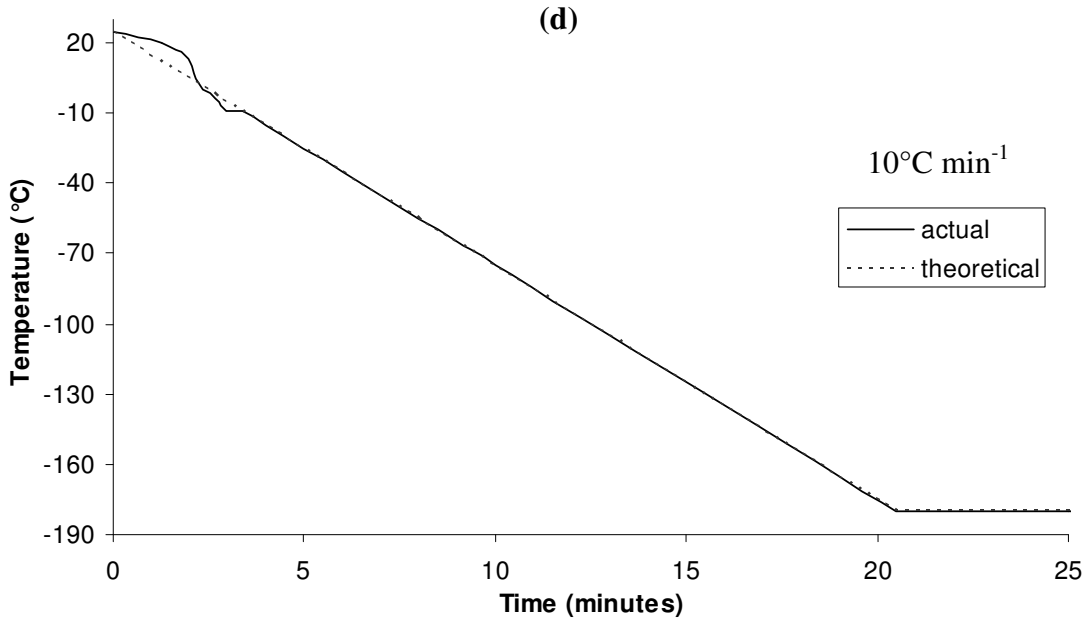


Figure 3.11c – d: Cooling time course (theoretical and actual course achieved) of axes of *Landolphia kirkii* excised with attached cotyledonary segments, flash-dried to 0.28±0.04 g g^{-1} (dmb) and cooled within a programmable freezer to -180°C (all graphs were reproduced from original print-outs generated from the Kryo 360-1.7 programmable freezer using computer software Delta T™). (c) Cooling rate of 5°C min^{-1}. (d) Cooling rate of 10°C min^{-1}

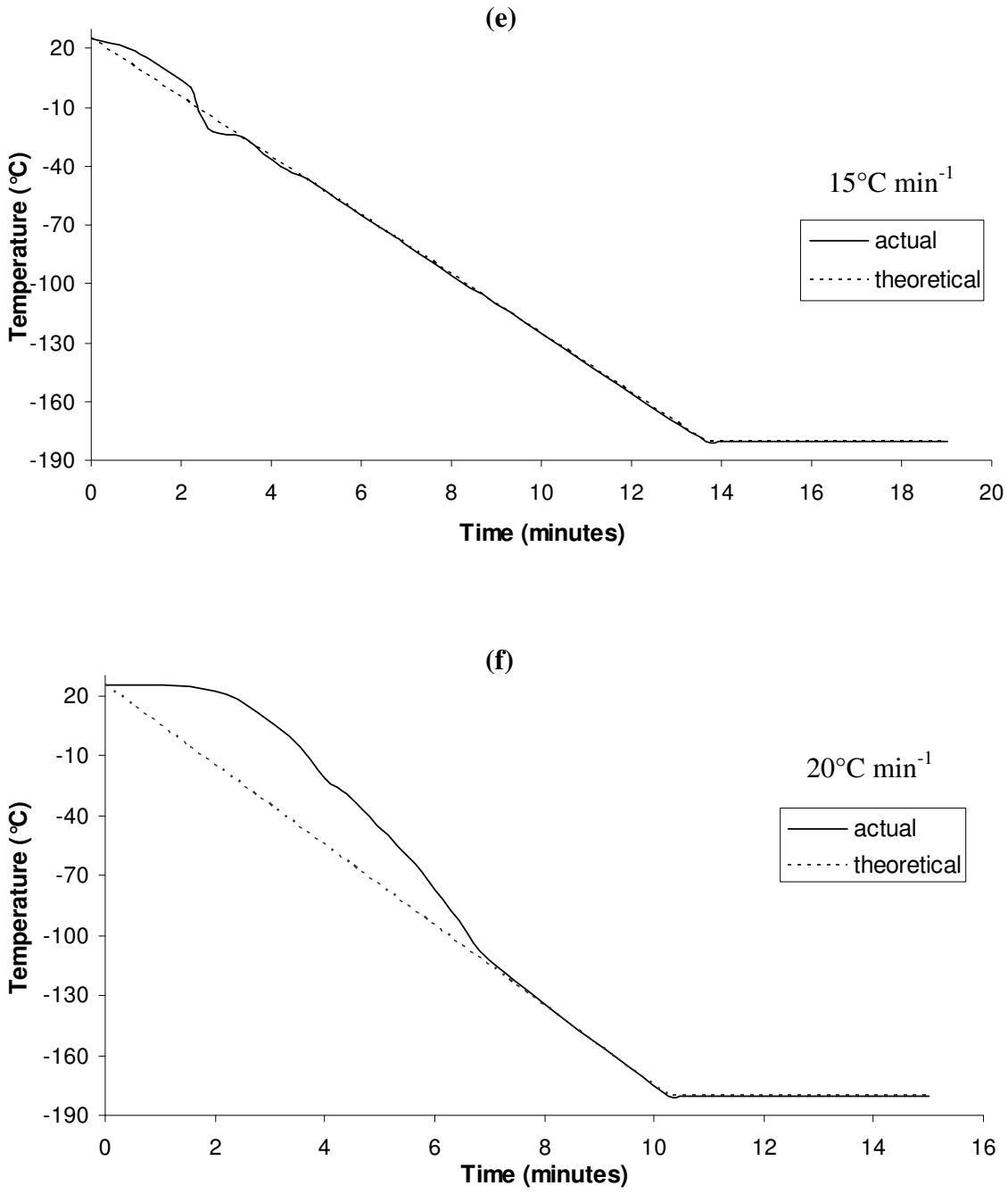


Figure 3.11e – f: Cooling time course (theoretical and actual course achieved) of axes of *Landolphia kirkii* excised with attached cotyledonary segments, flash-dried to 0.28±0.04 g g^{-1} (dmb) and cooled within a programmable freezer to -180°C (all graphs are reproduced from original print-outs generated from the Kryo 360-1.7 programmable freezer using computer software Delta T™). (e) Cooling rate of 15°C min^{-1}. (f) Cooling rate of 20°C min^{-1}

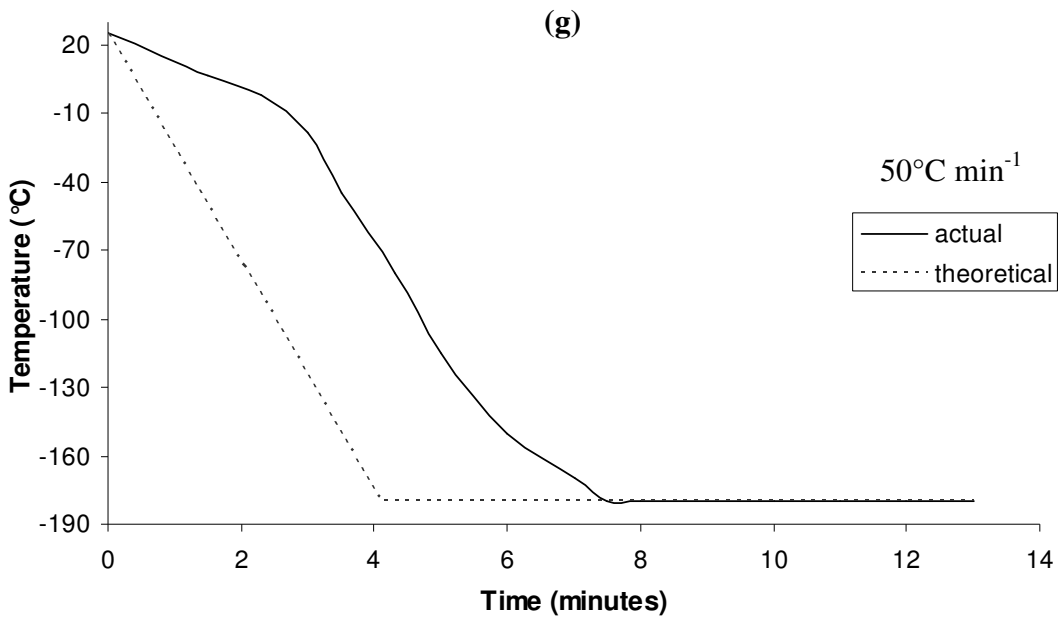


Figure 3.11g: Cooling time course (theoretical and actual course achieved) of axes of *Landolphia kirkii* excised with attached cotyledonary segments, flash-dried to 0.28±0.04 g g^{-1} (dmb) and cooled within a programmable freezer to -180°C (all graphs are reproduced from original print-outs generated from the Kryo 360-1.7 programmable freezer using computer software Delta T™). (g) Cooling rate of 50°C min^{-1}

The viability results obtained generally substantiated those of the previous cooling trials (compare Tables 3.6 and 3.7). A slow cooling rate (2°C min^{-1}) down to -70°C (Figure 3.11a) again produced the highest axes survival with 75% root and shoot development (Table 3.7). However, cooling at this rate down to cryogenic temperatures of -180°C (which was not previously achievable using Mr Frosty) (Figure 3.11b) resulted in no shoot development, with only 40% of axes producing roots (Table 3.7). Similarly, when a cooling rate of 5°C min^{-1} (Figure 3.11c) was employed, only 10% shoot production was recorded. Cooling rates of 10, 15 and 20°C min^{-1} down to -180°C (Figure 3.11d, e, f) were also tested as these rates approximated to that of embryonic axes of *L. kirkii* within cryovials plunged in LN (*c*. 14°C min^{-1}, as measured using a thermocouple). When

cooling rates of 10 (Figure 3.11d) and 15°C min^{-1} (Figure 3.11e) were utilised, survival was also very similar to that of axes within cryovials plunged in LN, to *viz.* ≥ 70% root protrusion for both, with 35 and 45% shoot development, respectively (Table 3.7). A higher cooling rate of 50°C min^{-1} (the highest achievable using this particular model of programmable freezer) (Figure 3.11g) was also tested. However, cooling rates higher than 20°C min^{-1} resulted in very low, or no survival when assessed by shoot development – i.e. the ability for seedling establishment (Table 3.7).

Table 3.7: The effect of different cooling rates, achieved by use of a programmable freezer, on root protrusion and shoot development by embryonic axes of *Landolphia kirkii* excised with attached cotyledon segments and subjected to rapid drying to a water content of 0.28±0.04 g g^{-1} (n=30)

Cooling rate (°C min^{-1}) to -180°C	**Root protrusion (%)**	**Shoot development (%)**
2 (to -70°C)	$75±8.7^{d}$	$75±8.7^{d}$
2	$40±0^{c}$	$0±0^{a}$
5	$25±13.2^{b}$	$10±8.7^{a}$
10	$75±5^{d}$	$35±8.7^{b}$
15	$70±8.7^{d}$	$45±5^{c}$
20	$25±8.7^{b}$	$5±5^{a}$
50	$0±0^{a}$	$0±0^{a}$

A Kolmogorov-Smirnov test was carried out which showed normal distribution of data. A one-way ANOVA was performed showing significant difference between treatments ($p<0.05$). Numbers followed by the same letter in the same columns are not significantly different according to the Duncan post-hoc test

3.9.3 Viability assessment after cryopreservation

Results obtained from cooling trials supported those of a previous study on *L. kirkii* (Vertucci *et al*., 1991), in that slow cooling down to -70°C was associated with the highest survival of embryonic axes (Tables 3.6 and 3.7). However, Vertucci *et al*. (1991) scored survival as expansion, greening and root development of axes of *L. kirkii*, and not, as in the current investigation, the ability for both root development and shoot production. It has been suggested that although a variety of techniques have been used to assess viability of seed material, the ultimate test is whether an independent functioning organism can be re-established after being subjected to various manipulations (Pammenter *et al*., 2002). These authors warned that although swelling and/or greening of embryonic axes suggests that it is not dead, it does not necessarily imply that the explant is capable of producing an independent, functional seedling: axes may often produce roots and so be scored as germinating, but the apical meristem tissue is so damaged that there is no subsequent shoot development and therefore the axis is unable to establish a viable seedling (Fu *et al*., 1993; Berjak *et al*., 1999; Berjak and Pammenter, 2004b). This has been shown to be the case predominately with tropical and sub-tropical species (particularly for dicotyledonous species with fleshy cotyledons), as axes survive to form roots and/or callus, but shoot production does not occur (Pammenter *et al*., 2011). Moreover, Berjak and Pammenter (2004b) cautioned that the shoot meristem in most embryonic axes is not as well protected as the root meristem and therefore appears to be more susceptible to damage. Those authors therefore suggested that the enlargement or greening of embryonic axes, subjected to the various manipulations required for cryopreservation, cannot be considered as a sign of germination and that protocols for assessing the response of axes needs to be focussed on both poles of the axis. These poles are required to retain a critical number of undamaged cells in order to produce a functional plantlet (Wesley-Smith *et al*., 2001a; Berjak and Pammenter, 2004b). Moreover, Pammenter and colleagues (2002; 2011) emphasised the problem of comparing current results with those of previous investigations, stating that older studies often do not provide data about seedling establishment and it is therefore not possible to ascertain what is meant by successful cryopreservation.

3.9.4 Excision damage

The survival (assessed by root and shoot production) of axes of *L. kirkii* at cryogenic temperatures (i.e. below -70°C) presently reported may be attributed to their excision with cotyledonary segments attached. This is emphasised in the current study, as axes excised with the cotyledons completely removed and then dehydrated and exposed to cryogenic temperatures, exhibited root protrusion but no shoot development, and therefore seedlings could not be established (data not shown). Earlier research, on axes of *L. kirkii* with their cotyledons completely removed, by Vertucci *et al*. (1991) reported axis survival at a temperature of -70°C. However, although the data were not published, axes did not survive exposure to -150°C (Wesley-Smith *et al*., 1992; Berjak, pers. comm.[3]).

As discussed in section 3.4 previous investigations on recalcitrant-seeded species (Goveia *et al*., 2004; Whitaker *et al*., 2010; Pammenter *et al*., 2011), showed that excising axes at the axis/cotyledon node or even with a 1 mm basal cotyledonous segment attached (Goveia *et al*., 2004), resulted in failure of shoot development which could only be avoided if a 2 mm segment of cotyledon was left attached (Goveia *et al*., 2004; reviewed by Berjak and Pammenter, 2004b; Pammenter *et al*., 2011). It has been suggested that the lack of shoot development is as a result of the generation of a wound-associated burst of ROS on excision of cotyledons (Minibayeva *et al*., 1998; Goveia *et al*., 2004; Roach *et al*., 2008; Whitaker *et al*., 2010; Pammenter *et al*., 2011). In addition to suggestions that the shoot meristem is more susceptible to damage than the root meristems due to a lack of adequate protection (Berjak and Pammenter, 2004b), the intensity and impact of the burst is also positively correlated to the proximity of the excision site to the apical meristem (Pammenter *et al*., 2011). This may be prevalent for embryonic axes of *L. kirkii* as cotyledons are attached close to the axis shoot meristems (see Figure 2.1). This ROS burst can damage the meristem, preventing shoot production and ROS may precondition the explants to successive damage as they pass through the various manipulations

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required for cryopreservation which, themselves may involve further ROS-mediated events (Whitaker *et al*., 2010; Pammenter *et al*., 2011) particularly during desiccation and rehydration of axes (Whitaker *et al*., 2010). Although ROS do play important roles in cell signalling in seeds (Bailly *et al*., 2008; Oracz *et al*., 2009), if their accumulation is uncontrolled there is consequential damage to a variety of macromolecules including those of membranes (Whitaker *et al*., 2010) and nucleic acids (Hendry, 1993; Bailly, 2004; Kranner and Birtic, 2005; Bailly *et al*., 2008). ROS-mediated damage has been associated with necrosis of the embryo shoot meristem following axis excision (Goveia *et al*., 2004; Pammenter *et al*., 2011).

However, in contrast to embryonic axes of *Trichilia dregeana* (Goveia *et al*., 2004; Berjak and Pammenter, 2004b), that showed considerable damage to the shoot apex when axes were excised from newly-shed seeds, complete severing of the cotyledons from the axis of *L. kirkii* did not, in itself, result in shoot apical meristem necrosis, as such excised axes retained the ability to produce shoots. Subsequent rapid dehydration also, did not preclude shoot development. However, when followed by exposure to cryogenic temperatures, no *L. kirkii* axes retained the ability for shoot production unless excised with a 3 mm segment of each cotyledon remaining attached. These results substantiate previous suggestions of the injurious effects of each step in a cryopreservation protocol being additive (e.g. Berjak *et al*., 1999; reviewed by Whitaker *et al*., 2010; Pammenter *et al*., 2011) or that there are interacting factors and differences in tolerance among tissue types.

3.9.5 Thawing

For the current investigation, thawing of embryonic axes of *L. kirkii* after exposure to cryogenic temperatures was undertaken by immersing naked axes directly into a CaMg at 40°C for 2 min. This rewarming regime resulted in normal growth and formation of seedlings from embryonic axes that survived the various manipulations of the

cryopreservation process. It has been suggested that rapid thawing limits the time spent in the temperature range that facilitates lethal ice formation (Wesley-Smith *et al*., 2004a; Benson, 2007) (discussed in section 1.7.1.6). Furthermore, the direct immersion of embryonic axes in a CaMg solution has also been suggested to limit the abnormal growth observed in many cryopreservation investigations (e.g. Kioko *et al*., 1998; Berjak *et al*., 1999; Mycock, 1999; Berjak and Mycock, 2004). The use of a CaMg solution as a thawing medium for desiccation-sensitive explants [e.g. *Quercus robur* (Berjak *et al*., 1999), *Trichilia emetica* (Kioko, 2003; Varghese *et al*., 2009), various amaryllid species (Naidoo, 2006) and *Phoenix reclinata* (Ngobese *et al*., 2010)] or its inclusion in the cryoprotection medium prior to freezing [e.g. *Pisum sativum* and *Phoenix dactylifera* (Mycock, 1999)] has been associated with improved post-thaw survival and the proportion of normal seedlings attained.

3.10 Electron microscopy

Ultrastructurally, radicle tip cells of fresh, mature untreated embryonic axes showed a marked degree of internal differentiation and organised compartmentation of the organelles (Figure 3.12a, b). Prominent nuclei with conspicuous nucleoli (meristem cell, Figure 3.12a), relatively large vacuoles occupying a considerable proportion of the cytomatrix in non-meristem cells (Figure 3.12b) and differentiated mitochondria were clearly discernible and characteristic of meristematic cells (Figure 3.12a, d) and their closely located derivatives (Figure 3.12b, c). The cytoplasm appears granular, dense and continuous (Figure 3.12a, b, d) and short profiles of endoplasmic reticulum with associated ribosomes, along with polysomes were also observed (Figure 3.12d). Plastids containing starch grains (Figure 3.12a, b, c), and lipid bodies (Figure 3.12a, b) were frequently observed.

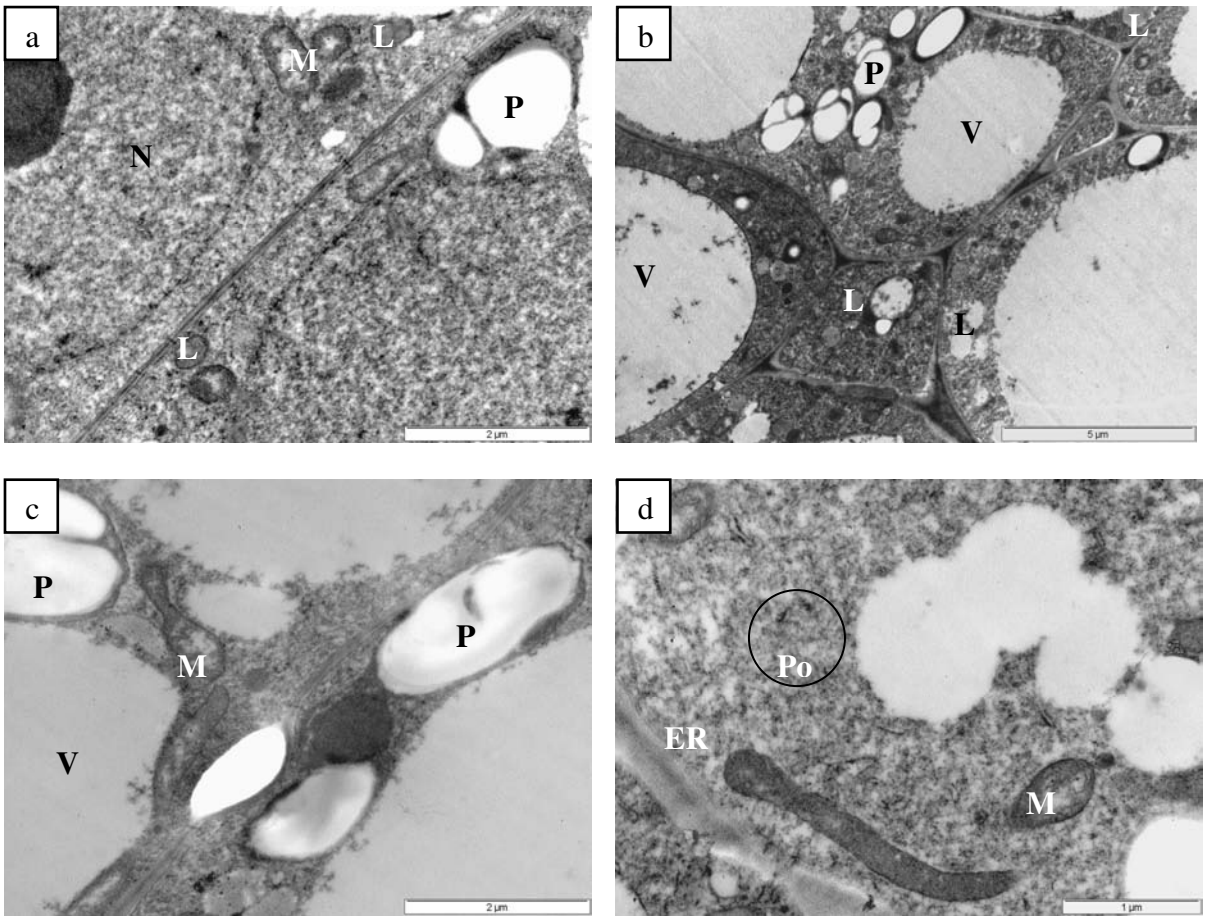

Figure 3.12a – d: Ultrastructural features of meristematic cells and their nearby derivatives are illustrated for fresh, mature embryonic axes of *Landolphia kirkii*. (a) Prominent nucleus and plastids within cells are evident. (b) The compartmentation, internal differentiation, occurrence of large vacuoles and aggregates of lipid bodies, were intracellular features of mature axes. (c) Large vacuoles occupying considerable proportion of cells, along with plastids showing a dense matrix and containing starch, are illustrated. (d) Well-developed mitochondria, rough endoplasmic reticulum and polysomes characterise the cells. ER, rough endoplasmic reticulum; L, lipid; M, mitochondrion; N, nucleus; P, plastid; Po, polysomes; V, vacuole. (a) X 20 000; (b) X 8000; (c) X 20 000; (d) X 30 000

The marked degree of internal differentiation, high proportion of the volume occupied by vacuolar compartments in cells external to the meristems, well-developed mitochondria in meristematic cells and their closely related derivatives, numerous profiles of rough endoplasmic reticulum and occurrences of polysomes (Figure 3.12) are consistent with the observations for embryonic axes of this (Berjak *et al*., 1992) and other recalcitrant-seeded species (e.g. Berjak *et al*., 1984; Farrant *et al*., 1989; Ajayi *et al*., 2006a; reviewed by Berjak and Pammenter, 2000). Although the proportion of volume occupied by the vacuolar compartments is relatively high in mature axes of *L. kirkii*, Berjak *et al*. (1992) did observe that mature axes of this species had undergone a reduction in these components during development. Furthermore, those authors suggested that the extensive *de novo* formation of vacuoles (and therefore its significant increase in extent) during early germination resulted in an increase in autophagic vacuolar activity, which may be a contributing factor to the increased desiccation sensitivity of embryonic axes of *L. kirkii* as germinative changes proceed. Similar observations were made for root meristematic and contiguous cells of axes from hydrated seeds of *Telfairia occidentalis* that had been stored at 25°C for two weeks (Ajayi *et al*., 2006a). Berjak *et al*. (1992) also found that plastid starch reserves were characteristic of mature axes of *L. kirkii*. High plastid starch reserves were also a prominent component within the radicle tip cells observed in this investigation (Figure 3.12b, c) as well as other studies on desiccation-sensitive species [e.g. *Araucaria angustifolia* (Farrant *et al*., 1989; Berjak and Pammenter, 2000)]. Mitochondrial development and deposition of material within plastids is attributed to increased cellular respiration and an advanced metabolic state (Berjak and Pammenter, 2000). Furthermore, Berjak and Pammenter (2000) stated that polysome formation, as evident in the current study (Figure 3.12d), is evidence of *de novo* protein synthesis. Therefore, the ultrastructure is a visible manifestation of the metabolic status of ongoing developmental or germinative metabolism in *L. kirkii* [and other recalcitrant embryos (e.g. Farrant *et al*., 1986; 1989; Berjak and Pammenter, 2000)].

Ultrastructural examination, while providing other information, was undertaken particularly to assess the responses of the lipid bodies to dehydration and subsequent cryogenic exposure, and to ascertain whether their responses could be involved in cell

death post-freezing. Specimens were rehydrated at 5, 25 or 45°C prior to processing for electron microscopy. Ultrastructural assessment of fully hydrated and dehydrated radicle tips from mature axes exposed to rapid cooling treatments, revealed massive intracellular destruction showing the destructive impacts of plunging naked axes into LN, or tumbling in nitrogen slush. Figure 3.13 shows loss of compartmentation and extensive degradation accompanying the death of all cells, when non-dried and axes flash-dried to 0.28±0.04 g g^{-1} were rapidly cooled to -196°C. Nuclei remained recognisable, but were considerably degraded (Figure 3.13a, b *cf*. Figure 3.12a) with possible evidence of ice crystal damage. Most organelles could no longer be differentiated (Figure 3.13a, b) and the remnants of cell contents including the fragmented plasmalemma were generally contracted well away from the walls (Figure 3.13c, d). However, the only subcellular components that appeared to have retained their morphology and remained intact, after fresh and axes flash-dried to 0.28±0.04 g g^{-1} were exposed to -196°C, were the lipid bodies, as illustrated in Figure 3.13a – d. However, the average diameter of the lipid bodies was approximately twice that of those seen in the fresh material (Figure 3.12.). It is therefore suggested that drying and/or cooling caused perturbation of the oleosin layer (which bounds lipid bodies), resulting in their spreading.

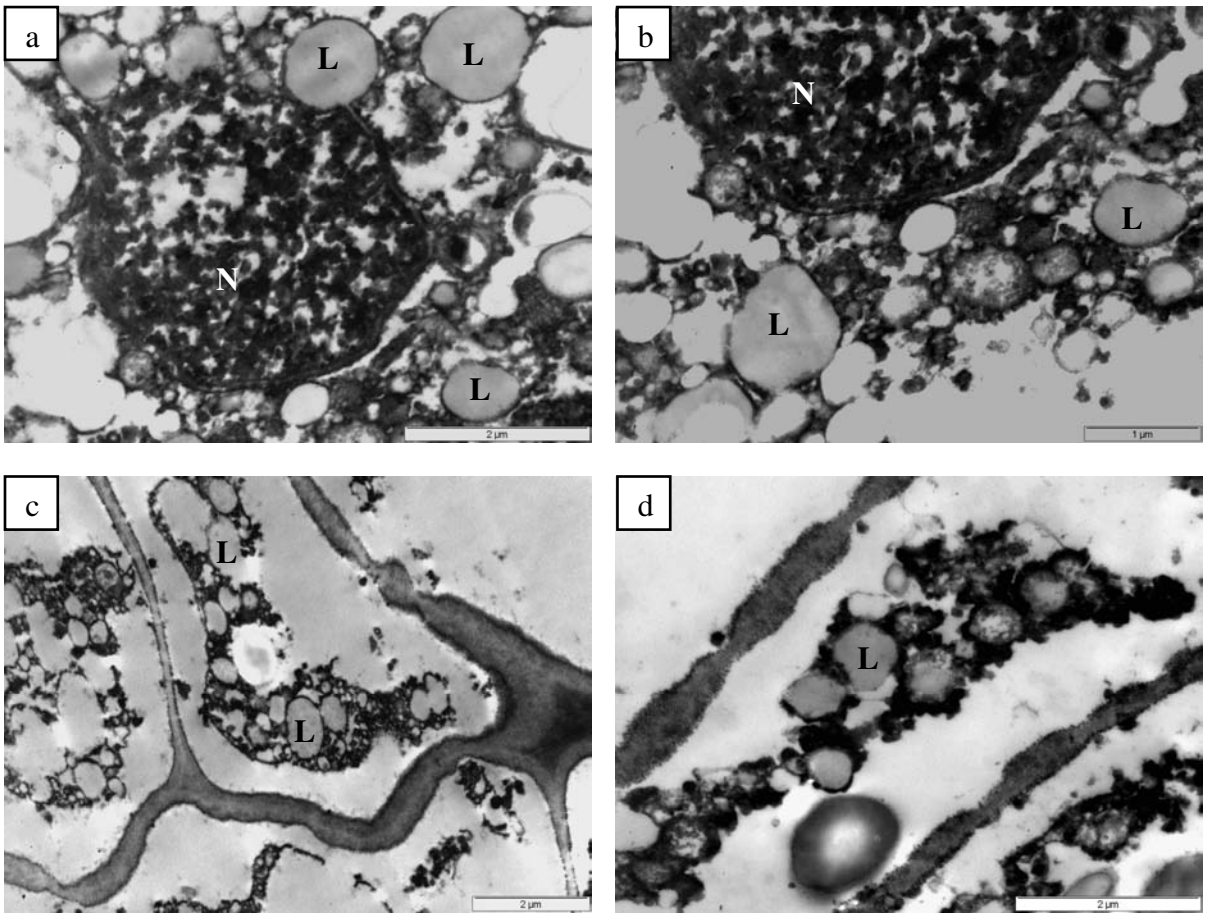

Figure 3.13a – d: Meristem cells from (a, c) fresh mature axes and (b, d) axes flash-dried to 0.28±0.044 g g^{-1}, all of which had been subjected to rapid cooling to -196°C in LN, thawed at 5°C and imbibed at 25°C, are illustrated. (a, b) Cooling resulted in the loss of intracellular integrity and compartmentation, (c) absence of differentiation among organelles. (a – d) Despite extensive ultrastructural degradation, lipid bodies retained morphological integrity. Note that as the subcellular situation was essentially similar for fresh axes and axes flash-dried to 0.28±0.04 g g^{-1}, thawed at 25 or 45°C and rapidly cooled in either LN or nitrogen slush, illustrative micrographs are not duplicated. L, lipid; N, nucleus. (a and d) X 20 000; (b) X 25 000; (c) X 12 000

When embryonic axes of *L. kirkii* were dehydrated to a 'detrimental' water content of 0.11±0.05 g g^{-1} and subsequently exposed to rapid cooling treatments, the effects of both desiccation and freezing damage is clearly evident (Figure 3.14); however it is difficult to identify and separate the damage caused by either process. As was the case with non-dried and axes flash-dried to 0.28±0.04 g g^{-1}, cytoplasm has been reduced to clumpy patches and most internal structures could not be positively deciphered (Figure 3.14a, b). It appeared that the vacuoles were no longer intact having lost their structure and shape with Figure 3.14b and c revealing the leakage of vacuolar contents into the cell, suggesting that the tonoplast had been damaged. This, in conjunction with loss of membrane integrity, may account for the increase in electrolyte leakage readings recorded for embryonic axes subjected to flash-drying and cooling trials, when compared to fresh axes that were only imbibed (Figure 3.12) (Wesley-Smith *et al*., 2001a). Plastids have been destroyed with only starch grains remaining (Figure 3.14a). There is visual evidence of potentially-lethal ice crystal formation within the nuclei (arrow), along with the swelling of the nuclear envelope (Figure 3.14d). Again, the only structures that appear to have remained intact after axes were dehydrated to a water content of 0.11±0.05 g g^{-1} and exposed to -196°C are the lipid bodies that are clearly visible in Figure 3.14b and c.

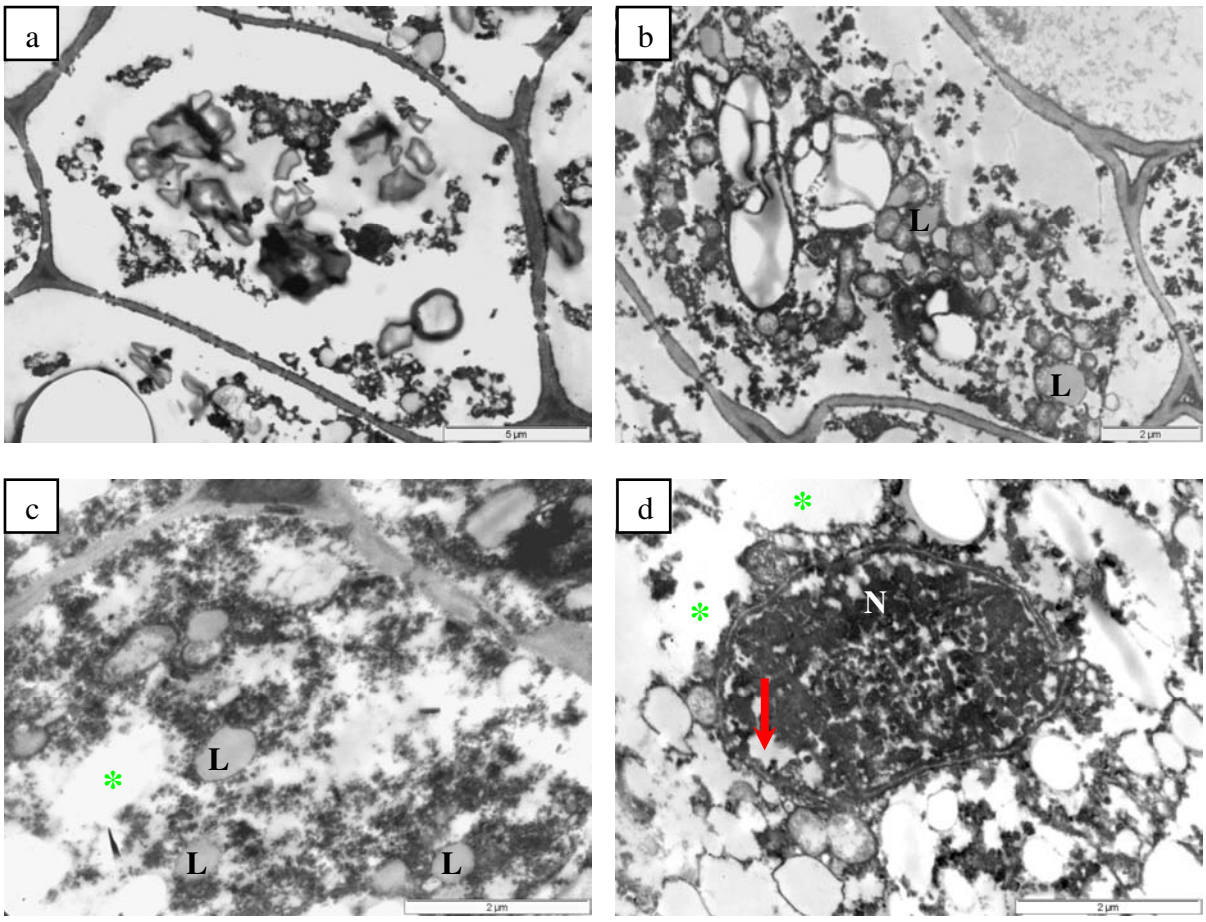

Figure 3.14a – d: Desiccation and freezing damage of meristematic cells and their nearby derivatives are illustrated for mature embryonic axes of *Landolphia kirkii* dehydrated to a water content of 0.11±0.05 g g^{-1}, and subjected to rapid cooling to in LN -196°C, prior to thawing at 5°C and imbibition at 25°C. (a) Deranged plastids, with only starch recognisable, (b, c) what are presumed to be vacuoles following tonoplast rupture (*) would have resulted in leakage of vacuolar contents into the cell and (d) ice crystal damage within the nucleus is apparent. (b, c, d) Lipid bodies however, remained prominent and unaffected by the sub-zero temperature treatments prior to thawing at 5°C. Note that as the subcellular situation was essentially similar for axes thawed at 25 or 45°C and rapidly cooled in either LN or nitrogen slush, illustrative micrographs are not duplicated. L, lipid; N, nucleus; S, starch. (a) X 6000; (b) X 10 000; (c) X 20 000; (d) X 20 000

Berjak and Pammenter (2000) suggested that the stress of mild desiccation would stimulate intracellular development, resulting in the strong development of mitochondria and polysomes, accumulation of material within the plastids and enhanced abundance of Golgi bodies. However, the complete loss of internal structure, made it impossible to decipher of most organelles for all embryonic axes of *L. kirkii*, including those flash-dried to 0.28±0.04 g g^{-1} (Figure 3.13d) and 0.11±0.05 g g^{-1} (Figure 3.14a – c). Ultrastructural evidence does suggest freezing damage within the nuclei of mature non-dried axes (Figure 3.13a), axes flash-dried to 0.28±0.04 g g^{-1} (Figure 3.13b) and 0.11±0.05 g g^{-1} (Figure 3.14d) and exposed to rapid cooling to -196°C. As discussed in section 1.6.2, it has been suggested that when seed tissue is dehydrated rapidly, the speed of the dehydration does not allow for water to equilibrate in the tissue and even though tissue has been dehydrated to a certain water content as a whole, the cells in the interior of the axes are likely to be at a higher water content than cells at the exterior (Pence, 1995; Pammenter *et al*., 1998; Wesley-Smith *et al*., 2001a). Previous studies (Berjak *et al*., 1986; Bruni and Leopold, 1992; Leubner-Metzger, 2005; reviewed by Berjak, 2006b) have also proposed localised pools within dehydrated cells to have higher water activity, with the suggestion that interior organelles such as mitochondria, plastids and *milieu* of chromatin may represent such loci (Berjak, 2006b). Therefore, bulk water contents of axes and seeds may mask such internal variations (Berjak, 2006b). This may be a potential explanation for the apparent swelling of the nuclear envelope and freezing damage within the nuclei of meristematic and contiguous cells of *L. kirkii* viewed in this study. It also serves to reiterate the thin line between desiccation damage and freezing damage when attempting cryopreservation of non-orthodox germplasm.

It was initially suspected that TAG crystallisation within lipid bodies might have resulted in their rapid disintegration upon rehydration, as has been demonstrated for embryos of the other species (Crane *et al*., 2003; 2006; Volk *et al*., 2006a; 2007) (discussed in sections 1.4.2.1 and 3.6). Vertucci (1989c) has also suggested that rapid cooling may induce lipid vitrification that may impart damage to seeds. However in all cases, whether axes had been subjected to dehydration or not, irrespective of the temperature of rewarming, lipid bodies within meristematic and other cells of *L. kirkii* remained intact,

with no evidence of disintegration having occurred (Figures 3.13a – d, and 3.14b – c). Mature axes that were exposed to cryogenic temperatures and rewarmed at 5°C before imbibition (which should not permit melting of crystallised lipid), retained their shape and ultrastructural integrity. Furthermore, lipid analyses of the non-polar fraction of *L. kirkii* axes (see section 3.6) revealed negligible occurrence of capric and lauric acids, the saturated fatty acids particularly implicated in crystallisation within the seeds of different *Cuphea* species. (Volk *et al*., 2006a).

Concluding remarks and future research

Previous research has recorded survival of mature embryonic axes of *Landolphia kirkii* when stored at -70°C, but no survival had been reported at any lower temperatures (Vertucci *et al*., 1991). Therefore, the present investigation was conducted with the intent of developing a successful cryopreservation protocol for recalcitrant embryonic axes of *L. kirkii* at temperatures lower than -140°C.

It was initially suspected that crystallisation of intracellular TAGs may occur during cooling of axes of *L. kirkii*. Previous studies have identified specific medium-chain fatty acids to play a major role in TAG crystallisation within seeds (Crane *et al*., 2003; 2006; Volk *et al*., 2006a; 2007). Therefore the lipid composition of embryos of this species was determined using gas chromatography and total lipid content was calculated by electron microscopic morphometry.

Evidence of desiccation and cooling induced injury in the intracellular environment was obtained through transmission electron microscopy. Rapid dehydration was achieved by flash-drying and water content most amenable to cryopreservation was established. Thereafter, the effects of various cooling techniques, cooling rates and the use of chemical cryoprotectants on the survival of mature axes, excised with cotyledons completely removed and with a portion of both cotyledons left attached, were evaluated. Viability was assessed by the TTZ test, electrolyte leakage readings and *in vitro* culture, with survival scored by the ability for both root development and shoot production.

The current study has shown that avoidance of excision injury and appropriate (slow) cooling rates have markedly beneficial effects on the survival of excised *L. kirkii* axes after cryogen exposure. Axes excised with no cotyledonary segments attached did not develop shoots when exposed to all the injurious steps in a cryopreservation protocol. However, shoot development (and therefore survival) was recorded when axes were excised with cotyledonary segments attached. Therefore the survival of axes after cryopreservation, as demonstrated in this study, may primarily be the result of a

decreased intensity of excision injury suggested to be the consequence of a ROS burst, as cotyledons were incised at least 3 mm away from the point of attachment to the axis. This may also have lessened the effects of further, presumably ROS-mediated events (e.g. Roach *et al*., 2008) that accompany subsequent procedures of cryopreservation. Future studies should therefore aim to assess the production of ROS [possibly through measuring extracellular superoxide ($O_2^{\cdot -}$) (e.g. Beckett *et al*., 2003; Goveia *et al*., 2004; Roach *et al*., 2008; Pammenter *et al*., 2011)] by embryonic axes of *L. kirkii*, with and without attached cotyledonary segments.

This study has also demonstrated the importance of cooling rate(s) that are appropriate to the embryonic axes concerned. When axes with attached cotyledonary segments were cooled, a slower rate was more effective than a more rapid one (assessed by the ability of recovered axes to form functional seedlings). Data showed that a cooling rate of 1 and 2°C min^{-1} resulted in the highest survival when explants were exposed to a temperature of -70°C. However, when axes were exposed to cryogenic temperatures (below - 180°C), cooling rates of 14 and 15°C min^{-1} showed the highest root and shoot production.

Controlling the formation of ice is critical for the survival of cryopreserved germplasm (Benson *et al*., 2005; Benson, 2008). The point of initiation of this ice formation is called ice-nucleation or 'seeding' and in most biological systems, generally occurs at or around -40°C (Benson, 2008). In future studies aimed at increasing plant explant survival of *L. kirkii* using a programmable freezer, seeding ice by incorporating holds of various durations for ice nucleation and the excursion of water from the cell at -40°C could be incorporated into control rate cooling protocols (Benson, 2005; 2008; Day *et* al., 2008; Reed and Uchendu, 2008). The addition of such a hold in the controlled rate cooling may optimise the survival of embryonic axes of *L. kirkii* (with attached cotyledonary segments).

Although the effects of chemical cryoprotectants on the survival of embryonic axes of *L. kirkii* was shown to be lethal when combined with rapid dehydration, their possible use in cryopreservation protocols of explants of this species should not be completely excluded.

Due to the lack of adequate seed material available at the time of this study, differing concentrations of cryoprotectants (and combinations thereof), exposure times and temperatures at exposure could not be assessed for the survival of these axes. Therefore, as suggested previously (see section 3.7), further research aimed at optimising cryopreservation survival of axes of *L. kirkii* should test the effects of cryoprotectant/cryoprotectant combinations at much lower concentrations than employed in the present investigation (possibly 1 and 2%). Furthermore, lowering the time of exposure as well as the temperature at exposure may result in improved axis survival post-dehydration and therefore increase the survival after cooling. Additionally, the use of vitrification solutions such as PVS2 (Sakai *et al*., 1990, as used by e.g. Cho *et al*., 2002; Gagliardi *et al*., 2002; Nadarajan, *et al*., 2007) in optimising the survival of embryonic axes of *L. kirkii* should not be dismissed. Cryoprotection and vitrification solutions and techniques need to be given priority for any prospective cryopreservation investigations on embryonic axes of *L. kirkii*.

Ultimately, this study has demonstrated the partial successful cryopreservation of embryonic axes of *Landolphia kirkii* with attached cotyledonary segments by rapid dehydration and slow and controlled rate cooling to temperatures below -180°C.

References

Abdelnour-Esquivel, A. & Engelmann, F. 2002. Cryopreservation of chayote (*Sechium edule* Jacq. Sw.) zygotic embryos and shoot-tips from *in vitro* plantlets. *CryoLetters* **23**: 299-308.

Acquaah, G. 2007. *Principles of Plant Genetics and Breeding*. Blackwell Publishing Ltd., Malden, U.S.A.

Ahmad, N., Faisal, M., Anis, M. & Aref, I.M. 2010. *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens* L. *South African Journal of Botany* **76**: 597-600.

Ahuja, M.R. 1991. Application of biotechnology to preservation of forest tree germplasm. *In*: M.R. Ahuja (ed.), *Woody Plant Biotechnology*, pp. 307-313. Plenum Press, New York, U.S.A.

Ajayi, S.A., Berjak, P., Kioko, J.I., Dulloo, M.E. & Vodouhe, R.S. 2006a. Responses of fluted pumpkin (*Telfairia occidentalis* Hook. F.) seeds to desiccation, chilling and hydrated storage. *South African Journal of Botany* **72**: 544-550.

Ajayi, S.A., Berjak, P., Kioko, J.I., Dulloo, M.E. & Vodouhe, R.S. 2006b. Observations on *in vitro* behaviour of the zygotic axes of fluted pumpkin. *African Journal of Biotechnology* **5**: 1397-1404.

Ajayi, S.A., Berjak, P., Kioko, J.I., Dulloo, M.E. & Vodouhe, R.S. 2007. Progress on the conservation of fluted pumpkin (*Telfairia occidentalis*) germplasm. *In*: R.S. Vodouhe, K. Atta-Krah, G.E. Achigan-Dako, O. Eyog-Matig & H. Avohou (eds), *Plant Genetic Resources and Food Security in West and Central Africa*, pp. 78-88. Regional Conference. Ibadan, Nigeria.

Amaral, R., Santos, M.F. & Santos, L.M.A. 2009. Overcoming recalcitrance in *Porphyridium aerugineum* Geitler employing encapsulation-dehydration cryopreservation methods. *CryoLetters* **30**: 462-472.

Arakawa, T. & Timasheff, S.N. 1982. Stabilisation of protein structure by sugars. *Biochemistry* **21**: 6536-6544.

Arakawa, T., Carpenter, J.F., Kita, Y.A. & Crowe, J.H. 1990. The basis for toxicity of certain cryoprotectants: a hypothesis. *Cryobiology* **27**: 401-415.

Ashburner, G.R., Thompson, W.K. & Burch, J.M. 1993. Effect of A-naphthaleneacetic acid and sucrose levels on the development of cultured embryos of coconut. *Journal of Plant Biotechnology* **35**: 157-163.

Ashwood-Smith, M.J. 1987. Mechanisms of cryoprotectant action. *In*: K. Bowler & B.J. Fuller (eds), *Temperature and Animal Cells*, pp. 395-406. Company of Biologists, Cambridge, U.K.

Assy-Bah, B. & Engelmann, F. 1992. Cryopreservation of mature embryos of coconut (*Cocos nucifera* L.) and subsequent regeneration of plantlets. *CryoLetters* **13**: 117-126.

Bailly, C. 2004. Active oxygen species and antioxidants in seed biology. *Seed Science Research* **14**: 93-107.

Bailly, C., El-Maarouf-Bouteau, H. & Corbineau, F. 2008. From intracellular signalling networks to cell death: the dual role of reactive oxygen species in seed physiology. *Comptes Rendus Biologies* **331**: 806-814.

Bajaj, Y.P.S. 1985. Cryopreservation of embryos. *In*: K.K. Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 227-242. CRC Press, Boca Raton, Florida, U.S.A.

Bald, W.B. 1987. *Quantitative cryofixation*. Adam Hilger, Bristol, U.K.

Beckett, R.P., Minibayeva, F.V., Vylegzhanina, N.V. & Tolpysheva, T. 2003. High rates of extracellular superoxide production by lichens in the suborder Peltigerineae correlates with indices of high metabolic activity. *Plant, Cell and Environment* **26**: 1827-1837.

Becwar, M.R., Stanwood, P.C. & Leonhardt, K.W. 1983. Dehydration effects on freezing characteristics and survival in liquid nitrogen of desiccation-tolerant and desiccation-sensitive seeds. *Journal of the American Society of Horticultural Science* **108**: 613-618.

Becwar, M.R., Stanwood, P.C. & Roos, E.E. 1982. Dehydration effects on imbibitional leakage from desiccation-sensitive seeds. *Plant Physiology* **69**: 1132-1135.

Benson, E.E. 1990. *Free Radical Damage in Stored Plant Germplasm*. International Board for Plant Genetic Resources, pp. 37-68. Rome, Italy.

Benson, E.E. 1993. Cryopreservation. *In*: R.A. Dixon & R.A. Gonzales (eds), *Plant Cell Culture: A Practical Approach*, pp. 147-167. IRL Press, Oxford, U.K.

Benson, E.E. 1995. Cryopreservation of shoot-tips and meristems. *In*: M. McClennan & J.G. Day (eds), *Methods in Molecular Biology, Volume 20, Cryopreservation and Freeze Drying Protocols*, pp. 121-132. Humana Press Inc., Totowa, New Jersey, U.S.A.

Benson, E.E. 1999. Cryopreservation *In*: E.E. Benson (ed.), *Plant Conservation Biotechnology*, pp. 83-95. Taylor and Francis, London, U.K.

Benson, E.E. 2004. Cryoconserving algal and plant diversity: historical perspectives and future challenges. *In*: B. Fuller, N. Lane & E.E. Benson (eds), *Life in the Frozen State*, pp. 299-328. CRC Press, Boca Raton, London, U.K.

Benson, E.E. 2007. Cryopreservation theory. *In*: B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide*, pp. 15-32. Springer, New York, U.S.A.
Benson, E.E. 2008. Cryopreservation of phytodiversity: a critical appraisal of theory and practice. *Critical Reviews in Plant Sciences* **27**: 141-219.

Benson, E.E. & Bremner, D. 2004. Oxidative stress in the frozen plant: a free radical point of view. *In*: B. Fuller, N. Lane & E.E. Benson (eds), *Life in the Frozen State*, pp. 205-241. CRC Press, Boca Raton, London, U.K.

Benson, E.E. & Lynch, P.T. 1999. Cryopreservation of rice tissue cultures. *In*: R.D. Hall (ed.), *Methods in Molecular Biology Volume 2 Plant Cell Culture Protocols*, pp. 83-94. Humana Press Inc., Totowa, New Jersey, U.S.A.

Benson, E.E., Harding, K. & Dumet, D. 2002. Cryopreservation of plant cells, tissues and organs. *In*: R.E. Spier (ed.), *Encyclopaedia for Plant Cell Technology*, pp. 627-635. Wiley Press, London, U.K.

Benson, E.E., Lynch, P.T. & Jones, J. 1992. Variation in free radical damage in rice cell suspensions with different embryogenic potentials. *Planta* **188**: 296-305.

Benson, E.E., Harding, K., Johnson, J. & Day, J.G. 2005. From ecosystems to cryobanks the role of cryo-conservation in the preservation and sustainable utilisation of global phyto-diversity. *In*: I.J. Bennett, E. Bunn, H. Clarke & J.A. McComb (eds), *'Contributing to a Sustainable Future'*, pp. 30-44. Proceedings of the Australian Branch of the IAPTC & B, September 21-24, 2005, Perth, Western Australia.

Benson, E.E., Johnston, J., Muthusamy, J. & Harding, K. 2006. Physical and engineering perspectives of *in vitro* plant cryopreservation. *In*: S. Dutta Gupta & Y. Ibaraki (eds), *Plant Tissue Culture Engineering, Volume 6*, pp. 441-476. Springer-Verlag, Dordrecht, Netherlands.

Berjak, P. 1996. The role of micro-organisms in deterioration during storage of recalcitrant and intermediate seeds *In*: A.-S. Ouédraogo, K.M. Poulsen & I. Stubsgaard (eds), *Intermediate/Recalcitrant Tropical Forest Seeds*, pp. 121-126. International Plant Genetic Resources Institute, Rome, Italy.

Berjak, P. 2000. Current status of cryopreservation research and future perspectives of its applications in South Africa. *In*: F. Engelmann & H. Takagi (eds). *Cryopreservation of Tropical Germplasm: Current Research Progress and Application*, pp. 315-319. International Plant Genetic Resources Institute, Rome, Italy.

Berjak, P. 2006a. The challenge of recalcitrant germplasm cryopreservation. *Journal of Horticultural Science and Biotechnology* **81**: 781-782.

Berjak, P. 2006b. Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms. *Seed Science Research* **16**: 1-15.

Berjak, P. & Dumet, D. 1996. Cryopreservation of seeds and isolated embryonic axes of neem (*Azadirachta indica*). *CryoLetters* **17**: 99-104.

Berjak, P. & Mycock, D.J. 2004. Calcium, with magnesium, is essential for normal seedling development from partially dehydrated recalcitrant axes: a study on *Trichilia dregeana* Sond. *Seed Science Research* **14**: 217-231.

Berjak, P. & Pammenter, N.W. 1994. Recalcitrance is not an all-or-nothing situation. *Seed Science Research* **4**: 263-264.

Berjak, P. & Pammenter, N.W. 1997. Progress in the understanding and manipulation of desiccation-sensitive (recalcitrant) seeds. *In*: R.H. Ellis, M. Black, A.J. Murdoch & T.D. Hong (eds), *Basic and Applied Aspects of Seed Biology: Proceedings of the Fifth International Workshop on Seeds*, pp. 689-703. Kluwer Academic Publishers, Reading, U.K.

Berjak, P. & Pammenter, N.W. 2000. What ultrastructure has told us about recalcitrant seeds. *Revista Brasileira de Fisiologia Vegetal* **12**: 22-50.

Berjak, P. & Pammenter, N.W. 2001. Seed recalcitrance - current perspectives. *South African Journal of Botany* **67**: 79-89.

Berjak, P. & Pammenter, N.W. 2003a. Understanding and handling desiccation-sensitive seeds. *In*: R.D. Smith, J.B. Dickie, S.H. Linington, H.W. Pritchard & R.J. Probert (eds), *Seed Conservation, Turning Science into Practice*, pp. 415-430. Cromwell Press Ltd., London, U.K.

Berjak, P. & Pammenter, N.W. 2003b. Orthodox and recalcitrant seeds. *In*: J.A. Vozzo (ed.), *Tropical Tree Seed Manual*, pp. 137-147. United States Department of Agriculture, Forest Service, Washington D.C., U.S.A.

Berjak, P. & Pammenter, N.W. 2004a. Recalcitrant seeds. *In*: R. Benech-Arnold & R. Sánchez (eds), *Seeds Physiology: Applications of Agriculture*, pp. 305-345. Haworth Press Inc., New York, U.S.A.

Berjak, P. & Pammenter, N.W. 2004b. Biotechnological aspects of non-orthodox seeds: an African perspective. *South African Journal of Botany* **70**: 102-108.

Berjak, P. & Pammenter, N.W. 2004c. Recalcitrance - current perspectives. *South African Journal of Botany* **67**: 79-89.

Berjak, P. & Pammenter, N.W. 2008. From *Avicennia* to *Zizania*: seed recalcitrance in perspective. *Annals of Botany* **101**: 213-228.

Berjak, P., Dini, M. & Pammenter, N.W. 1984. Possible mechanisms underlying the differing dehydration responses in recalcitrant and orthodox seeds: desiccation-associated subcellular changes in propagules of *Avicennia marina*. *Seed Science and Technology* **12**: 365-384.

Berjak, P., Farrant, J.M. & Pammenter, N.W. 1989. The basis of recalcitrant seed behaviour. Cell biology of the homoiohydrous seed condition. *In*: R. Taylorson (ed.), *Recent Advances in Development and Germination of Seeds*, pp. 89-108. Plenum Press, New York, U.S.A.

Berjak, P., Pammenter, N.W. & Vertucci, C.W. 1992. Homoiohydrous (recalcitrant) seeds: developmental status, desiccation sensitivity and the state of water in axes of *Landolphia kirkii* Dyer. *Planta* **186**: 911-917.

Berjak, P., Vertucci, C.W. & Pammenter, N.W. 1993. Effects of development status on dehydration rate on characteristics of water and desiccation sensitivity in recalcitrant seeds of *Camellia sinensis*. *Seed Science Research* **3**: 155-166.

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

Berjak, P., Kioko, J.I., Makhathini, A. & Watt, M.P. 2004. Strategies for field collection of recalcitrant seed and zygotic embryonic axes of the tropical tree *Trichilia dregeana* Sond. *Seed Science and Technology* **32**: 825-836.

Berjak, P., Walker, J.M., Watt, M.P. & Mycock, D.J. 1999. Experimental parameters underlying failure or success in plant germplasm cryopreservation: a case study on zygotic axes of *Quercus robur* L. *CryoLetters* **20**: 251-262.

Berjak, P., Campbell, G.K., Farrant, J.M., Omondi-Oloo, W. & Pammenter, N.W. 1995. Responses of seeds of *Azadirachta indica* (neem) to short-term storage under ambient or chilled conditions. *Seed Science and Technology* **23**: 779-792.

Berjak, P., Mycock, D.J., Wesley-Smith, J., Dumet, D. & Watt, M.P. 1996. Strategies of *in vitro* conservation of hydrated germplasm. *In*: M.N. Normah, M.K. Narimah & M.M. Clyde (eds), *In Vitro Conservation of Plant Genetic Resources*, pp. 18-28. Percetakan Watan, Kuala Lumpur, Malaysia.

Berjak, P., Mycock, D.J., Walker, M., Kioko, J.I., Pammenter, N.W. & Wesley-Smith, J. 2000b. Conservation of genetic resources naturally occurring as recalcitrant seeds. *In*: M. Black, K.J. Bradford & J. Vásquez-Ramos (eds), *Seed Biology: Advances and Applications*, pp. 223-228. CABI Publishing, Wallingford, Oxon, U.K.

Berjak, P., Walker, M., Mycock, D.J., Wesley-Smith, J., Watt, M.P. & Pammenter, N.W. 2000a. Cryopreservation of recalcitrant zygotic embryos. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 140-155. Japan International Research Centre for Agricultural Sciences, Tsukubu, Japan/International Plant Genetic Resources Institute, Rome, Italy.

Bewley, J.D. 1979. Physiological aspects of desiccation tolerance. *Annual Review of Plant Physiology* **30**: 195-238.

Bewley, J.D. & Black, M. 1994. *Seeds: physiology of development and germination, 2nd edition*, pp. 140-143. Plenum Press, New York, U.S.A.

Bhandal, I.S., Hauptmann, R.M. & Widholm, J.M. 1985. Trehalose as cryoprotectant for the freeze preservation of carrot and tobacco cells. *Plant Physiology* **78**: 430-432.

Bhatti, M.H., Percival, T., Davey, C.D.M., Henshaw, G.G. & Blakesley, D. 1997. Cryopreservation of embryogenic tissue of a range of genotypes of sweet potato (*Ipomoea batatas* (L) Lam.) using an encapsulation protocol. *Plant Cell Reports* **16**: 802-806.

Bilia, D.A., Marcos-Filho, J. & Novembre, A.D.C.L. 1999. Desiccation tolerance and seed storability of *Inga uruguensis* (Hook. et Arn.). *Seed Science and Technology* **27**: 77-89.

Bittencourt, S.R.M., Vieira, R.D. & Rodrigues, T.J.D. 1997. Criteria for peanut seed pre-conditioning for tetrazolium test. *Seed Science and Technology* **25**: 337-342.

Blakesley, D., Al-Mazrooei, S. & Henshaw, G.G. 1995. Cryopreservation of embryogenic tissue of sweet potato (*Ipomoea batatas*): use of sucrose and dehydration for cryoprotection. *Plant Cell Reports* **5**: 259-263.

Bligh, E.G. & Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**: 911-917.

Bonner, F.T. 1996. Responses to drying of recalcitrant seeds of *Quercus nigra* L. *Annals of Botany* **78**: 181-187.

Botkin, D.B., Saxe, H., Araújo, M.B., Betts, R., Bradshaw, R.H.W., Cedhagen, T., Chesson, P., Dawson, T.P., Etterson, J.R., Faith, D.P., Ferrier, S., Guisan, A., Hansen, A.S., Hilbert, D.W., Loehle, C., Margules, C., New, M., Sobel, M.J. & Stockwell, D.R.B. 2007. Forecasting the effects of global warming on biodiversity. *BioScience* **57**: 227-236.

Bowers, S.A. 1990. Long-term storage of *Narcissus* anthers and pollen in liquid nitrogen. *Euphytica* **48**: 275-278.

Bramalage, W.J., Leopold, A.C. & Parrish, D.J. 1978. Chilling stress to soybeans during imbibition. *Plant Physiology* **61**: 525-529.

Bruni, F. & Leopold, A.C. 1992. Pools of water in anhydrobiotic organisms. A thermally stimulated depolarization current study. *Biophysical Journal* **63**: 663-672.

Brush, S.B. 2000. The issue of *in situ* conservation of crop genetic resources. *In*: S.B. Brush (ed.), *Genes in the Field: On-Farm Conservation of Crop Diversity*, pp. 3-28. International Plant Genetic Resources Institute, Rome, Italy.

Bryant, G., Koster, K.L. & Wolfe, J. 2001. Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Science Research* **11**: 17-25.

Buitink, J. & Leprince, O. 2008. Intracellular glasses and seed survival in the dry state. *Comptes Rendus Biologies* **331**: 788-795.

Buitink, J., Leprince, O., Hemminga, M.A. & Hoekstra, F.A. 2000. Molecular mobility in the cytoplasm: an approach to describe and predict lifespan of dry germplasm. *Proceedings of the National Academy of Science* **97**: 2385-2390.

Buitink, J., Walters, C., Hoekstra, F.A. & Crane, J. 1998. Storage behaviour of *Typha latifolia* pollen at low water contents: interpretation on the basis of water activity and glass concepts. *Physiologia Plantarum* **103**: 145-153.

Burch, J. & Wilkinson, T. 2002. Cryopreservation of protonemata of *Ditrichum cornubicum* (Paton) comparing the effectiveness of four cryoprotectant pretreatments. *CryoLetters* **23**: 197-208.

Burke, M.J. 1986. The glassy state and survival of anhydrous biological systems. *In*: A.C. Leopold (ed.), *Membranes, Metabolism and Dry Organisms*, pp. 358-364. Cornell University Press, New York, U.S.A.

Calistru, C., Mclean, M., Pammenter, N.W. & Berjak, P. 2000. The effects of mycofloral infection on the viability and ultrastructure of wet-stored recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh. *Seed Science Research* **10**: 341-353.

Canavate, J.P. & Lubain, L.M. 1994. Tolerance of six marine microalgae to the cryoprotectants dimethyl sulphoxide and methanol. *Journal of Phycology* **30**: 559-565.

Chaitanya, K.S.K. & Naithani, S.C. 1994. Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertn.f. *New Phytologist* **126**: 623-627.

Chandel, K.P.S., Chaudhury, R., Radhamani, J. & Malik, S.K. 1995. Desiccation and freezing sensitivity in recalcitrant seeds of tea, cocoa and jackfruit. *Annals of Botany* **76**: 443-450.

Chapin III, F.S., Zavaleta, E.S., Eviner, V.T., Naylor, R.L., Vitousek, P.M., Reynolds, H.L., Hooper, D.U., Lavorel, S., SalaI, O.E., Hobbie, S.E., Mack, M.C. & Díaz, S. 2000. Consequences of changing biodiversity. *Nature* **405**: 234-242.

Chappell Jr, J.H. & Cohn, M.A. 2011. Corrections for interferences and extraction conditions make a difference: use of the TBARS assay for lipid peroxidation of orthodox *Spartina pectinata* and recalcitrant *Spartina alterniflora* seeds during desiccation. *Seed Science Research* **21**: 153-158.

Chaudhury, R. & Chandel, K.P.S. 1995. Cryopreservation of embryonic axes of almond (*Prunus amygdalus* Batsch.) seeds. *CryoLetters* **16**: 51-56.

Chaudhury, R. & Chandel, K.P.S. 1996. Cryopreservation of embryos/embryonic axes - a novel method for the long-term conservation of recalcitrant seed species. *In*: M.N. Normah, M.K. Narimah & M.M. Clyde (eds), *In Vitro Conservation of Plant Genetic Resources*, pp. 53-71. Percetakan Watan, Kuala Lumpur, Malaysia.

Chaudhury, R., Radhamani, J. & Chandel, K.P.S. 1991. Preliminary observations on the cryopreservation of desiccated embryonic axes of tea (*Camellia sinensis* (L.) O. Kuntze) seeds for genetic conservation. *CryoLetters* **12**: 31-36.

Chen, T.H.H., Kartha, K.K. & Gusta, L.V. 1985. Cryopreservation of wheat suspension culture and regenerable callus. *Plant Cell, Tissue and Organ Culture* **4**: 101-109.

Chen, T.H.H., Kartha, K.K., Constabel, F.C. & Gusta, L.V. 1984. Freezing characteristics of cultured *Catharanthus roseus* (L.) cells treated with dimethyl sulphoxide and sorbitol in relation to cryopreservation. *Plant Physiology* **75**: 720-725.

Chin, H.F. 1995. Storage of recalcitrant seeds. *In*: A.S. Basra (ed.), *Seed Quality: Basic Mechanisms and Agricultural Implications*, pp. 209-217. Food Products Press, New York, U.S.A.

Chin, H.F. & Krishnapillay, B. 1989. Cryogenic storage of some horticultural species. *Acta Horticulturae* **253**: 107-112.

Chin, H.F., Krishnapillay, B. & Alang, Z.C. 1988. Media for embryo culture of some tropical recalcitrant species. *Pertanika* **11**: 357-363.

Chin, H.F., Krishnapillay, B. & Stanwood, P.C. 1989. Seed moisture: recalcitrant vs orthodox seeds. *In*: P.C. Stanwood & M.B. McDonald (eds), *Seed Moisture,* pp. 15-22. Crop Science Society of America, Special Publication 14, Wisconsin, U.S.A.

Ching, T.M. & Schoolcraft, I. 1968. Physiological and chemical differences in aged seeds. *Crop Science* **8**: 407-409.

Cho, J.-S., Hong, S.-M., Joo, S.-Y., Yoo, J.-S. & Kim, D.-I. 2007. Cryopreservation of transgenic rice suspension cells producing recombinant hCTLA4Ig. *Applied Microbiology and Biotechnology* **73**: 1470-1476.

Cho, E.G., Hor, Y.L., Kim, H.H., Rao, V.R. & Engelmann, F. 2001. Cryopreservation of *Citrus madurensis* zygotic embryonic axes by vitrification: importance of pre-growth and preculture conditions. *CryoLetters* **22**: 391-396.

Cho, E.G., Hor, Y.L., Kim, H.H., Rao, V.R. & Engelmann, F. 2002. Cryopreservation of *Citrus madurensis* embryonic axes by vitrification: Importance of loading and treatment with vitrification solution. *CryoLetters* **23**: 317-324.

Christianson, M.L. 1998. A simple protocol for the cryopreservation of mosses. *The Bryologist* **101**: 32-35.

Corredoira, E., San-José, M.C., Ballester, A. & Vieitez, A.M. 2004. Cryopreservation of zygotic embryonic axes and somatic embryos of European chestnut. *CryoLetters* **25**: 33-42.

Costello, M.J. & Gulik-Krzywicki, T. 1976. Correlated x-ray diffraction and freeze-fracture studies on membrane model systems. Perturbations induced by freeze-fracture preparative procedures. *Biochimica et Biophysica Acta* **455**: 412.

Crane, J., Kovach, D., Gardner, C. & Walters, C. 2006. Triacylglycerols phase and 'intermediate' seed storage physiology: a study of *Cuphea carthagenensis*. *Planta* **223**: 1081-1089.

Crane, J., Miller, A.L., van Roekel, J.W. & Walters, C. 2003. Triacylglycerols determine the unusual storage physiology of *Cuphea* seeds. *Planta* **217**: 699-708.

Crowe, J.H. & Crowe, L.M. 1986. Stabilisation of membranes in anhydrobiotic organisms. *In*: A.C. Leopold (ed.), *Membranes, Metabolism and Dry Organisms*, pp. 188-209. Comstoc Publishing Associates, Ithaca, Greece.

Crutchfield, A.L.M., Diller, K.R. & Brand, J.J. 1999. Cryopreservation of *Chlamydomonas reinhardtii* (Chlorophyta). *European Journal of Phycology* **34**: 43-52.

Cyr, R. 2000. Cryopreservation: Roles in clonal propagation and germplasm conservation of conifers. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 261-268. Japan International Research Center for Agricultural Sciences, Tsukuba, Japan.

Dawar, S., Syed, F. & Ghaffar, A. 2007. Seed borne fungi associated with chickpea in Pakistan. *Pakistan Journal of Botany* **39**: 637-643.

Daws, M.I., Garwood, N.C. & Pritchard, H.W. 2005. Traits of recalcitrant seeds in a semi-deciduous tropical forest in Panamá: some ecological implications. *Functional Ecology* **19**: 8740-885.

Daws, M.I., Lydall, E., Chmielarz, P., Leprince, O., Matthews, S., Thanos, C.A. & Pritchard, H.W. 2004. Developmental heat sum influences recalcitrant seed traits in *Aesculus hippocastanum* across Europe. *New Phytologist* 162: **157**-166.

Daws, M.I., Cleland, H., Chmielarz, P., Gorian, F., Leprince, O., Mullins, C.E., Thanos, C.A., Vandvik, V. & Pritchard, H.W. 2006. Variable desiccation tolerance in *Acer pseudoplatanus* seeds in relation to developmental conditions: a case of phenotypic recalcitrance? *Functional Plant Biology* **33**: 59-66.

Day, J.G. & Harding, K. 2008. Cryopreservation of algae. *In*: B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide*, pp. 95-116. Springer-Verlag, New York, U.S.A.

Day, J.G., Fleck, R.A. & Benson, E.E. 2000. Cryopreservation-recalcitrance in microalgae: novel approaches to identify and avoid cryo-injury. *Journal of Applied Phycology* **12**: 369-377.

Day, J.G., Harding, K., Nadarajan, J. & Benson, E.E. 2008. Cryopreservation: conservation of bioresources at ultra low temperatures. *In*: J.M. Walker & R. Rapley (eds), *Molecular Biomethods Handbook*, pp. 915-945. Humana Press Inc., Clifton, New Jersey, U.S.A.

de Boucaud, M.T., Brison, M., Ledoux, C., Germain, E. & Lutz, A. 1991. Cryopreservation of embryonic axes of recalcitrant seed: *Juglans regia* L. cv. Franquette. *CryoLetters* **12**: 163-166.

de Camargo, I.P., de Carvalho, M.L.M. & Vieira, R.D. 1997. Evaluation of the deterioration in Brazil nut seeds by the tetrazolium test. *Pesquisa Agropecuária Brasileira* **32**: 835-839.

Demeulemeester, M.A.C., Vandenbussche, B. & de Proft, M.P. 1993. Regeneration of chicory plants from cryopreserved *in vitro* shoot tips. *CryoLetters* **14**: 57-64.

Diamond, J.M. 1989. The present, past and future of human-caused extinctions. *Philosophical Transactions of the Royal Society of London* **B325**: 469-477.

Dickie, J.B. & Pritchard, H.W. 2002. Systematic and evolutionary aspects of desiccation tolerance in seeds. *In*: M. Black & H.W. Pritchard (eds), *Desiccation and Survival in Plants: Drying Without Dying*, pp. 239-259. CABI Publishing, Wallingford, Oxon, U.K.

Drew, P.J., Pammenter, N.W. & Berjak, P. 2000. 'Sub-imbibed' storage is not an option for extending longevity of recalcitrant seeds of the tropical species, *Trichilia dregeana* Sond. *Seed Science Research* **10**: 355-363.

Dubochet, J., Lepault, J., Freeman, R., Beeriman, J.A. & Homo, J.-C. 1982. Electron microscopy of frozen water and aqueous solutions. *Journal of Microscopy* **128**: 219-237.

Dumet, D. & Benson, E.E. 2000. The use of physical and biochemical studies to elucidate and reduce cryopreservation-induced damage in hydrated/desiccated plant germplasm. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 43-56. International Plant Genetic Resources Institute, Rome, Italy.

Dumet, D. & Berjak, P. 1996. Cryopreservation of embryonic axes of recalcitrant species. *In*: R.H. Ellis, M. Black & T.D. Hong (eds), *Basic and Applied Aspects of Seed Biology*, pp. 771-776. Kluwer Academic Publishers, Dordrecht, Netherlands.

Dumet, D., Engelmann, F., Chabrillange, N., Dussert, S. & Duval, Y. 1994. Effect of various sugars and polyols on the tolerance to desiccation and freezing of oil palm polyembryonic cultures. *Seed Science Research* **4**: 243-250.

Dussert, S., Chabrillange, N., Engelmann, F., Anthony, F. & Hamon, S. 1998. Cryopreservation of seeds of four coffee species (*Coffea arabica*, *C. costatifructa*, *C. racemosa* and *C. sessiliflora*): importance of water content and cooling rate. *Seed Science Research* **8**: 9-15.

Dussert, S., Chabrillange, N., Vasquez, N., Engelmann, F., Anthony, F., Guyot, A. & Hamon, S. 2000. Beneficial effect of post-thawing osmoconditioning on the recovery of cryopreserved coffee (*Coffea arabica* L.) seeds. *CryoLetters* **21**:47-52.

Echlin, P. 1992. *Low-Temperature Microscopy and Analysis*. Plenum Press, New York, U.S.A.

Eggers, S., Erdey, D., Pammenter, N.W. & Berjak, P. 2007. Storage and germination response of recalcitrant seeds subjected to mild dehydration. *In*: S. Adkins (ed.), *Seed Science Research: Advances and Applications*, pp. 85-92. CABI Publishing, Wallingford, Oxon, U.K.

Ellis, R.H. & Roberts, E.H. 1980. Improved equations for the prediction of seed longevity. *Annals of Botany* **45**: 13-30.

Ellis, R.H., Hong, T.D. & Roberts, E.H. 1989. A comparison of the low-moisture-content limit to the logarithmic relation between seed moisture content and longevity in twelve species. *Annals of Botany* **63**: 601-611.

Ellis, R.H., Hong, T.D. & Roberts, E.H. 1990a. An intermediate category of seed storage behaviour? I. coffee. *Journal of Experimental Botany* **41**: 1167-1174.

Ellis, R.H., Hong, T.D. & Roberts, E.H. 1990b. Low moisture content limitations to relations between seed longevity and moisture. *Annals of Botany*: **493**-504.

Ellis, R.H., Hong, T.D. & Roberts, E.H. 1990c. Moisture content and the longevity of seeds of *Phaseolus vulgaris*. *Annals of Botany* **66**: 341-348.

Ellis, R.H., Hong, T.D. & Roberts, E.H. 1990d. An intermediate category of seed storage behaviour? II. Effects of provenance, immaturity, and imbibition on desiccation-tolerance in coffee. *Journal of Experimental Botany* **42**: 653-657.

Ellis, R.H., Hong, T.D. & Roberts, E.H. 1992. The low-moisture-content limit to the negative logarithmic relation between seed longevity and moisture content in three subspecies of rice. *Annals of Botany* **69**: 53-58.

Ellis, R.H., Osh-Bonsu, K. & Roberts, E.H. 1982. The influence of genotype, temperature and moisture on seed longevity in chickpea, cowpea and soybean. *Annals of Botany* **50**: 69-82.

Engelmann, F. 1993. Cryopreservation of embryos. *In*: Y. Dattée, C. Dumas & A. Gallais (eds), *Reproductive Biology and Plant Breeding*, pp. 281-290. Springer-Verlag, Berlin, Germany.

Engelmann, F. 1997. *In vitro* conservation methods. *In*: J.A. Callow, L. Ford & H.J. Newbury (eds), *Biotechnology and Plant Genetic Resources*, pp. 119-161. CABI Publishing, Oxford, U.K.

Engelmann, F. 2000. Importance of cryopreservation for the conservation of plant genetic resources. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 8-20. International Plant Genetic Resources Institute, Rome, Italy.

Engelmann, F. 2004. Plant cryopreservation: progress and prospects. *In Vitro Cellular and Developmental Biology - Plant* **40**: 427-433.

Engelmann, F. & Engels, J.M.M. 2002. Technologies and strategies for *ex situ* conservation. *In*: J.M.M. Engels, N.K. Rao, A.H.D. Brown & M.T. Jackson (eds), *Managing Plant Genetic Diversity*, pp. 89-104. CABI Publishing, New York, U.S.A.

Fahy, G.M. 1986. The relevance of cryoprotectant "toxicity" to cryobiology. *Cryobiology* **23**: 1-13.

Fahy, G.M., Takahashi, T. & Meryman, H.T. 1986. Practical aspects of ice-free cryopreservation. *In*: T.H. Smit-Sibinga & P.C. Das (eds), *Aspects of Ice-Free Cryopreservation*, pp. 111-122. Martinus-Nijhoff, Boston, U.S.A.

Fahy, G.M., MacFarlane, D.R., Angell, C.A. & Meryman, H.T. 1984. Vitrification as an approach to cryopreservation. *Cryobiology* **21**: 407-426.

Fahy, G.M., Wowk, B., Wu, J. & Paynter, S. 2004. Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology* **48**: 22-35.

Farnsworth, E. 2000. The ecology and physiology of viviparous and recalcitrant seeds. *Annual Review of Ecology and Systematics* **31**: 107-138.

Farrant, J.M. & Walters, C. 1998. Ultrastructural and biophysical changes in developing embryos of *Aesculus hippocastanum* in relation to the acquisition of tolerance to drying. *Physiologia Plantarum* **104**: 513-524.

Farrant, J.M., Berjak, P. & Pammenter, N.W. 1985. The effect of drying rate on viability retention of recalcitrant propagules of *Avicennia marina*. *South African Journal of Botany* **51**: 432-438.

Farrant, J.M., Berjak, P. & Pammenter, N.W. 1992. Proteins in development and germination of a desiccation sensitive (recalcitrant) seed species. *Plant Growth Regulation* **11**: 257-265.

Farrant, J.M., Pammenter, N.W. & Berjak, P. 1986. The increasing desiccation sensitivity of recalcitrant *Avicennia marina* seeds with storage time. *Physiologia Plantarum* **67**: 291-298.

Farrant, J.M., Pammenter, N.W. & Berjak, P. 1988. Recalcitrance - a current assessment. *Seed Science and Technology* **16**: 155-166.

Farrant, J.M., Pammenter, N.W. & Berjak, P. 1989. Germination associated events and the desiccation sensitivity of recalcitrant seeds - a study on three unrelated species. *Planta* **178**: 189-198.

Farrant, J.M., Pammenter, N.W. & Berjak, P. 1993. Studies on the desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* (Forssk.) Vierh.: the acquisition of germinability and response to storage and dehydration. *Annals of Botany* **51**: 432-438.

Farrant, J.M., Pammenter, N.W., Berjak, P. & Walters, C. 1997. Subcellular organisation and metabolic activity during the development of seeds attain different levels of desiccation tolerance. *Seed Science Research* **7**: 135-144.

Farrant, J.M., Pammenter, N.W., Berjak, P., Farnsworth, J. & Vertucci, C.W. 1996. Presence of dehydrin-like proteins and levels of abscisic acid in recalcitrant (desiccation-sensitive) seeds may be related to habitat. *Seed Science Research* **6**: 175-182.

Farrant, J.M., Walters, C., Lee, H., Morris, G.J. & Clarke, K.J. 1977. Structural and functional aspects of biological freezing techniques. *Journal of Microscopy* **11**: 17-34.

Finch-Savage, W.E. 1992. Embryo water status and survival in the recalcitrant species *Quercus robur* L.: evidence for a critical moisture content. *Journal of Experimental Botany* **43**: 663-669.

Finch-Savage, W.E. 1996. The role of developmental studies in research on recalcitrant and intermediate seeds. *In*: A.-S. Ouédraogo, K.M. Poulsen & F. Stubsgaard (eds), *Intermediate/Recalcitrant Tropical Forest Tree Seeds*, pp. 83-97. International Plant Genetic Resources Institute, Rome, Italy.

Finch-Savage, W.E. & Blake, P.S. 1994. Indeterminate development in desiccation-sensitive seeds of *Quercus robur* L. *Seed Science Research* **4**: 127-133.

Finch-Savage, W.E., Pramanik, S. & Bewley, J.D. 1994. The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. *Planta* **193**: 478-485.

Find, J.I., Krøgstrup, P., Moller, J.D., Noergaard, J.V. & Kristensen, M.M.H. 1993. Cryopreservation of embryogenic suspension cultures of *Picea sitchensis* and subsequent plant regeneration. *Scandinavian Journal of Forest Research* **8**: 156-162.

Finkle, B.J. & Ulrich, J.M. 1979. Effects of cryoprotectants in combination on the survival of frozen sugarcane cells. *American Society of Plant Biologists* **63**: 598-604.

Finkle, B.J., Zavala, M.E. & Ulrich, J.M. 1985. Cryoprotective compounds in the viable freezing of plant tissues. *In*: K.K. Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 75-113. CRC Press, Boca Raton, Florida, U.S.A.

Ford-Lloyd, B.V. & Jackson, M.T. 1991. Biotechnology and methods of conservation of plant genetic resources. *Journal of Biotechnology* **17**: 247-256.

Francisco-Ortega, J., Ellis, R.H., González-Feria, E. & Santos-Guerra, A. 1994. Overcoming seed dormancy in *ex situ* plant germplasm conservation programmes; an example in the endemic *Argytanthemum* (Asteraceae: Anthemideae) species from the Canary Islands. *Biodiversity and Conservation* **3**: 341-353.

Franks, F. 1985. *Biophysics and biochemistry at low temperatures*. Cambridge University Press, London, U.K.

Franks, J.R. 1999. *In situ* conservation of plant genetic resources for food and agriculture: a U.K. perspective. *Land Use Policy* **16**: 81-91.

Fridborg, G. & Erickson, T. 1975. Effect of activated charcoal on growth and morphogenesis in cell cultures. *Physiologia Plantarum* **34**: 306-308.

Fu, J.R., Xia, Q.H. & Tang, L.F. 1993. Effects of desiccation on excised embryonic axes of three recalcitrant seed species and studies on cryopreservation. *Seed Science and Technology* **21**: 85-95.

Fu, J.R., Jin, J.P., Peng, Y.F. & Xia, Q.H. 1994. Desiccation tolerance in two species with recalcitrant seeds: *Clausena lansium* (Lour.) and *Litchi chinensis* (Sonn.). *Seed Science Research* **4**: 257-261.

Fuller, B. 2004. Cryoprotectants: the essential antifreezes to protect life in the frozen state. *CryoLetters* **25**: 375-388.

Gagliardi, R.F., Pacheco, G.P., Valls, J.F.M. & Mansur, E. 2002. Cryopreservation of cultivated and wild *Arachis* species embryonic axes using desiccation and vitrification methods. *CryoLetters* **23**: 61-68.

George, E. 1993. *Plant Propagation by Tissue Culture. Part 1: The Technology*, pp. 3-93. Exegetics Ltd., Edington, England.

Gladis, T. 2000. Co-ordination of *ex-situ*, *in-situ* and on-farm conservation in Germany through networking. DSE-ZEL international workshop: Towards sustainable plant genetic resources programmes – policy, planning and coordination issues. Zschortau, May 10-18, 2001, pp. 254-255.

González-Benito, M.E. 1998. Cryopreservation as a tool for preserving genetic variability: its use with Spanish wild species with possible landscaping value. *Acta Horticulturae* **457**: 133-142.

González-Benito, M.E. & Pérez-Ruiz, C. 1992. Cryopreservation of *Quercus faginea* embryonic axes. *Cryobiology* **29**: 685-690.

González-Benito, M.E., Prieto, R.-M., Herradón, E. & Martín, C. 2002. Cryopreservation of *Quercus suber* and *Quercus ilex* embryonic axes: *in vitro* culture, desiccation and cooling factors. *CryoLetters* **23**: 283-290.

Goveia, M., Kioko, J.I. & Berjak, P. 2004. Developmental status is a critical factor in the selection of excised recalcitrant axes as explants for cryopreservation. *Seed Science Research* **14**: 241-248.

Grout, B.W.W. 1995. Introduction to the *in vitro* preservation of plant cells, tissues and organs. *In*: B.W.W. Grout (ed.), *Genetic Preservation of Plant Cells In Vitro*, pp. 1-20. Springer-Verlag, Berlin, Germany.

Grout, B.W.W., Shelton, K. & Pritchard, H.W. 1983. Orthodox behaviour of oil palm seed and cryopreservation of the excised embryo for germplasm conservation. *Annals of Botany* **52**: 381-384.

Gui, L., Deng, Z.-S. & Liu, J. 2004. Addition of cryoprotective agent into biological tissue through minimally invasive injection. *CryoLetters* **25**: 353-362.

Gullison, R.E., Frumhoff, P.C., Canadell, J.G., Field, C.B., Nepstad, D.C., Hayhoe, K., Avissar, R., Curran, L.M., Friedlingstein, P., Jones, C.D. & Nobre, C. 2007. Tropical forests and climate policy. *Science* **316**: 385-386.

Hammer, K., Gladis, T. & Diederichsen, A. 2003. *In situ* and on-farm management of plant genetic resources. *European Journal of Agronomy* **19**: 509-517.

Harding, K. 2004. Genetic integrity of cryopreserved plant cells: a review. *CryoLetters* **25**: 3-22.

Harding, K., Benson, E.E. & Roubelakis-Angelakis, K.A. 1996. Methylated DNA changes associated with the initiation and maintenance of *Vitis vinifera in vitro* shoot and callus cultures: a possible mechanism for age related changes. *Vitis* **2**: 79-85.

Harding, K., Day, J.G., Lorenz, M., Timmerman, H., Friedl, T., Bremner, D.H. & Benson, E.E. 2004. Introducing the concept and application of vitrification for the cryo-conservation of algae - a mini-review. *Nova Hedwigia* **79**: 207-226.

Harrington, J.F. 1973. Biochemical basis of seed longevity. *Seed Science and Technology* **1**: 453-461.

Hendry, G.A.F. 1993. Oxygen, free radical processes and seed longevity. *Seed Science Research* **3**: 141-153.

Heszky, L.E., Jekkel, Z. & Ali, A.-H. 1990. Effect of cooling rate, cryoprotectant and holding time at different transfer temperatures on the survival of cryopreserved cell suspension culture (*Puccinellia distans* (L.) Parl.). *Plant Cell, Tissue and Organ Culture* **21**: 217-226.

Hirai, D & Sakai, A. 1999. Cryopreservation of *in vitro* grown axillary shoot tips meristems of mint (*Mentha spicata* L.) by encapsulation-vitrification. *Plant Cell Reports* **19**: 150-155.

Hobbs, P.R. & Obendorf, R.L. 1972. Interaction of initial seed moisture and imbibitional temperature on germination and productivity of soybean. *Crop Science* **12**: 664-667.

Hoekstra, F.A., Golovina, E.A., van Aelst, A.C. & Hemminga, M.A. 1999. Imbibitional leakage from anhydrobiotes revisited. *Plant, Cell and Environment* **22**: 1121-1131.

Hong, T.D. & Ellis, R.H. 1990a. A comparison of mature drying, germination, and desiccation tolerance between developing seeds of *Acer pseudoplatanus* and *Acer platenoides* L. *New Phytologist* **116**: 589-596.

Hong, T.D. & Ellis, R.H. 1990b. Effect of moisture content and method of rehydration on the susceptibility of pea seeds to imbibition. *Seed Science and Technology* **1**: 131-137.

Hong, T.D. & Ellis, R.H. 1996. *A protocol to determine seed storage behaviour. IPGRI Technical Bulletin Number 1.* International Plant Genetic Resources Institute, Rome, Italy.

Hong, T.D. & Ellis, R.H. 1998. Contrasting seed storage behaviour among different species of Meliaceae. *Seed Science and Technology* **26**: 77-95.

Hor, Y.L., Stanwood, P.C. & Chin, H.F. 1990. Effects of dehydration on freezing characteristics and survival in liquid nitrogen of three recalcitrant seeds. *Pertanika* **13**: 309-314.

Ishikawa, M., Tandon, P., Suzuki, M. & Yamaguishi-Ciampi A. 1996. Cryopreservation of bromegrass (*Bromus inermis* Leyss) suspension cultured cells using slow prefreezing and vitrification procedures. *Plant Science* **120**: 81-88.

ISTA 1999. International rules for seed testing rules. *Seed Science and Technology* **27**: 33-35.

IUCN. 2010. IUCN Red List of Threatened Species. Version 2010.4. http://www.iucnredlist.org. (downloaded on 11 December 2010).

James, E. 1983. Low temperature preservation of living cells. *In*: S.H. Mantell & H. Smith (eds), *Plant Biotechnology*, pp. 163-186. Cambridge University Press, London, U.K.

Janeiro, L.V., Vieitez, A.M. & Ballester, A. 1996. Cryopreservation of somatic embryos and embryonic axes of *Camellia japonica* L. *Plant Cell Reports* **15**: 699-703.

Jentsch, A. & White, C.B.P.S. 2002. Scale, the dynamic stability of forest ecosystems, and the persistence of biodiversity. *Silva Fennica* **36**: 393-400.

Jörgensen, J. 1990. Conservation of valuable gene resources by cryopreservation in some forest tree species. *Journal of Plant Physiology* **136**: 373-376.

Kane, M.E. 2004. Shoot culture procedures. *In*: R.N. Gray & D.J. Gray (eds), *Plant Development and Biotechnology*, pp. 145-158. CRC Press Inc., Boca Raton, Florida, U.S.A.

Kappelle, M., Vuuren, M.M.I.V. & Baas, P. 1999. Effects of climate change on biodiversity: a review and identification of key research issues. *Biodiversity and Conservation* **8**: 1383-1397.

Kartha, K.K. 1985. *Cryopreservation of Plant Cells and Organs*. CRC Press Inc., Boca Raton, Florida, U.S.A.

Kartha, K.K. & Engelmann, F. 1994. Cryopreservation and germplasm storage. *In*: I. Vasil & T. Thorpe (eds), *Plant Cell and Tissue Culture*, pp. 195-230. Kluwer, Dordecht, Netherlands.

Kartha, K.K., Fowke, L.C., Leung, N.L., Caswell, K.L. & Hakman, I. 1988. Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*). *Journal of Plant Physiology* **132**: 529-539.

Kermode, A.R. 1990. Regulatory mechanisms involved in the transition from seed development to germination. *Critical Reviews in Plant Sciences* **9**: 155-195.

Kermode, A.R. 1997. Approaches to elucidate the basis of desiccation-tolerance in seeds. *Seed Science Research* **7**: 75-95.

Kermode, A.R. & Bewley, J.D. 1985. Developing seeds of *Ricinus communis L.* when detached and maintained in an atmosphere of high relative humidity, switch to a germinative mode without the requirement for complete desiccation. *Plant Physiology* **90**: 702-707.

Kermode, A.R. & Finch-Savage, W.E. 2002. Desiccation sensitivity in orthodox and recalcitrant seeds in relation to development. *In*: M. Black & H.W. Pritchard (eds), *Desiccation and Survival in Seeds: Drying Without Dying*, pp. 149-184. CABI Publishing, Wallingford., Oxon, U.K.

Kim, H.-H., Cha, Y.-S., Baek, H.-J., Cho, E.-G., Chae, Y.-A. & Engelmann, F. 2002. Cryopreservation of tea (*Camellia sinensis* L.) seeds and embryonic axes. *CryoLetters* **23**: 209-216.

Kim, H.-H., Yoon, J.-W., Park, S.-U., Kim, J.-H., Cho, E.-G. & Engelmann, F. 2005. Assessment of desiccation sensitivity of tea embryos for cryopreservation. *CryoLetters* **26**: 269-276.

Kim, H.-H., Lee, Y.-G., Shin, D.-J., Ko, H.-C., Gwag, J.-G., Cho, E.-G. & Engelmann, F. 2009. Development of alternative plant vitrification solutions in droplet-vitrification procedures. *CryoLetters* **30**: 320-334.

King, M.W. & Roberts, E.H. 1980. Maintenance of recalcitrant seeds in storage. *In*: H.F. Chin & E.H. Roberts (eds), *Recalcitrant Crop Seeds*, pp. 53-89. Tropical Press SDN, Kuala Lumpur, Malaysia.

Kioko, J.I. 2003. Aspects of post-harvest seed physiology and cryopreservation of the germplasm of three medicinal plants indigenous to Kenya and South Africa. Ph.D. Thesis, University of Natal, Durban, South Africa.

Kioko, J.I., Berjak, P. & Pammenter, N.W. 2003. Responses to dehydration and conservation of the non-orthodox seeds of *Warburgia salutaris*. *South African Journal of Botany* **69**: 532-539.

Kioko, J.I., Berjak, P. & Pammenter, N.W. 2006. Viability and ultrastructural responses of seeds and embryonic axes of *Trichilia emetica* to different dehydration and storage conditions. *South African Journal of Botany* **72**: 167-176.

Kioko, J.I., Berjak, P., Pammenter, N.W., Watt, M.P. & Mycock, D.J. 1998. Desiccation and cryopreservation of embryonic axes of *Trichilia dregeana*. *CryoLetters* **15**: 5-14.

Kirk, R.G. & Tosteson, D. 1973. Cation transportation and membrane morphology. *Journal of Membrane Biology* **12**: 273.

Kleinschmit, J. 1994. Efficiency of different conservation methods in forestry for conservation and utilisation. *In*: F. Begemann & K. Hammer (eds), *Integration of Conservation Strategies of Plant Genetic Resources in Europe*, pp. 181-186. Proceedings of International Symposium. Gatersleben, Germany.

Kobayashi, S., Sakai, A. & Oiyama, I. 1990. Cryopreservation and plant regeneration from embryogenic cultures of larch (*Larix eurolepsis*) and black spruce (*Picea mariana*). *Journal of Experimental Botany* **43**: 73-79.

Kobayashi, T., Niino, T. & Kobayashi, M. 2005. Simple cryopreservation protocol with an encapsulation technique for Tobacco by-2 suspension cell cultures. *Plant Biotechnology* **22**: 105-112.

Koster, K.L. 1991. Glass formation and desiccation tolerance in seeds. *Plant Physiology* **96**: 302-304.

Koster, K.L., Lei, Y.P., Anderson, M., Martins, S. & Bryant, G. 2000. Effects of vitrified and non-vitrified sugars on phosphatidylcholine fluid-to-gel transitions. *Biophysical Journal* **78**: 1932-1946.

Kovach, D.A. & Bradford, K.J. 1992. Imbibitional damage and desiccation tolerance of wild rice (*Zizania palustris*) seed. *Journal of Experimental Botany* **43**: 747-757.

Kranner, I. & Birtic, S. 2005. A modulating role for antioxidants in desiccation tolerance. *Integrative and Comparative Biology* **45**: 734-740.

Kundu, K. & Kachari, J. 2000. Desiccation sensitivity and recalcitrant behaviour of seeds of *Aquilaria agallocha* Roxb. *Seed Science and Technology* **28**: 755-760.

Leibo, S.P., McGrath, J.J. & Cravalho, E.G. 1978. Microscopic observation of intracellular ice formation in unfertilised mouse ova as a function of cooling rate. *Cryobiology* **15**: 257-271.

Leibo, S.P., Farrant, J.M., Mazur, P., Hanna, J., M.G. & Smith, L.H. 1970. Effects of freezing on marrow stem cell suspensions: interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol. *Cryobiology* **6**: 315-332.

Leprince, O. & Walters-Vertucci, C. 1995. A calorimetric study of the glass transition behaviours in axes of bean seeds with relevance to storage stability. *Plant Physiology* **109**: 1471-1481.

Leprince, O., Buitink, J. & Hoekstra, F. 1999. Axes and cotyledons of recalcitrant seeds of *Castanea sativa* Mill. exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity. *Journal of Experimental Botany* **50**: 1515-1524.

Leprince, O., Hendry, G.A.F. & McKersie, B.D. 1993. The mechanisms of desiccation tolerance in developing seeds. *Seed Science Research* **3**: 231-246.

Leprince, O., Aelst, A.V., Pritchard, H.W. & Murphy, D. 1998. Oleosins prevent oil-body coalescence during seed imbibition as suggested by a low-temperature scanning electron microscope study of desiccation-tolerant and -sensitive oilseed. *Planta* **204**: 109-119.

Leprince, O., Harren, J.M., Buitink, J., Alberda, M. & Hoekstra, F.A. 2000. Metabolic dysfunction and unabated respiration precede the loss of membrane integrity during dehydration of germinating radicals. *Plant Physiology* **122**: 597-608.

Leubner-Metzger, G. 2005. β-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *Plant Journal* **41**: 133-145.

Leunufna, S. & Keller, E.R.J. 2005. Cryopreservation of yams using vitrification modified by including droplet method: effects of cold acclimation and sucrose *CryoLetters* **26**: 93-102.

Levin, S.A. 1990. The seed bank as a source of genetic novelty in plants. *The American Naturalist* **135**: 563-572.

Li, C. & Sun, W.Q. 1999. Desiccation sensitivity and activities of free radical scavenging enzymes in recalcitrant *Theobroma cacao* seeds. *Seed Science Research* **9**: 209-217.

Li, Z., Traore, A., Maximova, S. & Guiltinan, M.J. 1998. Somatic embryogenesis and plant regeneration from floral explants of cocoa (*Theobroma cacao* L.) using thidiazuron. *In Vitro Cellular and Developmental Biology - Plant* **34**: 293-299.

Liang, Y. & Sun, W.Q. 2000. Desiccation tolerance of recalcitrant *Theobroma cacao* embryonic axes: the optimal drying rate and its physiological basis. *Annals of Botany* **51**: 1911-1919.

Liang, Y. & Sun, W.Q. 2002. Rate of dehydration and cumulative desiccation stress interacted to modulate desiccation tolerance of recalcitrant *Cocoa* and *Ginkgo* embryonic tissues. *Plant Physiology* **128**: 1323-1131.

Lin, T.-P. & Chen, M.-H. 1995. Biochemical characteristics associated with the development of the desiccation-sensitive seeds of *Machilus thubergii* Sieb. and Zucc. Annals of Botany **76**: 381-387.

Linington, S.H. & Pritchard, H.W. 2001. Genebanks. *In*: S.A. Levin (ed.), *Encyclopedia of Biodiversity, Volume 3*, pp. 165-181. Academic Press, San Francisco, U.S.A.

Liu, M.-S., Chang, C.-Y. & T-P., L. 2006 Comparison of phospholipids and their fatty acids in recalcitrant and orthodox seeds. *Seed Science and Technology* **34**: 443-452.

Loud, A.V. 1962. A method for the quantification of cytoplasmic structures. *Journal of Cell Biology* **15**: 481-487.

Lovelock, J.E. 1953. The haemolysis of human red blood cells by freezing and thawing. *Biochimica et Biophysica Acta* **10**: 414-426.

Luyet, B.J. 1960. On the mechanism of growth of ice crystals in aqueous solutions and on the effect of rapid cooling in hindering crystallisation. *In*: A.S. Parkes & A.U. Smith (eds), *Recent Research in Freezing and Drying*, pp. 3-22. Blackwell Scientific Publishing, Oxford, U.K.

Luyet, B.J. 1965. Phase transitions encountered in the rapid freezing of aqueous solutions. *Annals of the New York Academy of Sciences* **125**: 502-521.

Luyet, B.J., Tanner, J. & Rapatz, G. 1962. X-ray diffraction study of the structure of rapidly frozen gelatin solutions. *Biodynamica* **9**: 21-46.

Lynch, P.T. & Benson, E.E. 1991. Cryopreservation, a method for maintaining the plant regeneration capacity of rice suspension cultures. *Proceedings of the Second Rice Genetic Symposium*, pp. 321-332. IRRI, Los Banos, U.S.A.

MacKenzie, A.P. 1977. Non-equilibrium freezing behaviour of aqueous systems. *Philosophical Transactions of the Royal Society of London* **B278**: 167-189.

Makeen, M.A., Normah, M.N., Dussert, S. & Clyde, M.M. 2005. Cryopreservation of whole seeds and excised embryonic axes of *Citrus suhuiensis* cv. Limau langkat in accordance to their desiccation sensitivity. *CryoLetters* **26**: 259-268.

Malcolm, J.R., Liu, C., Neilson, R.P., Hansen, L. & Hannah, L. 2006. Global warming and extinctions of endemic species from biodiversity hotspots. *Conservation Biology* **20**: 538-548.

Marin M, L. & Duran-Vila, N. 1988. Survival of somatic embryos and recovery of plants of sweet orange (*Citrus sinensis* (L.) Osb.) after immersion in liquid nitrogen. *Plant Cell, Tissue Organ Culture* **14**: 51-57.

Martens, P., Rotmans, J. & Groot, D.D. 2003. Biodiversity: luxury or necessity? *Global Environmental Change* **13**: 75-81.

Martínez, M.T., Ballester, A. & Vieitez, A.M. 2003 Cryopreservation of embryogenic cultures of *Quercus robur* using desiccation and vitrification procedures. *Cryobiology* **46**: 182-189.

Maxted, N., Ford-Lloyd, B. & Hawkes, J.G. 1997. *Plant Genetic Conservation: The In Situ Approach*. Chapman and Hall, London, U.K.

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

Mazur, P. 1984. Freezing of living cells: mechanisms and applications. *American Journal of Physiology, Cell Physiology* **247**: C125-C142.

Mazur, P. 1990. Equilibrium, quasi-equilibrium, and non-equilibrium freezing of mammalian embryos. *Cell Biophysics* **17**: 53-92.

Mazur, P. 2004. Principles of cryobiology. *In*: B. Fuller, N. Lane & E.E. Benson (eds), *Life in the Frozen State*, pp. 3-65. CRC Press, Boca Raton, London, U.K.

McGann, L.E. 1978. Differing actions of penetrating and non-penetrating cryoprotective agents. *Cryobiology* **15**: 382-390.

McLaughlin, A. & Mineau, P. 1995. The impacts of agricultural practices on biodiversity agriculture. *Ecosystems and Environment* **55**: 201-212.

Menges, M. & Murray, J.A.H. 2004. Cryopreservation of transformed and wild-type *Arabidopsis* and tobacco cell suspension cultures. *The Plant Journal* **37**: 635-644.

Meryman, H.T. 1956. Mechanisms of freezing in living cells and tissue. *Science* **124**: 515-521.

Meryman, H.T. & Williams, R.J. 1980. Mechanisms of freezing injury and natural tolerance and the principles of artificial cryoprotection. *In*: L. Withers & J. Williams (eds), *Crop Genetic Resources - The Conservation of Difficult Material*, pp. 5-37. International Union of Biological Sciences, Série B42, Reading, U.K.

Meryman, H.T. & Williams, R. 1985. Basic principles of freezing injury to plant cells: natural tolerance and approaches to cryopreservation. *In*: K.K. Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 13-48. CRC Press, Boca Raton, Florida, U.S.A.

Metcalfe, L.D. & Wang, C.N. 1981. Rapid preparation of fatty acid methyl esters using organic base-catalysed transesterification. *Journal of Chromatographic Science* **19**: 530-535.

Minibayeva, F.V., Kolesnikov, O.P. & Gordon, L.K. 1998. Contribution of a plasma membrane redox system to the superoxide production by wheat root cells. *Protoplasma* **205**: 101-106.

Mix-Wagner, G., Schumacher, H.M. & Cross, R.J. 2002. Recovery of potato apices after several years of storage in liquid nitrogen. *CryoLetters* **24**: 33-41.

Morris, G.J. 1978. Cryopreservation of 250 strains of *Chlorococcales* by the method of two-step cooling. *British Phycological Journal* **13**: 15-24.

Morris, G.J. & Canning, C.E. 1978. The Cryopreservation of *Euglena gracilis*. *Journal of General Microbiology* **108**: 27-31.

Morriset, C., Gazeau, C., Hansz, J. & Dereuddre, J. 1993. Importance of actin cytoskeleton behaviour during preservation of carrot cell suspensions in liquid nitrogen. *Protoplasma* **173**: 35-47.

Munro, J. 1981. Monopolists and speculators: British investment in West African rubber, 1905-1914. *Journal of African History* **22**: 263-278.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497.

Mycock, D.J. 1999. Addition of calcium magnesium to a glycerol and sucrose cryoprotectant solution improves the quality of plant embryo recovery from cryostorage. *CryoLetters* **20**: 77-82.

Mycock, D.J. & Berjak, P. 1990. Fungal contaminants associated with several homoiohydrous (recalcitrant) seed species. *Phytophylactica* **22**: 413-418.

Mycock, D.J., Blakeway, F.C. & Watt, M.P. 2004. General applicability of *in vitro* storage technology to the conservation and maintenance of plant germplasm. *South African Journal of Botany* 70: 31-36.

Mycock, D.J., Watt, M.P. & Berjak, P. 1991. A simple procedure for the cryopreservation of hydrated embryonic axes of *Pisum sativum*. *Journal of Plant Physiology* **138**: 728-733.

Mycock, D.J., Wesley-Smith, J. & Berjak, P. 1995. Cryopreservation of somatic embryos of four species with and without cryoprotection pre-treatment. *Annals of Botany* **75**: 331-336.

Nadarajan, J., Staines, H.J., Benson, E.E., Mansor, M., Krishnapillay, B. & Harding, K. 2006. Optimisation of cryopreservation protocol for *Stericulia cordata* Blume. zygotic embryos using Taguchi experiments. *Journal of Tropical Forest Science* **18**: 166-172.

Nadarajan, J., Staines, H.J., Benson, E.E., Marzalina, M., Krishnapillay, B. & Harding, K. 2007. Optimization of cryopreservation for *sterculia cordata* zygotic embryos using vitrification techniques. *Journal of Tropical Forest Science* **19**: 79-85.

Naidoo, P. 2008. Development of explants potentially suitable for cryopreservation of the recalcitrant-seeded species *Theobroma cacao* L. and *Barringtonia racemosa* (L.) Roxb. MSc. Dissertation, University of KwaZulu-Natal, Durban, South Africa.

Naidoo, S. 2006. Investigations into the post-harvest behaviour and germplasm conservation of the seeds of selected Amaryllid species. MSc. Dissertation, University of KwaZulu-Natal, Durban, South Africa.

Naidoo, S., Pammenter, N.W. & Berjak, P. 2011. Effects of partial dehydration of recalcitrant *Haemanthus montanus* zygotic embryos on vigour of recovered seedlings. *South African Journal of Botany* **77**: 193-202.

Nevo, E. 1998. Genetic diversity in wild cereals: regional and local studies and their bearing on conservation *ex situ* and *in situ*. *Genetic Resources and Crop Evolution* **45**: 355-370.

Neya, O., Golovina, E., Nijsse, J. & Hoekstra, F. 2004. Ageing increases the sensitivity of neem (*Azadirachta indica*) seeds to imbibitional stress. *Seed Science and Technology* **14**: 205-217.

Ngobese, N.Z., Sershen, Pammenter, N.W. & Berjak, P. 2010. Cryopreservation of the embryonic axes of *Phoenix reclinata*, a representative of the intermediate seed category. *Seed Science and Technology* **38**: 704-716.

Nitzsche, W. 1983. Germplasm Preservation. *In*: D.A. Evans (ed.), *Handbook of Plant Cell Culture*, *Volume 1*, *Techniques for Propagation and Breeding*, pp.782-805. Macmillan Publishing Company, A division of Macmillan Incorporated, New York, U.S.A.

Nkang, A., Omokaro, D., Egbe, A. & Amanke, G. 2003. Variations in fatty acid proportions during desiccation of *Telfairia occidentalis* seeds harvested at physiological and agronomic maturity. *African Journal of Biotechnology* **2**: 33-39.

Normah, M.N. & Makeen, A.M. 2008. Cryopreservation of excised embryos and embryonic axes. *In*: B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide*, pp. 211-240. Springer-Verlag, New York, U.S.A.

Normah, M.N. & Vengadasalam, M. 1992. Effects of moisture content on cryopreservation of *Coffea* and *Vigna* seeds and embryos. *CryoLetters* **13**: 199-208.

Normah, M.N., Chin, H.F. & Hor, Y. 1986. Desiccation and cryopreservation of embryonic axes of *Hevea brasiliensis* Muell-Arg. *Pertanika* **9**: 299-303.

Normah, M.N., Reed, B.M. & Yu, X. 1994. Seed storage and cryoexposure behaviour in hazelnut (*Corylus avellana* L. cv. Barcelona). *CryoLetters* **15**: 315-322.

Nowshari, M.A., Nayudu, P.L. & Hodges, J.K. 1995. Effect of cryoprotectant and their concentration on post-thaw survival and development of rapid frozen-thawed pronuclear stage mouse embryos. *Human Reproduction* **10**: 3237-3242.

Ntuli, T.M., Finch-Savage, W.E., Berjak, P. & Pammenter, N.W. 2011. Increased drying rate lowers the critical water content for survival in embryonic axes of English oak (*Quercus robur* L.) seeds. *Journal of Integrative Plant Biology* **53**: 270-280.

Odion, D.C. & Sarr, D.A. 2007. Managing disturbance regimes to maintain biological diversity in forested ecosystems of the Pacific Northwest. *Forest Ecology and Management* **246**: 57-65.

Oliver, M.J., Tuba, Z. & Mishler, B.D. 2000. The evolution of vegetative desiccation tolerance in land plants. *Plant Ecology* **151**: 85-100.

Oracz, H., El-Maarouf-Bouteau, H., Kranner, I., Bogatek, R., Corbineau, F. & Bailly, C. 2009. The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signalling during germination. *Plant Physiology* **150**: 494-505.

Orthen, B., Popp, M. & Smirnoff, N. 1994. Hydroxyl radical scavenging properties of cyclitols. *Proceedings of the Royal Society of Edinburgh* **B102**: 269-272.

Ozudogru, E.A., Capuana, M., Kaya, E., Panis, B. & Lambardi, M. 2010. Cryopreservation of *Fraxinus excelsior* L. embryogenic callus by one-step freezing and slow cooling techniques. *CryoLetters* **31**: 63-75.

Pammenter, N.W. & Berjak, P. 1999. A review of recalcitrant seed physiology in relation to desiccation-tolerant mechanisms. *Seed Science Research* **9**: 13-37.

Pammenter, N.W. & Berjak, P. 2000. Aspects of recalcitrant seed physiology. *Revista Brasileira de Fisiologia Vegetal* **12**: 56-69.

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

Pammenter, N.W., Vertucci, C.W. & Berjak, P. 1993. Responses to dehydration in relation to non-freezable water in desiccation-sensitive and -tolerant seeds. *In*: D. Côme & F. Corbineau (eds), pp. 867-872. *Fourth International Workshop on Seeds: Basic and Applied Aspects of Seed Biology*. ASFIS, Paris, France.

Pammenter, N.W., Berjak, P., Wesley-Smith, J. & Vander Willigen, C. 2002. Experimental aspects of drying and recovery. *In*: M.H. Black & H.W Pritchard (eds), *Desiccation and Survival in Plants: Drying Without Dying*, pp. 93-110. CABI Publishing, New York, U.S.A.

Pammenter, N.W., Berjak, P., Farrant, J.M., Smith, M.T. & Ross, G. 1994. Why do stored hydrated recalcitrant seeds die? *Seed Science Research* **4**: 187-191.

Pammenter, N.W., Gregagains, V., Kioko, J., Wesley-Smith, J., Berjak, P. & Finch-Savage, W.E. 1998. Effects of differential drying rates on viability retention of recalcitrant seeds of *Ekerbergia capensis*. *Seed Science Research* **8**: 363-461.

Pammenter, N.W., Berjak, P., Goveia, M., Sershen, Kioko, J.I., Whitaker, C. & Beckett, R.P. 2011. Topography determines the impact of reactive oxygen species on shoot apical meristems of recalcitrant embryos of tropical species during processing for cryopreservation. *Acta Horticulturae* (in press).

Panis, B. & Lambardi, M. 2006. Status of cryopreservation technologies in plants (crops and forest trees). *In*: J. Ruane & A. Sonnino (eds), *The Role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources*, pp. 61-78. Food and Agricultural Organisation of the United Nations, Rome, Italy.

Park, Y.-J., Dixit, A., Ma, K.-H., Kang, J.-H., Rao, V.R. & Cho, E.-G. 2005. On-farm conservation strategy to ensure crop genetic diversity in changing agro-ecosystems in the Republic of Korea. *Journal of Agronomy and Crop Science* **191**: 401-410.

Parrish, D.J. & Leopold, A.C. 1978. On the mechanism of ageing in soybean seeds. *Plant Physiology* **61**: 365-368.

Pearce, R.S. 2004. Adaptation of higher plants to freezing. *In*: B.J. Fuller, E.E. Benson & N. Lane (eds), *Life in the Frozen State*, pp. 67-107. CRC Press, Boca Raton, London, U.K.

Pegg, D.E. 2002. The history and principles of cryopreservation. *Seminars in Reproductive Medicine* **20**: 5-13.

Pegg, D.E. 2007. Principles of cryopreservation. *In*: J.G. Day & G.N. Stacey (eds), *Methods in Molecular Biology, Volume 368: Cryopreservation and Freeze-Drying Protocols*, pp. 39-57. Humana Press Inc., Totowa, New Jersey, U.S.A.

Pence, V. 1990. Cryostorage of embryonic axes of several large-seeded temperate tree species. *Cryobiology* **27**: 212-218.

Pence, V. 1992. Desiccation and survival of *Aesculus*, *Castanea*, and *Quercus* embryos axes through cryopreservation. *Cryobiology* **29**: 391-399.

Pence, V. 1995. Cryopreservation of recalcitrant seeds. In: Y.P.S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry 32: Cryopreservation of Plant Germplasm 1*, pp. 29-50. Springer-Verlag, Berlin, Germany.

Pence, V. 2003. *In vitro* growth of embryo axes after long-term storage in liquid nitrogen. *In*: R.D. Smith, J.B. Dickie, S.H. Linington, H.W. Pritchard & R.J. Probert (eds), *Seed Conservation: Turning Science into Practice*, pp. 483-492. Cromwell Press Ltd, Kew, U.K.

Perán, R., Berjak, P., Pammenter, N.W. & Kioko, J.I. 2006. Cryopreservation, encapsulation and promotion of shoot production of embryonic axes of a recalcitrant species *Ekebergia capensis*, Sparrm. *CryoLetters* **27**: 5-16.

Perán, R., Pammenter, N.W., Naicker, J. & Berjak, P. 2004. The influence of rehydration techniques on the response of recalcitrant seed embryos to desiccation. *Seed Science Research* **14**: 179-184.

Percy, R.E., Klimaszewska, K. & Cyr, D.R. 2000. Evaluation of somatic embryogenesis for clonal propagation of western white pine. *Canadian Journal of Forest Research* **30**: 1867-1876.

Pérez, R.M., Navaro, L. & Duran-Vila, N. 1997. Cryopreservation and storage of embryogenic callus cultures of several *Citrus* species and cultivars. *Plant Cell Reports* **17**: 44-49.

Phartyal, S.S., Thapliyal, R.C., Koedam, N. & Godefroid, S. 2002. *Ex situ* conservation of rare and valuable forest tree species through seed gene-bank. *Current Science* **83**: 1351-1357.

Pita, J.M., Pérez-Garcia, F., Escudero, A. & de la Cuadra, C. 1998. Viability of *Avena sativa* L. seeds after 10 years of storage in base collection. *Field Crops Research* **55**: 183-187.

Pollock, B.M. 1969. Imbibition temperature sensitivity of lima bean seeds controlled by initial seed moisture. *Plant Physiology* **44**: 907-911.

Pooley, E. 1993. *The Complete Field Guide to Trees of Natal Zululand and Transkei*, pp. 430. Natal Flora Publications Trust, Durban, South Africa.

Potts, S.E. & Lumpkin, T.A. 1997. Cryopreservation of *Wasabia* spp. seeds. *CryoLetters* **18**: 185-190.

Poulsen, K.M. 1992. Sensitivity of desiccation and low temperatures (-196°C) of embryonic axes from acorns of the pedunculate oak (*Quercus robur* L.). *CryoLetters* **13**: 75-82.

Powell, A.A. & Matthews, S. 1978. The damaging effect of water on dry pea embryos during imbibition. *Journal of Experimental Botany* **29**: 1215-1229.

Prance, G.T. 1997. The conservation of botanical diversity. *In*: N. Maxted, B. Ford-Lloyd & J.G. Hawkes (eds), *Plant Genetic Conservation: The In Situ Approach*, pp. 3-14. Chapman and Hall, London, U.K.

Pritchard, H.W. 1991. Water potential and embryonic axis viability in recalcitrant seeds of *Quercus rubra*. *Annals of Botany* **67**: 43-49.

Pritchard, H.W. 2004. Classification of seed storage types for *ex situ* conservation in relation to temperature and moisture. *In*: E.O. Guerrant, K. Havens & M. Maunder (eds), *Ex Situ Plant Conservation: Supporting Species Survival in the Wild*, pp. 139-161. Island Press, Washington D.C., U.S.A.

Pritchard, H.W. & Dickie, J.B. 2003. Predicting seed longevity: the use and abuse of seed viability equations. *In*: R.D. Smith, J.B. Dickie, S.H. Linington, H.W. Pritchard & R.J. Probert (eds), *Seed Conservation Turning Science into Practice*, pp. 653-722. Cromwell Press Ltd., London, U.K.

Pritchard, H.W. & Manger, K.R. 1998. A calorimetric perspective on desiccation stress during preservation procedures with recalcitrant seeds of *Quercus robur* L. *CryoLetters* **19**: 23-30.

Pritchard, H.W. & Prendergast, F. 1986. Effects of desiccation and cryopreservation on the *in vitro* viability of the recalcitrant seed species *Araucaria hunsteinii* K. Shum. *Journal of Experimental Botany* **37**: 1388-1397.

Pritchard, H.W., Grout, B.W.W., Reid, D.S. & Short, K.C. 1982. The effects of growth under water stress on the structure, metabolism and cryopreservation of cultured sycamore cells. *In*: F. Franks & S.F. Mathias (eds), *Biophysics of Water*, pp. 315-318. John Wiley and Sons, New York, U.S.A.

Pritchard, H.W., Tompsett, P.B., Manger, K. & Smidt, W.J. 1995. The effect of moisture content on the low temperature responses of *Aruacaria hunsteinii* seed and embryos. *Annals of Botany* **76**: 79-88.

Pritchard, H.W., Daws, M.I., Fletcher, B.J., Gamene, C.S., Msanga, H.P. & Omondi, W. 2004. Ecological correlates of seed desiccation tolerance in tropical African dryland trees. *American Journal of Botany* **91**: 863-870.

Probert, R.J. & Longley, P.L. 1989. Recalcitrant seed storage physiology in three aquatic grasses (*Zizania palustris*, *Spartina anglica* and *Porteresia coarctata*). *Annals of Botany* **63**: 53-63.

Probert, R.J., Dawa, M.I. & Hay, F.R. 2009. Ecological correlates of *ex situ* seed longevity: a comparative study on 195 species. *Annals of Botany* **104**: 57-69.

Quain, M.D., Berjak, P., Acheampong, E. & Kioko, J.I. 2009. Sucrose treatment and explant water content: critical factors to consider in development of successful cryopreservation protocols for shoot tip explants of the tropical species *Dioscorea rotundata* (yam). *CryoLetters* **30**: 212-223.

Radhamani, J. & Chandel, K.P.S. 1992. Cryopreservation of embryonic axes of orange (*Poncirus trifoliata* (L.) RAF.). *Plant Cell Reports* **11**: 372-374.

Raja, K., Palanisamy, V., Selvaraju, P. & Shanmugasundaram, K.A. 2001. Desiccation sensitivity of avocado (*Persea americana* Mill) seeds. The project and handling and storage of recalcitrant and intermediate tropical forest seeds. *Newsletter* **8**: 22-24.

Rajasekaran, K. 1996. Regeneration of plants from cryopreserved embryogenic cell suspension and callus cultures of cotton (*Gossypium hirsutum* L.). *Plant Cell Reports* **15**: 859-864.

Rall, W.F. & Fahy, G.M. 1985. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* **313**: 573-575.

Rao, N.K. 2004. Plant genetic resources: advancing conservation and use through biotechnology. *African Journal of Biotechnology* **3**: 136-145.

Reed, B.M. 1990. Survival of *in vitro*-grown apical meristems of *Pyrus* following cryopreservation. *HortScience* **25**: 111-113.

Reed, B.M. 1996. Pretreatment strategies for cryopreservation of plant tissues. *In*: M.N. Normah, M. Narimah & M. Clyde (eds), *In Vitro Conservation of Plant Genetic Resources*, pp. 73-87. Percetakan watan, Kuala Lumpur, Malaysia.

Reed, B.M. 2008. Cryopreservation - practical considerations. *In*: B.M. Reed (ed.), *Plant Cryopreservation: A Practial Guide*, pp. 3-13. Springer-Verlag, New York, U.S.A.

Reed, B.M. & Lagerstedt, H.B. 1987. Freezing preservation of apical meristems of *Rubus* in liquid nitrogen. *HortScience* **22**: 302-303.

Reed, B.M. & Uchendu, E. 2008. Controlled rate cooling. *In*: B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide*, pp. 77-92. Springer-Verlag, New York, U.S.A.

Reed, B.M., Normah, M.N. & Yu, X. 1994. Stratification is necessary for successful cryopreservation of axes from stored hazelnut. *CryoLetters* **15**: 377-384.

Reed, B.M., Okut, N., D'Achino, J., Narver, L. & Denoma, J. 2003. Cold storage and cryopreservation of hops (*Humulus* L.) shoot cultures through application of standard protocols. *CryoLetters* **24**: 389-396.

Reidsma, P., Tekelenburg, T., Berg, M.V.D. & Alkemade, R. 2006. Impacts of land-use change on biodiversity: an assessment of agricultural biodiversity in the European Union. *Agriculture, Ecosystems and Environment* **114**: 86-102.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**: 208-212.

Roach, T., Ivanova, M., Beckett, R.P., Minibayeva, F.V., Green, I., Pritchard, H.W. & Kranner, I. 2008. An oxidative burst of superoxide in embryonic axes of recalcitrant sweet chestnut seeds as induced by excision and desiccation. *Physiologia Plantarum* **133**: 131-139.

Robards, A.W. & Sleytr, U.B. 1985. Low temperature methods in biological electron microscopy. *In*: A.M. Glauert (ed.), *Practical Methods on Electron Microscopy*, *Volume 10*. Elsevier, Amsterdam, Netherlands.

Roberts, E.H. 1973. Predicting the viability of seeds. *Seed Science and Technology* **1**: 499-514.

Roberts, E.H. 1991. Genetic conservation in seed banks. *Biological Journal of the Linnean Society* **43**: 23-29.

Roberts, E.H. & Ellis, R.H. 1989. Water and seed survival. *Annals of Botany* **63**: 39-52.

Roberts, E.H. & King, M.W. 1980. The characteristics of recalcitrant seeds. *In*: H.F. Chin & E.H. Roberts (eds), *Recalcitrant Crop Species*, pp. 1-5. Tropical Press, Kuala Lumpur, Malaysia.

Rubinsky, B. & Pegg, D.E. 1988. A mathematical model for the freezing process in biological tissue. *Proceedings of the Royal Society of London* **234**: 343-358.

Rudolph, A.S. & Crowe, J.H. 1985. Membrane stabilisation during freezing: the role of two natural cryoprotectants, trehalose and proline. *Cryobiology* **22**: 367-377.

Ruiz, M., Martin, I. & de la Cuadra, C. 1999. Cereal seed viability after 10 years of storage in active and base germplasm collections. *Field Crops Research* **64**: 229-236.

Ryan, K.P. & Purse, D.H. 1985. A simple plunge-cooling device for preparing biological specimens for cryo-techniques. *Mikroskopie* **42**: 247-251.

Sacandé, M., Hoekstra, F.A., van Pijlen, J.G. & Groot, S.P.C. 1998. A multifactorial study of conditions influencing the longevity of neem (*Azadirachta indica*) seeds. *Seed Science Research* **4**: 193-201.

Sackville Hamilton, N.R. & Chorlton, K.H. 1997. Regeneration of Accessions in Seed Collections: A Decision Guide. *In*: J.M.M. Engels (ed.), *Handbook for Genebanks Number 5*, pp. 1-75. Plant Genetic Resources Institute, Rome, Italy.

Sakai, A. 1985. Cryopreservation of shoot-tips of fruit trees and herbaceous plants. *In*: K.K. Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 135-158. CRC Press Inc., Boca Raton, Florida, U.S.A.

Sakai, A. 2000. Development of cryopreservation techniques. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 1-7. Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan/International Plant Genetic Resources Institute, Rome, Italy.

Sakai, A. 2004. Plant cryopreservation. *In*: B. Fuller, N. Lane & E.E. Benson (eds), *Life in the Frozen State*, pp. 329-345. CRC Press, Boca Raton, London, U.K.

Sakai, A. & Yoshida, S. 1967. Survival of plant tissue at super-low temperature VI. Effects of cooling and rewarming rates on survival. *Plant Physiology* **42**: 1695-1701.

Sakai, A., Kobayashi, S. & Oiyama, I. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* osb. Var. *Brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* **9**: 30-33.

Santarius, K. & Franks, F. 1998. Cryopreservation of lactate dehydrogenase - interactions among various cryoprotectants. *CryoLetters* **19**: 37-48.

Sax, D.F. & Gaines, S.D. 2003. Species diversity: from global decreases to local increases. *Trends in Ecology and Evolution* **18**: 561-566.

Schrijnemakers, E.W.M. & Van Iren, F. 1995. A two-step or equilibrium freezing procedure for the cryopreservation of plant cell suspensions. *In*: J.G. Day & M.R. McLellan (eds), *Methods in Molecular Biology, Volume 38*: *Cryopreservation and Freeze-Drying Protocols*, 103-111. Humana Press, Totowa, New Jersey, U.S.A.

Scocchi, A.M., Faloci, M., Medina, R., Olmos, S. & Mroginski, L. 2004. Plant recovery of cryopreserved apical meristem tips of *Melia azedarach* L. using encapsulation/dehydration and assessment of their genetic stability. *Euphytica* **135**:29-38.

Sershen, Berjak, P. & Pammenter, N.W. 2008. Desiccation sensitivity of excised embryonic axes of selected Amaryllid species. *Seed Science Research* **18**: 1-11.

Sershen, Berjak, P. & Pammenter, N.W. 2010. Effects of cryopreservation on recalcitrant *Amaryllis belladonna* zygotic embryos on vigour of recovered seedlings: a case of stress 'hangover?' *Physiologia Plantarum* **139**: 205-219.

Sershen, Berjak, P., Pammenter, N.W. & Wesley-Smith, J. 2011. Rate of dehydration, state of subcellular organisation and nature of cryoprotection are critical factors contributing to the variable success of cryopreservation: studies on recalcitrant zygotic embryos of *Haemanthus montanus*. *Protoplasma*. DOI 10.1007/s00709-011-0275-4.

Sershen, Pammenter, N.W., Berjak, P. & Wesley-Smith, J. 2007. Cryopreservation of embryonic axes of selected Amaryllid species. *CryoLetters* **28**: 387-399.

Shen, B., Hohman, S., Jensen, R.G. & Bohnert, H.J. 1999. Role of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. *Plant Physiology* **121**: 45-52.

Shimonishi, K., Karube, M. & Ishikawa, M. 2000. Cryopreservation of somatic embryos of sweet potato by slow prefreezing method. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 368-370. Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan.

Smith, M.T. & Berjak, P. 1995. Deteriorative changes associated with the loss of viability of stored desiccation-tolerant and desiccation-sensitive seeds. *In*: J. Kigel & G. Galili (eds), *Seed Development and Germination*, 701–746. Marcel Dekker Inc., New York, U.S.A.

Stanwood, P.C. 1985. Cryopreservation of seed germplasm for genetic conservation. *In*: K.K. Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 199-226. CRC Press, Boca Raton, Florida, U.S.A.

Steponkus, P.L., Langis, R. & Fugikawa, S. 1992. Cryopreservation of plant tissues by vitrification. *In*: P.L. Steponkus (ed.), *Advances in Low-Temperature Biology*, pp. 1-61. JAI Press, Greenwich, Connecticut, U.S.A.

Stolinski, C. & Breathnach, A.S. 1977. Freeze-fracture replication and surface sublimation of frozen collagen fibrils. *Journal of Cell Science* **23**: 325.

Storey, K. & Storey, J. 1996. Natural freezing survival in animals. *Annual Review of Ecology and Systematics* **27**: 365-386.

Sun, W.Q. & Leopold, A.C. 1993. Acquisition of desiccation tolerance in soybeans. *Physiologia Plantarum* **87**: 403-409.

Sun, W.Q. & Leopold, A.C. 1994. The role of sugar, vitrification and membrane phase transition in seed desiccation tolerance. *Physiologia Plantarum* **90**: 621-628.

Sun, W.Q. & Leopold, A.C. 1997. Cytoplasmic vitrification and survival of anhydrobiotic organisms. *Comparative Biochemistry and Physiology* **117**: 327-333.

Sunilkumar, K.K. & Sudhakara, K. 1998. Effect of temperature, media and fungicides on the storage behaviour of *Hopea parviflora* seeds. *Seed Science and Technology* **26**: 781-797.

Sutherland, J.R., Diekmann, M. & Berjak, P. 2002. *Forest Tree Seed Health*. IPGRI Technical Bulletin Number 6. International Plant Genetic Resources Institute, pp. 1-85. Rome, Italy.

Suzuki, K., Daws, M.I. & Pritchard, H.W. 2007. Responses of *Liriope platyphylla* F.T. Wang and T. Tang and *Ophiopogon japonicus* (L.f.) Ker Gawl. seeds to desiccation. *Seed Science and Technology* **35**: 129-133.

Suzuki, T., Kami, D., Oosawa, K. & McGann, L.E. 2005. Cryoprotection in plant tissues related to reduced volume expansion of cryoprotectant solution. *CryoLetters* **26**: 159-168.

Swan, T.W., O'Hare, D., Gill, R.A. & Lynch, P.T. 1999. Influence of preculture conditions on the post-thaw recovery of suspension cultures of Jerusalem artichoke (*Helianthus tuberosus* L.). *CryoLetters* **20**: 325-336.

Tao, D. & Li, P.H. 1986. Classification of plant cell cryoprotectants. *Journal of Theoretical Biology* **123**: 305-310.

Tessereau, H., Florin, B., Meschine, M.-C., Thierry, C. & Pétiard, V. 1994. Cryopreservation of somatic embryos: a tool for germplasm storage and commercial delivery of selected plants. *Annals of Botany* **74**: 547-555.

Theilade, I. & Petri, L. 2003. *Conservation of tropical trees ex situ through storage and use. Guidelines and Technical Notes Number 65*. Danida Forest Seed Centre, Humlebaek, Denmark.

Thierry, C., Tessereau, H., Florin, B., Meschine, M.-C. & Petiard, V. 1997. Role of sucrose for the acquisition of tolerance to cryopreservation of carrot somatic embryos. *CryoLetters* **18**: 283-292.

Tompsett, P.B. & Pritchard, H.W. 1993. Water status changes during development in relation to the germination and desiccation tolerance of *Aesculus hippocastanum* L. seeds. *Annals of Botany*: **107**-116.

Touchell, D.H. & Dixon, K.W. 1993. Cryopreservation of seed of western Australian native species. *Biodiversity and Conservation* **2**: 594-602.

Touchell, D.H., Chiang, V.L. & Tsai, C.-J. 2002. Cryopreservation of embryogenic cultures of *Picea mariana* (black spruce) using vitrification. *Plant Cell Reports* **21**: 118-124.

Towill, L.E. 1988. Survival of shoot tips from mint species after short-term exposure to cryogenic conditions. *HortScience* **23**: 839-841.

Towill, L.E. 1995. Cryopreservation by vitrification. *In*: B.W.W. Grout (ed.), *Genetic Preservation of Plant Cells In Vitro*, pp. 99-112. Springer-Verlag, Berlin, Germany.

Tuckett, R.E., Merritt, D.J., Rudall, P.J., Hay, F., Hopper, S.D., Baskin, C.C., Baskin, J.M., Tratt, J. & Dixon, K.W. 2010. A new type of specialised morphophysiological dormancy and seed storage behaviour in *Hydatellaceae*, an early-divergent angiosperm family. *Annals of Botany* **105**: 1053-1061.

Turner, M. 1996. Species loss in fragments of tropical rain forest; a review of the evidence. *Journal of Applied Ecology* **33**: 200-209.

Uchendu, E. & Reed, B.M. 2008. A comparative study of three cryopreservation protocols for effective storage of mint (*Mentha* spp.). *CryoLetters* **29**: 181-188.

van der Leede-Plegt, L.M., van de Ven, B.C.E., Bino, R.J., van der Salm, T.P.M. & van Tunen, A.J. 1992. Introduction and differential use of various promoters in pollen grains of *Nicotiana glutinosa* and *Lilium longiflorum*. *Plant Cell Reports* **11**: 20-24.

Vanoss, C.J., Giese, R.F. & Norris, J. 1991. Interaction between advancing ice fronts and erythrocytes - mechanism of erythrocte destruction upon freezing and influence of cryoprotective agents. *Cell Biophysics* **18**: 253-261.

van Slageren, M.W. 2003. The Millennium Seed Bank: building partnerships in arid regions for the conservation of wild species. *Journal of Arid Environments* **54**: 195-201.

Varghese, D., Berjak, P. & Pammenter, N.W. 2009. Cryopreservation of shoot tips of *Trichilia emetica*, a tropical recalcitrant-seeded species. *CryoLetters* **30**: 280-290.

Vendrame, A.W., Holliday, C.P., Montello, P.M., Smith, D.R. & Merkle, S.A. 2001. Cryopreservation of yellow-poplar and sweetgum embryogenic cultures. *New Forests* **21**: 283-292.

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

Vertucci, C.W. 1989b. The effect of water contents on physiological activities of seeds. *Physiologia Plantarum* **77**: 172-176.

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

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

Vertucci, C.W. & Farrant, J.M. 1995. Acquisition and loss of desiccation tolerance. *In*: J. Kigel & G. Galili (eds), *Seed Development and Germination*, pp. 237-272. Marcel Dekker Inc., New York, U.S.A.

Vertucci, C.W. & Leopold, A.C. 1987. The relationship between water binding and desiccation tolerance in tissues. *Plant Physiology* **85**: 232-238.

Vertucci, C.W. & Roos, E.E. 1990. Theoretical basis of protocols for seed storage. *Plant Physiology* **94**: 1019-1023.

Vertucci, C.W. & Roos, E.E. 1993. Theoretical basis of protocols for seed storage II. The influence of temperature on optimal moisture levels. *Seed Science Research* **3**: 201-213.

Vertucci, C.W., Roos, E.E. & Crane, J. 1994a. Theoretical basis of protocols for seed storage III. Optimum moisture contents for pea seeds stored at different temperatures. *Annals of Botany* **74**: 531-540.

Vertucci, C.W., Berjak, P., Pammenter, N.W. & Crane, J. 1991. Cryopreservation of embryonic axes of an homeohydrous (recalcitrant) seed in relation to calorimetric properties of tissue water. *CryoLetters* **12**: 339-350.

Vertucci, C.W., Crane, J., Porter, R.A. & Oelke, E.A. 1994b. Physical properties of water in *Zinzania* embryos in relation to maturity status, water content and temperature. *Seed Science Research* **4**: 211-224.

Vertucci, C.W., Crane, J., Porter, R.A. & Oelke, E.A. 1995. Survival of *Zizania* embryos in relation to water content, temperature and maturity status. *Seed Science Research* **5**: 31-40.

Villers, T.A. 1973. Ageing and the longevity of seeds in field conditions. *In*: W. Heydecker (ed.), *Seed Ecology*, pp. 265-288. Butterworth, London, U.K.

Volk, G.M. & Caspersen, A.M. 2007. Plasmolysis and recovery of different cell types in cryoprotected shoot tips of *Mentha piperita*. *Protoplasma* **231**: 215-226.

Volk, G.M. & Walters, C. 2006. Plant vitrification solution 2 lowers water content and alters freezing behaviour in shoot tips during cryoprotection. *Cryobiology* **52**: 48-61.

Volk, G.M., Harris, J.L. & Rotindo, K.E. 2006b. Survival of mint shoot tips after exposure to cryoprotectant solution components. *Cryobiology* **52**: 305-308.

Volk, G.M., Crane, J., Caspersen, A.M., Hill, L.M., Gardner, C. & Walters, C. 2006a. Massive cellular disruption occurs during early imbibition of *Cuphea* seeds containing crystallised triacylglycerols. *Planta* **224**: 1415-1426.

Volk, G.M., Crane, J., Caspersen, A.M., Kovach, D., Gardner, C. & Walters, C. 2007. Hydration of *Cuphea* seeds containing crystallised triacylglycerols. *Functional Plant Biology* **34**: 360-367.

von Teichman, I. & van Wyk, A.E. 1991. Trends in the evolution of dicotyledonous seeds based on character associations, with special reference to pachychalazy and recalcitrance. *Botanical Journal of the Linnean Society* **105**: 211-237.

von Teichman, I. & van Wyk, A.E. 1994. Structural aspects and trends in the evolution of recalcitrant seeds in dicotyledons. *Seed Science Research* **4**: 225-239.

Walker, M.J. 2000. Some investigations of the responses of *Quercus robur* and *Ekebergia capensis* embryonic axes to dehydration and cryopreservation. MSc. Dissertation, University of Natal, Durban, South Africa.

Walters, C. 1998. Understanding the mechanisms and kinetics of seed ageing. *Seed Science Research* **8**: 223-244.

Walters, C. 2000. Levels of recalcitrance in seeds. *Revista Brasileira de Fisiologia Vegetal* **12**: 7-21.

Walters, C. 2003. Optimising seed banking procedures. *In*: R.D. Smith, J.B. Dickie, S.H. Linington, H.W. Pritchard & R.J. Probert (eds), *Seed Conservation Turning Science Into Practice*, pp. 723-743. Cromwell Press Ltd., London, U.K.

Walters, C. 2004. Principles of preserving germplasm in gene banks. *In*: E.O. Guerrant, K. Havens & M. Maunder (eds), *Ex Situ Plant Conservation: Supporting Species Survival in the Wild*, pp. 113-138. Island Press, Washington D.C., U.S.A.

Walters, C. & Towill, L. 2004. *Seeds and pollen. Agricultural handbook number 66. The commercial storage of fruits, vegetables, and florist and nursery stocks.* USDA-ARS, National Center for Genetic Resources Preservation of Plant Germplasm Research, Fort Collins, U.S.A. http://www.ba.ars.usda.gov/hb66/153seeds.pdf. (downloaded on 14 January 2011)

Walters, C., Wheeler, L. & Grotenhuis, J.M. 2005. Longevity of seeds stored in a genebank: species characteristics. *Seed Science and Technology* **15**: 1-20.

Walters, C., Pammenter, N.W., Berjak, P. & Crane, J. 2001. Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. *Seed Science Research* **11**: 135-148.

Walters, C., Farrant, J.M., Pammenter, N.W., Berjak, P. & Crane, J. 2002a. Desiccation stress and damage. *In*: M. Black & H.W. Pritchard (eds), *Desiccation and Survival in Plants: Drying Without Dying*, pp. 263-291. CABI Publishing, Wallingford, Oxon, U.K.

Walters, C., Touchell, D.H., Power, P., Wesley-Smith, J. & Antolin, M.F. 2002b. A cryopreservation protocol for embryos of the endangered species *Zizania texana*. *CryoLetters* **23**: 291-298.

Walters, C., Wesley-Smith, J., Crane, J., Hill, L., Chmielarz, P., Pammenter, N.W. & Berjak, P. 2008. Cryopreservation of recalcitrant (i.e. desiccation-sensitive) seeds. *In*: B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide*, pp. 465-484. Springer-Verlag, New York, U.S.A.

Wang, B.S.P., Charest, P.J., & Downie, B. 1993. *Ex situ storage of seeds, pollen and in vitro cultures of perennial woody plant species, FAO Forestry paper 113*. Food and Agriculture Organisation of the United Nations, Rome, Italy.

Wang, Z., Lehmann, D., Bell, J. & Hopkins, A. 2002. Development of an efficient plant regeneration system for Russian wild rye (*Psathyrostachys juncea*). *Plant Cell Reports* **20**: 797-801.

Weibel, E.R. & Bolender, R.P. 1973. Stereological techniques for electron microscope morphometry. *In*: M.A. Hayat (ed.), *Principles and Techniques of Electron Microscopy: Biological Applications*, *Volume 3*, pp. 237-296. Van Nostrand-Reinhold, New York, U.S.A.

Wesley-Smith, J. 2002. Investigations into the responses of axes of recalcitrant seeds to dehydration and cryopreservation. PhD. Thesis. University of Natal, Durban, South Africa.

Wesley-Smith, J., Pammenter, N.W. & Vertucci, C.W. 1995. Ultrastructural evidence for the effects of freezing in embryonic axes of *Pisum sativum* L. at various water contents. *Annals of Botany* **76**: 59-64.

Wesley-Smith, J., Pammenter, N.W., Berjak, P. & Walters, C. 2001a. The effects of two drying rates on the desiccation tolerance of embryonic axes of recalcitrant jackfruit (*Artocarpus heterophyllus* Lamk.) seeds. *Annals of Botany* **88**: 653-664.

Wesley-Smith, J., Vertucci, C.W., Berjak, P. & Pammenter, N.W. 1999. A method for the cryopreservation of embryonic axes at ultra-rapid cooling rates. *In*: M. Marzalina, K. Khoo, N. Jayanthi, F.Y. Tsan & B. Krishnapillay (eds), *IUFRO Symposium 1998 Recalcitrant Seeds*, pp. 132-139. FRIM, Kuala Lumpur, Malaysia.

Wesley-Smith, J., Walters, C., Berjak, P. & Pammenter, N.W. 2004b. Non-equilibrium cooling of *Poncirus trifoliata* (L.) embryonic axes at various water contents. *CryoLetters* **25**: 121-128.

Wesley-Smith, J., Walters, C., Pammenter, N.W. & Berjak, P. 2001b. Interactions among water contents, rapid (non-equilibrium) cooling to -196°C and survival of embryonic axes of *Aesculus hippocastanum* L. seeds. Cryobiology **42**: 196-206.

Wesley-Smith, J., Walters, C., Pammenter, N.W. & Berjak, P. 2004a. The influence of water content, cooling and warming rate upon survival of embryonic axes of *Poncirus trifoliata* (L.). *CryoLetters* **25**: 129-138.

Wesley-Smith, J., Vertucci, C.W., Berjak, P., Pammenter, N.W. & Crane, J. 1992. Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. *Journal of Plant Physiology* **140**: 596-604.

Whitaker, C., Beckett, R.P., Minibayeva, F.V. & Kranner, I. 2010. Production of reactive oxygen species in excised, desiccated and cryopreserved explants of *Trichilia dregeana*. *South African Journal of Botany* **76**: 112-118.

White, P.S. & Jentsch, A. 2001. The search for generality in studies of disturbance and ecosystem dynamics. *Progress in Botany* **62**: 399-450.

Wikefeldt, P. 1971. Growth of an ice phase in frozen tissue studied by proton NMR-spectroscopy. *Cryobiology* **8**: 589-593.

Williams, J. 1997. Technical and political factors constraining reserve placements. *In*: N. Maxted, B. Ford-Lloyd & J.G. Hawkes (eds), *Plant Genetic Conservation: The In Situ Approach*, pp. 88-98. Chapman and Hall, London, U.K.

Withers, L.A. 1978. Freeze-preservation of cultured cells and tissues. *In*: T.A. Thorpe (ed.), *Frontiers of Plant Tissue Culture 1978*, pp. 297-306. International Association for Plant Tissue Culture/Calgary University Press, Calgary, Canada.

Withers, L.A. 1979. Freeze preservation of somatic embryos and clonal plantlets of carrots (*Daucus carota* L.). *Plant Physiology* **63**: 460.

Withers, L.A. 1985. Cryopreservation of cultured plant cells and protoplasts. *In*: K.K. Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 243-267. CRC Press, Boca Raton, Florida, U.S.A.

Withers, L.A. 1988. Germplasm preservation. *In*: G. Bock & J. Marsh (eds), *Application of Plant Cell and Tissue Culture*, pp. 163-177. John Wiley and Sons Ltd., Chichester, U.K.

Withers, L.A. & Engelmann, F. 1998. *In vitro* conservation of plant genetic resources. *In*: A. Altman (ed.), *Agricultural Biotechnology*. Marcel Dekker Inc., New York, U.S.A.

Withers, L.A. & Engels, J.M.M. 1990. The test tube genebank - a safe alternative to field conservation. *IBPGR Newsletter for Asia and the Pacific* **3**: 1-2.

Withers, L.A. & King, M. 1980. A simple freezing unit and routine cryopreservation methods for plant cell cultures. *CryoLetters* **1**: 213-220.

Yadav, S.S., McNeil, D. & Stevenson, P.C. 2007. *Lentil: An Ancient Crop for Modern Times*. Springer-Verlag, New York, U.S.A.

Yancey, P.H. 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *The Journal of Experimental Biology* **208**: 2819-2830.