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Development of strategies towards the cryopreservation of germplasm of *Ekebergia capensis* Sparrm. – an indigenous species that produces recalcitrant seeds

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Development of strategies towards the cryopreservation of germplasm of *Ekebergia capensis* Sparrm. – an indigenous species that produces recalcitrant seeds

By

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ABSTRACT

The conservation of germplasm of indigenous plant species is vital not only to preserve valuable genotypes, but also the diversity represented by the gene pool. A complicating factor, however, is that a considerable number of species of tropical and sub-tropical origin produce recalcitrant or otherwise non-orthodox seeds. Such seeds are hydrated and metabolically active when shed and cannot be stored under conventional conditions of low temperature and low relative humidity. This poses major problems for the long-term conservation of the genetic resources of such species. Presently, the only strategy available for the long-term conservation of species that produce recalcitrant seeds is cryopreservation.

Ekebergia capensis is one such indigenous species that produces recalcitrant seeds. The aim of the present study was to develop methods for the cryopreservation of germplasm of this species. Different explant types were investigated for this purpose, *viz.* embryonic axes (with attached cotyledonary segments) excised from seeds, and two *in vitro*-derived explants, i.e. 'broken' buds excised from *in vitro*-germinated seedlings and adventitious shoots generated from intact *in vitro*-germinated roots. Suitable micropropagation protocols were developed for all explant types prior to any other experimentation.

Before explants could be cryopreserved it was necessary to reduce their water content in order to limit damaging ice crystallisation upon cooling. All explants tolerated dehydration (by flash drying) to 0.46 – 0.39 g g^{-1} water content (dry mass basis) with survival ranging from 100 – 80%, depending on the explant. In addition, penetrating and non-penetrating cryoprotectants were used to improve cryo-tolerance of explants. The cryoprotectants tested were sucrose, glycerol, DMSO and a combination of sucrose and glycerol. Explant survival following cryoprotection and dehydration ranged from 100 – 20%. Cryoprotected and dehydrated explants were exposed to cryogenic temperatures by cooling at different rates, since this factor is also known to affect the success of a cryopreservation protocol. The results showed that 'broken' buds could not tolerate cryogen exposure. This was likely to have been a consequence of the large size

of explants and their originally highly hydrated condition. Adventitious shoots tolerated cryogenic exposure slightly better with 7 – 20% survival after cooling in sub-cooled nitrogen. Limited shoot production (up to 10%) was obtained when axes with attached cotyledonary segments were exposed to cryogenic temperatures. In contrast, root production from axes cooled in sub-cooled nitrogen remained high (67 – 87%). Adventitious shoots were subsequently induced on roots generated from cryopreserved axes by applying a protocol developed to generate adventitious shoots on *in vitro*-germinated roots. In this manner, the goal of seedling establishment from cryopreserved axes was attained.

Each stage of a cryopreservation protocol imposes stresses that may limit success. To gain a better understanding of these processes the basis of damage was investigated by assessing the extracellular production of the reactive oxygen species (superoxide) at each stage of the protocol, as current thinking is that this is a primary stress or injury response. The results suggested that superoxide could not be identified as the ROS responsible for lack of onwards development during the cryopreparative stages or following cryogen exposure.

The stresses imposed by the various stages of a cryopreservation protocol may affect the integrity of germplasm. Since the aim of a conservation programme is to maintain genetic (and epigenetic) integrity of stored germplasm, it is essential to ascertain whether this has been achieved. Thus, explants (axes with cotyledonary segments and adventitious shoots) were subjected to each stage of the cryopreservation protocol and the epigenetic integrity was assessed by coupled restriction enzyme digestion and random amplification of DNA. The results revealed little, if any, DNA methylation changes in response to the cryopreparative stages or following cryogen exposure.

Overall, the results of this study provided a better understanding of the responses of germplasm of *E. capensis* to the stresses of a cryopreservation protocol and two explant types were successfully cryopreserved. Future work can be directed towards elucidating the basis of damage incurred so that more effective protocols can be developed. Assessment of the integrity of DNA will give an indication as to the suitability of

developed protocols, or where changes should be made to preserve the genetic (and epigenetic) integrity of germplasm.

PREFACE

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

This study represents original work by the author, and no part of this work has been submitted in any form to another university. Where use has been made of work of other authors, it has been duly acknowledged in the text.

_____

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DECLARATION 1 - PLAGIARISM

I, Elliosha Hajari, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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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: Hajari, E, Berjak, P, Pammenter, N.W. and Watt, M.P. 2009. Micropropagation of recalcitrant-seeded *Ekebergia capensis* Sparrm. using *in vitro* nodal segments and roots. *Journal of Horticultural Science and Biotechnology* **84**: 87 – 91.

I carried out all the experimental work and wrote this paper and my supervisors provided input on the draft. Chapter 2 was written subsequently, with the paper as a basis.

Publication 2: Hajari, E, Berjak, P, Pammenter, N.W. and Watt, M.P. 2011. A novel means for cryopreservation of germplasm of the recalcitrant-seeded species, *Ekebergia capensis*. *CryoLetters – in press.*

I carried out all the experimental work and wrote Chapter 3 first. Then I worked with Prof. Berjak on this paper, and subsequently dealt with the reviewer's comments.

Signed:

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ABA	abscisic acid
AFLP	amplified fragment length polymorphism
AMP PCR	amplified DNA methylation polymorphism
ANOVA	analysis of variance
AOS	active oxygen species
BAP	6-benzylaminopurine
$CaCl_2.2H_2O$	calcium chloride dihydrate
CaMg	calcium magnesium rehydration solution
$Ca(OCl)_2$	calcium hypochlorite
CAT	catalase
CPU	computer processing unit
CRED-RA	coupled restriction enzyme digestion and random amplification
CTAB	cetyltrimethyl ammonium bromide
D	direct shoot regeneration
2,4-D	2,4-dichlorophenoxyacetic acid
dmb	dry mass basis
$DMSO/Me_2SO$	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DSE	direct somatic embryogenesis
DTT	dithiothreitol
FDA	fluorescein diacetate
$g\ g^{-1}$	gram per gram
$HgCl_2$	mercuric chloride
HPCE	high pressure capillary electrophoresis
HPLC	high pressure liquid chromatography
I	indirect shoot regeneration
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2-iP	2-isopentyladenine
ISE	indirect somatic embryogenesis
kb	kilobase
kPa	kilopascal

LEAs	Late embryogenic accumulating/abundant proteins
LS	Linsmaier and Skoog
LUMA	Luminometric Methylation Assay
MDA	malondialdehyde
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
MPa	Megapascal
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog salts and vitamins (1962)
MSAP	methylation-sensitive amplified polymorphism
Ms-SnuPe	methylation-sensitive single-nucleotide primer extension
n	sample size
NAA	napthaleneacetic acid
NaOCl	sodium hypochlorite
nmol g^{-1} s^{-1}	nanomole per gram per second
np	non-penetrating
NT	not tested
O	orthodox seeds
p	penetrating
p value	probability value
PCR	polymerase chain reaction
PGRs	plant growth regulators
PPFD	photosynthetic photon flux density
PPM	plant preservative mixture
PVP	polyvinlypyrrolidone
PVS	plant vitrification solution
R	recalcitrant seeds
*R	minimally recalcitrant seeds
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RITA®	Rėcipient à Immersion Temporaire Automatique
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SH	Schenk and Hildebrandt
SOD	superoxide dismutase

spp.	species
SSR	simple sequence repeats
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
TBE	tris boric acid disodium ethylene diamine tetra acetic acid
Tg	glass transition temperature
TDZ	thidiazuron
TTZ	2,3,5-triphenyl tetrazolium chloride
$\mu mol\ m^{-2}\ s^{-1}$	micromole per metre squared per second
U	units
UV	ultraviolet
V	volts
v/v	volume per volume
WC	water content
w/v	weight per volume

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CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Plant biodiversity uses and conservation

It is well known that plant and animal biodiversity is under threat as a result of habitat modification due to anthropogenic activities such as farming, developments associated with industrialisation and urbanisation and consequent changing patterns in global climate (Thomas *et al.*, 2004). Plant breeding strategies have also contributed to the loss of genetic diversity by the cultivation of a few high-yielding varieties of crops, characterised by narrow genetic bases (Staritsky, 1997; Hancock, 1998). In South Africa (refer to Figure 1.1), there is the additional factor that a large proportion of the human population (70 – 80%) relies on traditional medicine, using plants for the treatment of a variety of ailments, illnesses and disease (Cunningham, 1993; Mander *et al.*, 1996). Even before 2000, this had resulted in approximately 19 500 tonnes of plant material being traded per year, with a market value of approximately R 273 million (Mander, 1998). In the KwaZulu-Natal Province alone over 400 species are used in the plant trade but very few are cultivated for that purpose (Mander *et al.*, 1996). As most plants used in the indigenous plant trade are harvested from naturally-occurring populations (Mander, 1998), and the collection of various plant parts (e.g. roots, tubers, bark) is not done in a sustainable manner (Dold and Cocks, 2002), natural populations are being severely depleted.

Concern about high levels of biodiversity loss resulted in the establishment of the Convention on Biological Diversity, which was signed at the United Nations Conference on Environment and Development in Rio de Janeiro in June, 1992. This represents an international agreement designed to curb biodiversity loss significantly and ensure sustainable and fair use of biodiversity (Convention on Biological Diversity, 2005). In 2002, the Global Strategy for Plant Conservation was adopted which aimed to reduce biodiversity loss drastically by 2010. Towards this end, 16 outcome-oriented global targets were outlined, including conservation of at least 10% of the world's ecological regions, establishment of *in situ* and *ex situ* collections for 60% of the world's threatened species and conservation of 70% of the genetic diversity of socio-

economically important crops and plant species. This policy outlined a framework for the implementation of conservation efforts at local, national, regional and global levels (Global Strategy for Plant Conservation online, 2002).

Figure 1.1: Map of South Africa (Source: D.K. World Atlas, 2008).

1.2 Strategies for conservation of plant germplasm

There are two broad approaches to the conservation of plant germplasm, *viz. in situ* and *ex situ* conservation. The former is the conservation of species in their natural habitats as uncultivated plant communities in nature reserves, natural parks or protected areas (Krøgstrup *et al*., 1992; Withers and Engelmann, 1998; Rao, 2004), the advantage being that it allows for natural evolution to occur (Arora and Paroda, 1991). However, the disadvantages are that large areas of land are required, the maintenance of parks and reserves are expensive, a large number of individuals are required to maintain viable

populations and *in situ* collections are prone to natural disasters such as pests and fires (Engelmann, 1991; Moss, 1994; Berjak *et al*., 1996; Phartyal *et al*., 2002).

Ex situ conservation is the conservation of plants outside their natural habitats. By this means germplasm can be stored as collections in botanic gardens, arboreta, field genebanks, seedbanks or *in vitro* cultures (Krøgstrup *et al*., 1992; Razdan and Cocking, 1997). The disadvantages include land space requirements and labour costs; however, this is the only available conservation strategy in cases where the natural habitat of a plant species has been destroyed (Li *et al*., 2002) or natural populations are at risk from human activity. Currently a significant additional threat has been recognised, which demands *ex situ* conservation measures: this is habitat destruction as a consequence of climate change (Hannah *et al*., 2002). Germplasm conserved under *ex situ* conditions can be stored as either active or base collections (Krøgstrup *et al*., 1992; Ruiz *et al*., 1999). Active collections provide germplasm for evaluation and distribution (stores of germplasm for the short-to medium-term) and are most commonly kept as seed stocks, but also in the field (Withers and Engelmann, 1998) and greenhouse (Krøgstrup *et al*., 1992; Staritsky, 1997). A base collection stores germplasm in the long-term and acts as a back up if material is lost from the active collection (Towill, 2000).

1.3 Conservation of indigenous African species: a study on *Ekebergia capensis* Sparrm.

As indigenous plant species are being non-sustainably harvested for traditional medicinal purposes, there is a clear need for the conservation of such species. One such example is the species under investigation in the present study, *viz. Ekebergia capensis* Sparrm., an indigenous African species of the family, Meliaceae. It is a semi-deciduous to evergreen tree that is distributed from the southern Cape through KwaZulu-Natal to Ethiopia in the north with some trees also found inland in Zambia and Angola (White, 1986; Pooley, 1993).

Ekebergia capensis is traded for a variety of traditional medicinal purposes (von Ahlefeldt *et al*., 2003). Preparations of the bark are used to treat skin conditions such as

abscesses, boils and pimples (Pujol, 1990), as an emetic to purify the blood (Pujol, 1990), for the relief of heartburn (Watt and Breyer-Brandwijk, 1962), to treat exhaustion, and as a charm to counteract witchcraft (Pujol, 1990; Hutchings *et al.*, 1996). Decoctions of the roots are used to treat dysentery (Pooley, 1993) and relieve headaches and coughs (Watt and Breyer-Brandwijk, 1962; Palgrave and Drummond, 1984); the leaves have been used as an antihelmintic for the treatment of intestinal worms and for scabies, and the bark is also used for tanning (Watt and Breyer-Brandwijk, 1962; Palgrave and Drummond, 1984). In addition, this species provides an attractive street tree (Palgrave and Drummond, 1984). Although *E. capensis* is not harvested as intensively as some other indigenous species, demand remains high and the legal status of *E. capensis* in KwaZulu-Natal is listed as controlled (von Ahlefeldt *et al.*, 2003). Further, *E. capensis* produces recalcitrant seeds (Pammenter *et al.*, 1998), which cannot be stored using conventional approaches (see below). This poses major problems for the long-term conservation of the germplasm of such species (Berjak and Pammenter 2001; 2004a).

1.4 Seed storage characteristics

Seeds were originally separated into two broad categories based on differences displayed in their physiology and post-harvest behaviour, *viz.* orthodox (desiccation tolerant) and recalcitrant seeds (desiccation sensitive) (Roberts, 1973). A third category was also identified, *viz.* intermediate seeds, to describe those exhibiting characteristics intermediate between those of orthodox and recalcitrant seeds, i.e. they can tolerate a greater degree of dehydration than recalcitrant seeds but not as much as orthodox seeds, and are generally not long lived (Ellis *et al.*, 1990; Hong and Ellis, 1996). However, variation within categories has since led to the suggestion that post-harvest seed behaviour should be viewed as a continuum, with seeds displaying characteristics of extreme orthodoxy on one end, and those displaying the highest degree of recalcitrance on the other end, with some (perhaps many) species falling variously between the two extremes (Berjak and Pammenter, 1994; 1997; 2001).

A characteristic of orthodox seeds is that they acquire desiccation tolerance, with most naturally undergoing the process of maturation drying while still on the parent plant. During this process, seeds dry down to a relatively low water content of 20% (0.25 g g^{-1} dry mass basis [dmb]) or less (Roberts and King, 1980). They are usually shed or harvested at this point and are capable of further dehydration down to water contents of around 5% (0.053 g g^{-1} dmb), without displaying signs of damage (Roberts and King, 1980; Vertucci and Farrant, 1995). In contrast, recalcitrant seeds do not acquire desiccation tolerance and consequently do not undergo maturation drying. As a result, seeds are hydrated and metabolically active when shed (water contents range from 0.3 to 4.0 g g^{-1} or more depending on the species) and remain desiccation sensitive, losing viability if the water content is reduced below some relatively high value (Roberts and King, 1980; Pammenter *et al*., 1998; Berjak and Pammenter, 2001). As demonstrated by Pammenter *et al*. (1998) the water content at which viability is lost, depends on the drying rate, thus one cannot define unqualified 'critical' water contents.

Plant species that produce recalcitrant seeds often occur in wet or humid environments in tropical and sub-tropical regions (Roberts and King, 1980; Tweddle *et al*., 2003). However, such species also occur in some temperate regions (Berjak *et al*., 1999; Corredoira *et al*., 2004) and occasionally dry tropical zones in Africa (Danthu *et al*., 2000; Gaméné *et al*., 2004). Seed recalcitrance (basically desiccation sensitivity) does not appear to be a common phenomenon, although it does occur across a range of plant taxa (Dickie and Pritchard, 2002). According to Tweddle *et al*. (2002), of 6 919 species examined, only 514 (7.4%) have been reported to produce (or be likely to produce) recalcitrant seeds. Some examples include species of commercial importance such as cocoa (*Theobroma cacao*), rubber (*Hevea brasiliensis*), mango (*Mangifera indica*), litchi (*Litchi sinensis*) and jackfruit (*Artocarpus heterophyllus*) (King and Roberts, 1980; Chaudhury and Malik, 1998; Berjak and Pammenter, 2001). Besides *E. capensis*, other meliaceous trees producing recalcitrant seeds include *Trichilia* spp., as do some gymnospermous species such as yellowwood (*Podocarpus henkelii*) (Berjak and Pammenter, 2001), and the cycads *Encephalartos gratus* and *E. natalensis* (Woodenberg *et al*., 2007). A few monocotyledonous species have so far been identified

as being recalcitrant-seeded such as coconut (*Cocos nucifera*), some other palms (Daws *et al*., 2007) and amaryllids (Sershen *et al*., 2008).

1.4.1 Developmental, morphological and chemical characteristics of recalcitrant seeds

The development of orthodox seeds is characterised by three distinct stages: histodifferentiation, reserve deposition and expansion, and maturation drying (e.g. Bewley and Black, 1994; Kermode, 1995; Kermode and Finch-Savage, 2002). During histodifferentiation, extensive cell division follows zygote formation, followed by differentiation to form the multicellular embryo. This stage is characterised by an increase in seed fresh mass and a high water content (Kermode and Finch-Savage, 2002). During the expansion stage, cell division ceases and the laying down of reserves and intensive growth accompanies cell enlargement (Bewley and Black, 1994; Kermode and Finch-Savage, 2002). Some water is lost as reserve deposition occurs when reserves replace water in storage tissues, with embryo dry mass increasing (Kermode and Finch-Savage, 2002). The ability to tolerate desiccation is then acquired, preceding the final stage, maturation drying, during which seed tissues lose water and the embryo enters a metabolically inactive or quiescent state. The maturation drying stage is characterised by a reduction in fresh weight effected by the substantial decrease in water content (Bewley and Black, 1994; Kermode and Finch-Savage, 2002).

In recalcitrant seeds, the histodifferentiation stage occurs in a similar manner to that in orthodox seeds (Farrant *et al*., 1992). Following this, reserve deposition and cell expansion occur with a corresponding gain in dry mass (Farrant *et al*., 1992). In contrast to orthodox seed development, however, dry matter continues to accumulate in recalcitrant seeds, which generally do not lose fresh weight and show no substantial decline in water content (Farrant *et al*., 1992; Finch-Savage *et al*., 1992). A slight decline in water content during development of some temperate recalcitrant-seeded species has been observed in, for example *Quercus robur* (Finch-Savage, 1992), *Aesculus hippocastanum* (Tompsett and Pritchard, 1993) and some sub-tropical species such as *Machilus thunbergii* (Lin and Chen, 1995). However, the observed decline in

water content has, in some cases, been attributed to the increase in seed dry weight relative to total tissue water during the latter stages of reserve deposition, rather than a loss of water from the seed (Tompsett and Pritchard, 1993). While the seeds of some species may tolerate a slight decline in water content, recalcitrant seeds never acquire a substantial degree of desiccation tolerance and further dehydration is lethal (Finch-Savage, 1992).

Considering the number of similarities that exist during development of orthodox and recalcitrant seeds, Finch-Savage and Blake (1994) suggested that seed recalcitrance might occur as a result of incomplete or truncated development. Using *Q. robur* as an example, those authors suggested that seed development in recalcitrant seeds ends before true tolerance to desiccation develops. Consequently, those authors suggested that development in recalcitrant seeds might be described as indeterminate (Finch-Savage and Blake, 1994). Similarly, Vertucci and Farrant (1995) suggested that recalcitrance might be a consequence of early interruption of development. However, desiccation tolerance of recalcitrant seeds could not be increased by manipulating seed developmental conditions, thereby implying that there are inherent limitations to the development of desiccation tolerance (Kermode and Finch-Savage, 2002).

Stated simply, the underlying characteristic of recalcitrant seeds is that they are desiccation sensitive, although, the extent of desiccation tolerated varies among different species (Berjak and Pammenter, 2008). This variation may be due to a number of factors such as seed size, structure, chemical composition, developmental status, etc. (Berjak and Pammenter, 2001; 2004a). In addition, differences may exist in the water content of different components of seeds. Tompsett and Pritchard (1993) reported that the water content of axes of *A. hippocastanum* was higher than that of the seed storage tissues. Similar results were reported for *M. thunbergii* (Lin and Chen, 1995), *Araucaria hunsteinii* (Pritchard *et al*., 1995), *T. cacao* (Li and Sun, 1999), *E. capensis* (Erdey *et al*., 2004) and *Warburgia salutaris* (Kioko *et al*., 2004).

Inter- and intra-seasonal variation in characteristics of recalcitrant seeds are common (Berjak and Pammenter, 1997; 2004a). For example, individual seeds within the same

batch may have different water contents; the water content of seeds may vary depending on the time during the season they are harvested (intra-seasonal variation); and seeds harvested in consecutive years may have considerably different water contents (inter-seasonal variation). Further, seeds produced late in the season are reported to be of poor quality and may be heavily fungally infected (Berjak *et al.*, 1996; Berjak and Pammenter, 1997; 2004a).

Daws *et al*. (2004) provided a possible explanation that might relate to the poor quality of late season seeds. Those authors suggested that environmental conditions influenced the development of *A. hippocastanum* seeds so that those that developed under warmer conditions (i.e. that had a higher cumulative heat sum) were more developed, had accumulated more storage reserves and were relatively more desiccation tolerant than seeds that developed under cooler climatic conditions. Berjak and Pammenter (2008) applied this logic to the situation that may occur during the end of the season in non-equatorial zones. Those authors suggested that flowers produced late in the season produce fruit that were exposed to lower air temperatures and shorter days at the end of summer. As a result, fruits developing during this time were subject to a lower heat sum (i.e. below the optimal levels that were achieved earlier in the season). Consequently, fruit and seed development may be adversely affected with poor quality seeds being produced, i.e. they are less developed and less tolerant to desiccation than seeds produced earlier in the season.

Another characteristic typical of recalcitrant seeds is that in addition to being hydrated when shed, they are also metabolically active (Berjak *et al.*, 1989; Finch-Savage, 1996). This characteristic of ongoing metabolism is suggested to be the most significant factor underlying the response of recalcitrant seeds to desiccation (Pammenter and Berjak, 1999). The extent of metabolic activity may vary depending on the species, developmental status and water content at which seeds are shed and, although there may be a slight decline in metabolic rate as seeds mature (as reported by Finch-Savage, 1996), there is no cessation of metabolism. In contrast, orthodox seeds undergo an extensive decline in metabolic activity preceding and during the maturation drying stage (Bewley and Black, 1994; Kermode and Finch-Savage, 2002).

As a result of ongoing metabolism, recalcitrant seeds progress towards germination, even in the absence of an externally supplied source of water (Berjak *et al.*, 1989). Depending on the relative developmental state of recalcitrant seeds, well differentiated axes may be poised for germination-associated events on, or soon after, shedding, while those axes that are relatively undifferentiated will proceed through the necessary developmental stages after seed shed, before germination can occur (Berjak *et al.*, 1989). Considering this, there is considerable variation in the extent of dehydration that such seeds can tolerate since tolerance to desiccation varies at different seed developmental stages (Kermode and Finch-Savage, 2002). Chandel *et al.* (1995) found differences in the response of recalcitrant seeds to dehydration at different developmental (maturity) stages for tea, jackfruit and cocoa. In general, it appears that recalcitrant seeds are most tolerant to dehydration when seed metabolic rate is at its lowest, which commonly (although not always) coincides with natural shedding (Berjak *et al.*, 1999). As the metabolic events associated with germination proceed, seeds again become increasingly sensitive to dehydration.

A problem often experienced when working with recalcitrant seeds is the difficulty in identifying when seeds are physiologically mature (Berjak and Pammenter, 2004a). Orthodox seeds mature and are generally shed after maturation drying (Bewley and Black, 1994). Considering that recalcitrant seeds progress from development to germination without obvious punctuation, there are no macroscopic indicators of the termination of development (Berjak and Pammenter, 2004a). As highlighted by Berjak *et al*. (1999), seeds are generally harvested on the basis of fruit colour as an indicator of seed maturity. However, this is not an accurate indicator since a batch of seeds hand-harvested in this manner could contain pre-mature seeds, mature seeds as well as seeds that have already initiated germination associated events, while collection of seeds that have already abscised from parent plants may include those at various post-shedding developmental stages (Berjak *et al.*, 1996). This presents further problems as the presence of a range of developmental stages in any one batch of seeds can result in unpredictable variation in the response of axes to dehydration and cryostorage within an individual sample of seeds, and from one sample to the next.

1.5 The role of water in seeds

Considering that recalcitrant seeds are desiccation sensitive, it is important to understand the function of water in seeds and the consequences of water loss. Water plays a vital role in biological processes as a solvent for diffusion of substrates, a reactant or product in a range of biochemical reactions and structurally, in filling cellular spaces and stabilising macromolecules and intra-cellular structures by hydrophilic and hydrophobic interactions (Wolfe and Bryant, 2001; Koster and Bryant, 2005). Hence, water plays an essential role in controlling the metabolic activity of seeds (Clegg, 1978; Leopold and Vertucci, 1989). The removal of water from seeds results in physical and mechanical stresses as cells lose turgidity. In metabolically active, recalcitrant seeds, metabolism is held to become unbalanced, accompanied by uncontrolled free radical generation (Walters *et al*., 2001). Further, undesirable lipid phase transitions may occur, and the situation may be exacerbated by attack by free radicals and subsequent lipid peroxidation (discussed by Vertucci and Farrant, 1995 and references therein). Consequently, membranes are often the primary site of damage accompanying dehydration and cryogen exposure (Wolfe and Bryant, 2001). Some changes resulting from water loss may be reversible upon rehydration; however, desiccation damage is exhibited by the inability of cells to resume normal functioning upon rehydration (Walters *et al*., 2002a).

It has been suggested that the properties of water are different depending on the hydration level of tissues and that water present at particular hydration levels enables particular functions (Vertucci, 1990). Consequently, the loss of water corresponding to a particular hydration level may lead to a corresponding loss of function. Considering this, five hydration levels (or types of water) have been identified based on the interaction between water molecules and cell constituents (Vertucci, 1990; Vertucci and Roos, 1990). Type 5 water (hydration level V) is dilute solution water and occurs at water potentials lower than -2 MPa (water content 0.6 – 0.9 g g^{-1}). This is typical of fully hydrated seeds and Vertucci (1990) suggested that type 5 water maintains turgor of seeds. In highly recalcitrant seeds of *Avicennia marina*, the removal of this type of water is lethal (Farrant *et al*., 1993). Type 4 water (hydration level IV) is concentrated

solution water (capillary water) that may be present at water potentials ranging from -2 to -4 MPa (water content 0.7 – 0.45 g g^{-1}). This hydration level represents the permanent wilting point for vegetative cells and removal of this type of water is lethal to all immature embryos and seedlings.

Type 3 water (hydration level III) occurs at water potentials between -4 and -11 MPa (water content 0.45 – 0.25 g g^{-1}) and this is suggested to form bridges over hydrophobic entities of macromolecules. Orthodox seeds (and some recalcitrant seeds) can survive dehydration to within the third hydration level, provided they are not held at this hydration level for extended periods. In the case of recalcitrant embryonic axes, temporary survival of this degree of dehydration is dependent upon water being lost rapidly (Pammenter *et al*., 1998). If all type 3 water is lost, then changes in membrane structure are likely (Bryant and Wolfe, 1989; 1992). Recalcitrant seeds do not survive removal of *all* type 3 water (Pammenter *et al*., 1991; Berjak *et al*., 1992; 1993). Type 2 water (hydration level II) occurs between water potentials of -12 and -150 MPa (water content 0.25 – 0.05 g g^{-1}) and is suggested to interact strongly with polar surfaces of macromolecules and hydroxyl groups of solutes. Type 1 water (hydration level I) occurs at water potentials less than -150 MPa (water content less than 0.08 g g^{-1}) and theoretically indicates the water that binds to macromolecules as structural components (Vertucci and Roos, 1990; Walters *et al*., 2005). Intermediate seeds are killed if this type of water is removed (Ellis *et al*., 1990; 1991) and the long-term viability of orthodox seeds and pollen may also be affected (Vertucci and Roos, 1990; Hoekstra *et al*., 1992; Walters and Engels, 1998). One factor contributing to differences in tolerance of orthodox and recalcitrant seeds to desiccation is likely to be the ability of orthodox seeds to tolerate the removal of a larger proportion of hydration water than recalcitrant seeds (Berjak *et al*., 1984; Vertucci and Leopold, 1987; Pammenter *et al*., 1991; Grange and Finch-Savage, 1992).

1.5.1 Dehydration stress and tolerance mechanisms

Desiccation tolerance in seeds (at least in orthodox seeds) is a consequence of a series of programmed events that occur during seed development, such that if development

proceeds naturally, desiccation tolerance is an inevitable consequence. Therefore in the context of orthodox seed behaviour, tolerance is the outcome of a series of interacting processes under coordinated multi-genic control (Berjak and Pammenter, 2004a). When this situation is compared with recalcitrant seed development, it is suggested that the phenomenon of recalcitrance may be at least partly explained by the incomplete expression or interaction of factors necessary to confer tolerance (Pammenter and Berjak, 1999), as discussed below.

a) Intracellular physical characteristics and metabolic shut down

Dehydration results in the loss of water from cells and a consequent reduction in cell volume (Farrant, 2000). Desiccation-tolerant orthodox seeds can withstand the mechanical stress resulting from volume reduction by reducing the volume of vacuoles, accumulating insoluble storage reserves within and outside of vacuoles and by the organised disassembly of the cytoskeleton and minimisation of membrane surface area preceding and during dehydration, and reorganisation upon rehydration (Farrant *et al*., 1997; Faria *et al*., 2005). Desiccation tolerant organisms must also protect DNA during dehydration and be able to repair damage upon rehydration, so that a functional genome is maintained (Osborne *et al*., 2002). In contrast, recalcitrant seeds do not display most or all of these characteristics (Farrant *et al*., 1997; Boubriak *et al*., 2000; Gumede *et al*., 2003), which must contribute to their intolerance of dehydration (Mycock *et al*., 2000).

A characteristic trait of orthodox seeds is the observed intracellular de-differentiation that occurs preceding and during the maturation drying stage that leads to a reduction in physiological and metabolic activity (Klein and Pollock, 1968; Farrant *et al*., 1999). Hence, intracellular de-differentiation protects organelles and contributes to desiccation tolerance, and its maintenance by a controlled shutdown of activities. In orthodox seeds, upon rehydration, re-differentiation occurs accompanied by a resumption of metabolic activity (Dasgupta *et al*., 1982). In contrast, in the highly recalcitrant seeds of *A. marina*, and in somewhat less sensitive *Aesculus hippocastanum*, organelles remain highly differentiated and their morphology is indicative of ongoing metabolism (Farrant *et al*., 1992; 1997). If metabolism is not shut down, then unregulated metabolism is

presumed to occur, which can result in the uncontrolled production and escape of damaging free radicals.

b) Free radicals, reactive oxygen species and antioxidant systems

Plants are aerobic organisms and oxygen plays a vital role in their metabolism, electron transfer reactions and oxidative processes (Bailly, 2004). Although oxygen is regarded as a slightly reactive molecule (Hendry, 1993), it can generate highly reactive and potentially harmful intermediates during the electron transport process (Fridovich, 1998). Such intermediates have been described in a number of ways, for example, as reactive oxygen species (ROS), active oxygen species (AOS), reactive oxygen intermediates (ROI) and free radicals (Hendry, 1993; Mittler, 2002; Bailly, 2004; Benson and Bremner, 2004). The addition of protons and electrons to oxygen can produce the highly reactive singlet oxygen, di-oxygenal radical, the superoxide radical, peroxide anion, hydrogen peroxide, as well as water. The superoxide radical is not very reactive and is usually short-lived; however, if it further reacts with hydrogen peroxide, the highly reactive hydroxyl radical is produced, which is the most strongly oxidising radical species (Hendry, 1993; Bailly, 2004; Benson and Bremner, 2004).

Under normal physiological conditions, ROS are generated at low levels in chloroplasts, mitochondria and peroxisomes (Suzuki and Mittler, 2006) as a result of metabolism, respiration and photosynthesis. Notwithstanding the signalling role of ROS (Vranová *et al.*, 2002; op den Camp *et al.*, 2003; Van Breusegem *et al.*, 2008), under conditions of stress normal cellular homeostasis is disrupted and there may be an increase in ROS production (Suzuki and Mittler, 2006). Such stresses include heat, cold, dehydration, salinity, high light intensities and pathogen attack (O'Kane *et al.*, 1996; Niyogi, 1999; Larkindale and Knight, 2002). Reactive oxygen species (in particular the hydroxyl radical) can oxidise DNA and may result in strand breaks, mutagenesis, carcinogenesis and ageing (Breen and Murphy, 1995; Halliwell and Gutteridge, 1999). Hydroxyl radicals can initiate lipid peroxidation and also affect the structure and functioning of proteins including enzymes (refer to Halliwell and Gutteridge, 1999 for an overview of the interaction of hydroxyl radicals with biomolecules). However, there are additional

roles for ROS not related to oxidative damage, including as signalling molecules in programmed cell death (Pellinen *et al*., 2002), response to wounding (Orozco-Cardenas *et al*., 2001), root gravitropism (Joo *et al*., 2001), ABA-mediated stomatal closure (Zhang *et al*., 2001a) and plant defence (Wisniewski *et al*., 1999).

Considering those diverse roles of ROS, e.g. as damaging toxic chemical species but important in signal response pathways, it is clear that there is a requirement for plant cells to generate ROS at strictly controlled levels or to quench excess ROS, thus preventing oxidative damage (Bailly, 2004; Suzuki and Mittler, 2006). In metabolically active, hydrated tissue, a suite of antioxidants including superoxide dismutase (SOD), catalase (CAT), glutathione, ascorbate, α–tocopherol and peroxiredoxins (Stacy *et al*., 1999; Finkle and Holbrook, 2000; Mittler, 2002) function to quench and/or regulate ROS production (Hendry, 1993; Leprince *et al*., 1993). Bailly *et al.* (2001) found that various antioxidants occurred at different levels during seed development. During the desiccation sensitive (immature) stage, high levels of CAT and glutathione reductase and low levels of SOD and ascorbate peroxidase were found, while the reverse was true for mature (desiccation tolerant) seeds.

In recalcitrant seeds, metabolism is ongoing throughout development and upon dehydration, such processes continue until water content becomes limiting. As a result, it has been suggested that metabolism becomes unbalanced or unregulated and this results in intracellular damage (metabolism-linked damage), which is lethal to seeds or embryos at high water contents under conditions of slow dehydration (Pammenter *et al*., 1998; Pammenter and Berjak, 1999; Walters *et al*., 2001; 2002a). Such metabolism-linked damage is suspected to be closely associated with the production of ROS and the inability of recalcitrant seeds to scavenge them. Hendry *et al*. (1992) and Finch-Savage *et al*. (1994) reported the production of free radicals and lipid peroxidation when mature recalcitrant seeds of *Q. robur* were dried, which corresponded to a decline in viability.

A number of researchers have proposed that desiccation tolerance may be related (in part) to the ability to scavenge ROS produced and thereby prevent harmful consequences of unbalanced metabolism (Hendry *et al*., 1992; Leprince *et al*., 1993;

Vertucci and Farrant, 1995). Recalcitrant seeds do possess antioxidant defence systems against ROS, as reported by Hendry *et al*. (1992) and Varghese *et al*. (2011). However, the commonly-held view is that even though such protective systems exist in recalcitrant seeds, they may function suboptimally to the point of being ineffective. This is likely to be the consequence of the intensity of ROS production associated with unregulated metabolism that is suggested to occur during dehydration (Smith and Berjak, 1995).

c) Accumulation of protective molecules

Late embryogenic accumulating/abundant proteins (LEAs) refer to a subset of proteins reported to accumulate in seeds at high levels during the later stages of embryogenesis, when orthodox seeds acquire desiccation tolerance (Dure and Chlan, 1981; Galau and Dure, 1981). Consequently, LEA proteins have been implicated in contributing to tolerance of desiccation (Kermode, 1997; Oliver and Bewley, 1997). These proteins have been classified into groups, families or classes based on similarities in their amino acid sequence (or on peptide profile analysis [Wise and Tunnacliffe, 2004]). To date, seven groups of LEAs have been identified, of which the dehydrins are the most studied group (discussed in Kalemba and Pukacka, 2007; Tunnacliffe and Wise, 2007; Battaglia *et al*., 2008), probably because of the availability of an antibody to them, but not the other, groups of LEAs.

The exact functions of LEA proteins are not completely known. Most of the information on LEA structure derives from studies of the mRNA, therefore intracellular functions of these proteins have been largely inferred (reviewed by Berjak *et al*., 2007). However, the basis of the evidence suggesting a role for LEAs in desiccation tolerance (and associated stresses) is convincing. In particular, dehydrins have been suggested to protect against desiccation stress (Bravo *et al*., 2003) by preventing the aggregation of dehydration-sensitive proteins (Goyal *et al.*, 2005), thereby acting as molecular shields (Wise and Tunnacliffe, 2004). These proteins may also function to prevent cell collapse by acting as space filling molecules (Tunnacliffe and Wise, 2007) and may stabilise membranes and the cytoskeleton during dehydration (Wise and Tunnacliffe, 2004;

Tolleter *et al*., 2007). Other proposed roles of LEA proteins (particularly dehydrins) are that they may function to bind or sequester ions and they may also act as hydration buffers (McCubbin *et al*., 1985). Walters *et al*. (1997) suggested that LEAs achieve the latter function by association with sugars (see later).

Regardless of their functions in response to dehydration and other stresses, it is accepted that LEAs (or dehydrin-like proteins) on their own are insufficient to confer desiccation tolerance (Blackman *et al*., 1991; Finch-Savage *et al*., 1994; Šunderlíkova *et al*., 2009). A number of researchers have identified LEAs (dehydrin-like proteins in particular) in orthodox seeds (Wechsberg *et al*., 1994; Han *et al*., 1997) and in recalcitrant seeds from temperate regions (Finch-Savage *et al*., 1994; Gee *et al*., 1994; Farrant *et al*., 1996). However, dehydrin-like proteins were absent from a range of recalcitrant seeds of tropical wetland species (Farrant *et al*., 1996). Current work in our laboratory focused on determining whether two types of orthodox seed-specific LEA1 (*viz*. XhLEA1-4 and XhLEA0797) were present in the recalcitrant seeded species *Avicennia marina* and *Castanospermum australe*. However, no LEAs were found after drying, chilling or exogenous ABA application (unpublished results). The presence of dehydrins does not imply tolerance to desiccation as dehydrins can be produced in recalcitrant seeds of certain species, which are desiccation sensitive (Gee *et al*., 1994; Farrant *et al*., 1996). However, it must be noted that there has been a bias towards the dehydrins as noted above, and that studies on the spectrum of other LEAs in recalcitrant seeds are required, before any valid generalisations can be made.

Farrant *et al*. (1996) suggested that the absence of LEAs may provide an indication of the inability of seeds to survive desiccation. For example, *A. marina* does not contain any dehydrin-like proteins and is also extremely desiccation sensitive. Those authors further suggested that the presence of dehydrin-like proteins in mature recalcitrant seeds may have functional significance in terms of the extent of dehydration tolerated. For example, some recalcitrant seeds can tolerate a decline in water content during their development such as *Q. robur* (Finch-Savage, 1992) and *A. hippocastanum* (Tompsett and Pritchard, 1993) and these species also expressed dehydrin-like proteins (Farrant *et al*., 1996).

Sugars accumulate in response to dehydration, thereby suggesting their role in desiccation tolerance (Koster and Leopold, 1988; Williams and Leopold, 1989). There are a number of mechanisms by which sugars are reported to confer desiccation tolerance. The original proposal was that of glass formation (Williams and Leopold, 1989). According to this proposal, as water is removed from desiccation tolerant tissues, solutes – primarily sucrose – in the cytoplasm become concentrated resulting in an increase in cytoplasmic viscosity until the cytoplasm vitrifies and exists in a glassy state (Koster and Leopold, 1988; Williams and Leopold, 1989). According to Sun and Leopold (1993) and Sun (1997), glasses reduce the rate of molecular diffusion, minimise the deleterious effects of unbalanced metabolism and also act as space fillers and may prevent cellular collapse (Buitink and Leprince, 2004). Further, intracellular glasses may have different densities (Burke, 1986; Angell, 1995), resulting in brittle glass in some localities, while in others the glass may be fluid enough to permit some chemical reactions, in dry desiccation tolerant tissues (Berjak, 2006). Leopold *et al*. (1994) proposed that the function of the glassy state is not to confer desiccation tolerance *per se*, but rather to preserve the viability of stored, dry seeds.

A proposal about the mode of action of sugars, primarily trehalose (animal cells) and sucrose (seed tissues) was the water replacement hypothesis (Clegg, 1986; Crowe *et al*., 1992), suggesting that sugars bind to polar head groups of phospholipids and so replace lost water molecules during dehydration. This was held to maintain the original spacing between phospholipid molecules, so preventing the liquid to gel phase transition. However, *in vivo* supporting evidence for this proposal has not been forthcoming (discussed by Berjak, 2006).

The third proposal is based on hydration forces. Accordingly, elevated sucrose (or other compatible solutes) can help to restrict damage upon water loss by keeping molecules sufficiently separated to avoid harmful interactions (Bryant and Wolfe, 1992; Wolfe and Bryant, 1999). As water is removed, the solute concentration increases, resulting in increased osmotic pressure (and lowered hydration force). Those solutes positioned between membrane bilayers (interlamellar solutes) are suggested to maintain separation of bilayers at low water content by a volumetric effect, i.e. the bilayers remain

physically separated by the molecular volume of the solutes (Bryant and Wolfe, 1992; Wolfe and Bryant, 1999). Accordingly, interlamellar solutes are suggested to reduce the stresses present in membranes during low water availability, thus preventing gel phase transitions (Bryant *et al*., 2001).

However, the accumulation of sugars does not imply desiccation tolerance as a number of recalcitrant seeds have been found to accumulate sugars (Berjak *et al*., 1989; Farrant *et al*., 1993; Finch-Savage *et al*., 1993) but are nevertheless desiccation sensitive. Vertucci and Farrant (1995) and Berjak and Pammenter (2008) suggested that sucrose is unable to protect recalcitrant seeds against desiccation damage (as it does orthodox seeds), as recalcitrant seeds would already have lost viability at water contents far above those at which the benefits of sucrose protection could be realised. For example, glasses are formed below 0.3 g g^{-1} water content (Hoekstra and van Roekel, 1988) while during slow drying under ambient conditions, recalcitrant seeds are reported to lose viability in the water content range of 0.8 – 1.0 g g^{-1} (Berjak *et al*., 1992; 1993; Pammenter *et al*., 1993). Similarly, for water to be replaced by sugars, the water content attained would have to be less than 0.3 g g^{-1} (Hoekstra and van Roekel, 1988), which again is below the lethal limit for recalcitrant seeds upon slow water loss.

An alternative view of the role of sugars in glasses in desiccation tolerance is to consider the interaction of sugars with other intracellular molecules. A number of researchers have suggested that intracellular glasses may be stabilised by LEAs in the dry state (Walters *et al*., 1997; Oliver *et al*., 2001). Supporting evidence for this was provided by Wolkers *et al*. (2001) who showed that the α-helical conformation of a LEA protein was promoted by dehydration in the presence of sucrose. Further, those authors found that a LEA-sugar glass had a higher glass transition temperature (T_g) and stronger hydrogen bonds than a sugar glass. They concluded that LEAs and sucrose interact synergistically during glass formation and in so doing, both factors promote the long-term stability of intracellular glasses in dry, desiccation tolerant organisms. Considering this, Berjak (2006) highlighted that the consensus on the intracellular glassy state has evolved from the initial perception of intracellular glasses being just sugar glasses to the current view that sugars and residual water interact with proteins

(specifically LEAs) and probably other intracellular constituents. However, as noted above, these proposals and conjectures generally lack relevance to recalcitrant seeds – except perhaps under conditions of very rapid dehydration (flash drying [Berjak *et al.*, 1990]) of excised embryonic axes which facilitates transient viability retention at water contents sufficiently low for cryopreservation (Berjak and Pammenter, 2008), as is discussed later.

1.6 Methods of seed storage

1.6.1 Storage of orthodox seeds

Orthodox seeds can be stored in the short-term (from harvest to sowing in the next season) at ambient temperatures, provided they have been adequately dried and are kept free of insects (Schmidt, 2000). In the long-term, however, such seeds should be stored at reduced temperature and relative humidity (Schmidt, 2000). The storage longevity of orthodox seeds is reported to increase logarithmically with decreasing moisture (water) content (Ellis and Roberts, 1980) down to a certain limit, below which a further reduction is not beneficial (Ellis and Hong, 2006) and may even be damaging (Walters, 1998; Walters and Engels, 1998). In general, orthodox seeds should be stored in airtight containers at -18^{o}C or lower and at a water content of 5 – 7 % (0.053 – 0.075 g g^{-1}) (Schmidt, 2000). Most orthodox seeds should survive under these conditions for a century or longer, with little decline in viability as long as seeds are of good quality at the outset, i.e. showing high vigour and germinability (Ellis, 1991; Withers and Engelmann, 1998; Ellis and Hong, 2006). Eventually, orthodox seeds stored under conventional conditions (even at -18^{o}C) will deteriorate and die, therefore cryopreservation can be considered as an alternative since it can facilitate storage of seeds for hundreds (to over a thousand) years (Walters *et al.*, 2004). Since orthodox seeds are intrinsically desiccation tolerant, those of most species should be able to be successfully cryopreserved simply by dehydration to a low water content before cooling (González-Benito *et al.*, 1995; Panis and Lambardi, 2005; Pritchard, 2007). Pritchard (2007) provides a list of desiccation tolerant seeds that have been successfully cryopreserved from 1995 to 2005.

1.6.2 Storage of non-orthodox (recalcitrant) seeds

As discussed, recalcitrant seeds are hydrated, desiccation sensitive and metabolically active when shed. In addition, many species, particularly those from the tropics, may also be chilling sensitive and some cannot be stored at temperatures below 15^{o}C. Consequently, recalcitrant seeds cannot be stored under conditions used for the storage of orthodox seeds (Pammenter and Berjak, 1999), which poses serious problems for the long-term conservation of such germplasm. Good quality recalcitrant seeds can be maintained in the short- to medium-term under conditions of hydrated storage (wet storage) where seeds are stored at the water content at which they were shed (Berjak *et al.*, 1989; Berjak and Pammenter, 2004a). However, storage below shedding water content was found to be unfavourable (Drew *et al*., 2000; Eggers, 2007).

A problem that is often encountered in recalcitrant seed storage is the proliferation of mycoflora on and in the seeds (Mycock and Berjak, 1990). Under hydrated storage conditions, seeds are kept at high water content and above chilling temperatures, which are ideal conditions for the proliferation of seed associated mycoflora (Mycock and Berjak, 1990). For *A. marina*, Calistru *et al*. (2000) showed that if fungi were allowed to proliferate in stored seeds, then the hydrated storage lifespan was considerably reduced, and that the hydrated storage lifespan was extended when fungal activity was curtailed by treatment with appropriate fungicides. Mycock and Berjak (1995) reported that in most cases, the fungicidal treatments attempted do not eliminate internal mycoflora, instead they curtail the spread of infection. Consequently, seed loss will still occur, but it may be more limited and/or delayed. Work is presently underway in our laboratory to assess a range of systemic fungicides as well as biological and other control agents to attempt to eliminate or reduce seed-associated mycoflora.

Recalcitrant seeds can be stored only in the short-term under conditions of hydrated storage, but the time for which this is effective varies depending on species characteristics, ranging from 2 – 3 weeks (or less) for some tropical species (Farrant *et al*., 1989) to 2 – 3 years for chilling tolerant temperate species (Pritchard *et al*., 1996). However, it is inevitable that recalcitrant seeds stored under hydrated conditions will

eventually die. Pammenter *et al*. (1994) suggested that the loss of viability of wet stored recalcitrant seeds is probably a result of a mild, but prolonged water stress experienced by the seeds. Stored recalcitrant seeds show signs of extensive vacuolation and increase in cell size, which is indicative of germinative metabolism (Pammenter *et al*., 1994; Farrant *et al*., 1997). These processes require additional water and as under conditions of hydrated storage there is no external supply of water; the seeds are consequently exposed to water stress. Although the intensity of this water stress is not as severe under conditions of hydrated storage as that which would be imposed by dehydration, the duration of the stress is extended. During this time, damage accumulates which will ultimately result in deterioration of seeds and viability loss (Pammenter *et al*., 1994). Once recalcitrant seeds begin to deteriorate in storage, fungi are often observed to proliferate (Mycock and Berjak, 1990).

Considering that germinative metabolism proceeds in hydrated storage, it is not uncommon for tropical recalcitrant seeds to germinate in storage (Farrant *et al*., 1985; Berjak *et al*., 1992; 1993). Recalcitrant seeds from temperate regions have also been reported to germinate in storage (Finch-Savage *et al*., 1993), although this process occurs after a longer duration than that observed for tropical species (Pammenter *et al*., 1994). In both cases, however, the nascent seedlings deteriorate and will die. This suggests that the deteriorative metabolic events that occur during hydrated storage of temperate and tropical recalcitrant seeds may be similar, although they may occur at a reduced rate in the former (Pammenter *et al*., 1994). However, it must be emphasised that if conditions for hydrated storage of recalcitrant seeds can be optimised, then this provides a very useful method for maintaining planting stock for immediate planting programmes (Berjak and Pammenter, 2004a). It does not, however, provide an option for long-term conservation.

1.7 Cryopreservation for the long-term conservation of recalcitrant-seeded germplasm

By definition, cryobiology is the study of biological material or systems subjected to temperature below normal (ranging from cryogenic temperatures to moderately

hypothermic conditions [www.societyforcryobiology.org]). Although -18ºC constitutes a cryogenic temperature, cryopreservation is generally understood as the storage of biological material at ultra-low temperature, either in liquid nitrogen at -196°C or preferably, in the vapour phase above it at approximately -140 to -160°C depending how far or near from the surface of the liquid nitrogen (Kartha, 1981; Sakai, 1997; Razdan, 2003; Berjak and Pammenter, 2004a; Walters *et al*., 2008). At the temperature of liquid nitrogen, cellular and metabolic activities are arrested and, theoretically, material can be stored indefinitely (Kartha, 1981; Chen and Kartha, 1987; Engelmann, 1991; Chandel and Pandey, 1996; Mandal, 2005). The only biological changes that may occur are those related to background radiation (Henshaw and Blakesley, 1995), but Benson and Bremner (2004) draw attention to the free radical potential even in cryostorage.

Cryopreservation allows for the storage of a wide variety of genotypes so that the genetic variability of species can be conserved and the gene pool is not lost (Benson, 2008a). Hence, it is a valuable tool to preserve genetic resources for future use. It is also the only strategy presently available for the long-term storage of species that produce recalcitrant seeds (Dumet *et al*., 1997; Engelmann, 2004; Mandal, 2005; Walters *et al*., 2008). Other specific applications of cryopreservation are that it can be used to back-up important embryogenic cultures; those cultures that produce valuable metabolites; or to back-up clonally propagated plants having selected, superior genotypes (Henshaw *et al*., 1985; Reed, 2008). Cryopreservation can also be used to store germplasm of plants that are susceptible to diseases, specific climatic conditions, insect pests or other environmental threats, as well as wild varieties or cultivars not currently used for farming (Reed, 2008).

Another advantage of cryopreservation is that it allows for the conservation of many samples in a small space for extended periods (Engelmann, 1991; 2004; Mandal, 2005). As long as suitable protocols for regrowth have been developed, no maintenance is required once explants have been cryopreserved (Mix-Wagner and Schumacher, 2003; Mandal, 2005). Also, considering that biochemical and physiological processes cease at cryogenic temperatures, as long as explants are not damaged when initially exposed to

cryogenic temperatures, further damage should be essentially curtailed as long as the temperature is maintained (Wolfe and Bryant, 2001).

1.7.1 The theory underlying cryopreservation of plant germplasm

The control or avoidance of ice crystallisation is vital for successful cryopreservation (Sakai and Yoshida, 1967; Krishnapillay, 2000; Volk and Walters, 2006; Benson, 2008a). Therefore to understand cryopreservation theory, it is necessary to understand the physical process of ice crystallisation. In order for ice to form, a nucleation site must be present (Meryman and Williams, 1985; Mazur, 2004; Benson *et al*., 2006). There are two types of nuclei, termed heterogenous and homogenous. Heterogenous nuclei are regions on non-aqueous surfaces (such as container walls, dust, macromolecules, etc.) that provide templates for ice crystal growth (Meryman and Williams, 1985; Wolfe and Bryant, 1999; Mazur, 2004; Benson *et al*., 2006). When heterogenous nuclei are not present, water can supercool to the point of homogenous ice nucleation at approximately -40°C (Benson, 2008b). Below this temperature, water can self-nucleate and form an ice embryo of a critical size which can then grow into an ice crystal (Meryman, 1957; Meryman and Williams, 1985; Benson, 2008a). Ice formation proceeds in two steps, i.e. first by the formation of nuclei and subsequently by the growth of ice crystals as a result of the aggregation of additional molecules (Chaudhury, 2005; Benson, 2008b).

a) The effect of cooling rate on ice crystal formation

The rate of cooling will determine the size of ice crystals that form and their location, which are critical factors in the cryostorage and subsequent recovery of biological samples (Pegg, 2001; Wesley-Smith *et al*., 2004; Benson *et al*., 2006). When the rate of cooling is slow (in the region of 1°C min^{-1}), ice forms in extracellular spaces as a result of heterogenous ice nucleation (Meryman and Williams, 1985) and can invade the plant cell wall. Ice crystals increase in size at the expense of water extracted from the extracellular solution, which consequently becomes concentrated (Muldrew *et al*., 2004). This results in an increase in osmotic potential which results in water from inside

cells migrating to the outside, causing cell contents to become concentrated (Meryman and Williams, 1985; Chaudhury, 2005). If tissues are cooled sufficiently slowly, there is enough time for water inside cells to diffuse to the outside where extracellular ice is forming. If this occurs, then the system can approach vapour pressure equilibrium and provided the cell membrane is not damaged, intracellular ice crystallisation can be avoided. Ice will continue to form as the temperature is reduced until the eutectic point is reached, where the entire system exists in a solid state (Muldrew *et al*., 2004; Benson *et al*., 2006).

If the cooling rate is increased (rapid cooling) many ice nuclei form (since when one ice nucleus forms, there is insufficient time for the first nucleus to grow and propagate before a second nucleus forms and so on). There is insufficient time for osmotic dehydration to occur since the rate of ice crystal growth is faster than the rate of cellular dehydration (Muldrew *et al*., 2004). As a result, intra- and extracellular ice formation may occur (Benson *et al*., 2006); however intracellular, ice formation is reported to occur more frequently (Muldrew *et al*., 2004).

b) Damage caused by ice crystals

In order to develop successful cryopreservation protocols, it is important to understand the physical and biological processes that occur during cooling and their injurious effects. Early reviews (Luyet and Gehenio, 1940) highlighted cooling injury as being caused predominantly by direct physical damage by ice crystals. Subsequent work by Lovelock (1953a; b) suggested that cell dehydration (accompanied by increase in extracellular solute concentration) also play a role. In 1965, Mazur combined both these ideas and described the two-factor hypothesis for freezing injury, which stated that freezing injury was caused either by the concentration of solutes by extracellular ice (producing solution effects) or by the presence of ice itself. Hence, the overall effect of ice formation is considered to be two-fold: firstly it can physically damage cells by mechanical injury (Benson, 2008a) or by the volumetric expansion of ice crystals (Wolfe and Bryant, 2001) and secondly, it can dehydrate cells as it results in the

conversion of liquid water into ice either intracellularly or extracellularly (Mazur, 2004). Since ice crystallisation reduces the amount of available water in cells, this results in an increase in the concentration of solutes as the temperature is lowered, which can cause detrimental osmotic and pH changes (Mazur, 1984; Kartha, 1985; Franks, 1990) and may affect the secondary and tertiary structure of enzymes (Wolfe and Bryant, 2001).

Intact plasma membranes are an essential feature contributing to survival after cooling as the plasma membrane prevents extracellular ice from propagating into cells; similarly, intact organelle membranes preserve cell compartments (Wolfe and Bryant, 2001; Muldrew *et al*., 2004). Plasma membranes can be damaged during osmotic contractions and may even rupture during cooling and warming. Cells that have been severely dehydrated may also exhibit signs of damage such as lipid phase transitions upon warming (Wolfe and Bryant, 2001). Generally, intracellular ice formation is regarded as a lethal event (Meryman and Williams, 1985; Benson *et al*., 2006). However, there is contrasting evidence that suggests that if ice crystals remain below a critical size, i.e. 0.05 to 0.1 μm (Nei, 1973; Shimada and Asahina, 1975), or if they form in particular compartments, then intracellular ice crystals may not compromise viability (Wesley-Smith, 2002). Further, Acker and McGann (2003) suggested that intracellular ice may be innocuous and may even have a cryoprotective effect since once it is formed, there is no further efflux of water out of cells, thus preventing further cell volume changes.

Both rapid and slow cooling can cause injury to cells. Rapid cooling injury is most often associated with the formation of intracellular ice while slow cooling injury is associated with solution effects (concentration of solutes) (Meryman *et al*., 1977; Benson *et al*., 2007). It is important to note that these types of injury may overlap and seem to be related to the osmotic pressure gradient across the plasma membrane (Muldrew *et al*., 2004). Considering this, Muldrew *et al*. (2004) identified different orders of cryoinjury, *viz*. first order injury or events that lead directly to cell death, and second order injuries which are sub-lethal, as cells can repair that damage upon resumption of metabolism. Hence, for cryopreservation, an optimal cooling rate must be attained that will avoid

lethal intracellular ice formation while limiting the detrimental consequences of solution effects (Muldrew *et al*., 2004). There are two broad approaches to the cryopreservation of plant germplasm, *viz*. those that involve ice formation (e.g. controlled rate cooling) and those that allow for cryostorage in the absence of ice (vitrification techniques) (Benson *et al*., 2002).

1.7.2 Approaches to cryopreserve plant germplasm

a) Controlled rate cooling

Controlled rate cooling has also been referred to as classical (Mandal, 2005), conventional (Dereuddre and Kaminski, 2005), traditional (Benson, 2008c) and two-step cooling (Volk *et al*., 2006). This cooling strategy has been successfully applied to germplasm of species from temperate regions but has not been similarly successful with species of tropical origin (Sakai, 1960; Haskins and Kartha, 1980; Bagniol *et al*., 1992). The technique involves cooling explants at a controlled rate of 0.25 – 5°C min^{-1} (Benson, 2008b) down to a defined prefreezing temperature, usually approximately -40°C (Dereuddre and Engelmann, 1987; Reed and Uchendu, 2008). Explants can be held at this temperature but are generally subjected to lower temperatures, usually by direct plunging into liquid nitrogen (Benson, 2008b; Reed and Uchendu, 2008).

Recent research has suggested that if the cytoplasmic contents can be sufficiently dehydrated during freeze-induced dehydration, then vitrification could occur upon immersion in liquid nitrogen (Benson, 2008a; Reed and Uchendu, 2008). The extent of dehydration is dependent on the rate of cooling and the prefreezing temperature (Engelmann, 2000; Reed and Uchendu, 2008). Under optimal conditions, most of the intracellular solution water is lost, thereby substantially decreasing the risks of intracellular ice formation when explants are plunged into liquid nitrogen. However, excessive freeze-induced dehydration can have damaging or even lethal effects as a result of concentration of intracellular solutes (Meryman *et al*., 1977; Benson *et al*., 2007; Reed and Uchendu, 2008). Consequently, determination of the appropriate parameters for cooling is essential for explant survival.

Controlled rate cooling can be performed by using a programmable freezer (Reed and Uchendu, 2008), or within a liquid nitrogen Dewar vessel by holding cryovials at different distances above liquid nitrogen (Withers and King, 1980) or by using the more convenient, commercially available Mr Frosty™ (Nalgene®, USA) unit (Benson, 2008b). Advantages of controlled rate cooling are that if a computer-based programmable freezer is used, then many samples can be cryopreserved simultaneously and record keeping is efficient as all data is electronically stored (Benson, 2008b). The disadvantage of such a system is that a computer-based programmable freezer is very expensive (Engelmann, 2000; Benson, 2008b). A cheaper option is the Mr Frosty unit (Benson, 2008b); however, only a few samples can be processed at a time (18 cryovials) and, by using this device, the rate of cooling cannot exceed 1°C min^{-1} (Benson, 2008b).

The controlled rate cooling method has been successfully employed for a variety of explants including cell suspension cultures (Kartha and Engelmann, 1994), callus cultures (Withers and Engelmann, 1998), de-differentiated cultures (Heine-Dobbernack *et al*., 2008), embryogenic cultures (Gale *et al*., 2007) and shoot-tip meristems (Reed and Uchendu, 2008). However, a disadvantage of this technique is that many other forms of germplasm are not amenable to this type of storage, particularly those that are well differentiated with different tissue-types and varying water contents (Benson, 2008b).

b) Vitrification-based techniques

A range of vitrification techniques was developed during the 1990s, to cryopreserve species of tropical origin, in which cells were dehydrated (by air desiccation or exposure to cryoprotectant solutions or media) and then rapidly cooled in liquid nitrogen (Engelmann, 2000; 2003). Using this method, intracellular ice formation may be avoided as cell contents should vitrify when rapidly cooled (Engelmann, 2000). Vitrification is a physical process that occurs when an aqueous solution undergoes a phase transition from a liquid to an amorphous glassy state (Fahy *et al*., 1984), which takes place at the glass transition temperature and avoids ice formation (Sakai, 2000). Advantages of vitrification-based techniques are that there is no requirement for

expensive equipment (such as programmable freezers), and that these techniques are suitable for cryopreserving complex tissues with a range of cell types (Steponkus *et al.*, 1992). Such techniques have proved successful in cryopreserving germplasm from tropical species, which could not be cryopreserved using controlled rate cooling (Sakai and Engelmann, 2007).

Seven vitrification-based procedures have been developed for cryopreserving plant germplasm (Engelmann, 2000), which are discussed in greater detail below. Engelmann (1997) highlighted that the critical step in all these methods is the dehydration step (in contrast to controlled cooling, where rate is the critical step).

i) Desiccation

This is the simplest and least expensive of the vitrification-based techniques, as explants are simply dehydrated and then rapidly cooled in liquid nitrogen (Engelmann, 2000). Explants can be dehydrated in a sterile laminar air flow (Chandel *et al.*, 1995), over a saturated salt solution (Normah and Makeen, 2008) or by flash drying (Berjak *et al.*, 1990; Pammenter *et al.*, 2002a). The most precise, reproducible and rapid method of dehydration is by flash drying and this is the method of choice when dehydrating germplasm from recalcitrant seeds (Berjak *et al.*, 1999). The desiccation technique has been successfully used to cryopreserve zygotic and embryonic axes excised from seeds of tropical forest trees (Normah and Marzalina, 1996; Engelmann, 1997) and other species (Pammenter *et al.*, 1991; Vertucci *et al.*, 1991; Wesley-Smith *et al.*, 1992; Berjak *et al.*, 1993). A number of parameters can be optimised using this technique to increase survival after cooling (refer to section 1.7.3).

ii) Pregrowth

This method (also called preculture) involves culturing explants *in vitro* (for a few days to weeks) on medium supplemented with elevated levels of sugars (Dumet *et al.*, 1993; Suzuki *et al.*, 1998), sugar alcohols, amino acids or other cryoprotectants (Thierry *et al.*, 1997). Following pregrowth, explants are rapidly cooled in liquid nitrogen (Engelmann,

2000). Some explants that have been successfully cryostored using this method include proliferating meristem cultures (Panis *et al*., 2000), *in vitro* axillary buds (Suzuki *et al*., 1998) and somatic embryos (Thierry *et al*., 1997).

iii) Pregrowth-desiccation

This method is a combination of pregrowth and desiccation where explants are first cultured on a medium supplemented with specific additives, then desiccated, for example within a laminar airflow cabinet, on a laboratory bench, or over silica gel (Cho *et al*., 2002a), and cooled rapidly in liquid nitrogen (Engelmann, 2000). Some explants successfully cooled using this technique include somatic embryos (Dumet *et al*., 1993), embryogenic suspension cultures (Zhang *et al*., 2001b), shoot tips (Normah and Tan, 2000) and embryonic axes (Cho *et al*., 2002a).

iv) Encapsulation-dehydration

The first step of the encapsulation-dehydration protocol is the pre-conditioning, where stock plants are cultured under conditions that may promote freezing tolerance. Pre-conditioning may be carried out at reduced temperature (Wu *et al*., 2001a) and/or on sucrose-supplemented medium (Grospietsch *et al*., 1999). Explants are then excised and precultured (Reed *et al*., 2005). In the next step, explants are encapsulated (in a hydrosoluble gel matrix) in sodium (González-Arnao and Engelmann, 2006) or calcium (Cho *et al*., 2002b) alginate beads. Encapsulated explants are then osmoprotected in liquid sucrose medium for a few hours (Niino and Sakai, 1992) or days (Fabre and Dereuddre, 1990). Alternatively, species sensitive to immediate high sucrose concentrations may be exposed to a progressive increase in sucrose concentration over a few days (Plessis *et al*., 1991). Beads are then submitted to evaporative dehydration and then rapidly cooled in liquid nitrogen. The combination of sucrose preculture followed by dehydration is likely to remove most of the freezable water from cells so that upon rapid cooling, vitrification may occur without intracellular ice formation (Engelmann, 1997).

Following cooling, regeneration of explants is usually direct (i.e. without an intervening callus stage) and rapid (Engelmann, 2000), suggested to be because encapsulation preserves integrity of cells (Engelmann, 1997; 2000). Further, encapsulation allows explants to be precultured under conditions of high sucrose concentration and dehydrated to low water contents – treatments that may otherwise cause damage to unencapsulated explants (Engelmann *et al.*, 2008).

Other advantages of the encapsulation-dehydration method are that non-toxic cryoprotectants can be used and warming is simple (Reed, 2001). Further, the glasses that are formed by this method are reported to be very stable (González-Arnao and Engelmann, 2006) once optimal desiccation has been achieved. Its disadvantages are that it is applicable only to explants that are tolerant to dehydration and it is labour intensive and time consuming, involving a number of steps over a few days. All cryopreservation protocols involve dehydration, whether explants are desiccation tolerant or not and an important parameter regarding this is the time factor. For desiccation-sensitive explants, rapid dehydration is essential to limit metabolism-linked damage (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999). If dehydration is slow, generally lethal consequences are seen at water contents too high for cryopreservation. Also, although desiccation-sensitive explants can be appropriately dried, their survival in the dehydrated state is transient, unless they are immediately exposed to cryogenic temperatures (Walters *et al.*, 2001). Consequently, only a limited number of samples can be processed at a time (Benson, 2008b). However, this disadvantage is partially counteracted when the explants are encapsulated in beads, which makes handling them easier (Engelmann *et al.*, 2008).

Encapsulation-dehydration has been successfully used to cryopreserve germplasm from plants of temperate provenances (Dereuddre *et al.*, 1991; Plessis *et al.*, 1991) and a range of species of tropical origin. Engelmann *et al.* (2008) provide a list of 87 species that have been successfully cryopreserved by this method. Material cryopreserved using this method includes cell suspension cultures (Bachiri *et al.*, 1995), callus (Shibli *et al.*, 2009), anthers (Marassi *et al.*, 2006), embryonic axes (Kaviani, 2007), shoot tips (Verleysen *et al.*, 2005), adventitious shoots (Burrit, 2008), hairy roots (Hirata *et al.*,

1998), protocorms (Flachsland *et al*., 2006), somatic embryos and axillary buds (Pawłowska, 2008).

v) Vitrification

Vitrification is a freeze-avoidance mechanism circumventing the damaging effects of both intra- and extracellular ice by promoting supercooling followed by the phase transition of a highly concentrated cryoprotectant solution from liquid to amorphous glass during cooling (Luyet, 1937; Fahy *et al*., 1984). Vitrification can be achieved by controlled rate cooling (partial vitrification) and by rapid cooling (complete vitrification) (Sakai *et al*., 2008). In controlled rate cooling, as samples are slowly cooled, the unfrozen fraction of the cytoplasm and the suspending solution become sufficiently concentrated to allow for partial vitrification once samples are transferred to liquid nitrogen. Complete vitrification may be achieved when the controlled rate cooling step is eliminated and samples are instead exposed to highly concentrated cryoprotectant solutions (7 – 8 M) (Sakai *et al*., 2008). The latter method is different from partial vitrification, as highlighted by Sakai and Engelmann (2007).

The first step of the vitrification procedure is to preculture explants on appropriate media, followed by a step where the explants are osmoprotected with a loading solution, usually containing 2 M glycerol and 0.4 M sucrose (Sakai *et al*., 2008). This solution, which contains a lower concentration of cryoprotectants than the vitrification solution, is used to minimise any deleterious effects of the vitrification solution applied in the next step (Matsumoto *et al*., 1998; Sakai *et al*., 2008). The plant vitrification solution, PVS2, comprising 30% (v/v) glycerol, 15% (v/v) ethylene glycol, 15% (v/v) DMSO and 0.4 M sucrose (Sakai *et al*., 1990; 1991), is most commonly used. However, alternate vitrification solutions have been developed as DMSO is reportedly toxic to some species and there are concerns regarding the possible mutagenic effects of this cryoprotectant (Takagi, 2000). Examples of alternate solutions are PVS3 comprising 40% (v/v) glycerol and 40% (w/v) sucrose (Nishizawa *et al*., 1993) and PVS4 comprising 35% (v/v) glycerol, 20% (v/v) ethylene glycol and 0.6 M sucrose (Takagi, 2000). After dehydration with a PVS solution, explants are placed in cryovials with

fresh PVS and then plunged into liquid nitrogen. This is followed by rapid warming, removal of the vitrification solution by unloading in a solution such as 1.2 M sucrose (Sakai *et al*., 1991) and then recovery (Takagi, 2000; Sakai *et al*., 2008).

For success, cells must be sufficiently dehydrated by the vitrification solutions without adversely affecting viability so that vitrification can occur upon immersion in liquid nitrogen (Sakai and Engelmann, 2007). Therefore, a critical step is to assess the tolerance of explants to PVS (Sakai and Engelmann, 2007). The disadvantages of the vitrification technique are that the successive steps need to be implemented quickly and accurately while handling small explants (Matsumoto *et al*., 1995). Further, vitrification solutions have been reported to be toxic to some species (Volk *et al*., 2006). However, it appears that this problem can be minimised by careful pretreatment and addition of PVS (Sakai *et al*., 2008) or dilution of PVS (Kami *et al*., 2008).

The vitrification technique has proved to be successful for cryostoring specimens of a range of plant species (Sakai *et al*., 2008) and a variety of explants such as cell suspension cultures and protoplasts (Chen and Wang, 2003), embryogenic callus cultures (Lambardi *et al*., 2005), microspores (Custódio *et al*., 2004), embryonic axes (Cho *et al*., 2002c), somatic embryos (Corredoira *et al*., 2004), zygotic embryos (Ishikawa *et al*., 1997), seeds (Thammasiri, 2000), nucellar cells (Sakai *et al*., 1990), callus (Hao *et al*., 2002), nodal segments (Benelli *et al*., 2001), protocorm-like bodies (Yin and Hong, 2009) and roots (Jung *et al*., 2001). The greatest success has been achieved with meristems (Takagi, 2000; Sakai and Engelmann, 2007).

vi) Encapsulation-vitrification

The encapsulation-vitrification protocol, which combines the encapsulation-dehydration and vitrification protocols, was developed to process a large number of samples simultaneously using the vitrification procedure (Matsumoto *et al*., 1995). Encapsulation-vitrification includes preculture, encapsulation of explants, loading, PVS dehydration and rapid cooling. Following cryostorage, beads are rapidly warmed, unloaded and plated for recovery (Matsumoto *et al*., 1995). The encapsulation-

vitrification technique significantly reduces the time for the procedure to be completed as it eliminates the necessity for the desiccation step (Sakai and Engelmann, 2007). It has been applied to a range of plant species (Takagi, 2000; Sakai and Engelmann, 2007) and explants including somatic embryos (Shibli and Al-Juboory, 2000), hairy roots (Phunchindawan *et al*., 1997), shoot tips (De Carlo *et al*., 2000), cell suspension cultures (Wang and Perl, 2006), seeds (Thammasiri, 2008) and protocorm-like bodies (Yin and Hong, 2009). The disadvantages of this method are the potential toxicity of vitrification solutions and the requirement for careful timing of the various steps (Sakai and Engelmann, 2007).

vii) Droplet-vitrification

Droplet-freezing, by which shoot tips were immersed in drops of cryoprotectant and slowly cooled in a programmable freezer, was first developed in 1982 (Kartha *et al*., 1982). This method was adapted in the 1990s (Schäfer-Menuhr *et al*., 1996) and termed droplet-vitrification. Using this method, meristems are loaded, and then placed individually in droplets (5 – 10 µl) of PVS2 on a strip of aluminium foil which is then plunged into liquid nitrogen or inserted into cryovials containing PVS2 which are then plunged into liquid nitrogen (Panis *et al*., 2005). For warming, foil strips are placed in liquid medium containing 1.2 M sucrose and the explants are then cultured for recovery (Panis *et al*., 2005). The main advantage of this technique is that very high cooling rates are possible as a result of the small volume of cryoprotectant used and the good conductivity of aluminium (Sakai and Engelmann, 2007; Benson, 2008b). Further, it has proved to be applicable to explants of a wide range of crop plants (Leunufna and Keller, 2003; 2005; Kim *et al*., 2006; Kryszczuk *et al*., 2006; Sant *et al*., 2008). Explants that have been successfully cryopreserved using this technique include unripe inflorescences (Kim *et al*., 2007a), somatic embryos (Martinez-Montero *et al*., 2008), bulbil primordia (Haeng-Hoon *et al*., 2007), shoot tip meristems (Panis *et al*., 2005), seeds (Jitsopakul *et al*., 2008) and hairy roots (Kim *et al*., 2007b).

A disadvantage of the vitrification, encapsulation-dehydration and droplet-vitrification methods is that they require that explants be placed in a suspending PVS2 solution

within cryovials which are then plunged into liquid nitrogen. This raises safety and phytosanitary concerns because if liquid nitrogen enters cryovials (which is very likely) the cryovials may explode upon warming as the gas expands (Benson, 2008b). Further, liquid nitrogen has been reported to harbour microbial contaminants which may infect germplasm stored in cryovats (Fountain *et al.*, 1997), and as we have found in our laboratory (unpublished observations). Such hazards can be partially ameliorated by storing samples in the vapour phase of liquid nitrogen where possible (Pegg, 1999) and/or by double containment, using a specially developed sealant to enclose cryovials (Nunc^{TM}, 2005; 2008).

1.7.3 Factors to be considered for the practical application of cryopreservation

Successful cryopreservation depends on the optimisation of a number of physiological and physical parameters, e.g. choice of a suitable explant, procedure and culture conditions, rate of dehydration, which is critical for desiccation-sensitive material, cryoprotectant treatments, rate of cooling, warming, assessment of recovery and genetic integrity after cryopreservation (Engelmann, 1991; Chawla, 2004). The composition of the rehydration medium also appears to be critical (Berjak *et al.*, 1999; Berjak and Mycock, 2004). Of these parameters, choice of explant and optimal culture conditions for onwards development need to be optimised before cryopreservation trials can be attempted.

a) Explants for cryopreservation

For successful cryopreservation, intracellular ice formation must be avoided or minimised during cooling (Sakai and Yoshida, 1967) thus explants require to be partially dehydrated. This places constraints on the size of explants for cryopreservation (Walters *et al.*, 2008). Large explants which have a small surface area to volume ratio, will dry unevenly and too slowly to avoid metabolism-linked damage: considering that most recalcitrant seeds are large, they are generally precluded as suitable explants for cryopreservation (Berjak and Pammenter, 2008; Normah and Makeen, 2008). For this

reason, a number of workers have used embryonic axes excised from seeds as explants for cryopreservation (Normah *et al*., 1986; Pritchard and Prendergast, 1986; Pence, 1990; Vertucci *et al*., 1991; Radhamani and Chandel, 1992; Goveia *et al*., 2004; Perán *et al*., 2006; Berjak and Pammenter, 2008; Normah and Makeen, 2008). Excised embryonic axes theoretically provide ideal explants for cryopreservation since they are organised structures capable of regenerating whole plants (Chandel *et al.*, 1995; Malik and Chaudhury, 2005). Also because they are usually small, embryonic axes can be rapidly and sufficiently dehydrated to facilitate cryopreservation (Berjak *et al*., 1999), with minimal injurious ice crystal damage during cooling. Embryonic axes also offer the same advantages with respect to conservation of genetic diversity as whole seeds. However, especially for tropical species, problems have been apparent when using excised axes as explants (Pammenter *et al*., 2011), each of which requires solution before universal, or even general cryopreservation protocols can be developed.

The selection of embryonic axes at the correct developmental stage is one critical factor for successful cryostorage (Goveia *et al*., 2004). Embryonic axes, at similar water contents but different developmental stages, have been shown to be differentially tolerant to dehydration and cryopreservation. In most cases, immature axes are less tolerant to cryopreservation than those that are mature (Chandel *et al*., 1995; Kim *et al*., 2002). A complicating factor is that it is not always easy to identify when recalcitrant seeds are physiologically mature (see above).

It is not always possible to use embryonic axes as explants for cryopreservation. In some species the axis itself may be too large or hypertrophied (yet undeveloped) as in the case of the economically important species Brazil nut and mangosteen (Berjak, 2000). In such cases, explants alternative to embryonic axes must be used, such as *in vitro* nodal segments (González-Benito and Perez, 1997), shoot tips (Abdelnour-Esquivel and Engelmann, 2002), meristem explants (Panis *et al*., 2005), adventitious shoots (Burritt, 2008), somatic embryos (Scocchi *et al*., 2007), cell suspension cultures (Mikuła, 2006), callus (Popova *et al*., 2009), pollen (Tyagi and Hymowitz, 2003), protoplasts (Gazeau *et al*., 1992), etc. However, in a conservation programme, not only should genetic diversity per species be represented, but also the aim is to maintain

genetic fidelity. Hence, it is best to use explants that do not develop through a callus stage since this type of organogenesis is associated with a high risk of somaclonal variation (Bayliss, 1980; Scowcroft *et al.*, 1987). This aspect is further considered later.

b) Dehydration of explants

The water content of recalcitrant embryonic axes is generally too high for cryopreservation without lethal ice formation (Wesley-Smith *et al.*, 2001a; Berjak and Pammenter, 2004a). If axes were cooled at such typically high water contents, the cooling rate would be reduced with an extended period in the temperature range that facilitates ice crystallisation (Wesley-Smith *et al.*, 2001a). Therefore, axes need to be dehydrated as rapidly as possible to water contents that would not cause damage due to desiccation, but will allow for non-injurious cooling (Pammenter and Berjak, 1999). Besides size, other factors that can influence the response of explants to dehydration are the developmental stage, dehydration rate achievable, the time the explant is kept in the partially dehydrated state and the method of rehydration and recovery (Pammenter *et al.*, 2002a).

For recalcitrant axes in particular, the more rapidly they are dehydrated, the lower the water content to which they can be dried before viability is lost (Berjak *et al.*, 1990; Pammenter *et al.*, 1991). In this manner, rapid dehydration allows axes to pass swiftly through the intermediate water contents (approximately 1.0 – 0.3 g g^{-1}) where aqueous-based metabolism linked damage (i.e. unbalanced metabolism, ROS production and associated damage) is likely to occur (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001). In this way damage accumulation and viability loss are minimised (Pammenter *et al.*, 1998). However, regardless of the rate of dehydration, there is a lower limit, below which desiccation-sensitive tissue will not survive [Pammenter *et al.* (2002a) reported that the minimum water content tolerated is approximately 0.2 g g^{-1}]. This limit is generally at the level where the water remaining is structure associated (non-freezable water). The damage that occurs when structure-associated water is lost is called desiccation damage *sensu stricto* and is different from the damage that results from unbalanced metabolism at intermediate water contents

(Pammenter *et al*., 1991; 1998; Pammenter and Berjak, 1999; Walters *et al*., 2001). According to Pammenter *et al*. (2002a), the lowest water content tolerated by desiccation sensitive tissues is not nearly as low as that which characterises dry, desiccation tolerant seeds (< 0.05 g g^{-1}water content). Further, desiccation-sensitive tissue will survive at low water contents only if immediately cryopreserved. If the material is kept partially dry (at room temperature), viability will be rapidly lost (Walters *et al*., 2001).

c) Cryoprotectants

Explants can be exposed to cryoprotective compounds prior to cooling (Benson, 2008a) with the objective of increasing freeze tolerance by controlling the potentially lethal effects of concentrated extracellular solutions and ice formation (Elmoazzen *et al*., 2005). Explants can be treated with cryoprotectants either by immersion in a solution (Shimonishi *et al*., 2000) or by being exposed to cryoprotective agents in culture media (Thierry *et al*., 1997). Cryoprotectants are separated into two categories, *viz.* penetrating (permeating or colligative) and non-penetrating (non-permeating or osmotic) cryoprotectants (Day *et al*., 2008).

Penetrating cryoprotectants include methanol, acetamide, dimethylsulphoxide (DMSO/Me_2SO), ethanol and a range of glycols (Finkle *et al*., 1985; Meryman and Williams, 1985; Benson, 2008a; Day *et al*., 2008). For colligative cryoprotection, cryoprotectants must be able to penetrate cells and must be non-toxic at the concentrations required for them to afford protection (Benson, 2008b). Penetrating cryoprotectants increase the intracellular concentration (Wolfe and Bryant, 2001) and consequently viscosity so that intracellular ice formation and further dehydration may be inhibited (Meryman and Williams, 1985; Wolfe and Bryant, 2001). Such cryoprotectants also reduce freezing point, essentially maintaining a proportion of water in the unfrozen state at subzero temperatures such that solutes do not accumulate to toxic levels intra- and extracellularly (Finkle *et al*., 1985; Benson, 2008a). Hence, penetrating cryoprotectants counteract excessive or insufficient dehydration (Benson, 2008a). An example of a cryoprotectant capable of penetrating cell membranes very

rapidly is DMSO (Wolfe and Bryant, 2001). However, a disadvantage of a rapidly penetrating cryoprotectant (such as DMSO) is that it must be either membrane- or water-soluble: once penetration into the plasma membrane occurs, the latter may be structurally altered, causing lethal damage to the cell. Primarily for this reason, penetrating cryoprotectants are often toxic (Wolfe and Bryant, 2001).

Non-penetrating cryoprotectants include sugars and sugar alcohols, betaine, urea, sarcosine, amino acids, polyols, polyethylene $glycol_{6000}$, polyvinylpyrrolidone and trimethylamine oxide (Finkle *et al.*, 1985; Meryman and Williams, 1985; Tao and Li, 1986). Glycerol has variably been reported as both a penetrating (Tao and Li, 1986; Wolfe and Bryant, 2001; Benson, 2008a; Day *et al.*, 2008) and non-penetrating (Finkle *et al.*, 1985) cryoprotectant, although the former view is generally favoured (as reviewed by Naidoo, 2011). Non-penetrating cryoprotectants remain outside affording protection in a different way from penetrating types. High molecular weight, non-penetrating cryoprotectants act osmotically (Finkle *et al.*, 1985; Wolfe and Bryant, 2001), their extracellular accumulation resulting in efflux of water from cells. This means that less water is available for intracellular ice formation or this process may be delayed or even prevented (Finkle *et al.*, 1985). A further effect of non-penetrating cryoprotectants is to keep the extracellular solution dilute (Finkle *et al.*, 1985). However, if excessive water loss occurs first by osmotic dehydration by non-penetrating cryoprotectants followed by water removal during extracellular ice crystal formation, two lethal outcomes are possible; either excessive water loss which can result in accumulation of intracellular solutes to toxic levels (solution effects) and/or the difference in osmotic pressure across the plasma membrane may cause cell collapse (Finkle *et al.*, 1985).

Although cell permeation is often used to describe the action of cryoprotectants, there is still ongoing debate as to the exact functioning and sites of protection of cryoprotectants. For example, penetrating cryoprotectants have been reported to be effective after exposure for times that were likely to be too brief to allow adequate protection to occur (Lovelock and Bishop, 1959).

d) Cooling rate

The cooling rate of explants is dependent upon the water potential, water content, explant size and efficient moisture and heat transfer (Walters *et al*., 2008). Different cooling techniques can be used to achieve varying cooling rates; for example, explants within cryovials placed in the vapour above liquid nitrogen cool at a rate of approximately 10°C min^{-1} while plunging cryovials into liquid nitrogen produces a cooling rate of approximately 100 – 200°C min^{-1} (Vertucci, 1989). A faster cooling rate can be achieved by plunging naked explants into sub-cooled nitrogen slush (-210°C), which results in a cooling rate of approximately 1 000°C s^{-1} (Vertucci, 1989). The cooling rate can be further increased by forcibly propelling samples into nitrogen slush using a spring-loaded device (Wesley-Smith *et al*., 2001a) or compressed air (Wesley-Smith *et al*., 2004) which can produce a cooling rate of up to 1 282°C s^{-1}. Those authors showed that the rate of cooling was dependent on water content when axes of *Poncirus trifoliata* were rapidly cooled (non-equilibrium cooling), that study showing that the higher the water content, the faster the cooling rate that is required to limit damage associated with ice crystallisation. At lower water contents, intracellular viscosity is increased, thereby slowing ice crystallisation and tending to make survival independent of cooling rate (Wesley-Smith *et al*., 2004). Obviously, therefore, a range of factors must be considered to achieve an optimal cooling rate.

e) Warming

Cryopreserved explants must be warmed before culturing for regrowth assessment or the potential for onwards development. The rate of warming has been reported to be linked to the rate of cooling (Mazur, 2004; Wesley-Smith *et al*., 2004). Generally, when explants are rapidly cooled, they should also be rapidly warmed (Walters *et al*., 2008). When cooling rates are sufficiently high for intracellular ice formation to occur, the warming rate also needs to be rapid to prevent recrystallisation of small ice crystals into larger crystals during warming (Walters *et al*., 2008). Rapid warming is usually carried out by immersing cryovials into a water bath at 40°C (Chen and Kartha, 1987) or by direct immersion of explants into a warming solution (Perán *et al*., 2004). For

recalcitrant seeds, a warming and rehydration solution containing 0.5 µM Ca^{2+} and 0.5 mM Mg^{2+} has been shown to promote seedling development (Berjak and Mycock, 2004).

f) *In vitro* regeneration protocols for cryopreserved material

In intact seeds, embryonic axes rely on cotyledons and/or endosperm to provide nutrients for growth and development. When axes are excised from cotyledons, they similarly require an external source of nutrients for further growth, thus they must be cultured *in vitro* on an appropriate medium that contains the required carbon source and mineral salts (Grosser, 1994; Kartha and Engelmann, 1994; George, 2008). It is therefore necessary to develop a suitable and reliable *in vitro* regeneration protocol for excised axes (or other material) prior to any other experimentation (Engelmann, 2004), as the goal is to generate functional plants after cryopreservation (Berjak *et al*., 1996). In some cases onwards development may be promoted by including plant growth regulators in the medium. Examples inlcude α-napthaleneacetic acid (Krishnapillay, 2000), 6-benzylaminopurine (Abdelnour-Esquivel and Engelmann, 2002), zeatin and indole-3-acetic acid (Adkins *et al*., 1995), but this may not be necessary for onwards development of mature embryonic axes (Walters *et al*., 2008). In addition, some researchers have suggested specific modifications to media to promote onwards development following retrieval from liquid nitrogen, such as the reduction or omission of ammonium nitrate (Decruse and Seeni, 2002), the addition of activated charcoal (Kim *et al*., 2002) and even the addition of a haemoglobin solution (Erythrogen^{TM}) which protects against free radical damage (Al-Forkan *et al*., 2001).

Culture conditions may also require modification to minimise damage and promote survival; for example, cultures may need to be incubated in the dark (Touchell and Walters, 2000) or under reduced light intensities (Benson *et al*., 1989). In addition, it is necessary to establish an appropriate decontamination protocol so that fungal and/or bacterial inoculum can be eliminated before culturing explants *in vitro*. An inappropriate medium and/or too harsh a decontamination procedure may lead to an over-estimation of the damage caused by cryo-exposure (Berjak *et al*., 1999; Reed,

2008; Walters *et al*., 2008) since damage that may be incurred during the decontamination process may be wrongfully attributed to cryo-exposure damage.

A common problem encountered in work on tropical and sub-tropical species is the lack of shoot production when excised axes are used as explants, even before dehydration and cooling (discussed by Engelmann, 1998; Goveia *et al*., 2004; Walters *et al*., 2008). Seedling development is emphasised because germination *sensu stricto* embodies events culminating in radicle protrusion (Bewley and Black, 1994). In most of the species tested in our laboratories, seedling establishment has not occurred, seemingly as a consequence of excision injury to the shoot meristem (Goveia *et al*., 2004; Walters *et al*., 2008; Pammenter *et al*., 2011). In fact, published reports often do not report survival following cryostorage in terms of seedling development; rather, greening, root development, callus production or germination is reported to indicate survival (Table 1.1). As a result, it is difficult to assess the success and efficiency of many reported cryopreservation protocols.

Table 1.1: Summary of examples of research articles on cryopreservation of embryonic axes of tropical, sub-tropical and temperate species indicating reported criteria for survival. R = recalcitrant, I = intermediate, *R = minimally recalcitrant, O = orthodox.

Species	Seed category	Survival	Reference
Araucaria hunsteinii	R	Roots	Pritchard and Prendergast, 1986
Araucaria hunsteinii	R	Callus	Pritchard *et al.*, 1995
Artocarpus heterophyllus	R	Survival	Thammasiri, 1999
Artocarpus heterophyllus	R	Survival	Krishnapillay, 2000
Azadirachta indica	*R	Axis elongation	Berjak and Dumet, 1996
Camellia sinensis	R	Greening, roots	Wesley-Smith *et al*., 1992
Castanea sativa	R	Callus, roots	Pence, 1990
Castanea sativa	R	Growth	Corredoira *et al*., 2004
Cocos nucifera	R	Gemmules	Assy-Bah and Engelmann, 1992
Coffea arabica,	I	Germination	Abdelnour-Esquivel *et al*., 1992
C. canephora,			
C. arabica x	I		

C. canephora	I		
Corylus avellana	O	Roots	González-Benito and Perez, 1994
Gossypium hirsutum	O	Germination	Wheeler, 2000
Hevea brasiliensis	R	Greening, roots, callus	Yap *et al.*, 1998
Juglans regia	R	Callus, regrowth	de Boucaud *et al.*, 1991
Lansium domesticum,	R	Viability	Normah *et al.*, 2000
Baccaurea motleyana,	R		
B. polyneura	R		
Mangifera indica,	R	Germination, leaf production	Fu *et al.*, 1990
Litchi chinensis,	R		
Euphoria longan	R		
Melia azedarach	O	Viability	Kaviani, 2007
Panax ginseng	O	Roots	Kim *et al.*, 2008
Passiflora edulis,	I	Viability	Ospina *et al.*, 2000
P. ligularis	O		
Piper nigrum	R	Roots	Chaudhury and Chandel, 1994
Poncirus trifoliata	R	Greening, roots	Wesley-Smith *et al.*, 2004
Pterocarpus indicus	O	Viability, germination	Krishnapillay *et al.*, 1994
Quercus falcata,	R	Callus	Pence, 1992
Castanea sativa	R	Roots	Pence, 1992
Sechium edule	R	Germination	Abdelnour-Esquivel and Engelmann, 2002
Sterculia cordata	I/R	Germination	Nadarajan *et al.*, 2006
Theobroma cacao	R	Callus	Pence, 1991
Trichilia dregeana	R	Greening, callus	Kioko *et al.*, 1998
Zizania palustris	R	Coleoptile elongation	Touchell and Walters, 2000
Zizania texana	R	Coleoptile extension	Walters *et al.*, 2002b

As mentioned, one major reason for the observed lack of seedling development has emerged as damage during excision from cotyledons. In dicotyledonous seeds, common

practice has been for the embryonic axis to be removed by cutting through the cotyledonary attachments flush with its surface (Kioko *et al*., 1998; Berjak *et al*., 1999; Goveia *et al*., 2004). It has been shown that a burst of reactive oxygen species (ROS) occurs in response to the wounding caused by excision (Roach *et al*., 2008; Whitaker *et al*., 2010; Pammenter *et al*., 2011). If wound sites are close to the shoot meristem (as is the case for *E. capensis*), then an injury-related ROS burst might cause the necrosis, so obviating shoot development (Perán *et al*., 2006). Furthermore, Whitaker *et al*. (2010) have shown that ROS emission accompanies dehydration and rehydration following cryo-exposure. Thus each step of a protocol for cryopreservation, has the potential to cause uncontrolled ROS-mediated damage, under conditions where the endogenous antioxidants (Varghese *et al*., 2011) of the axes are inadequate to counteract the ROS generated (Berjak *et al*., 2011b).

Starting with excision injury, it is necessary to devise strategies to overcome effects of ROS production. One option is to leave small pieces of each cotyledon attached to the axis. In this way, the shoot meristem region of the axis may be protected from the potential harmful effects of ROS accompanying the excision process, as the axis itself would not be wounded. However, the disadvantage of this is the increase in explant size, which may slow down the rate of dehydration and will have an adverse effect on cooling, perhaps promoting ice crystal formation (Pammenter *et al*., 2002a). The problem of ROS production and its consequences at each preparative stage of a cryopreservation protocol, demands urgent attention if cryopreservation is to emerge as a really successful means for tropical/sub-tropical germplasm conservation using excised embryonic axes. In this regard, the newly-developed approach of using the cathodic fraction of an electrolysed CaMg solution, is highly promising (Berjak *et al*., 2011b). However, the work presented in this thesis preceded the development of cathodic amelioration.

g) Assessment of genetic integrity

Cryopreservation and the cryopreparative stages impose a number of stresses on plant tissues, which may affect the genetic integrity of the germplasm. Further, the necessity

of an *in vitro* stage for onwards development may also have an effect as somaclonal variation can occur. The term somaclonal variation has been used to describe variation that occurs in plants as a result of the tissue culture process (Larkin and Scowcroft, 1981; Ammirato, 1986) and includes both genetic and epigenetic changes (Bayliss, 1980; Bednarek *et al*., 2007). Although the exact mechanism of somaclonal variation is not clearly understood, the genetic changes have been identified as point mutations, karyotype changes, chromosome rearrangements, altered sequence copy number, transposable elements, somatic crossing over, sister chromatid exchange, gene amplification and deletion (Cocking, 1986; Scowcroft *et al*., 1987; Phillips *et al*., 1994; Karp, 1995; Jain and de Klerk, 1998; Kaeppler *et al*., 2000; reviewed by Sahijram *et al*., 2003). The advantages of somaclonal variation have been harnessed to generate novel sources of variation for plant breeding (Evans *et al*., 1984; Karp, 1995; Jayasankar, 2005; Singh *et al*., 2008; Rajeswari *et al*., 2009), but variation is detrimental in conservation programmes (Towill, 1991) or in clonal multiplication systems where genetic uniformity is required (Jain and de Klerk, 1998; Sahijram *et al*., 2003; Mulwa and Bhalla, 2007).

Somaclonal variants will not invariably be generated by *in vitro* culture manipulations (Karp, 1995), but there are certain factors that increase their likelihood. The major factors that affect the nature and frequency of variation are genotype, ploidy, tissue source, tissue culture procedure, culture environment and medium composition (Karp, 1992; 1995; Zucchi *et al*., 2002; Etienne and Bertrand, 2003; Sahijram *et al*., 2003; Bednarek *et al*., 2007). A significant factor in the generation of variants is the degree of departure from organised growth, i.e. the greater the departure for example a callus stage, and the longer the time spent in this condition, the greater the likelihood of generating somaclonal variants (Bayliss, 1980; Karp, 1995; Pontaroli and Camadro, 2005). The type and concentration of plant growth regulators can also influence the production of somaclonal variants (Karp, 1992); in particular, 2, 4-dichlorophenoxyacetic acid (2,4-D) has been singled out as affording a high risk of generating somaclonal variants (Karp, 1995; Etienne and Bertrand, 2003; Jayasankar, 2005; Pontaroli and Camadro, 2005; George, 2008).

Somaclonal variation pertains to genetic and phenotypic variation (Bayliss, 1980; Sahijram *et al*., 2003). It can be manifested either somatically (where the variation is not heritable) or meiotically (where the variation is heritable and can affect subsequent generations) (Kaeppler *et al*., 2000). Epigenetic variation refers to variation that occurs by changes other than in the primary DNA sequences. Such mechanisms, characterised by methylation, acetylation and phosphorylation, are active at the level of the nucleosome and affect DNA-protein interactions (Harding and Millam, 2000). Methylation particularly has been suggested to be the basis of epigenetic phenomena (Finnegan *et al*., 2000). In higher organisms, this process involves the methylation of cytosine residues yielding 5-methylcytosine (Akimoto *et al*., 2007). DNA methylation in plants has been suggested to affect processes associated with morphogenesis and development (Finnegan *et al*., 2000), particularly cell differentiation, inactivation of chromatin, genomic imprinting, differential gene expression and gene silencing (Kaeppler *et al*., 2000; Paszkowski and Whitham, 2001; Bird; 2002; Bender, 2004), as well as to occur in response to stress (Tariq and Paszkowski, 2004; Johnston *et al*., 2005). Although epigenetic changes do not involve changes to primary DNA, there is a concern that they may persist and result in altered phenotypes of recovered plants (Peredo *et al*., 2008). In this way, epigenetic variation could contribute to somaclonal variation (Bednarek *et al*., 2007).

In his review, Harding (2004) lists a range of techniques that can be used to detect abnormalities following cryopreservation. The most basic method is to assess the morphological development of plants (Rodriguez and Vendrame, 2003) to determine if cryopreservation treatments result in altered phenotypes (Wu *et al*., 2001b). Biometric studies can also be performed to assess the extent of phenotypic variation (Harding, 2004) by statistically analysing qualitative and quantitative morphological features (Harding and Staines, 2001). Histological and cytological analyses can be performed to assess the chromosomal stability of plants (Harding, 2004). Regenerated plants can also be analysed by assessing specific metabolites, proteins or enzymes (Harding, 2004). Proteins and enzymes can be electrophoretically analysed while changes in metabolite profiles can be assessed using particular biochemical assays (Tyagi and Yusuf, 2005).

In addition, there is a range of molecular biological techniques that have been developed to assess genetic integrity. These can be separated into three broad categories, i.e. polymerase chain reaction (PCR) techniques [such as microsatellites or simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD)], non-PCR techniques [e.g. restriction fragment length polymorphism (RFLP)] and a third category which utilises principles of both PCR- and non-PCR based techniques. Since their development, PCR techniques have been widely used as they are generally reliable and usually require small quantities of DNA for analysis (except where otherwise stated in Table 1.2). However, disadvantages are that differing DNA profiles may be encountered among different DNA extracts, PCR machines, technicians, laboratories and sources of Taq DNA polymerase (Harding, 1996). Table 1.2 provides a brief summary of the most commonly used techniques to assess genetic integrity of plant germplasm and their associated advantages and disadvantages. For an overview of molecular DNA marker techniques suitable for analysing the genetic integrity of micropropagated woody plants, the reader is referred to Rani and Raina (2003) and references therein. Regardless of the specific technique selected for analysis, Harding (2004) and Bhat (2005) have advised that it should be reliable, reproducible, relatively simple and quick to perform, non-hazardous and as cost effective as possible.

Table 1.2: Summary of the most commonly used techniques to assess genetic integrity of germplasm and their associated advantages and disadvantages.

Technique	Advantages	Disadvantages
Morphological analysis	simple and easy to observe traits; considers the effect of whole genome interactions on phenotype	requires regular assessment until maturity – impractical for long-lived trees; genetic changes may not be phenotypically expressed and may therefore be undetected; expression of phenotypes may be influenced by environmental changes and may not be heritable
Histological and cytological analysis	technically simple; identifies changes in ploidy	not suitable for polyploid species with high chromosome numbers or species with very small chromosomes
Enzyme, protein and metabolite analysis	easy to perform; inexpensive; isozymes provide a good estimation of gene expression	provides information only on regions of the genome that code for soluble proteins; sensitive to developmental and environmental conditions
Molecular Biological Techniques		
RFLP	provides information on the nature and extent of variation at specific loci; good reproducibility among laboratories	technically complex; necessary to use radioisotopes for detection; requires large amounts of good quality DNA; expensive to construct cDNA library; analysis is limited to those sequences used as a probe; not possible to automate, therefore time consuming
Microsatellites (SSR, inter–SSR)	robust; reproducible	construction of genomic library and synthesis of specific primers is time consuming and expensive
AFLP	sensitive marker; samples genome widely; cost per data point cheaper than RFLP or RAPD; no need for radioisotopes for detection; reportedly more efficient than RFLP or RAPD; reliable;	requires large amounts of DNA per reaction; point mutations that occur outside of sampled priming regions may not be identified; requires specialised technical knowledge to identify problems

	reproducible	
RAPD	no blotting or hybridisation steps; no requirement for prior information of primer sequences; can be automated	reaction conditions must be precisely standardised to obtain reproducible results

The information in Table 1.2 was compiled using the following references: Leroy *et al*. (2000); Rani and Raina (2000; 2003); Turner *et al*. (2001a); Rodriguez and Vendrame (2003); Harding (2004); Bhat (2005); Tyagi and Yusuf (2005); Mulwa and Bhalla (2007).

A problem with the molecular biological techniques described above is that such techniques analyse only a fraction of the genome, approximately 0.001% (Harding, 2004), and despite the various advantages of PCR-based techniques, small deletions or insertions that occur in chromosomes outside of sampled primer sites will not be detected (Harding and Benson, 2000). Therefore, even when researchers find no changes in genetic profiles, a *caveat* is that this does not provide conclusive evidence that no genetic changes have occurred in cryostored germplasm (Turner *et al*., 2001a; Liu *et al*., 2004). It is also possible that such techniques are biased to detect stability of selected sequences, rather than instability in the genome (Harding, 2004). Certain techniques (described in Table 1.2) can be modified to include methylation-sensitive restriction enzymes (e.g. the methylation-sensitive amplified polymorphism assay); however, such techniques require specialised information on the sequence of the genome of the species under investigation in order to design specific primers. However, as is the case with many indigenous African species that produce recalcitrant seeds, very little (if any) previous molecular biological analyses have been performed. Therefore, any protocol that requires designing of specific primers is not suitable. An option to overcome this problem is to use commercially available universal primers, as was done in the present study. In this way, methylation-sensitive restriction enzymes can be used to cleave DNA and these products can be PCR-amplified and the products run on an agarose gel to provide an indication of changes in the methylation status of DNA.

Considering the number of stages in a cryopreservation protocol, it would be useful to identify at which stage variation (particularly epigenetic variation) is most likely to occur. Kaity *et al*. (2009) suggested that the cooling, warming or cryoprotection stages

may have induced DNA methylation changes in pawpaw, while Peredo *et al.* (2008) suggested that for hop, the micropropagation stage is likely to generate the most variation with cryopreservation also playing a role. Johnston *et al*. (2009) suggested for *Ribes* genotypes that the sucrose-simulated cold acclimation step may cause the largest change in the DNA methylation status. Hence, it is important to determine the status of germplasm in terms of epigenetic changes at each stage of a cryopreservation protocol, and also after acclimatisation.

1.8 Aims and objectives of the present study

Conservation of germplasm of indigenous plant species is vital not only to preserve valuable genotypes, but also the diversity represented by the gene pool. Presently, the only strategy available for the long-term conservation of species that produce recalcitrant seeds is cryopreservation. *Ekebergia capensis* is an indigenous species that produces recalcitrant seeds (Pammenter *et al*., 1998) and the aim of the present study was to develop a protocol for cryopreservation of germplasm of this species. Different explant types were investigated for this purpose, *viz.* embryonic axes (with attached cotyledonary segments) excised from seeds, and two types of *in vitro*-derived explants, i.e. nodal segments excised from shoots of *in-vitro*-germinated seedlings and adventitious shoots generated from intact roots developed by *in-vitro*-germinated axes.

Suitable micropropagation protocols needed to be developed for all explant types prior to any other experimentation. Further, before explants could be cryopreserved it was necessary to reduce their water content in order to limit damaging ice crystallisation upon cooling. In addition, penetrating and non-penetrating cryoprotectants were used to improve the tolerance of explants to cryogen exposure. Trials were also performed at different cooling rates, since this factor is also known to affect the success of a cryopreservation protocol.

Each stage of a cryopreservation protocol imposes stresses that may limit success. To gain a better understanding of these processes the effects of the stress needed to be investigated by assessing the extracellular production of ROS (in particular, superoxide)

at each stage of the protocol, in line with current thinking that this is a primary stress or injury response (see above). This would give an indication whether ROS contribute to viability loss at each stage. In addition, imposed stresses may affect the genome. Since the aim of a conservation programme is to maintain genetic and epigenetic fidelity of stored germplasm, it is essential to ascertain whether this has been achieved. Thus, DNA was isolated from material after each stage of the cryopreservation protocol and analysed by restriction enzyme digestion of DNA and PCR-amplification of the products, with assessment being made of the methylation status, in terms of possible epigenetic changes. Overall, the investigations of this study were aimed both at developing a successful cryopreservation protocol for *E. capensis*, and at providing a better understanding of the responses of germplasm of *E. capensis* to the stresses of a cryopreservation protocol.

CHAPTER 2: STRATEGIES FOR THE MICROPROPAGATION OF *Ekebergia capensis* Sparrm.

2.1 INTRODUCTION

The origin of micropropagation can be traced back to the pioneering work of Gottlieb Haberlandt (1902) who first cultured isolated plant cells and tissues in nutrient solutions in 1902 (loc. cit. Laimer and Rücker, 2003; Vasil, 2008). Since then, research on the establishment of suitable media for regeneration and elucidation of the role of plant growth regulators have contributed significantly to the development of micropropagation techniques (Bhowmik and Matsuiz, 2001; Vasil, 2008). Micropropagation is the propagation of small selected explants using *in vitro* culture techniques (George, 1993; George and Debergh, 2008). In contrast, traditional macropropagation, which is practised by the horticultural and forestry industries, is performed using larger plant pieces (George and Debergh, 2008). Today, *in vitro* culture and its associated micropropagation techniques are established and practised in many research and commercial laboratories throughout the world (e.g. de Fossard, 2000; Bhowmik and Matsuiz, 2001; Loyola-Vargas and Vázquez-Flota, 2006; Vasil, 2008).

There are two developmental pathways or regeneration routes for micropropagation *in vitro*, i.e. embryogenesis or organogenesis (Ahuja, 1993; Ramage and Williams, 2002; Razdan, 2003). Somatic (asexual) embryogenesis is the production of embryo-like structures from somatic cells in response to applied plant growth regulators (Hansen and Wright, 1999) and the stages are considered to be similar to the process of zygotic embryogenesis (Razdan, 2003; Mujib *et al*., 2005). Somatic embryos do not have any vascular connection to the parent tissue and are bipolar structures that have a separate root and shoot pole (Ahuja, 1993; Bhowmik and Matsuiz, 2001; Ramage and Williams, 2002). Organogenesis is a developmental process where organ primordia are initiated on explants in response to the application of plant growth regulators. This process is initiated by cell division, followed by the formation of meristems and thereafter organs (Bhowmik and Matsuiz, 2001; Ramage and Williams, 2002). In contrast to somatic

embryos which are bipolar, organogenesis results in the formation of a unipolar structure with either a root or shoot primordium (Bhowmik and Matsuiz, 2001).

Both somatic embryogenesis and organogenesis can occur either directly or indirectly. As the term implies, direct somatic embryogenesis occurs when embryos originate directly from the cells of an explant *in vitro* (Cheliak and Rogers, 1990; Watt *et al*., 1995) and direct organogenesis takes place when regeneration occurs directly on the parent explant from pre-existing meristems of shoots, roots, leaf initials, inflorescences or seeds (George, 2008). In contrast, indirect embryogenesis and organogenesis occur when regeneration takes place indirectly, usually via an unorganised callus stage (Razdan, 2003). A callus is a mass of undifferentiated cells, which can be induced to form shoots, roots or somatic embryos when the correct type and ratio of plant growth regulators (PGRs) are applied (George, 2008; George and Debergh, 2008). However, a significant problem with indirect routes of regeneration via callus is that it carries a risk of generating somaclonal variants (Bayliss, 1980; Scowcroft *et al*., 1987; Karp, 1995; Pontaroli and Camadro, 2005) which are not desirable in conservation programmes or for clonal propagation where the aim is to maintain, and not alter, the genetic integrity of germplasm (Towill, 1991; George and Debergh, 2008). Therefore, in the present study, only direct routes of micropropagation were investigated. Two of the common direct micropropagation techniques presently used are axillary bud multiplication and adventitious shoot production (George and Debergh, 2008). However, any micropropagation protocol can be separated into distinct stages, which are discussed below.

There are five stages in a micropropagation protocol, *viz*. selection of parent plants, initiation of aseptic cultures, multiplication, elongation and rooting of shoots (although it may be necessary to separate this into two steps) and finally acclimatisation (discussed by George, 1993; Preil, 2003; George and Debergh, 2008). During stage one, stock plants can be maintained in a greenhouse to limit the subsequent proliferation of contaminants *in vitro*. This is common practice when axillary bud explants are initiated from parent plants (Taji *et al*., 2002a; Wilhelm, 2003). During stage two, selected explants are excised and treated to eliminate or reduce microbial contaminants.

Commonly used decontaminants include sodium and calcium hypochlorite (Niedz and Bausher, 2002), mercuric chloride (Bhat *et al*., 1992), ethanol (Leifert and Waites, 1994) and various fungicides (Leifert and Waites, 1990). If bacterial contamination persists *in vitro*, antibiotics can be applied (Reed *et al*., 1998; Leifert and Cassells, 2001); however, care must be taken to avoid phytotoxicity and to guard against the indiscriminate use of antibiotics as a prophylactic treatment (Falkiner, 1990; Niedz, 1998). To avoid this situation, other compounds, such as the isothiazolone plant preservative mixture (PPM), may be used to curb bacterial proliferation *in vitro* (Niedz, 1998; Niedz and Bausher, 2002).

After decontamination, explants are cultured *in vitro* on a suitable regeneration medium. This medium has to be experimentally determined for each species and explant-type but is usually comprised of a carbon source, gelling agent (such as agar or Gelrite), a nutrient formulation (containing salts, macronutrients, micronutrients and vitamins) and appropriate PGRs that are required for morphogenesis and development. Some examples of developed nutrient formulations include Hoagland and Snyder medium (1933), White's medium (1943), Nitsch's medium (1951), the MS medium of Murashige and Skoog (1962), the LS medium of Linsmaier and Skoog (1965), B5 medium (Gamborg *et al*., 1968), the SH medium of Schenk and Hildebrandt (1972), Woody Plant Medium (McCown and Lloyd, 1981) and DKW medium (Driver and Kuniyuki, 1984). The various media formulations have different concentrations of salts and nutrients that may be suitable for different types of plant cultures (George, 2008).

The type and concentration of PGRs present in a culture medium will determine the developmental pathway that is promoted during each stage of a micropropagation protocol (Skoog and Miller, 1957; Thorpe, 1980; Minocha, 1987; Machakova *et al*., 2008). The five groups of PGRs most commonly used in plant tissue culture are cytokinins, auxins, gibberellins, ethylene and abscisic acid, of which, the first two are essential for the regulation of plant development *in vitro* (Gaspar *et al*., 1996; Machakova *et al*., 2008). Cytokinins play a role in cell division and shoot initiation and are therefore used to induce bud break and for shoot multiplication in stages two and three (Gaspar *et al*., 1996; Razdan, 2003). Examples of cytokinins commonly used for

micropropagation are kinetin, 2-isopentyladenine (2-iP), 6-benzylaminopurine (BAP), zeatin (Haberer and Kieber, 2002; Taji *et al*., 2002b; Razdan, 2003; Machakova *et al*., 2008) and the cytokinin-like compound thidiazuron (TDZ) (Huetteman and Preece, 1993). Auxins influence processes such as cell division, elongation, vascular differentiation and can promote either unorganised growth (callus) or give rise to roots (Gaspar *et al*., 1996; Razdan, 2003; Machakova *et al*., 2008). Examples of commonly used auxins are napthaleneacetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-amino-3,5,6-trichloropicolonic acid (picloram) (Gaspar *et al*., 1996; Taji *et al*., 2002b; Razdan, 2003; Machakova *et al*., 2008). However, it is the ratio of auxin to cytokinin that will determine the type of organogenesis (Taji *et al*., 2002b; Razdan, 2003); i.e. when the ratio of auxin to cytokinin is relatively high then roots are initiated and when the ratio of cytokinin to auxin is high, then shoots are initiated (Skoog and Miller, 1957; Minocha, 1987; Machakova *et al*., 2008; van Staden *et al*., 2008).

The final stage of a micropropagation protocol (stage five) is acclimatisation where rooted plantlets are transferred from *in vitro* conditions to the *ex vitro* environment, usually in a mist tent or greenhouse (George and Debergh, 2008), and it is this stage that will eventually determine the success of a protocol (Hazarika, 2003; 2006). Micropropagation protocols are often hampered by poor survival when *in-vitro*-produced plantlets are transferred to *ex vitro* conditions (reviewed by Hazarika, 2003; Rohr *et al*., 2003; Loyola-Vargas and Vázquez-Flota, 2006). This may be attributed to the fact that while *in vitro* plantlets are grown under conditions of high humidity (Pospíšlová *et al*., 1999; Chen, 2004) and are provided with the carbohydrates and nutrients needed for survival (Hazarika, 2003; 2006), during the acclimatisation process, they need to adapt to a number of changes including those that relate to the control of water loss and autotrophic growth (Pospíšlová *et al*., 1999; Marin, 2003). Hence, in order to limit mortality during this stage, *in-vitro*-produced plants should be gradually exposed to conditions of lowered relative humidity until they can survive under ambient conditions (Hazarika, 2003). Alternatively, plants can be grown under photoautotrophic conditions *in vitro* (Nguyen and Kozai, 2005) where they are cultured on medium

without carbohydrates and under conditions that promote photosynthesis (by, for example, increased or forced ventilation). Micropropagated plants cultured under conventional conditions are photoheterotrophic or photomixotrophic and display characteristics such as defective stomata and limited epicuticular wax, which have been reported to hamper acclimatisation (Afreen, 2005; Hazarika, 2006), while those plants cultured under photoautotrophic conditions reportedly do not exhibit such features (Afreen, 2005) and therefore can be more successfully acclimatised. However, a disadvantage of photoautotrophic culture is that it is expensive to purchase even simple gas-permeable filter discs, and there is significantly more expense involved in setting up forced ventilation chambers.

Many explants that are used for micropropagation are also suitable for cryopreservation since both applications require the use of small specimens. In the present study, axillary buds and adventitious shoots were used as explants for micropropagation and cryopreservation. As mentioned, axillary buds develop from pre-existing meristems, while adventitious shoots occur on explants that lack pre-existing meristems (Gahan and George, 2008). Adventitious shoots do not develop from meristems, but they can be induced on a range of organs including stems (Wang *et al*., 2008), petioles (Geneve, 2005), leaves (Ainsley *et al*., 2000), cotyledons (Canli and Tian, 2009), ovaries (Williams *et al*., 1998) and roots (Gahan and George, 2008). Adventitious regeneration can occur directly on explants or indirectly via a callus phase (Gahan and George, 2008). Once adventitious shoots have been produced *in vitro* they must be multiplied, elongated, rooted and acclimatised (Karim *et al*., 2007), as is done in any micropropagation protocol. From an examination of the literature, direct production of adventitious shoots from intact roots is scarcely reported. Most often, adventitious shoot production has been reported from excised root segments, as indicated in the reports listed in Table 2.1.

Table 2.1: Summary of reports on adventitious shoot production from roots. D = direct shoot regeneration, I = indirect shoot regeneration, DSE = direct somatic embryogenesis, ISE = indirect somatic embryogenesis.

Species	Explant	Regeneration	Reference
Acacia albida	Root segments	D	Ahée and Duhoux, 1994
Aeschynomene sensitiva	Root segments	D and I	Nef-Campa *et al*., 1996
Albizzia julibrissin	Intact roots	I	Sankhla *et al*., 1994
Albizzia julibrissin	Root segments	I	Sankhla *et al*., 1996
Albizzia julibrissin	Root segments and intact roots	D and I	Hosseini-Nasr and Rashid, 2000
Aralia elata	Root segments	D	Karim *et al*., 2007
Citrus aurantifolia	Root segments	D and I	Bhat *et al*., 1992
Clitoria ternatea	Root segments	D and I	Shahzad *et al*., 2007
Comptonia peregrina	Root segments	D	Goforth and Torrey, 1977
Convolvulus arvensis	Root segments	D	Bonnett and Torrey, 1965
Gymnocladus dioicus	Root segments	D	Geneve, 2005
Helianthus annuus x *H. tuberosus*	Intact roots	DSE	Fambrini *et al*., 2003
Hypericum perforatum	Root segments	D	Zobayed and Saxena, 2003
Melia azedarach	Root segments	D and I	Vila *et al*., 2005
Populus alba x P. grandidentata	Root segments	D	Son and Hall, 1990
Populus tremula	Root segments and intact roots	D	Vinocur *et al*., 2000
Linum usitatissimum	Root segments	I	Xiang-Can *et al*., 1989
Spinacia oleracea	Root segments	D	Knoll *et al*., 1997
Tylophora indica	Root segments	D and ISE	Chaudhuri *et al*., 2004

At present, there are no published protocols for the micropropagation of *E. capensis*. The main aim of the current investigation was to cryopreserve germplasm of *E. capensis*, which is an indigenous South African species that produces non-storable recalcitrant seeds (Pammenter *et al.*, 1998). Considering this, and the fact that significant difficulties have been experienced in obtaining shoot survival from cryopreserved seed explants including excised isolated axes of *E. capensis* (Perán *et al*., 2006), it was decided to investigate alternative *in-vitro*-derived explants for this purpose. The aim of this aspect of the study, therefore, was to establish direct organogenesis protocols for *in vitro* propagation of *E. capensis* using: 1) embryonic axes with 2 mm^3 blocks of cotyledonary tissue attached and, 2) vegetative buds, for subsequent work which would involve cryopreservation of such explants. In the case of vegetative explants, excised nodal segments from seedlings and adult plants, as well as adventitious buds induced *in vitro* from root explants, were assessed and the yield of plantlets generated from them compared. In addition, such an *in vitro* protocol is useful for supporting efforts towards conservation of the species through clonal multiplication and subsequent cultivation of superior genotypes for the traditional medicine trade.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Saplings of *E. capensis* grown from seed were maintained in the greenhouse at the University of KwaZulu-Natal, Durban (29°52'S, 30°59'E; 25°C day/18°C night). Plants were sprayed with fungicides and fertilisers on a weekly basis, the former as a mixture of 2 g l^{-1} Mancozeb (Dithane®; Efekto, South Africa) and 1 ml l^{-1} Chlorothalonil (Bravo®; Shell, South Africa) applied as a foliar spray, and a mixture of 1 g l^{-1} Prochloraz manganese chloride (Sporgon®; Hoechst Schering AgrErvo, South Africa) and 1.25 ml l^{-1} Tebuconazole (Folicur®; Bayer, South Africa) applied to the soil. The fertilisers used were a foliar spray of 2.5 ml l^{-1} trace element solution (18 Fe, 4 Cu, 2 Zn, 1 B and 0.4 Mo, all in g l^{-1}) (Trelmix; Hubers, South Africa) and 1 g l^{-1} Mondi Orange 1N: 2P: 1K (Harvest Chemicals, South Africa) applied to the soil, in alternate weeks. In addition, every five weeks, 5 ml l^{-1} of the organic fertiliser Seagro® (50 N,

9 P, 22 K, 16 S and 14 Mg, all in g l^{-1}) (Premier Fishing, South Africa) was added to the soil.

Ripe (red) *E. capensis* fruits were collected from three provenances along different latitudinal gradients (refer to Figure 3.1, page 99), *viz*. Mtunzini (28°58'S), St Lucia (28°22'S) and Port Elizabeth (33°54'S) and immediately transported to the laboratory, where the seeds were cleaned of fruit pulp, decontaminated by a 10-min immersion in 1% (w/v) sodium hypochlorite (NaOCl) and rinsed three times with sterile distilled water. They were then stored under hydrated conditions in plastic buckets, suspended on a grid above a few sheets of paper towel moistened with water and 3.5% (w/v) NaOCl. The lid of the bucket was lined with a layer of paper towel to prevent condensate from dripping onto stored seeds. The buckets were maintained at 16^{o}C for 2 – 3 w.

2.2.2 Explant preparation

a) Embryonic axes with attached cotyledonary segments

Embryonic axes were excised with 2 mm^3 blocks of each cotyledon attached (see Figure 2.1B), decontaminated by immersion in 1% (w/v) NaOCl for 10 min, rinsed three times in sterile distilled water and then cultured *in vitro*. A range of studies was performed to identify a suitable medium for onwards development of axes. These are summarised in Table 2.2. All media were prepared using ¼ MS (Murashige and Skoog, 1962) salts and vitamins (Walker, 2000). To ascertain the effects of excision, a total of 20 axes were plated, 5 per Petri dish (65 mm diameter) on 20 – 25 ml medium. The best medium for germination of explants (to yield both shoots and roots) comprised ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 3 μM (0.6 mg l^{-1}) pyridoxine and 8 g l^{-1} agar.

Table 2.2: Summary of additives used to promote germination of axes. The basal medium comprised ¼ MS salts and vitamins, 30 g l^{-1} sucrose and 8 g l^{-1} agar. n = 20.

Additive	Concentration (mg l^{-1})
BAP	1
Ascorbic acid	1.76
BAP + Ascorbic acid	1 + 1.76
2iP	0.5, 2, 5
BAP	0.5, 2, 5
kinetin	0.5, 2, 5
zeatin	0.5, 2, 5
TDZ	0.005, 0.02, 0.05
Thiamine	1
Calcium pantothenate	1.4
Nicotinic acid	0.4
Myoinositol	1.4
Pyridoxine	0.6
Glutamine	250, 500
Casein hydrolysate	250, 500, 1000

b) Seedling explants

To generate a supply of *in vitro* seedlings, embryonic axes with small cotyledonary segments were germinated (using the best conditions previously ascertained [section 2.2.2.a]) to produce seedlings in 4 – 6 w. Germinated seedlings were subcultured onto MS salts and vitamins incorporating 30 g l^{-1} sucrose and 8 g l^{-1} agar in 100 ml culture bottles (2 seedlings with 30 ml medium per bottle). When seedlings reached a height of 40 – 55 mm with 5 – 7 nodes, segments incorporating one node were excised and used for axillary bud break experiments.

c) Nodal explants from saplings

Stem segments (10 – 15 mm incorporating one node) were excised from parent plants maintained in the greenhouse. Two decontamination protocols were tested. In the first, explants were soaked for 30 min in a fungicide cocktail [1 g l^{-1} Benomyl® (Dow AgroSciences, South Africa), 1 g l^{-1} boric acid and 0.5 ml l^{-1} Bravo® (Grovida, South Africa)] followed serially by a 2-min immersion in 0.2 g l^{-1} $HgCl_2$ and 8 g l^{-1} $Ca(OCl)_2$, with sterile water rinses between solutions; in the other, the fungicide cocktail step was excluded. Following decontamination, explants (nodal segments) were trimmed to remove all leaves and most of the stem material and placed onto bud break medium (see below) in culture tubes.

d) Root explants for adventitious bud production

Root explants for adventitious bud production were produced by decontamination of excised embryonic axes as above, followed by culture on medium comprising ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 1 mg l^{-1} BAP and 8 g l^{-1}, agar for 4 – 6 w. A total of 25 axes were plated, 5 per Petri dish (65 mm diameter) on 20 – 25 ml medium. Intact roots produced from germinated embryonic axes were used for adventitious bud production, as described below. In the first instance, material from Mtunzini was used, and later the protocol was applied to explants from seeds derived in Port Elizabeth and St Lucia.

2.2.3 Bud break

Two explant sizes (2 and 5 mm) from excised nodal segments of *in vitro*-germinated seedlings were investigated for bud break. Each explant incorporated one node with a portion of the stem above and below. Four different bud break media were tested, *viz.* MS salts and vitamins, 20 g l^{-1} sucrose and 8 g l^{-1} agar (medium A); ¼ MS salts and vitamins, 20 g l^{-1} sucrose, 1 mg l^{-1} BAP and 8 g l^{-1} agar (medium B); MS salts and vitamins, 20 g l^{-1} sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.04 mg l^{-1} NAA, 0.11 mg l^{-1} BAP, 0.05 mg l^{-1} kinetin and 8 g l^{-1} agar (medium C); and MS salts

and vitamins, 20 g l^{-1} sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.01 mg l^{-1} NAA, 0.2 mg l^{-1} BAP and 8 g l^{-1} agar (medium D). For bud break from saplings, only medium D was tested.

2.2.4 Adventitious bud production

Intact, whole roots (15 – 25 mm long) produced from *in-vitro*-germinated excised embryonic axes were placed in a RITA® bioreactor (Rècipient à Immersion Temporaire Automatique, CIRAD, France) for 24 or 48 h with medium containing ¼ MS salts and vitamins, 30 g l^{-1} sucrose and 0, 1, 3 or 6 mg l^{-1} BAP. There were a total of 25 roots with 200 ml liquid medium per vessel, set at an immersion interval of 30 s flush and 10 min rest. After 24 or 48 h in the RITA® bioreactor, roots were cultured (5 per 65 mm diameter Petri dish) on 20 – 25 ml medium containing ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 1 mg l^{-1} BAP and 8 g l^{-1} agar for 6 w.

2.2.5 Multiplication, elongation and rooting

Shoots that developed by axillary bud break from nodal segments of *in-vitro*-germinated seedlings and greenhouse-grown saplings were placed on ½ MS salts and vitamins, 20 g l^{-1} sucrose, 0.33 mg l^{-1} IBA, 1.7 mg l^{-1} BAP and 8 g l^{-1} agar for multiplication for two cycles of 6 w each. As these shoots elongated on the bud break medium during the 4 – 6 w in culture, a subsequent elongation step was not required. The clumps of adventitious buds produced from root explants were elongated on medium containing MS salts and vitamins, 30 g l^{-1} sucrose and 8 g l^{-1} agar for 4 w.

Shoots produced from all explant types (a total of 30) were rooted in culture tubes (one shoot per tube) on 10 ml medium containing ½ MS salts and vitamins, 30 g l^{-1} sucrose and 8 g l^{-1} agar supplemented with 0, 0.1 or 0.5 mg l^{-1} IBA.

2.2.6 Decontamination of adventitious shoots

Surface decontamination of explant type 2 (adventitious shoots) was by immersion in either 1% (w/v) NaOCl or 1% (w/v) calcium hypochlorite [$Ca(OCl)_2$] with a few drops of Tween 20, for either 5 or 10 min. Explants were then rinsed three times with sterile distilled water.

2.2.7 Culture conditions

All media were adjusted to pH 5.6 – 5.8, prior to autoclaving for 20 min at 120^{o}C and 121 kPa. All cultures were maintained in a growth room under a 16-h photoperiod at a photosynthetic photon flux density (PPFD) of 37 μmol m^{-2} s^{-1} (lateral lighting) provided by Philips tubes (58W) at 23^{o}C day/21^{o}C night.

2.2.8 Acclimatisation

Rooted plantlets were acclimatised in pots (55 x 55 mm) or in polystyrene seedling trays (330 x 130 x 60 mm) containing a mix of 1:1:1 potting soil, vermiculite and peat. Pots containing plantlets were either tightly sealed in transparent plastic bags (160 x 280 mm) using a heat sealer or were loosely enclosed in sandwich bags (145 x 180 mm), which allowed for some gaseous exchange. The seedling tray was enclosed in a single, large plastic bag closed with an elastic band. The plastic bag was not in direct contact with plantlets, being suspended above plantlets by a rigid metal framework such that there was a 40 – 60 mm headspace. All material was kept in the laboratory (at approximately 21 to 25°C). In all cases, after two weeks the humidity of the microclimate was reduced gradually by punching holes in the bags and by the fourth week the plastic bags were completely removed. These three protocols were tested using at least 20 rooted plantlets generated from nodal explants of *in-vitro*-germinated seedlings. The best protocol was applied to adventitious shoots generated from *in-vitro*-germinated roots.

2.2.9 Photography and data analysis

Photographs were recorded with a Nikon FM2 camera fitted with a 60 mm Mikro macro lens and a Nikon Coolpix® digital camera attached to a Wild stereo microscope. Data were analysed using One or Two Way Analysis of Variance (ANOVA) and means were contrasted using Scheffe's multiple range test (95% confidence interval). Alphabetical values were assigned to the mean values recorded per treatment. Mean values that did not share the same letter were recognised as being significantly different.

2.3 RESULTS AND DISCUSSION

2.3.1 Establishment of a medium for germination of axes with cotyledonary attachments

The present aspect of the study reports and compares direct organogenesis protocols for *in vitro* propagation of *E. capensis*. Micropropagation protocols have been developed for other species in the family Meliaceae, e.g. regeneration in *Azadirachta indica* has been reported from leaf explants (Eeswara *et al*., 1998), nodal segments (Chaturvedi *et al*., 2004; Quraishi *et al*., 2004; Srinidhi *et al*., 2008) and cotyledonary nodes (Reddy *et al*., 2006); in *Melia azedarach*, regeneration has been reported from nodal segments (Thakur *et al*., 1998; Husain and Anis, 2009), somatic embryos (Sharry *et al*., 2006), immature zygotic embryos (Vila *et al*., 2003), shoot apical meristems (Vila *et al*., 2002), roots (Vila *et al*., 2005) and leaf explants (Vila *et al*., 2004); plantlets were produced from nodal segments of *Toona ciliata* (Mroginski *et al*., 2003) and *Cedrela fissilis* (da Costa Nunes *et al*., 2002). To our knowledge there have been no reports on the micropropagation of *E. capensis*. Perán *et al*. (2006) described a medium for germination of isolated axes, but did not investigate subsequent stages of a micropropagation protocol.

The first explant under consideration comprised the embryonic axis with 2 mm^3 blocks of each cotyledon. Figure 2.1A shows a halved *E. capensis* seed with Figure 2.1B

illustrating the explant comprising the axis with attached cotyledonary segments. Goveia *et al*. (2004) established that to ensure shoot production by excised axes of *T. dregeana*, it was necessary to leave cotyledonary segments attached. This recommendation was followed in the present study since significant difficulties were experienced in achieving shoot production from axes of *E. capensis* from which cotyledons had been completely severed, irrespective of the germination medium or pre-treatment strategy applied. The size of the cotyledonary attachments was, however, reduced as much as possible since these explants were targeted for subsequent cryopreservation, for which explant volume and thermal mass are critical factors (Berjak and Pammenter, 2004b).

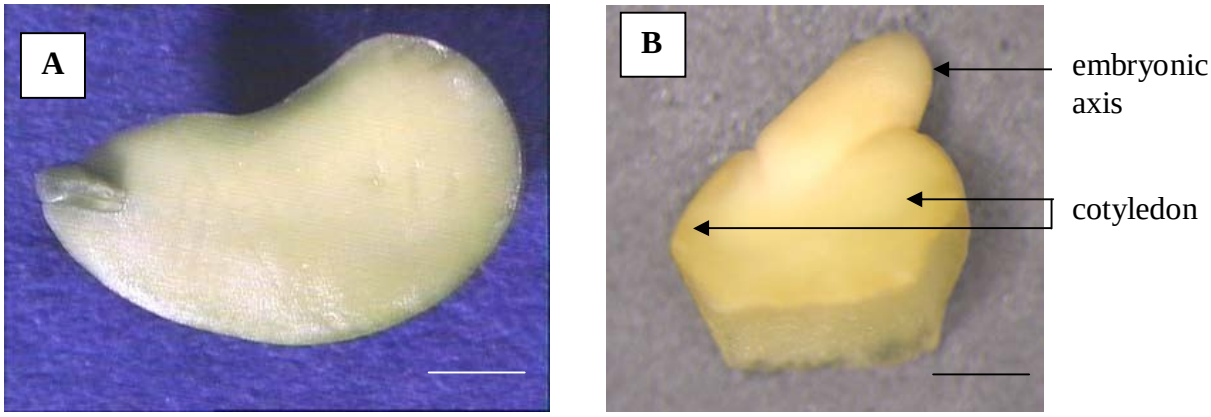

Figure 2.1: Seed-derived explants. A: Halved *E. capensis* seed showing relative size of axis and one cotyledon, bar = 10 mm; **B: Explant comprising the embryonic axis with attached cotyledonary segments**, bar = 8 mm.

The first task was to establish a suitable and reliable germination medium for the selected explant. The most definitive assessment of viability after cryopreservation (and the cryopreparative stages) is examination of onwards development of explants (Engelmann, 1991). Thus, in the present study the ability to germinate was used to assess viability with success reported as percentage of axes producing both a root and shoot (refer to Chapter 3). Previous work done in our laboratory (Walker, 2000) established that the most suitable nutrient formulation for *in vitro* onwards development

of isolated axes and whole seeds of *E. capensis* was MS (Murashige and Skoog, 1962) medium; however, that study considered germination successful if just roots were produced. That study also identified immersion of explants in a 1% (w/v) solution of sodium hypochlorite for 10 min as providing adequate decontamination. With these procedures as starting points, the present study focused on investigations towards the establishment of a suitable medium for *in vitro* seedling formation, with particular emphasis placed on the effect of additives commonly used to promote this. To this end, a range of media additives was investigated, including various cytokinins (BAP, zeatin, kinetin, 2-iP and TDZ), sources of nitrogen (glutamine and casein hydrolysate) and vitamins (ascorbic acid, citric acid, thiamine hydrochloride, calcium pantothenate, nicotinic acid, myoinositol and pyridoxine hydrochloride) provided in various concentrations (summarised in Table 2.2). After extensive investigation, it was found that the only protocol that yielded root and shoot production by 100% of the axes was when the culture medium included 3 µM pyridoxine (0.6 mg l^{-1}). Hence, for the present study, (¼) MS salts and vitamins supplemented with 3 µM pyridoxine was identified as the most suitable germination medium for excised axes and was used in all subsequent studies. Figure 2.2 below is an illustration of seedlings formed by axis explants (refer to Materials and Methods, section 2.2.2.a) germinated on this medium.

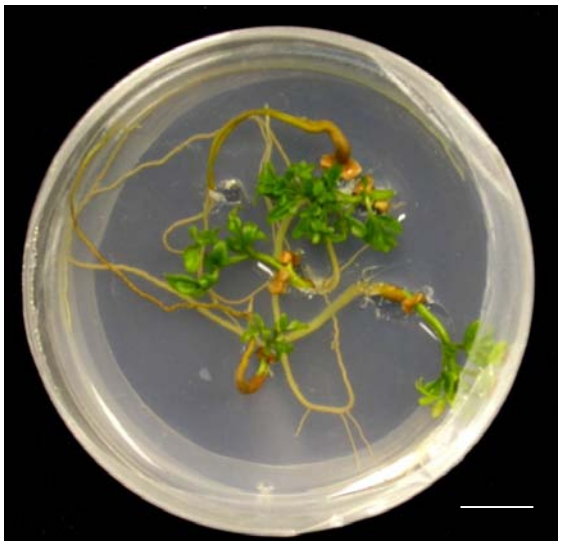

Figure 2.2: Germination (shoots and roots) of explants comprising the embryonic axis with small cotyledonary attachments after 6 weeks in culture, bar = 10 mm.

Pyridoxine is a water-soluble vitamin (B_6), that is often added to culture media to promote a range of processes including seed germination (Dolatabadian *et al*., 2008; Schuelter *et al*., 2009), production of somatic embryos (McKently, 1991), adventitious shoots (Schuelter *et al*., 2009) and to induce axillary buds (Sudhersan *et al*., 2003). Pyridoxine has been reported to function as a cofactor in a range of enzymatic reactions, importantly in those relating to the biosynthesis of amino acids (Schneider *et al*., 2000; Mittenhuber, 2001), hence pyridoxine could be an essential additive to the medium. Interestingly, research within the last decade has revealed another role of pyridoxine, in terms of the activity of this compound as an antioxidant in response to oxidative stress (Chen and Xiong, 2005; Dolatabadian *et al*., 2008). In this regard, Denslow *et al*. (2005) discussed a range of gene regulation and metabolic studies that provided evidence for the role of pyridoxine as an antioxidant.

Pyridoxine activity as an antioxidant is of particular relevance to the present study, as the process of excision of explants from the rest of the seed causes wounding and a consequence of wounding is the production of reactive oxygen species (Roach *et al*., 2008; Pammenter *et al*., 2011), a phenomenon that was suggested by Goveia *et al*. (2004) in relation to excision in axes of *T. dregeana*. Therefore, the addition of pyridoxine to culture medium could help to minimise the injurious effects of such an oxidative burst, as it has been shown to efficiently quench singlet oxygen (Bilski *et al*., 2000) and superoxide (Denslow *et al*., 2005) and also to prevent the peroxidation of lipids (Denslow *et al*., 2005) and denaturation of proteins (Dolatabadian *et al*., 2008).

Embryonic axes with attached cotyledonary blocks have been used in cryopreservation studies, e.g. for *Quercus faginea* (González-Benito and Perez-Ruiz, 1992), *Juglans cinerea* (Beardmore and Vong, 1998), *Camellia sinensis* (Kim *et al*., 2002) and *Citrus suhuiensis* (Makeen *et al*., 2005). In the present study, conditions were investigated to establish a germination medium for selected explants since viability after cooling would be assessed by onwards development of explants. Other tests are available to predict explant viability such as fluorescein diacetate (FDA) staining (Engelmann, 1991), 2,3,5-triphenyl tetrazolium chloride (TTZ) staining (Iborra *et al*., 1992), spectrophotometric assessment of TTZ, measurement of electrolyte leakage and malondialdehyde (MDA)

determination (Verleysen *et al*., 2004). However, such methods are destructive and the results require careful interpretation, as these tests are predictive and not definitive (Verleysen *et al*., 2004). For these reasons, such methods were not considered in the present study.

2.3.2 Shoot production from nodal segments and *in vitro*-germinated roots

The present study investigated the suitability of using nodal segments from *in-vitro*-germinated seedlings and from saplings, explant sizes (2 and 5 mm nodal segments from *in-vitro*-germinated seedlings) and media composition (A, B, C and D) on bud break and multiplication of *E. capensis* (Table 2.3). Bud break occurred on all four media and with both explant sizes when nodal segments from *in-vitro*-germinated seedlings were used (Figure 2.3A). The effect of explant size (2 and 5 mm) on percentage bud break was investigated using nodal segments excised from *in-vitro*-germinated seedlings to determine the smallest explant that was capable of further growth and development, which would best facilitate subsequent cryopreservation. The 5 mm explants consistently showed a higher percentage bud break than the smaller (2 mm) explants on each medium tested, but statistically this result was not significantly different with the exception of explants on medium C. However, as the p value obtained for this analysis was very close to the cut-off point of $p \leq 0.05$, with more replicates this relationship between explant size and percentage bud break would be more confidently resolved. The observed bud break using 2 mm explants (up to 93% depending on medium; Table 2.3) was deemed acceptable for future applications in cryopreservation studies.

Table 2.3: Effect of explant type and, size, and medium composition on percentage bud break and multiplication of nodal explants from *in-vitro*-germinated seedlings and saplings. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 10 – 30).

		Bud break		Multiplication
Explant type	Medium	Explant size (mm)	% bud break	(no. shoots/bud)
Seedlings	A	2	83[a]	NT
		5	86[a]	2.2[a]
	B	2	60[b]	NT
		5	70[b]	2.5[a]
	C	2	63[b]	NT
		5	97[a]	2[a]
	D	2	93[a]	NT
		5	100[a]	2.2[a]
Saplings	D	10 – 15	90[a]	0[b]

Unless stated all media contained: MS, 20 g l^{-1} sucrose and 8 g l^{-1} agar (A):

B: ¼ MS, 1 mg l^{-1} BAP;

C: 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.04 mg l^{-1} NAA, 0.11 mg l^{-1} BAP, 0.05 mg l^{-1} kinetin;

D: 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.01 mg l^{-1} NAA, 0.2 mg l^{-1} BAP;

Multiplication medium: ½ MS, 0.33 mg l^{-1} IBA and 1.7 mg l^{-1} BAP; and

NT: Not tested.

The components of culture media are important in influencing shoot production and multiplication from micropropagated explants (Gamborg, 2002; Ramage and Williams, 2002). In the present study, medium B produced the lowest bud break compared with the other media tested (Table 2.3). Even though there was no significant difference in percentage bud break using media A and D, explants incubated on medium D showed a higher percentage bud break (93% and 100% with 2 mm and 5 mm explants, respectively), than medium A. For this reason, medium D (MS salts and vitamins, 20 g l^{-1} sucrose, 8 g l^{-1} agar, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.01 mg l^{-1} NAA and 0.2 mg l^{-1} BAP) was identified as the most appropriate medium for bud break using nodal segments excised from *in-vitro*-germinated seedlings, and

was thus used to assess bud break using nodal segments from greenhouse-grown saplings. Because of sapling size, only larger explants (10 – 15 mm) were available for bud break. The results (Table 2.3) indicated that the percentage bud break obtained for sapling-derived material (90%) was not significantly different from that obtained using media A, C and D for seedlings. Therefore, medium D was considered suitable to promote bud break from nodal segments excised from the saplings (Figure 2.3B).

The proliferation of axillary buds (multiplication stage) is an important step of any micropropagation protocol since, when successfully performed, it results in the mass production of selected genotypes (Phillips and Hubstenberger, 1995; Khan *et al.*, 2004; Cui *et al.*, 2009). In the present study, shoots produced via bud break from nodal segments excised from *in vitro*-germinated seedlings (5 mm explants only) and saplings were placed on medium containing ½MS salts and vitamins, 20 g l^{-1} sucrose, 0.33 mg l^{-1} IBA, 1.7 mg l^{-1} BAP and 8 g l^{-1} agar for multiplication. From Table 2.3 it can be seen that similar levels of multiplication (2 – 2.5 shoots per bud) was achieved for shoots derived from nodal segments of *in-vitro*-germinated seedlings. Further, no multiplication was observed when shoots regenerated from saplings were used (Table 2.3). Other researchers working on woody species have similarly reported that shoots were more readily multiplied when explants were derived from seedlings compared with those derived from mature plants, e.g. for European mountain ash (Chalupa, 2002) and oak (Kartsonas and Papafotiou, 2007). In the present study, only one multiplication medium was tested, therefore further work is required to optimise multiplication. In addition, multiplication of 2 mm explants was not assessed as shoots from both 2 and 5 mm explants were of essentially similar size at the end of the bud break stage.

A protocol established in this study involved the production of adventitious shoots from intact roots from *in-vitro*-germinated seedlings (Table 2.4). Although root tissues reportedly have a high regenerative potential, records of adventitious shoot generation from roots are limited to a few species (refer to Table 2.1). However, this is not a new concept since adventitious bud production from roots was reported more than 40 years ago by Bonnett and Torrey (1965; 1966). Although some researchers have used roots as a source of explants to produce adventitious shoots for both woody and non-woody

species, they generally employed root segments as explants (Table 2.1). Relatively few reports (only 4 of 19) described shoot regeneration from intact roots; these were for *Helianthus annuus* x *H. tuberosus* (Fambrini *et al*., 2003), *Albizzia julibrissin* (Sankhla *et al*., 1994; Hosseini-Nasr and Rashid, 2000) and *Populus tremula* (Vinocur *et al*., 2000), none of which are of the Meliaceae, the family to which *E. capensis* belongs.

In the present investigation, intact roots produced by *in vitro* germination of excised embryonic axes were exposed to a 24 h treatment with 0 – 3 mg l^{-1} BAP present in the liquid medium within RITA® bioreactors, and were subsequently cultured on semi-solid medium in which 1 mg l^{-1} BAP was incorporated. This facilitated bud and then shoot production by 85 – 95% of the roots, with 12 – 17 shoots per root (Table 2.4; Figure 2.3C). Including BAP in culture medium in the bioreactors did not significantly increase adventitious shoot yields compared with the bioreactor treatment with no BAP present (Table 2.4). The explants that were not exposed to the RITA® conditions (control) yielded the significantly lowest percentage roots forming buds (12%) and number of shoots per root (3). Consideration of all the results presented in Table 2.4 indicates that it was the 24 h RITA® conditions and not BAP which significantly promoted shoot production (control vs 0 BAP), in terms of both the percentage explants forming buds and number of buds induced per explant.

Table 2.4: Effect of BAP treatment (for 24 h in a RITA® bioreactor) on adventitious bud production from intact seedling roots. As a comparison, control explants were maintained on semi-solid medium only and were not exposed to the RITA® treatment. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 20 – 25).

BAP (mg l^{-1})	% roots forming buds	No. shoots/root
Control	12^{a}	3^{a}
0	85^{b}	13^{b}
1	95^{b}	17^{b}
3	95^{b}	12^{b}

In contrast, other researchers have reported the promotion of *in vitro* adventitious shoot regeneration from roots of tree species by using plant growth regulators, for example, TDZ (Sankhla *et al*., 1994; Vinocur *et al*., 2000; Geneve, 2005), BAP (Vila *et al*., 2005) and zeatin (Son and Hall, 1990). In addition, there are only two reports on the use of liquid culture systems to promote adventitious bud formation by root segments: Zobayed and Saxena (2003) used a number of bioreactors including a RITA® system and Vinocur *et al*. (2000) used liquid cultures kept on a shaker and a disposable plastic bioreactor. In both those studies, higher bud yields were reported when the bioreactor systems were used. Generally, the major applications of *in vitro* culture of roots (particularly using liquid medium in a bioreactor) has been restricted to the production of secondary metabolites (Kevers *et al*., 2005) or as a system to increase biomass of hairy roots rapidly (Paek *et al*., 2001). Hence, the present study reports an additional application of culturing *in vitro* roots in a bioreactor, *viz*. to promote direct adventitious shoot regeneration.

The high yields obtained in the present study when using the RITA® bioreactor (Table 2.4) – 12 to 17 shoots produced per root compared with the 3 shoots produced per root using the semi-solid system – may therefore be attributed to the unique culture conditions provided by the temporary immersion system, i.e. direct contact of all parts of explants with the culture medium, immersion times that are automated so that anoxic conditions are avoided, explants that do not dry out since a thin film of medium covers them when they are not immersed and a continuous renewal of the atmosphere within the bioreactor effected by forced ventilation (Etienne *et al*., 1999; Etienne and Berthouly, 2002; Etienne *et al*., 2006). Other researchers have suggested various factors to promote adventitious shoot generation, such as genotype (Xiang-Can *et al*., 1989), age of cultures (Nef-Campa *et al*., 1996), excision of roots (Bonnett and Torrey, 1966) and the distance of the excised root segment from the root tip (Hosseini-Nasr and Rashid, 2000; Vinocur *et al*., 2000).

2.3.3 Rooting and acclimatisation

Indole-3-butyric acid (0, 0.1 and 0.5 mg l^{-1}) was used to promote *in vitro* rooting of shoots generated from *in-vitro*-germinated seedlings (Figure 2.3D), saplings and *in vitro* roots (Table 2.5). Shoots generated from nodal explants of *in-vitro*-germinated seedlings rooted readily on ½ MS medium with 0, 0.1 or 0.5 mg l^{-1} IBA (Table 2.5; 83 – 90% rooting), as did shoots generated from roots on medium containing 0 or 0.1 mg l^{-1} IBA (Table 2.5; 75 – 80% rooting). With the exception of adventitious shoots generated from roots on medium containing 0.5 mg l^{-1} IBA, percentage rooting was not significantly different for shoots produced from seedlings or roots (Table 2.5). In contrast, shoots generated from explants derived from saplings proved difficult to root *in vitro* with the significantly lowest percentage rooting of just 10% obtained on ½ MS medium without provision of IBA (Table 2.5). For these explants, incorporation of IBA in the medium appeared inhibitory. Other researchers working on woody species have reported similar poor rooting in explants derived from older, compared with younger, material, e.g. for *Eucalyptus nitens* (Gomes and Canhoto, 2003) and *Acacia mangium* (Monteuuis, 2004).

Three acclimatisation protocols were tested using shoots generated from seedling material, all resulting in 100% plantlet survival. The protocol in which pots containing plantlets that were tightly enclosed in heat-sealed plastic bags was used for adventitious shoots generated from roots. Using this method, plantlets were acclimatised to *ex vitro* conditions by gradually reducing the humidity of the microclimate by punching holes in the plastic bags until, after 4 weeks, the bags were removed, with a resultant survival of 93% of the plantlets (Table 2.5). This was not significantly different from the 100% survival after acclimatisation obtained with the plantlets generated from nodal explants (Figure 2.3E). Shoots generated from saplings were not acclimatised considering the low percentage rooting obtained.

Table 2.5: The effect of IBA concentration on the percentage rooting, and subsequent survival after acclimatisation of plants generated from *in vitro*-germinated seedlings, saplings and roots. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 20 – 41).

Explant type	IBA (mg l^{-1})	% rooting	% survival after acclimatisation
Seedlings – nodal	0	83[a]	100[a]*
	0.1	90[a]	
	0.5	90 [a]	
Saplings – nodal	0	10[b]	NT
	0.1	0[b]	
	0.5	0[b]	
Roots – adventitious buds	0	75[a]	93[a]*
	0.1	80[a]	
	0.5	20[b]	

***** all rooted plantlets for each explant type were combined for acclimatisation

NT: not tested

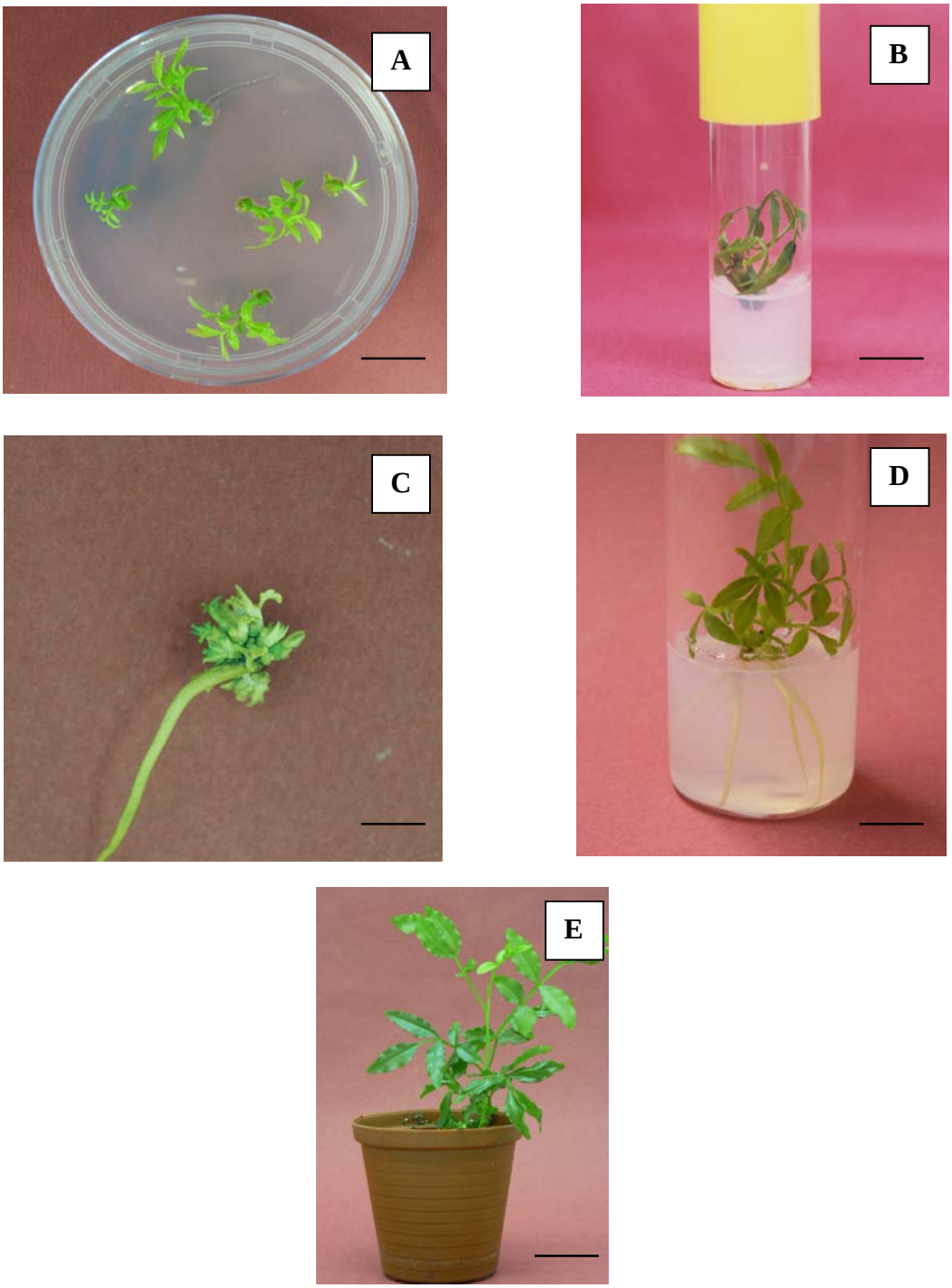

Figure 2.3: Different stages of the developed protocols. A) Bud break from nodal segments of *in vitro*-germinated seedlings, bar = 9 mm; B) bud break from nodal segment of adult plant, bar = 10.3 mm; C) adventitious shoots regenerated from intact *in vitro* root, bar = 10 mm; D) *in vitro* rooted shoot, bar = 11 mm and E) acclimatised plant, bar = 18 mm.

2.3.4 Testing the micropropagation protocol to produce adventitious shoots from *in vitro*-germinated roots

a) Using germplasm from different provenances

The value of any developed protocol is increased if it can be successfully applied to a range of genotypes (or to germplasm from other provenances) (Bhatti *et al*., 1997; Panis *et al*., 2005). This is of particular importance in conservation programmes where a variety of genotypes are stored (Reed *et al*., 2005; Ozden-Tokatli *et al*., 2010). Therefore, the micropropagation protocol that was developed to generate adventitious shoots on intact *in vitro*-germinated roots using germplasm from the Mtunzini provenance (Table 2.4) was applied to germplasm from the two other provenances, *viz.* Port Elizabeth and St Lucia (Table 2.6). The results showed that the protocol could be successfully applied to germplasm from other provenances, albeit with different (lesser) degrees of success. Examination of the results indicates that germplasm from both the Port Elizabeth and St Lucia provenances appeared to have a lower shoot regeneration potential (36 – 40% of roots producing shoots with 3 – 6 shoots produced per root for Port Elizabeth seeds and 30 – 48% of roots generating shoots with 5 – 7 shoots produced per root for St Lucia seeds) compared with germplasm from Mtunzini (85 – 95% of roots producing shoots with 12 – 17 shoots developing from the buds produced per root). Figure 2.4A – C shows representative images of shoots produced from adventitious buds induced directly on roots using germplasm from all three provenances.

Figure 2.4: Adventitious shoots regenerated from buds produced directly on *in vitro*-germinated roots. Adventitious shoot regeneration from material collected from: A) Mtunzini, bar = 7.7 mm; B) Port Elizabeth, bar = 4.6 mm; C) St Lucia, bar = 5.3 mm.

The results (Table 2.6) suggested that while germplasm from the Port Elizabeth and St Lucia provenances had the potential for adventitious bud production, this was lower than that of germplasm from Mtunzini. It could be that this variation in the level of

adventitious shoot production by germplasm of *E. capensis* from different provenances may reside in genomic differences among populations of parent trees (Vishal Bharuth, unpublished data) or be a consequence of the marked inter-seasonal variability that can occur in any one locality that may influence seed characteristics and behaviour. However, the differences in capacities for adventitious shoot induction by material from Mtunzini and St Lucia are presently difficult to explain.

From Table 2.6 it can be seen that in all cases, 12 – 16% of control root explants (no exposure to temporary immersion [RITA®]) generated buds which formed shoots, with 2 – 3 shoots produced per root across material from all three provenances. Therefore, even in the absence of the RITA® treatment, adventitious shoots were generated, but when root explants were incubated in the RITA® temporary immersion system for 24 h with 0 – 3 mg l^{-1} BAP, more root explants generated shoots (Table 2.6). In addition, more shoots were produced per root following the temporary immersion (RITA®) treatment (although this relationship was not always significant for material from St Lucia and Port Elizabeth). Increasing the concentration of BAP in the bioreactor did not yield significantly more shoots. This served to confirm that it was the unique conditions and advantages of the RITA® system that promoted an increase in adventitious shoot production for germplasm from all provenances.

A possible explanation for this could be related to the root tip being the primary site of cytokinin biosynthesis (Haberer and Kieber, 2002; Arigita *et al.*, 2005) and one of the proposed effects of cytokinins is to induce adventitious buds and shoots (Werner *et al.*, 2001; Howell *et al.*, 2003; Shani *et al.*, 2006). Thus, it may be suggested that the endogenous cytokinins present in the roots (in conjunction with the 1 mg l^{-1} BAP supplied for 6 weeks thereafter in the semi-solid adventitious shoot promoting medium) may have been sufficient to induce a low level of adventitious bud morphogenesis, with subsequent shoot development (Table 2.6; no RITA®). In the present study, the unique conditions provided by the RITA® system amplified the ability of root explants for the induction of adventitious shoots compared with those root explants not exposed to the RITA® conditions (Table 2.6). Further, there may have been an additive effect between the applied exogenous cytokinin, BAP, and the endogenous cytokinins present in the

roots that brought about adventitious bud formation and shoot production (Gaspar *et al*., 1996; Nikolić *et al*., 2006). Considering this, it is possible that explants from the three provenances may be characterised by different levels of endogenous cytokinins. Yıldırım and Turker (2009) worked on root segments of *Filipendula ulmaria* and noted that different plant tissues are likely to be characterised by different levels of endogenous hormones. Lombardi *et al*. (2007) suggested that since roots are the site of cytokinin biosynthesis, endogenous cytokinin levels are likely to be high in roots therefore a careful balance is required for shoot production as when too much exogenous cytokinin was supplied to roots of *Passiflora cincinnata*, shoot production was inhibited. Further, it has been reported that differences in the response of differing varieties (genotypes) to the same applied *in vitro* conditions may occur as a result of differences in uptake, transport and metabolism of exogenous cytokinins (Strnad *et al*., 1997; van Staden *et al*., 2008). Consideration of all these factors may explain differences in the extent of adventitious shoot generation observed in the germplasm from the three provenances (Table 2.6).

Table 2.6: Effect of BAP concentration in the RITA® temporary immersion system on adventitious shoot production using germplasm from three provenances, *viz*. Mtunzini, Port Elizabeth and St Lucia. *In vitro*-germinated roots were placed in RITA® temporary immersion systems for 24 h during which they were exposed to medium supplemented with different concentrations of BAP and then cultured on the established semi-solid adventitious shoot regeneration medium incorporating 1 mg l^{-1} BAP. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 25).

	Provenance					
	Mtunzini (28°58'S)		Port Elizabeth (33º54'S)		St Lucia (28°22'S)	
BAP (mg l^{-1})	% roots forming shoots	no.shoots/root	% roots forming shoots	no.shoots/root	% roots forming shoots	no.shoots/root
No RITA (0)	12^{a}	3^{a}	14^{a}	2^{a}	16^{a}	2^{a}
0	85^{b}	13^{b}	40^{b}	3^{a}	30^{ab}	5^{ab}
1	95^{b}	17^{b}	36^{b}	6^{a}	44^{b}	7^{b}
3	95^{b}	12^{b}	40^{b}	5^{a}	48^{b}	5^{ab}

*Results for Mtunzini provenance same as shown in Table 2.4.

As a result of the differing seasonality of fruiting, trials for Mtunzini and St Lucia provenances were performed in February while trials for Port Elizabeth seeds were carried out in June.

In the present study, regardless of the regeneration capacity of explants, care was taken to ensure that adventitious bud and shoot production always occurred directly without an intervening callus stage, which was achieved for the germplasm from all three provenances (Figure 2.4A – C). To minimise the risk of callus formation, intact roots were used, thereby reducing the possibility of its formation in response to wounding. An option to increase levels of adventitious shoot production by material from Port Elizabeth and St Lucia provenances was to cut roots into segments, as was done by Sankhla *et al.* (1996), Zobayed and Saxena (2003), Vila *et al*. (2005) and Shahzad *et al*. (2007), but this strategy was not employed in order to guard against the risk of callus induction and the subsequent generation of genetically altered shoots (Karp, 1992; 1995; Pontaroli and Camadro, 2005). Also, the only plant growth regulator applied was BAP, which has been reported to promote direct adventitious bud and shoot production from roots of *Filipendula ulmaria* (Yıldırım and Turker, 2009) and epicotyl explants of three *Citrus* species (Costa *et al*., 2004). Other plant growth regulators afford a higher risk of generating somaclonal variants; for example, Yıldırım and Turker (2009) reported that callus induction was promoted in root explants when they were cultured on medium supplemented with TDZ, IBA, 2,4-D and IAA, while this was not the case when explants were cultured on medium supplemented with BAP.

The adventitious shoots generated using germplasm from the three provenances were elongated in culture for four weeks on a medium devoid of plant growth regulators. Once shoots had elongated, they were induced to form roots by culturing on media supplemented with different concentrations of IBA (0, 0.1 and 0.5 mg l^{-1}). A high percentage of shoots producing roots was obtained for germplasm from all three provenances (Table 2.7; 75 – 90%), except for shoots from the Mtunzini provenance that were cultured in the presence of 0.5 mg l^{-1} IBA (20%). This indicated that root induction by shoots from material of the Mtunzini provenance was inhibited by high concentrations of IBA (> 0.1 mg l^{-1}), but the shoots formed from Port Elizabeth and St Lucia germplasm were not similarly affected, suggesting the possibility of differing genotypes and/or physiological responses of *E. capensis* germplasm derived from the different provenances. Rooted plantlets from all three provenances were successfully acclimatised

(Table 2.7) with survival ranging from 87% for the Port Elizabeth and St Lucia material to 93% for germplasm from Mtunzini. Although the acclimatisation stage often results in high levels of plantlet mortality (Hazarika, 2003; 2006), such difficulties were not encountered in the present study as evidenced by the high levels of plantlet survival (Table 2.7).

The results (Tables 2.6 and 2.7) confirmed that the protocol developed to produce adventitious shoots from *in-vitro*-germinated roots of seeds derived from *E. capensis* growing in Mtunzini could be successfully applied to germplasm from two other provenances. When estimations of potential acclimatised plantlet yields are compared, germplasm from the Mtunzini provenance promised the greatest yields. If 100 initial root explants were used then it would have been possible to obtain 1200 acclimatised plantlets from Mtunzini seeds, 183 from Port Elizabeth seeds and 223 from seeds derived from St Lucia. It must be noted that the restricted fruiting season of *E. capensis,* together with erratic productivity on an annual basis, precluded the possibility of repeating this comparison during the present study. Nevertheless, as marked inter-seasonal variability has been reported for seeds of the same species (Berjak and Pammenter, 2004b), it is not possible to predict that the trends presently observed would be consistent for material from the three provenances. Also, the possibility exists that the shoot generation potential of roots from seeds of Port Elizabeth and St Lucia could be promoted by optimising culture conditions for these explants, which may have somewhat different requirements for bud and shoot generation, such as different plant growth regulator concentrations (Gomes *et al.*, 2010; Magyar-Tábori *et al.*, 2010).

Table 2.7: Effect of IBA concentration on percentage rooting and subsequent survival after acclimatisation of adventitious shoots generated from *in-vitro*-germinated roots of *E. capensis* from different provenances. Elongated adventitious shoots were cultured on root induction medium containing different concentrations of IBA. Rooted shoots were acclimatised following the developed protocol. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30).

Provenance	IBA (mg l^{-1})	% rooting	% plantlet survival after acclimatisation
Mtunzini	0	75[a]	93[a]
	0.1	80[a]	
	0.5	20[b]	
Port Elizabeth	0	83[a]	87[a]
	0.1	90[a]	
	0.5	90[a]	
St Lucia	0	80[a]	87[a]
	0.1	83[a]	
	0.5	83[a]	

*Results for material from Mtunzini the same as in Table 2.5.

All rooted plantlets were combined for acclimatisation studies

b) Investigation of conditions to promote adventitious shoot production by low-yielding material

An attempt was made to increase levels of adventitious shoot production using Port Elizabeth seeds, which previously showed the lowest yield of adventitious shoots (Table 2.6). This was done by consideration of the factors that affect the process of organogenesis, and manipulation of conditions to promote it. Organogenesis is generally divided into three stages beginning with the ability of cells to respond to an organogenic stimulus (competence), followed by the cellular process of organ induction and formation (determination) and concluding with the morphological differentiation and subsequent development of organs (morphogenesis) (Christianson and Warnick, 1985; Sugiyama, 1999). Two factors were considered in the present study, *viz.* that the adventitious shoot

induction phase (determination) is reported to occur within the first three days (Mercier *et al*., 2003) and that the process of morphogenesis is not dependent on exogenously supplied plant growth regulators (Sugiyama, 1999). Thus, *in-vitro*-germinated roots were cultured in the RITA® temporary immersion system for up to 48 h (to allow a longer period of time for induction of shoot organogenesis under conditions which had been shown to promote adventitious shoot generation in the present study; Table 2.4) and the concentration of BAP in the RITA® system was increased to 6 mg l^{-1} (Table 2.8).

Table 2.8: Effect of BAP concentration on adventitious shoot production from roots using germplasm from the Port Elizabeth provenance. *In-vitro*-germinated roots were placed in the RITA® temporary immersion system with 0 – 6 mg l^{-1} BAP for 24 or 48 h and then cultured on the established semi-solid adventitious shoot regeneration medium containing 1 mg l^{-1} BAP. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30).

	Culture period in RITA® system			
	24 hours		48 hours	
BAP (mg l^{-1})	% roots forming buds	no. shoots/root	% roots forming buds	no. shoots/root
0	40[a]	3[a]	24[ab]	4[a]
1	36[a]	6[a]	32[a]	4[a]
3	40[a]	5[a]	32[a]	5[a]
6	40[a]	6[a]	17[b]	5[a]

*Data for 24 h RITA® for 0, 1 and 3 mg l^{-1} BAP same as shown for Port Elizabeth seeds in Table 2.6.

However, none of the conditions promoted adventitious shoot yields. In fact, exposure of roots to the high concentrations of BAP (6 mg l^{-1}) appeared to inhibit shoot formation. There are reports of elevated BAP concentrations being inhibitory to adventitious shoot production, e.g. by roots of *Passiflora cincinnata* (Lombardi *et al*., 2007) and epicotyl explants of *Citrus* species (Costa *et al*., 2004).

Since the conditions tested, as reported in Table 2.8, did not increase adventitious shoot production from germplasm from Port Elizabeth, the experiment was not extended to material from St Lucia. Further trials to attempt to increase adventitious shoot generation from roots germinated from seeds obtained from St Lucia and Port Elizabeth were not undertaken because of the seasonality and limitation of seeds. However, even though the procedure might be improved upon for material from Port Elizabeth and St Lucia, it was established that the protocol developed could be applied to germplasm from provenances other than that from Mtunzini (Tables 2.6 and 2.7).

2.3.5 Decontamination of adventitious shoots for subsequent cryopreservation studies

The adventitious shoots regenerated from roots were identified as suitable explants for cryopreservation since they were sufficiently small. Since no previous attempts had been made to cryopreserve adventitious shoots of *E. capensis*, a protocol had first to be developed for decontamination since both processing and the cryopreservation process itself necessitate the removal of explants from *in vitro* conditions before subsequent *in vitro* culturing. Two commonly-used hypochlorite-based decontamination solutions were selected for this purpose, *viz.* sodium and calcium hypochlorite (Table 2.9). Decontamination solutions can be toxic to plant tissues, thus it was vital to determine whether either of the solutions could be used, and, if so, to identify suitable concentrations and duration of exposure. From the data in Table 2.9, it can be seen that use of sodium hypochlorite resulted in a significantly lower percentage survival (50 – 30% after immersion for 5 and 10 min, respectively) than when shoots were decontaminated in calcium hypochlorite (100% survival for both immersion times).

Table 2.9: Effect of type of decontamination solution and exposure time on survival of adventitious shoots. Adventitious shoots (2 – 2.5 mm) were decontaminated by immersion in 1% (w/v) NaOCl or 1% (w/v) $Ca(OCl)_2$ for 5 or 10 min, rinsed three times with sterile distilled water and cultured on the established adventitious shoot regeneration medium. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, $n = 30$).

Decontamination solution	Exposure time (min)	% survival
NaOCl	5	50[a]
	10	30[a]
$Ca(OCl)_2$	5	100[b]
	10	100[b]

Other researchers have found calcium hypochlorite to be effective for decontamination of buds of tree species, e.g. *Prunus dulcis* (Ainsley *et al*., 2000) and *Larix decidua* (Chalupa, 2004) as well as the shrub species *Lonicera caerulea* (Dziedzic, 2008). Hence, prior to culture following each step in the cryopreservation protocol, adventitious shoots were decontaminated by immersion in 1% (w/v) calcium hypochlorite for 10 min.

2.4 CONCLUDING COMMENTS

The present aspect of the work reports on direct organogenesis protocols for the *in vitro* propagation of *E. capensis,* using different explants. Axes with attached cotyledonary segments could be successfully germinated on medium comprising ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 0.6 mg l^{-1} pyridoxine and 8 g l^{-1} agar (for 6 weeks). Nodal segments excised from *in-vitro*-germinated seedlings were propagated by culturing explants on bud break medium (MS salts and vitamins, 20 g l^{-1} sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1}calcium pantothenate, 0.01 mg l^{-1} NAA, 0.2 mg l^{-1} BAP and 8 g l^{-1} agar for 2 weeks); multiplied on medium comprising ½ MS salts and vitamins, 20 g l^{-1} sucrose, 0.33 mg l^{-1} IBA, 1.7 mg l^{-1} BAP and 8 g l^{-1} agar (for two cycles of 6 weeks each) and rooted on ½ MS salts and vitamins, 30 g l^{-1} sucrose and 8 g l^{-1} agar containing 0.1 or 0.5 mg l^{-1}

IBA (for 4 weeks). Adventitious shoots were regenerated on root explants that were cultured in a RITA® bioreactor for 24 h and subsequently cultured on semi-solid medium comprising ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 1 mg l^{-1} BAP and 8 g l^{-1} agar (for 6 weeks). Shoots were successfully elongated on MS salts and vitamins, 30 g l^{-1} sucrose and 8 g l^{-1} agar (for 4 weeks) and rooted on ½ MS salts and vitamins, 30 g l^{-1} sucrose, 8 g l^{-1} agar containing 0.1 – 0.5 mg l^{-1} IBA (for 4 weeks). Shoots from all protocols could be successfully acclimatised using the method developed.

A vital requirement for cryopreservation is the development of appropriate *in vitro* regeneration protocols (Krishnapillay, 2000), which require to be established *a priori*. In the present investigation, micropropagation protocols were developed for three types of explants, *viz.* axes with attached cotyledonary segments, nodal segments and adventitious shoots from roots. All three explant types are small and should be suitable for cryopreservation of the germplasm of this species. The protocol for micropropagation using nodal segments from saplings (classed as being non-juvenile) proved considerably less effective. Associated advantages of using *in vitro* material for cryopreservation are that explants can be obtained which are small, theoretically free of superficial contaminants and pathogens (Engelmann, 1991; Liao *et al.*, 2006; Mehrotra *et al.*, 2007) and are physiologically uniform and available whenever required (unlike seeds which are seasonal) as they can be maintained as proliferating cultures (Kartha, 1984). Also, the protocols developed using vegetative explants can be used for multiplication of selected genotypes as part of conservation programmes. In addition, the protocols established avoid an intervening callus stage, and therefore the risk of somaclonal variation (Skirvin *et al*., 1994) is minimised.

CHAPTER 3: CRYOPRESERVATION OF SELECTED EXPLANTS

3.1 INTRODUCTION

3.1.1 Cryopreservation of recalcitrant-seeded germplasm

Cryopreservation is presently the only method available for the long-term conservation of germplasm of species that produce recalcitrant seeds. The various approaches currently available for cryopreservation as well as their associated advantages and disadvantages have been discussed in Chapter 1. Although there have been many reports on the development of protocols for the cryopreservation of germplasm of orthodox seeds (Pritchard, 2007), far less work has been done on recalcitrant seeds (Engelmann, 2011). This is likely to be due to the difficulties encountered in working with recalcitrant seeds (Berjak *et al*., 2011a) and also because the majority of the species that produce such seeds occur in tropical and/or sub-tropical regions of the world (Engelmann, 2011), predominantly in developing countries where this type of research is of low priority and awarded limited funding (Noor *et al*., 2011). As a result, there is little information on the biology of such seeds and they remain largely unstudied but for the work of a few groups of researchers (Engelmann, 2000; 2011). In Africa (including South Africa) the fact that many recalcitrant-seeded species are being unsustainably harvested for use in traditional medicine, has prompted the need to develop protocols to conserve the germplasm of such species (discussed in Chapter 1). Consequently, efforts in our laboratory have focused on cryopreservation of germplasm of indigenous species that produce recalcitrant seeds (Berjak *et al*., 2011a) with the present investigation being focused on one such species, *viz*. *Ekebergia capensis.*

3.1.2 Criteria governing explant selection for cryopreservation of germplasm of *E. capensis*

The selection of suitable explants for cryopreservation is dependent on the morphology of seeds and intrinsic explant characteristics (Berjak *et al*., 2011a; Pammenter *et al*., 2011).

Ekebergia capensis is a dicotyledonous species and the morphology of seeds is such that close to the shoot apical meristem the embryonic axis is connected to a pair of fleshy cotyledons. To cryopreserve seed explants, the cotyledons need to be removed from the axis, which entails cutting through the cotyledonary attachments flush with the axis surface. Recent work has shown that in other meliaceous species, this excision process is associated with a wounding injury to the shoot apical meristem concomitant with, and considered likely to be caused by, a burst of ROS (Goveia, 2007; Whitaker *et al*., 2010; Cassandra Naidoo *et al*., 2011). As a consequence, the apical meristem becomes necrotic (Goveia *et al*., 2004; Perán *et al*., 2006), precluding shoot production. Pammenter *et al.* (2011) have attributed shoot meristem necrosis to the proximity of the lesions resulting from severing of the cotyledons, and hence the origin of the ROS burst, to the shoot apex. A suggestion made by Goveia *et al*. (2004) to obviate this problem was to excise axes with small cotyledonary attachments to buffer the shoot meristem from the adverse effects of the ROS.

a) *Ekebergia capensis* explants suitable for cryopreservation

One of the difficulties in working with recalcitrant seeds is their restricted seasonal availability, as, at best, they are available for only a few months annually. Furthermore, recalcitrant seeds cannot be effectively stored and the quantity and quality of seeds harvested in successive years has repeatedly been found to be inconsistent (Berjak *et al*., 2011a). Considering this, it is advisable to establish more than one source of explants for cryopreservation of germplasm from seeds. In this regard, vegetative *in-vitro*-derived explants offer a suitable option, as one of the advantages is that material is available (or can be generated) throughout the year (Engelmann, 1991). Furthermore, recalcitrant seeds must be used quickly after collection as they cannot be stored for extended periods without physiological changes, followed (more or less rapidly, depending on the species) by vigour and viability loss (Berjak and Pammenter, 2004a). Previously reported studies (Chapter 2) identified two potential *in vitro*-derived explants presently considered for cryostorage, *viz*. explants excised from nodal segments and adventitious shoots produced by *in-vitro*-germinated roots.

3.1.3 Explant survival and onwards development after cryostorage

The process of cryopreservation encompasses a number of steps, each of which is potentially injurious and therefore requires optimisation in order to achieve successful cryostorage (Berjak *et al*., 2011a; detailed in Chapter 1). Two outcomes are possible following retrieval of explants from cryostorage: either explants may be irrevocably damaged eventually culminating in death, or cells of the explant may recover from damage incurred and resume metabolic activity (Benson and Noronha-Dutra, 1988). The best assessment of cryopreservation success is post-warming onwards development (Noor *et al*., 2011). If a critical proportion of cells of the explant survive, onwards development following warming and rehydration can range from swelling of explants (Bandupriya *et al*., 2010), greening (Wesley-Smith *et al*., 2004), callus formation (Pritchard *et al*., 1995), root production (Kim *et al*., 2008) to plantlet formation and seedling establishment (e.g. Sershen *et al*., 2007; Ngobese *et al*., 2010; Berjak *et al*., 2011b). However, for cryostorage to be of practical use, it is essential that functional plants are ultimately produced.

In many cases, shoot production following cryopreservation of axes does not occur (reviewed by Engelmann, 1998; Harding *et al*., 2009) seemingly as a consequence of injury to the shoot meristem (Berjak *et al*., 2011a; Pammenter *et al*., 2011). This is a significant obstacle and efforts need to be focused to overcome this. To this end, one or both of two options should be considered: firstly, the cryopreservation protocol can be revisited and modifications made or different cryopreservation procedures can be attempted (Noor *et al*., 2011); or secondly, the potential reasons for the incomplete development following cryostorage could be determined (Panis and Lambardi, 2005) and consequently methods to overcome them developed. In the present study, the investigation was focused on a novel way to induce adventitious shoots from cryopreserved explants and exploring some aspects of the possible basis of damage that could contribute to lack of success in attaining shoot and root development from axes. A number of factors can contribute to the failure of cryopreservation protocols, not only damage arising from the formation of ice crystals, but also biochemical or oxidative

damage as a consequence of the uncontrolled production of ROS during the various steps of a cryopreservation protocol (Berjak *et al*., 2011a). The two aspects were dealt with simultaneously, i.e. the development of a method to overcome lack of shoot production from axes by applying the procedure described in Chapter 2 to induce adventitious shoots from roots, and assessing superoxide production at each stage as an indication of possible ROS-mediated damage at each procedural stage.

a) Consideration of novel methods to induce seedling development after cryostorage

With conservation of genetic diversity in mind, seedling development from seed-derived germplasm following cryopreservation is the primary goal of cryopreservation efforts (Berjak *et al*., 2011a). However, as mentioned above, this does not always occur. In some cases, callus formation is the only form of post-warming survival reported (Pence, 1990; Pritchard *et al*., 1995; Goveia, 2007; Whitaker *et al*., 2010). If the callus formed is organogenic or embryogenic, then under the appropriate conditions, organs (shoots and roots) or somatic embryos can be induced. In this manner, plantlet establishment may be achieved post-warming. However, this is not ideal when the risks of generating somaclonal variants are considered (Karp, 1995). Alternatively, for those explants that swell, green or produce roots, adventitious shoots may be induced directly on explants under the appropriate conditions. For example, Hargreaves *et al*. (2005) reported the production of adventitious shoots from cryopreserved cotyledons of *Pinus radiata* and Perán *et al*. (2006) reported adventitious shoot production in *E. capensis*, from lesion sites where cotyledons were severed from the axis. To promote onwards development after cryopreservation, novel supplements may also be added to *in vitro* culture media either as a preculture step before cryopreservation or in recovery medium after retrieval from cryostorage. In this regard, various additives have been applied to promote successful onwards development of cryopreserved rice cells such as the iron chelating agent, desferrioxamine (Benson *et al*., 1995), the oxygen-carrying perfluorochemical liquids, perfluorocarbon and pluronic F-68 (Anthony *et al*., 1997), and a haemoglobin solution, Erythrogen™ (Al-Forkan *et al*., 2001). Thus, novel applications must be considered in cases where explant recovery post-warming is a problem.

b) Implications of uncontrolled ROS generation

Oxidative damage caused by ROS has been reported to contribute to lack of survival of cryopreserved axes (e.g. Whitaker *et al.*, 2010) and also represents one of the factors that contribute to viability loss of recalcitrant seeds (Cheng and Song, 2008; Varghese and Naithani, 2008). Such damage can arise not only from oxidative stress, but also from the inability of cells to repair or replace damaged biomolecules (Halliwell, 2006). Oxidative stress occurs as a result of an imbalance between pro-oxidative and antioxidative processes. The accumulation of ROS to toxic levels is associated with a range of deleterious processes, since particularly the hydroxyl radical, reacts indiscriminately with cellular components (proteins, sugars, lipids and nucleic acids) to initiate reaction cascades. This results in DNA lesions and mutations, peroxidation of lipids causing leaky membranes (Halliwell and Gutteridge, 1999) and the eventual formation of volatile hydrocarbons and aldehydes, as well as damage and inactivation of proteins including enzymes, and ion channels (Valenzuela, 1991). If uncontrolled, these processes ultimately result in metabolic dysfunction, disruption of the plasmalemma, and eventual cell death (Halliwell, 2006; Van Breusegem and Dat, 2006). However, this situation could be avoided if ROS were to be detoxified by enzymatic (superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and catalase) and non-enzymatic scavengers (ascorbate, glutathione, tocopherol, flavonoids, alkaloids and carotenoids) (Apel and Hirt, 2004). In addition, a range of enzymes, including monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase, is needed to regenerate active forms of the antioxidants (Blokhina *et al.*, 2003). Under normal circumstances this is achieved by a complex network of feed-forward and feed-back loops between ROS and antioxidants, that function in the various subcellular compartments (Van Breusegem and Dat, 2006).

The production of ROS must be carefully interpreted, since although ROS can cause harmful oxidative damage when present at high levels (Vranová *et al.*, 2002; Pukacka and Ratajczak, 2006), at relatively low concentrations they are known to function as signalling molecules which elicit a range of responses. Examples of these include

regulation of plant growth and development (Gapper and Dolan, 2006); generation and activity of ROS as a consequence of wounding (Ross *et al*., 2006) and pathogen infection (Bolwell *et al*., 2002); induction of programmed cell death (Kawai-Yamada *et al*., 2005); and adjustment of gene expression to effect adaptive responses to stress (Bailly *et al*., 2008). ROS are thus suggested to form part of a complex signal network that brings about responses by interaction with other signalling networks including mitogen-activated protein kinases (Apel and Hirt, 2004), and by redox signalling via the oxidation or reduction of redox-sensitive proteins (Vranová *et al*., 2002). In order for ROS to function as signalling molecules, a tightly controlled balance must be maintained between the liberation of ROS and their quenching (Bailey-Serres and Mittler, 2006). Further, the timing and location of liberated ROS are critical if they are to play a role in signal transduction (Bailey-Serres and Mittler, 2006).

c) Investigation of the possibility of epigenetic changes

Conservation of plant germplasm is essential to maintain biodiversity and to preserve selected genotypes for agriculture, forestry and horticulture (Jokipii *et al*., 2004; Li *et al*., 2007; Gao *et al*., 2010). It is therefore imperative that the techniques employed to conserve germplasm maintain genetic integrity. Variation can arise from changes in DNA sequence (genetic variation) as well as from changes not directly related to alteration in DNA sequences (epigenetic changes). Epigenetic changes describe alterations in the patterns of expression of information in the genome (Jaenisch and Bird, 2003; Korochkin, 2006; Smulders and de Klerk, 2011). Such changes are brought about by covalent modification of nucleotides (Saze, 2008). In this respect, DNA methylation is suggested to be the major epigenetic modification (Vanyushin, 2006; Gehring and Henikoff, 2007).

The primary target for DNA methylation is cytosine residues in the 5’ position of the pyrimidine ring. This process is mediated by DNA methyltransferases that transfer a methyl group from S-adenosylmethionine to cytosine (Goll and Bestor, 2005). Methylated DNA can also be demethylated either passively where methylated cytosines are replaced with unmethylated equivalents during DNA replication, or actively by

enzymatic removal of the methyl group by glycosylases (Morales-Ruiz *et al*., 2006). DNA methylation plays a vital role in plant growth and development by regulation of gene expression (Finnegan *et al.*, 2000). In this manner, genes that are methylated are generally silenced while demethylation is usually associated with gene activation (Jones and Takai, 2001; Zhang *et al*., 2010). Thus, DNA methylation is a dynamic process as cycles of methylation and demethylation are associated with various plant developmental processes (Gehring and Henikoff, 2007; Valledor *et al*., 2007).

It has been reported that DNA methylation levels may be influenced by biotic and abiotic stresses (Chinnusamy and Zhu, 2010). In the context of the present study, the tissue culture process and the various stages of the cryopreservation protocol all constitute stresses that could influence the methylation status of DNA. In this respect, altered DNA methylation levels have been reported in tissue-cultured plants (Xu *et al.*, 2004; Peredo *et al*., 2009; Baránek *et al*., 2010), in plants recovered from slow growth (Harding, 1994), cold storage (Peredo *et al*., 2008) and cryopreservation (Hao *et al*., 2001; Kaity *et al*., 2008; Wang and He, 2008). Johnston *et al.* (2009) suggested that transient DNA methylation changes may play a role in eliciting adaptive responses to stresses incurred. Most epigenetic changes are temporary and reversible; however, there are concerns that such changes can persist and even be transferred during sexual propagation (Brettell and Dennis, 1991) leading to heritable epimutations (Saze, 2008; Jullien and Berger, 2010). This emphasises the need to assess the epigenetic status of germplasm retrieved from cryostorage.

The first step in probing DNA methylation status is to find a suitable method to extract good quality, high molecular weight DNA from plant material. This can be done using classical methods such as the cetyltrimethyl ammonium bromide method (CTAB; Doyle and Doyle, 1990) or the sodium dodecyl sulphate method (SDS; Dellaporta *et al*., 1983). More recently, a range of commercially available kits has been developed for this purpose, such as the Nucleon Phytopure Plant DNA Extraction Kit (Amersham Biosciences, United Kingdom), the Viogene Plant Genomic DNA Extraction System (Viogene, California, USA), the DNeasy Plant Mini Kit (Qiagen, Germany), etc.

There are many techniques presently available to assess the methylation status of DNA. A prerequisite for any technique is the requirement for a way to detect methylated cytosines. This can be achieved by the use of methylation-sensitive restriction enzymes and/or sodium bisulphite treatment, followed by PCR amplification, and if necessary, sequencing (Ibrahim, 2010). Examples of some published methods include sodium bisulphite conversion of DNA (Kneip *et al*., 2009), methylation-sensitive AFLP (metAFLP; Mikuła *et al*., 2011), coupled restriction enzyme digestion and random amplification (CRED-RA; Temel *et al*., 2008), amplified DNA methylation polymorphism (AMP PCR; Kaity *et al*., 2008), amplified fragment length polymorphism (AFLP; Xu *et al*., 2004), methylation-sensitive amplified polymorphism (MSAP; Peredo *et al*., 2008), high pressure liquid chromatography (HPLC; Johnston *et al*., 2005), high pressure capillary electrophoresis (HPCE; Viejo *et al*., 2010), methylation-sensitive single-nucleotide primer extension (Ms-SnuPE; Gonzalgo and Jones, 1997), Luminometric Methylation Assay (LUMA; Karimi *et al*., 2006) and DNA Methylation Analysis by MethyLight Technology (Trinh *et al*., 2001). In addition, a non-PCR based method has also been described using Southern Blotting (Kumar *et al*., 1999). Table 3.1 provides a summary of research articles that have assessed DNA methylation levels in plants using the various methods.

Table 3.1: Summary of examples of research articles that have assessed DNA methylation changes in plants.

Method	Conclusion	Reference
CRED-RA	Inhibition of shoot induction by demethylating agents involves hypomethylation in petunia	Prakash and Kumar, 1997
Southern blots	One cold stored sample and one sample treated with BAP showed methylation changes in strawberry	Kumar *et al.*, 1999
AFLP	Methylation changes in micropropagated apple	Xu *et al.*, 2000
MSAP	Cryopreservation-induced demethylation in apple	Hao *et al.*, 2001
MSAP	Hypermethylated DNA in micropropagated banana	Peraza-Echeverria *et al.*, 2001
MSAP	Increased methylation in *in vitro* potato leaves with mature leaf morphology	Joyce and Cassells, 2002
MSAP	Methylation changes in citrus callus recovered from slow growth	Hao *et al.*, 2004
MSAP	DNA demethylation during germination of pepper seeds	Portis *et al.*, 2004
AFLP	Demethylation during somatic embryogenesis of rose	Xu *et al.*, 2004
HPLC	Demethylation in micropropagated plants of cedar	Renau-Morata *et al.*, 2005
AFLP	Progressive DNA methylation during plant development in *Arabidopsis*	Ruiz-García *et al.*, 2005
MSAP	Methylation polymorphisms present in cotton	Keyte *et al.*, 2006
MSAP	Tissue culture induced methylation changes in *Codonopsis lanceolata*	Guo *et al.*, 2007
MSAP	Demethylation in tissue cultured barley	Li *et al.*, 2007
CRED-RA	Demethylation in adult trees of crab apple	Hafiz *et al.*, 2008
AMP PCR	Cryopreservation-induced methylation changes in papaya	Kaity *et al.*, 2008
HPLC	Shoots with a juvenile-like morphology have higher methylation levels than mature-like ones in sequoia	Monteuuis *et al.*, 2008
MSAP	Cryopreservation and cold storage induced methylation changes in hops	Peredo *et al.*, 2008
MSAP	Methylation alterations detected in grapevine somaclones	Schellenbaum *et al.*, 2008

CRED-RA	Tissue culture induced methylation changes in barley callus and mature embryos	Temel *et al*., 2008
MSAP	Cryoprotection and cryopreservation altered methylation status in *Arabidopsis*	Wang *et al.*, 2008
CRED-RA	In rice, petunia and spinach, methylation-dependent changes corresponded with vernalisation	Anuntalabhochai *et al*., 2009
HPCE	Low levels of methylation during somatic embryo development in pine	Noceda *et al*., 2009
MSAP	Hypermethylation in variant *Doritaenopsis*	Park *et al*., 2009
MSAP	Demethylation in micropropagated hops	Peredo *et al*., 2009
MSAP	Change in methylation levels in response to *in vitro* culture and thermotherapy in grapevine	Baránek *et al*., 2010
MSAP	Tissue culture of *Freesia* flowers induced methylation changes	Gao *et al*., 2010
HPCE	Demethylation after fertilisation, increased methylation during seed development and induction of somatic embryogeneis occurred after demethylation in chestnut	Viejo *et al*., 2010
metAFLP	No methylation changes in control and cryopreserved proembryogenic masses of gentian	Mikuła *et al.*, 2011

The selection of a suitable technique to assess DNA methylation is governed by a number of factors. In the present study, the primary constraints were that a limited amount of material was available for DNA isolation (as a consequence of seasonal availability of seeds) and that the species under investigation had not been extensively studied at the molecular biological level. Thus, there was limited information available on suitable primers for PCR-based analysis. In the present study, therefore, commercially available random primers were used. The technique used to assess DNA methylation levels was digestion of DNA with methylation-sensitive restriction enzymes and random amplification of DNA, i.e. CRED-RA (Cai *et al*., 1996). This is the simplest method to detect DNA polymorphisms (Rafalski and Tingey, 1993; Powell *et al.*, 1996), and has been used to detect DNA methylation changes in various plant species (refer to Table 3.1).

d) Consideration of seed provenance

Seeds collected from different provenances may display differential responses to cryogenic temperatures (Wen *et al.*, 2010). Considering this factor, the present study also investigated the response of seeds collected from two provenances within South Africa, *viz*. Port Elizabeth and St Lucia. Figure 3.1 shows the location of Port Elizabeth relative to St Lucia, both of which are on the east coast of South Africa, but at different latitudes. St Lucia is located further north and is sub-tropical, with high relative humidity in mid to late summer, while Port Elizabeth is characterised by cool winters and mild summers. These geographic and meteorological parameters are potentially important, as the environmental conditions of parent plants can influence seed characteristics, including tolerance to dehydration and cooling (Dussert *et al.*, 2000; Khurana and Singh, 2001; Daws *et al.*, 2004; Li and Pritchard, 2009). Of particular relevance is that current studies in our laboratory have shown that *E. capensis* seeds from St Lucia are chilling-sensitive, whereas those from Port Elizabeth are not (Bharuth, 2011).

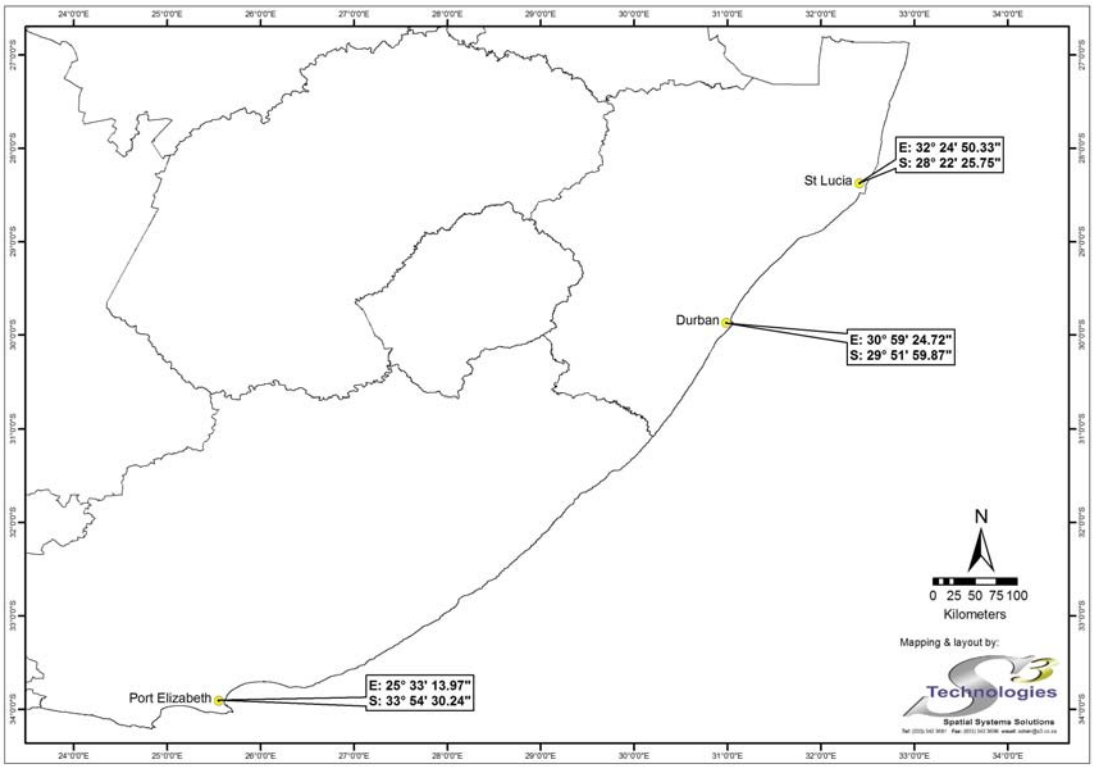

Figure 3.1: Map of eastern region of South Africa showing locations of Port Elizabeth and St Lucia (Source: S^3 Technologies).

3.1.4 Aims and objectives of the present study

The aim of the present study was to develop a protocol for cryopreservation of germplasm of *E. capensis* using the explant-types for which micropropagation protocols were established (Chapter 2). Cryopreparative stages (dehydration and cryoprotection) and different cooling rates were investigated for each explant type, *viz.* isolated 'broken' buds, adventitious shoots and embryonic axes with 2 mm^3 attached cotyledonary segments.

Each stage of a cryopreservation protocol imposes stresses that may limit success. To gain a better understanding of these processes the extracellular production of superoxide

was assessed at each stage of the protocol as this is considered to be a primary stress or injury response (Whitaker *et al*., 2010). Although limited to only one type of ROS, it was considered that superoxide production would give an indication as to the basis of damage incurred and whether ROS contribute to viability loss at each stage. In addition, assessment was made of changes in DNA methylation status of recovered plants after each cryopreparative stage and following cooling in sub-cooled nitrogen. Towards this end, suitable protocols had to be identified for DNA isolation, restriction enzyme digestion and PCR amplification of products. Overall, these investigations aimed to develop a protocol for cryostorage of germplasm of *E. capensis*, and where success was not achieved, to attempt to characterise the basis of damage incurred so that future studies could be directed towards ameliorating such effects.

3.2 MATERIALS AND METHODS

3.2.1 Explants for cryopreservation

The investigations were performed using germplasm of *Ekebergia capensis* derived from seeds from provenances at different latitudes along the East coast of South Africa, St Lucia (28°22'S) and Port Elizabeth (33°54'S) which are characterised by differing climatic conditions, *viz*. sub-tropical and warm temperate, respectively (Figure 3.1).

Three types of explants were tested for their responses to cryogen exposure: 1) Isolated 'broken' buds (approximately 2 mm in length) produced by culturing buds while attached to 2 – 4 mm stem pieces incorporating the nodal region, on MS (Murashige and Skoog, 1962) salts and vitamins with 20 g l^{-1} sucrose and 8 g l^{-1} agar; 2) Adventitious shoots (approximately 2 – 2.5 mm with 1 – 2 nodes) generated from intact roots derived from *in vitro*-germinated material; 3) Embryonic axes with 2 mm^3 attached cotyledonary segments (type 3 explants). The final, novel protocol developed, employed type 3 explants after retrieval from liquid nitrogen and *in vitro* culture to develop rooted plantlets from adventitious buds produced by seedling roots (refer to Chapter 2).

3.2.2 Explant decontamination

Surface decontamination protocols that were used prior to cryostorage were described in Chapter 2. After retrieval from cryostorage, explant types 1 (*in vitro* 'broken' buds) and 3 (embryonic axes with small cotyledonary attachments) were surface decontaminated by immersion in 1% (w/v) sodium hypochlorite (NaOCl) with a few drops of Tween 20® for 10 min, while explant type 2 (adventitious shoots) were decontaminated by immersion in 1% (w/v) calcium hypochlorite [$Ca(OCl)_2$] with a few drops of Tween 20, for 10 min. All explants were then rinsed three times with sterile distilled water.

3.2.3 Culture conditions

All media (refer to section 3.2.7b and d) were adjusted to pH 5.6 – 5.8, prior to autoclaving for 20 min at 120°C at 121 kPa. Unless otherwise stated, all cultures were maintained in a growth room under a 16-h photoperiod at a photosynthetic photon flux density (PPFD) of 37 μmol m^{-2} s^{-1} and at 23°C day/21°C night.

3.2.4 Gravimetric determination of water content

The water contents of explants from all experimental treatments were determined gravimetrically. Dry mass was recorded after 48 h at 80°C. Water contents were determined individually for 5 explants and expressed on a dry mass basis (g H_20 g^{-1} dry mass [g g^{-1}]).

3.2.5 Cryopreparative procedures

i) Dehydration

All three explant types were dehydrated by flash drying, which entailed exposing them to a stream of silica-gel-dehydrated air generated by a small computer processing unit (CPU) fan within a closed container (Pammenter *et al*., 2002a). *In vitro* 'broken' buds and adventitious shoots were flash dried for up to 80 min while embryonic axes with

small cotyledonary attachments were dehydrated for up to 60 min. All three explant-types were assessed for *in vitro* regeneration after dehydration. Vegetative explants (*in vitro* 'broken' buds and adventitious shoots) were not directly rehydrated after dehydration, but were decontaminated (see 3.2.2) and placed directly on medium for onwards development (see below). Axes with attached cotyledonary segments were rehydrated in a solution of $CaCl_2.2H_2O$ (0.5 μM) and $MgCl_2.6H_2O$ (0.5 mM) for 30 min in the dark (Berjak *et al.*, 1999; Mycock, 1999) before decontamination and plating.

ii) Cryoprotection

The cryoprotectant solutions tested were sucrose, glycerol, DMSO and a combination of sucrose and glycerol. All explants (30 of each type) were immersed in solutions of cryoprotectants in a stepwise manner, i.e. in a 5% (w/v or v/v) followed by a 10% (w/v or v/v) solution for 15, 30 or 60 min in each. *In-vitro*-derived explants were exposed to cryoprotectants for 15 or 30 min in each solution while explants excised directly from seeds were exposed for 30 or 60 min in each solution. After cryoprotection, 5 explants were blotted to remove solution from the surface, and used for water content determination. The remaining explants (30) were surface decontaminated and plated to ascertain survival and potential for onwards development. In all cases where explants were cryoprotected only, such material was not previously dehydrated.

iii) Sucrose preculture

Adventitious shoots were the only explants that were exposed to a sucrose preculture treatment. Shoots (30, in clumps of 3) were excised and precultured on medium containing 0.15 M sucrose for 3 d followed by medium containing 0.3 M sucrose for a further 3 d. All sucrose preculture media contained MS salts and vitamins and 8 g l^{-1} agar, with 20 – 25 ml medium per Petri dish (65 mm diameter). The water content, survival and potential for onwards development of adventitious shoots was assessed after the 3 d preculture period on each of the sucrose-containing media.

iv) Combinations of cryopreparative procedures

The three explant-types were subjected to combinations of the cryopreparative procedures described above (i – iii). Table 3.2 provides a summary of the individual, or combinations of treatments applied to each explant-type.

Table 3.2: Summary of cryopreparative procedures used to prepare the three types of explants for cryopreservation. n = 30.

Explant type	Treatment no.	Flash drying	Cryoprotection	Sucrose preculture
In vitro	1	+		
'broken' buds	2		+	
(2 mm)	3	+	+	
Adventitious shoots (2 – 2.5 mm)	4	+		
	5		+	
	6	+	+	
	7			+
	8	+		+
	9	+	+	+
Axes with	10	+		
cotyledonary	11		+	
attachments	12	+	+	
(2 mm^3)				

v) Additional procedures applied to *in vitro* nodal segments

1) Dehydration over a saturated salt solution

Explants were dried over on a grid suspended over 5 ml of saturated sodium chloride solution [75% (w/v)] in a closed 30 ml bottle. There were 7 explants per bottle, which were sampled each morning and afternoon over a period of 5 d to assess water content and the potential for onwards development.

2) Dehydration over silica gel

Explants (30) were placed on a sheet of filter paper over 50 g of activated silica gel in a 100 ml culture bottle (ten explants per culture bottle). Explants were sampled at 1 h intervals over a period of 6 h to assess water content and potential for onwards development.

3) Sucrose preculture

Explants (30 per treatment) were precultured on media supplemented with sucrose by exposing them to increasing sucrose concentrations at 24 h intervals (0.2, 0.4, 0.6 and 0.8 M sucrose). In addition, explants were exposed to culture medium incorporating a single sucrose concentration for a period for 3 or 7 days for each sucrose concentration. All sucrose preculture media contained MS salts and vitamins and 8 g l^{-1} agar.

4) ABA preculture

Explants (30 per treatment) were precultured on media supplemented with ABA at 2.5 or 5 mg l^{-1} for 3, 6 or 9 days. All preculture media incorporating ABA contained MS salts and vitamins, 30 g l^{-1} sucrose and 8 g l^{-1} agar.

5) Plant vitrification solutions

Explants (30 per treatment) were exposed to loading solution (MS salts and vitamins, 2 M glycerol and 0.4 M sucrose), PVS2 (MS salts and vitamins, 30% glycerol, 15% ethylene glycol, 15% DMSO and 0.4 M sucrose) and unloading solution (MS salts and vitamins and 1.2 M sucrose). In further trials, these solutions were modified by reducing the sucrose concentration to 0.2 M. All solutions were applied at 10-min intervals up to 60 min.

3.2.6 Cryopreservation methods

Explants subjected to the various cryopreparative procedures were then exposed to cryogenic temperatures using three cooling rates. For the slowest rate, explants were exposed to the two-step cooling method, being placed into 2 ml polypropylene cryovials (5 explants per cryovial with 6 cryovials per treatment) and cooled at 1°C min^{-1} down to -40°C in a -70°C freezer using the Mr Frosty® apparatus (Nalgene, Thermo Scientific, USA) with 250 ml isopropyl alcohol in the outer reservoir, after which the cryovials were plunged into liquid nitrogen. A faster cooling rate, (3 – 10°C s^{-1} [Vertucci, 1989; Wesley Smith, 2002]) was achieved by plunging 2 ml cryovials containing 5 explants per vial directly into liquid nitrogen (6 cryovials per treatment). The explants remained in liquid nitrogen for at least 1 h before further processing. The fastest cooling rate (up to 1 000°C s^{-1}) involved tumbling explants (30) directly into sub-cooled nitrogen (nitrogen slush) at -210°C (Echlin, 1992), prepared by exposing liquid nitrogen (within a polystyrene container) to a vacuum until slush was formed. Before exposure to the vacuum, the liquid nitrogen was placed under an ultraviolet light for 15 min to inactivate microbial inoculum generally present within liquid nitrogen (Naidoo, 2006). Explants remained within slush for approximately 10 min as sub-cooled nitrogen slush returned to the liquid state and were immediately processed for recovery as described below.

3.2.7 Recovery of explants after exposure to cryogenic temperatures

a) Warming and rehydration

Two means of warming were investigated. In the first, cryovials were rapidly transferred to a water bath held at 40°C for 2 min. Explants were then removed from the cryovials and immersed in a solution containing 0.5 µM calcium chloride and 0.5 mM magnesium chloride (CaMg [Mycock, 1999]) for 30 min in the dark at 23°C. For the second method, explants were immersed directly in the CaMg solution at 40°C for 2 min and then transferred to fresh CaMg solution at room temperature for 30 min and held in the dark for rehydration.

b) *In vitro* culture

After warming and rehydration, 30 explants per treatment were surface decontaminated (see section 3.2.2) and then cultured *in vitro* on 20 – 25 ml in 65 mm diameter Petri dishes of the medium established for each type (5 explants per Petri dish), as follows: *In vitro* 'broken' buds were cultured on a medium comprising MS salts and vitamins, 20 g l^{-1} sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.01 mg l^{-1} NAA, 0.2 mg l^{-1} BAP and 8 g l^{-1}agar. Adventitious shoots were cultured on ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 1 mg l^{-1} BAP and 8 g l^{-1} agar. Embryonic axes with cotyledonary segments were cultured on ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 3 µM (0.6 mg l^{-1}) pyridoxine and 8 g l^{-1}agar. All explants (30 per treatment) were kept in the dark (at 23°C day/21°C night) until growth was observed. Thereafter, cultures were maintained under the standard photoperiod conditions (see 3.2.3). Results for all treatments were recorded after 6 weeks.

All shoots were rooted by maintenance for 4 weeks on media containing ½ MS salts and vitamins, 30 g l^{-1} sucrose and 8 g l^{-1} agar supplemented with 0, 0.1 or 0.5 mg l^{-1} IBA. Cultures were kept under the standard photoperiod conditions described in section 3.2.3.

c) Acclimatisation

Rooted plantlets were acclimatised in pots (55 x 55 mm) containing a mix of 1:1:1 potting soil, vermiculite and peat, initially in tightly sealed transparent plastic bags (160 x 280 mm). Material was kept at room temperature in the laboratory. In all cases, after two weeks the humidity of the microclimate was reduced gradually by punching holes in the bags and by the fourth week the plastic bags were completely removed.

d) Application of the protocol (Chapter 2; Hajari *et al.*, 2009) to generate adventitious shoots from roots developed by cryopreserved type-3 explants

Explants comprising the embryonic axis with attached cotyledonary segments were cryopreserved, warmed upon retrieval from the cryogen, and plated (on ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 3 µM pyridoxine and 8 g l^{-1}agar) for root production in culture. Intact roots were then placed in a RITA® temporary immersion system (flushed for 30 s every 10 min with medium comprising ¼ MS salts and vitamins, 30 g l^{-1} sucrose and 1 mg l^{-1} BAP) for 24 h and then cultured on the established adventitious shoot generation medium, comprising ¼ MS salts and vitamins, 30 g l^{-1} sucrose, 1 mg l^{-1} BAP and 8 g l^{-1} agar, for 6 weeks (see Chapter 2, section 2.2.4 and Hajari *et al.*, 2009).

3.2.8 Quantification of superoxide as an indication of the possible implication of ROS associated with damage

For adventitious shoots and axes, quantification of superoxide (a ROS commonly produced as a stress response [Minibayeva *et al.*, 1998]), was performed after each cryopreparative stage and also after cryopreservation. Extracellular superoxide was estimated using a colorimetric assay that measures the oxidation of epinephrine to adrenochrome spectrophotometrically by absorbance at 490 nm (Misra and Fridovich, 1972). Five explants were placed into each of three 15 ml vials and 3 ml of 1 mM epinephrine (Sigma, Germany) solution at pH 7.0 was added to each vial. The vials were then shaken at 45 revolutions min^{-1} on a rotary shaker in the dark for 15 min. The absorbance of each sample was measured (against the standard unreacted epinephrine solution) at 490 nm using a Cary 50 Ultraviolet-Visible spectrophotometer (Varian) coupled with a single cell Peltier accessory. The control comprised the epinephrine solution without any explants. There were three replicates per treatment.

3.2.9 Assessment of changes in DNA methylation status

a) Plant material

i) Axes with attached cotyledonary segments

Explants were exposed to the cryopreparative stages: 1) after excision; 2) dehydration; 3) cryoprotection; and 4) a combination of cryoprotection and dehydration, and cryopreservation (refer to section 3.2.5 – 3.2.7 for details), after which DNA was extracted from *in-vitro*-recovered material using either shoots or roots. As the control, DNA was extracted from shoots produced from seeds germinated on moistened filter paper kept under ambient conditions in the laboratory.

ii) Adventitious shoots

Isolated adventitious shoots were exposed to the cryopreparative stages and cooling (refer to section 3.2.5 – 3.2.7 for details), after which DNA was isolated from recovered shoots that developed *in vitro*.

b) DNA extraction

i) Modified mini CTAB method

The modified mini CTAB method described by Rogers and Bendich (1988) was initially used to extract genomic DNA from shoots. Additional modifications were included to optimise DNA yields, *viz.* the fresh weight of starting material was adjusted, the volume of CTAB precipitation solution was increased, leaves were ground in 50 or 100 mg of insoluble polyvinylpyrrolidone (PVP), and/or dithiothreitol (DTT) at 0.1 and 0.2 mg ml^{-1} was added to the extraction buffer, as well as combinations of these modifications. However, even after additional modifications were made, the concentration of extracted DNA remained very low (2 – 6 ng μl^{-1}) and samples were heavily contaminated with phenolics, carbohydrates and RNA (results not shown).

ii) DNeasy Plant Mini Kit (Qiagen, Germany)

Genomic DNA was extracted from *in vitro*-germinated shoots, roots and adventitious shoots using the Qiagen DNeasy Plant Mini Kit. The manufacturer's instructions were slightly modified to optimise DNA yield from shoots in that the volume of lysis and precipitation buffers were doubled. Using this method, the concentration of extracted DNA – 61 ng μl^{-1} from shoots and 83 – 480 ng μl^{-1} from roots – was much higher than that obtained by the modified mini CTAB method, and was not contaminated.

c) Genomic DNA quantification

The final concentration of DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). The quality of genomic DNA was assessed using a 1% (w/v) agarose gel containing 0.05 mg ml^{-1} ethidium bromide. Agarose gels were prepared and electrophoresis was conducted in TBE (0.89 M Tris-HCl, 0.89 M boric acid and 0.02 M ethylenediaminetetra-acetic acid, pH 8.0). A molecular weight marker (Fermentas, O'Gene Ruler DNA Ladder Mix, 1 kb) was loaded onto each gel. Electrophoresis was performed at 85 V for 90 min and DNA bands were visualised using low radiation UV light. This confirmed that extracted DNA was of high molecular weight and good quality and could therefore be used in downstream applications. It was thus established that for *E. capensis*, the method of choice to extract DNA from plant material was to use the commercially available Qiagen DNeasy Plant Mini Kit.

To remove traces of elution buffer before restriction enzyme digestion, all DNA samples were subjected to evaporation for 20 min at 60°C using an Eppendorf Concentrator attached to a vacuum (Eppendorf, Germany). The resultant DNA pellet was resuspended in 10 µl of PCR-grade water to a final concentration of 2 µg.

d) Restriction enzyme digestion

Genomic DNA (2 µg) from all treatments was digested using 10 U of the methylation-sensitive fast-digesting endonucleases *HpaII* and *MspI* (Fermentas Life Sciences, Canada) in a final reaction volume of 20 µl. According to the manufacturer, these enzymes can completely digest 2 µg of DNA in 5 min at 37°C; however, in the present study, genomic DNA was digested for 8 min to ensure that the process was complete, which is essential to prevent false positive results from incomplete DNA digestion (Ariel, 2002).

e) Detection of DNA methylation by RAPD analysis

PCR was carried out using digested and undigested DNA from all treatments following the parameters and primers outlined in two published protocols (details below). All reactions were performed in a 50 µl volume containing 1 ng template DNA, 3 mM $MgCl_2$ (KapaBiosystems, SA), 0.5 mM dNTPs (Roche, Germany), 0.5 mM primer, 5 U Taq (Kapa Taq DNA Polymerase, KapaBiosystems, SA) in a 10x PCR buffer (High Yield Reaction Buffer, KapaBiosystems, SA). PCR was performed using an Applied Biosystems GeneAmp® PCR System.

i) Primers and PCR conditions (*from Prakash and Kumar, 1997)*

The following arbitrary 10-mer primers were tested: OPU5 (TTGGCGGCCT), OPU8 (GGCGAAGGTT), OPU 14 (TGGGTCCCTG) and OPU15 (ACGGCCAGT) (Operon Technologies, Alameda, USA). The cycling parameters were: 94°C for 2 min initial denaturation followed by 40 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min.

ii) Primers and PCR conditions (from Temel et al*., 2008)*

The following arbitrary 10-mer primers (Operon Technologies, Alameda, USA) were tested: OPA19 (CAAACGTCGG), OPA22 (TGCCGAGCTG), OPB22 (TGATCCCTGG), OPC8 (TGGACCGGTG), OPC12 (TGTCATCCCC), GB7 (GGTGACGCAG) and GB8 (GTCCACACGG). The cycling parameters were: 94°C for 5 min initial denaturation followed by 40 cycles of 94°C for 1 min, 34°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min.

PCR products were run on a 1.5% (w/v) agarose gel under the conditions described in section 3.2.9c above.

3.2.10 Photography and data analysis

Images were captured with Nikon Coolpix® digital camera attached to a Wild stereo microscope. Polymorphisms were scored per treatment using a presence (1) or absence (0) matrix and recorded per primer. Percent polymorphisms were then calculated from the total number of monomorphic bands. Data were analysed using a One Way Analysis of Variance (ANOVA) or Pearson correlation and where not normally distributed, data were transformed. Means were contrasted using Scheffe's multiple range test (95% confidence interval). Alphabetical values were assigned to the mean values recorded per treatment. Mean values that do not share the same letter are recognised as being significantly different.

3.3 RESULTS

3.3.1 Explant type 1: *in vitro* 'broken' buds

a) Cryopreparative procedures: dehydration and cryoprotection

The explants initially targeted for cryopreservation were nodal segments excised from *in-vitro*-germinated seedlings. It was necessary first to dehydrate explants to limit ice formation during cooling (Suzuki *et al*., 1998; Hitmi *et al*., 1999). Towards this end, an extensive series of investigations was performed, including: 1) use of different rates of dehydration (over a saturated salt solution, over silica gel and by flash drying); 2) preculture of explants on sucrose-supplemented media (factors that were investigated included varying the sucrose concentration, duration of preculture and a stepwise increase in sucrose concentration); 3) preculture of explants on media supplemented with abscisic acid (ABA) (where the ABA concentration and duration of preculture were varied); 4) exposure of explants to a range of cryoprotectants (sucrose, glycerol, a combination of sucrose and glycerol and DMSO); and 5) the application of plant vitrification solutions (testing both PVS solutions as published, and modifications thereof). However, in none of those investigations could nodal segments (buds subtended above and below by 2 – 4 mm stem pieces) be sufficiently dehydrated without a significant decline in survival (results not shown).

Thus, a variation was attempted in the use of slightly more developed nodal segments for cryopreparative trials, on the basis that such explants may be more tolerant of water loss (Padaychee *et al*., 2008). Hence, nodal segments were excised and cultured on medium devoid of plant growth regulators to facilitate bud break. These 'broken' (sprouted) buds were isolated and then dehydrated by flash drying (Table 3.3). This method was selected because preliminary trials (results not shown) showed that it was the most successful for dehydrating nodal segments to approximately 1.83 g g^{-1} with 60% survival.

Table 3.3: Effect of dehydration by flash drying on water content and onwards development of 'broken' buds. Nodal segments were excised from *in-vitro*-germinated seedlings and cultured on medium devoid of plant growth regulators. 'Broken' buds were flash dried for 0 – 80 min, decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on bud break medium. Values represent the mean ± standard deviation. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for onwards development and 5 for water content).

Time (min)	Water content (g g^{-1})	% explants showing growth
0	3.55 ± 1.26^{a}	90^{a}
40	1.32 ± 0.35^{b}	93^{a}
50	1.43 ± 0.56^{b}	100^{a}
60	1.05 ± 0.50^{b}	90^{a}
70	0.60 ± 0.26^{c}	100^{a}
80	0.45 ± 0.13^{c}	100^{a}

Table 3.3 illustrates the results obtained when 'broken' buds were flash dried. Such explants could be dehydrated from an initial water content of 3.55 ± 1.26 g g^{-1} to 0.45 ± 0.13 g g^{-1}, with 100% shoot production. To our knowledge, this is the first report of *in vitro* 'broken' buds of *E. capensis* tolerating dehydration to a level that may be considered suitable for cryopreservation, with no adverse effects on survival.

In order to promote tolerance of 'broken' buds to cooling, prior to flash drying they were immersed in various penetrating and non-penetrating cryoprotectant solutions (Table 3.4). No adverse effects on survival were apparent following any of the treatments, as indicated by resumption of growth in all cases (Table 3.4).

Table 3.4: Effect of cryoprotectants on water content and resumption of growth of 'broken' buds. Nodal segments were excised from *in-vitro*-germinated seedlings and cultured on medium devoid of plant growth regulators. 'Broken' buds were immersed in 5% and 10% solutions of sterile cryoprotectants for 15 min in each and then cultured on bud break medium. Values represent the mean ± standard deviation. a = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for onwards development and 5 for water content).

Cryoprotectant (np and/or p)*	Water content (g g^{-1})	% explants showing growth
Control	3.68 ± 0.40^a	93^a
Sucrose (np)	3.45 ± 0.79^a	100^a
Glycerol (p)	3.93 ± 1.01^a	100^a
Sucrose and glycerol (np + p)	3.61 ± 0.80^a	100^a
DMSO (p)	3.23 ± 0.60^a	100^a

*np = non-penetrating, p = penetrating

Since the water content of the cryoprotected explants remained high (Table 3.4), explants were subsequently flash dried (Table 3.5) to reduce it to an appropriate level to facilitate subsequent non-injurious cooling. All explants survived these treatments with onwards development ranging from 80 – 100% (Table 3.5). A trend that was observed was that as the water content of sucrose-cryoprotected explants was lowered to 0.34 ± 0.25 g g^{-1}, survival also declined from 100 – 80%. Since the water content of explants was considered to be sufficiently reduced with acceptable levels of survival (Table 3.5), all cryoprotected and flash dried explants were subjected to cryopreservation trials.

Table 3.5: Effect of cryoprotection followed by flash drying on water content and onwards development of 'broken' buds. Nodal segments were excised from *in-vitro*-germinated seedlings and cultured on medium devoid of plant growth regulators. 'Broken' buds were immersed in 5% and 10% solutions of cryoprotectants for 15 min in each. Following cryoprotection, explants were flash dried for 80 min, decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and then cultured on bud break medium. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for onwards development and 5 for water content).

Cryoprotectant (np and/or p)*	Water content (g g^{-1})	% explants showing growth
Control	4.21 ± 0.40^{a}	93^{a}
Sucrose (np)	0.34 ± 0.25^{b}	80^{a}
Glycerol (p)	0.53 ± 0.09^{b}	100^{a}
Sucrose and glycerol (np + p)	0.44 ± 0.35^{b}	90^{a}
DMSO (p)	0.49 ± 0.16^{b}	90^{a}

*np = non-penetrating, p = penetrating

b) Exposure of explants to cryogenic temperatures

In no case did the cryoprotected, flash-dried 'broken' bud explants of *E. capensis* survive cryogen exposure. *In vitro* 'broken' buds were cryoprotected, flash dried and cooled by two-step cooling, within cryovials and in sub-cooled nitrogen (results not shown). However, none of the cooling rates applied supported any survival. The inability of nodal segments to survive dehydration (results not shown) was overcome by using slightly more developed explants (i.e. 'broken' buds), which were shown to survive the stresses imposed by dehydration (Table 3.3), cryoprotection (Table 3.4) and a combination of cryoprotection and dehydration (Table 3.5), but the damage incurred during cryopreservation and/or warming and/or rehydration was lethal. For these, and other reasons (see Discussion), it was decided to focus efforts towards the use of alternative explants derived from *in vitro* material, *viz.* adventitious shoots, for cryopreservation of germplasm of this species.

3.3.2 Explant type 2: adventitious shoots generated from intact roots derived from *in vitro*-germinated material

a) Cryopreparative procedures: dehydration and cryoprotection

As a necessary prerequisite for cryopreservation, adventitious shoots were dehydrated to lower their water content (Table 3.6). Flash drying for 20 min did not significantly reduce the water content of adventitious shoots, which declined from 3.57 ± 0.17 to 3.28 ± 0.24 g g^{-1}, with 100% and 80% survival, respectively. However, there was a significant decline in water content after dehydration for 40 min (to 1.60 ± 0.25 g g^{-1}), with 70% explant survival. A further significant decline in water content to 0.46 ± 0.17 g g^{-1}, with 80% viability retention was obtained when explants were dehydrated for 60 min, beyond which there was no significant reduction in water content but there was a significant decline in percentage survival (Table 3.6). The water content of adventitious shoots after 60 min flash drying was within the range for cryopreservation and the survival obtained was deemed acceptable, as Towill (1988) recommended that survival of at least 80% be attained for a method to be considered suitable for cryogenic storage of germplasm. Hence, in the present study, the cut-off level was chosen as 80% and any cryopreparative treatment that resulted in lower survival was considered unsuitable.

Table 3.6: Effect of dehydration by flash drying on survival (onwards growth) after 6 weeks in culture, and water content immediately after flash drying of adventitious shoots. Following flash drying for 0 – 80 min, adventitious shoots (2 – 2.5 mm) were decontaminated in 1% (w/v) $Ca(OCl)_2$ for 10 min, rinsed and cultured on adventitious shoot regeneration medium. Values represent the mean ± standard deviation. a – d = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for survival and 5 for water content).

Time (min)	Water content (g g^{-1})	% survival
0	3.57 ± 0.17^{a}	100^{a}
20	3.28 ± 0.24^{a}	80^{ab}
40	1.60 ± 0.25^{b}	70^{b}
60	0.46 ± 0.17^{c}	80^{ab}
70	0.46 ± 0.14^{c}	40^{c}
80	0.35 ± 0.14^{c}	10^{d}

To attempt to improve survival of dehydration, and ultimately freeze tolerance, adventitious shoots were immersed in solutions of cryoprotectants. The shoots were exposed to the penetrating and non-penetrating cryoprotectants for varying times after which the effect on water content and survival was assessed (Table 3.7). For each single cryoprotectant or the combination of glycerol and sucrose, explants were placed in 5% solutions for 15 or 30 min followed by 10% solutions for a further 15 or 30 min. Adventitious shoots were found to survive after a 15 min exposure to all cryoprotectant treatments (Table 3.7). However, in all cases, a 30 min exposure to cryoprotectants resulted in significantly decreased shoot survival (60 – 27%), therefore no further trials were done using the longer exposure period. Undiminished survival relative to the control was obtained when explants were cryoprotected using sucrose and glycerol in combination for 15 min (Table 3.7). However, as there was no significant difference in percentage survival for the various 15 min cryoprotectant treatments and they all yielded at least 80% survival, all four cryoprotectants tested were used in subsequent trials.

Table 3.7: Effect of cryoprotectants on survival (onwards growth) after 6 weeks in culture, and water content immediately after cryoprotection of adventitious shoots. Shoots (2 – 2.5 mm) were immersed in 5% solutions of sterile cryoprotectants followed by 10% solutions for 15 or 30 min in each, and then cultured on adventitious shoot regeneration medium. Values represent the mean ± standard deviation. a – d = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for survival and 5 for water content).

	Exposure time			
	15 min		30 min	
Cryoprotectant (np and/or p)*	WC (g g^{-1})	% survival	WC (g g^{-1})	% survival
Control	4.17 ± 0.69^{a}	100^{a}	4.17 ± 0.69^{a}	100^{a}
Sucrose (np)	2.91 ± 0.72^{bc}	80^{a}	2.23 ± 0.25^{b}	27^{b}
Glycerol (p)	2.58 ± 0.56^{c}	90^{a}	2.96 ± 0.55^{ab}	30^{bd}
Sucrose+glycerol (np + p)	3.67 ± 0.47^{ab}	100^{a}	3.32 ± 0.72^{ab}	60^{c}
DMSO (p)	3.83 ± 0.35^{ab}	80^{a}	3.23 ± 0.83^{ab}	47^{cd}

*np = non-penetrating, p = penetrating

The application of cryoprotectant solutions for 15 min at each concentration did lower the water content of adventitious shoots but, in all cases, the water content was considerably too high for cooling the *E. capensis* explants without lethal ice formation (Perán *et al*., 2006). Hence, cryoprotected adventitious shoots were flash dried (Table 3.8) in an attempt to lower the water content of explants to levels appropriate for non-injurious cooling and subsequent warming. The combination of cryoprotection followed by flash drying significantly reduced the water content of adventitious shoots from 3.30 ± 0.23 to 0.38 ± 0.10 g g^{-1} (Table 3.8). However, the only treatments that resulted in a reasonably high survival (80%) were flash drying alone and when adventitious shoots were cryoprotected for 15 min in the combination of sucrose and glycerol, and then flash dried. All other cryoprotectant treatments significantly lowered shoot survival, which ranged from 27 – 20% (Table 3.8). Nevertheless, the 80% survival of suitably dehydrated shoots without the use of cryoprotectants, and after exposure to sucrose and glycerol and 60 min flash drying indicated that there were procedures that might facilitate successful cryogen exposure. Thus, the only treatments that were used in subsequent cooling trials were when explants were cryoprotected with the combination of 5 and then 10% sucrose and glycerol for 15 min each and then flash dried, or flash dried only.

Table 3.8: Effect of cryoprotection followed by flash drying on survival (onwards growth) after 6 weeks and water content (immediately after cryoprotection and flash drying) of adventitious shoots. Shoots (2 – 2.5 mm) were cryoprotected by immersion in 5% and 10% solutions of cryoprotectants for 15 min in each. Following cryoprotection, shoots were flash dried for 60 min, decontaminated in 1% (w/v) $Ca(OCl)_2$ for 10 min, rinsed and cultured on adventitious shoot regeneration medium. Control explants were not exposed to any cryoprotectant or flash drying treatments. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for survival and 5 for water content).

Cryoprotectant (np and/or p)*	Flash dry	Water content (g g^{-1})	% survival
Control	–	3.30 ± 0.23^{a}	100^{a}
No cryoprotectant	+	0.46 ± 0.17^{b}	80^{a}
Sucrose (np)	+	0.55 ± 0.18^{b}	27^{b}
Glycerol (p)	+	0.50 ± 0.21^{b}	20^{b}
Sucrose+glycerol (np + p)	+	0.46 ± 0.29^{b}	80^{a}
DMSO (p)	+	0.38 ± 0.10^{b}	20^{b}

*np = non-penetrating, p = penetrating
For results of flash drying alone, refer to Table 3.6

b) The effects of cooling rate on explant survival after retrieval from liquid nitrogen

Three methods of cooling which achieved different rates were investigated, *viz.* two-step cooling, cooling within cryovials plunged into liquid nitrogen and cooling directly in sub-cooled nitrogen. No survival was obtained when adventitious shoots were cooled with the first two methods (results not shown), indicating that for these shoot explants, equilibrium and/or slower cooling are not options. The only cooling rate that facilitated any survival was when adventitious shoots were exposed to sub-cooled nitrogen. However, in this case the only cryopreparative treatments that afforded survival were when shoots were flash dried only (7% shoot survival) or when shoots were cryoprotected in a combination of sucrose and glycerol and then flash dried (20% survival).

The few (7 – 20%) shoots that survived cryostorage developed further (Figure 3.2) without any callus formation. No morphological abnormalities were observed in the shoots generated after cryostorage, as similarly noted by Maruyama *et al*. (1998) and Burritt (2008) for *Guazuma crinita* and *Begonia* x *erythrophylla*, respectively. Results having a bearing on the retention of epigenetic fidelity of these shoots are presented in section 3.3.5.

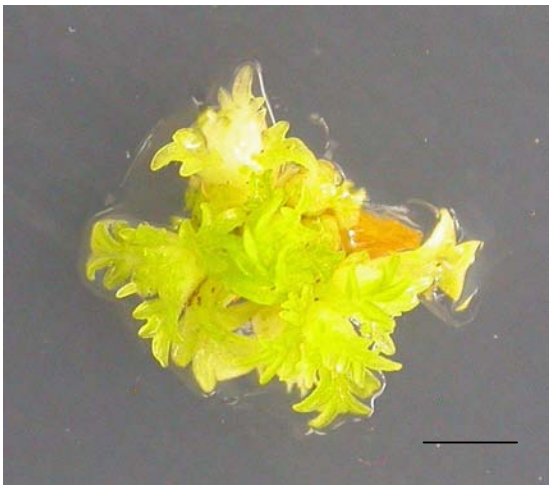

Figure 3.2: Adventitious shoot growth and development 6 weeks after retrieval from cryostorage. Adventitious shoots were cryoprotected using the combination of sucrose and glycerol for 15 min at each concentration, flash dried for 60 min and cooled in nitrogen slush; bar = 10 mm.

c) Attempts to promote freeze tolerance in adventitious shoots by sucrose preculture

Considering that there was survival, although the percentage was low (≤ 20%), attempts were made to promote freeze tolerance of adventitious shoots by including a sucrose preculture step (Table 3.9). The concentrations of sucrose (0.15 and 0.3 M) in sequential increase, but no higher concentrations, were used, since in an earlier trial (results not shown), *in vitro* nodal segments of *E. capensis* were found to be very sensitive to higher sucrose concentrations in preculture media. In that study, explants were found to tolerate

sucrose preculture only if there was a stepwise increase in concentration, and none tolerated concentrations greater than 0.4 M.

Table 3.9: Effect of sucrose preculture on survival (onwards growth) after 6 weeks, and water content of adventitious shoots. Shoots (2 – 2.5 mm) were precultured on medium incorporating 0.15 M sucrose for 3 d followed by 0.3 M sucrose for a further 3 d and then cultured on adventitious shoot regeneration medium. Survival was assessed as onwards growth after preculture on 0.15 M sucrose alone and followed by 0.3 M sucrose. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for survival and 5 for water content).

Sucrose (M)	Water content (g g^{-1})	% survival
Control	3.27 ± 0.12^{a}	100
0.15	3.92 ± 0.41^{b}	100
0.15 and 0.3	2.80 ± 0.37^{a}	100

Adventitious shoots tolerated preculture on the sucrose-supplemented media with no decline in survival (Table 3.9), indicating that, as was the case for nodal segments, adventitious shoots survive a stepwise increase in sucrose concentration up to 0.3 M. As sucrose preculture on its own has been shown to be insufficient to afford protection or adequate dehydration for cryopreservation (González-Arnao *et al*., 1996), adventitious shoots that were precultured on sucrose-supplemented media were also cryoprotected and flash dried. This was necessary to lower the water content of sucrose-precultured explants and also to determine if adventitious shoots could tolerate the cumulative effect of all three cryopreparative stresses by maintaining adequate levels of survival. These results are presented in Table 3.10.

Table 3.10: Effect of sucrose preculture followed by cryoprotection and flash drying on survival (onwards growth after 6 weeks) and water content (immediately after treatment) of adventitious shoots. Shoots (2 – 2.5 mm) were precultured on 0.15 M sucrose for 3 d followed by 0.3 M sucrose for a further 3 d and were then cryoprotected with 5% and 10% solutions of sucrose and glycerol for 15 min in each. Shoots were then flash dried for 60 min, decontaminated in 1% (w/v) $Ca(OCl)_2$ for 10 min, rinsed and cultured on adventitious shoot regeneration medium. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for survival and 5 for water content).

Preculture	Cryoprotect	Flash dry	Water content (g g^{-1})	% survival
Control	–	–	3.30 ± 0.23^{a}	100^{a}
+	–	–	0.61 ± 0.15^{b}	100^{a}
+	+	–	3.67 ± 0.59^{a}	90^{a}
+	+	+	0.41 ± 0.15^{b}	83^{a}

After sucrose preculture, cryoprotection and flash drying, 83 – 100% survival was obtained (Table 3.10), indicating that shoots could tolerate the combined stresses of all three applied cryopreparative stages. The water content reached after sucrose preculture, cryoprotection and flash drying was within the range considered suitable to facilitate cryopreservation (i.e. 0.41 ± 0.15 g g^{-1}). Therefore, in subsequent studies, adventitious shoots treated in this manner were cooled in nitrogen slush.

However, despite the encouraging outcomes preceding exposure to sub-cooled nitrogen, none survived cryogenic temperatures.

3.3.3 Explant type 3: embryonic axes with attached cotyledonary segments

i) Provenance 1: Port Elizabeth

a) Cryopreparative procedures: dehydration and cryoprotection

With few exceptions, the method of choice for the dehydration of germplasm from recalcitrant seeds is flash drying (Berjak *et al*., 1989; 1990; Walters *et al*., 2008). Thus, an extensive dehydration trial was carried out in the present study where embryonic axes, excised with 2 mm^3 cotyledonary blocks, were flash dried and sampled at 10-min intervals up to 60 min (Table 3.11). Survival declined as the water content decreased from 0.82 ± 0.19 g g^{-1} to 0.12 ± 0.03 g g^{-1}, the latter after 60 min flash drying. An examination of the water contents obtained after 30 to 50 min flash drying showed that although these remained essentially similar (Table 3.11; 0.21 ± 0.10 g g^{-1} to 0.20 ± 0.09 g g^{-1}), the percentage of axes producing shoots declined from 60 to 7%, with axes producing roots decreasing from 87 to 40%. However, at the water content reached after 20 min of flash drying (0.39 ± 0.05 g g^{-1}), shoot and root production by axes was 87% and 93%, respectively (Table 3.11). This water content was within the recommended range for cryopreservation of this species (Perán *et al*., 2006) and the survival presently obtained as indicated by seedling production, was considered acceptable. Thus, for subsequent experiments explants were flash dried for 20 min.

Table 3.11: Effect of dehydration of explants (embryonic axis with cotyledonary attachments) by flash drying, on germination (root and shoot production) and water content. Explants were excised, flash dried for up to 60 min and rehydrated in a solution containing 0.5 µM calcium and 0.5 mM magnesium chloride for 30 min in the dark. Explants were then surface decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on germination medium. Values represent the mean ± standard deviation. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for germination and 5 for water content).

Time (min)	Water content (g g^{-1})	% axes producing roots	% axes producing shoots
0	0.82 ± 0.19^{a}	93^{a}	100^{a}
10	0.49 ± 0.10^{b}	93^{a}	80^{a}
20	0.39 ± 0.05^{b}	93^{a}	87^{a}
30	0.21 ± 0.10^{c}	87^{a}	60^{b}
40	0.18 ± 0.07^{c}	37^{b}	20^{c}
50	0.20 ± 0.09^{c}	40^{b}	7^{c}
60	0.12 ± 0.03^{c}	27^{b}	0^{c}

In an attempt to promote freeze tolerance, explants were cryoprotected in solutions of sucrose, glycerol, a combination of sucrose and glycerol, or DMSO (Table 3.12). The data indicate that in general a higher percentage shoot and root production was obtained after cryoprotection for 30 min in each solution compared with exposure for 60 min. The only exceptions were when explants were cryoprotected with sucrose, where percentage root production was higher after 60-min (but shoot development was severely compromised) than after 30-min exposure, and when a combination of sucrose and glycerol was used as a cryoprotectant, where no differential effects resulted (Table 3.12).

Table 3.12: Effect of cryoprotectants on germination (root and shoot production) and water content of explants (embryonic axis with cotyledonary attachments). Explants were excised and cryoprotected with 5% and 10% solutions of cryoprotectants for either 30 or 60 min in each. Explants were then decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on germination medium. Control explants were not exposed to any cryoprotectants. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for germination and 5 for water content).

	30 min cryoprotection			60 min cryoprotection		
		% axes producing			% axes producing	
Cryoprotectant (np and/or p)*	WC (g g^{-1})	roots	shoots	WC (g g^{-1})	roots	shoots
Control	0.89 ± 0.17^{ab}	100^{a}	100^{a}	0.89 ± 0.17^{a}	100^{a}	100^{a}
Sucrose (np)	1.12 ± 0.24^{a}	80^{a}	87^{a}	0.73 ± 0.11^{ab}	93^{a}	27^{b}
Glycerol (p)	0.66 ± 0.09^{b}	93^{a}	80^{a}	0.58 ± 0.09^{ab}	87^{a}	47^{b}
Sucrose+glycerol (np + p)	0.70 ± 0.16^{ab}	93^{a}	80^{a}	0.44 ± 0.04^{b}	93^{a}	80^{a}
DMSO(p)	0.86 ± 0.12^{ab}	93^{a}	87^{a}	0.58 ± 0.14^{ab}	60^{b}	33^{b}

*np = non-penetrating, p = penetrating

The application of cryoprotectants generally results in the loss of water from cells (Karlsson and Toner, 1996; Gonzalez-Arnao *et al*., 2008). However, presently the degree of dehydration after cryoprotection for 30 min was insufficient to facilitate cryopreservation, the water contents remaining too high for explant exposure to cryogenic conditions (Table 3.12; 1.12 ± 0.24 to 0.66 ± 0.09 g g^{-1}). Hence, explants were further dehydrated by flash drying for 20 min after cryoprotection (Table 3.13). After cryoprotection and flash drying (Table 3.13), the water content of explants was reduced to the range 0.44 ± 0.06 to 0.34 ± 0.03 g g^{-1} which was considered to be suitable for cooling. The corresponding percentage of axes producing roots ranged from 100 – 67% and shoots ranged from 87 – 80% (Table 3.13). The only anomaly observed (which is presently inexplicable) was the 67% of axes that produced roots after cryoprotection with glycerol and then flash dried (Table 3.13). However, 80% of these explants produced shoots, which was at the cut-off point recommended by Towill (1988).

Table 3.13: Effect of cryoprotection followed by flash drying on germination (root and shoot production) and water content of explants (embryonic axis with cotyledonary attachments). Explants were excised, cryoprotected with 5% and 10% solutions of cryoprotectants for 30 min in each and flash dried for 20 min. Explants were then rehydrated in 0.5 μM calcium and 0.5 mM magnesium chloride for 30 min in the dark, decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on germination medium. Control explants were not exposed to any cryoprotectant or flash drying treatments. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for germination and 5 for water content).

Cryoprotectant (np and/or p)*	WC (g g^{-1})	% axes producing roots	% axes producing shoots
Control	0.89 ± 0.17^{a}	100^{a}	100^{a}
Sucrose (np)	0.36 ± 0.09^{b}	93^{a}	87^{a}
Glycerol (p)	0.44 ± 0.08^{b}	67^{b}	80^{a}
Sucrose+glycerol (np + p)	0.34 ± 0.03^{b}	93^{a}	87^{a}
DMSO (p)	0.44 ± 0.06^{b}	100^{a}	87^{a}

*np = non-penetrating, p = penetrating

b) Exposure of explants to cryogenic temperatures

Explants were cryoprotected, flash dried and then cooled using the three procedures mentioned previously. No explants survived the two-step cooling process (results not shown). Some success was, however, obtained when explants were cooled within cryovials, and root production, ranging from 87 to 67% was generally good when cryoprotected axes were exposed directly to nitrogen slush (Table 3.14).

Table 3.14: Effect of cooling rate on germination (root and shoot production) and water content of explants (embryonic axis with cotyledonary attachments). Explants were excised, cryoprotected with 5% and 10% solutions of cryoprotectants for 30 min in each and flash dried for 20 min before cooling in cryovials or nitrogen slush. Explants were then warmed (at 40°C for 2 min) and rehydrated (at room temperature) in a solution containing 0.5 µM calcium and 0.5 mM magnesium chloride for 30 min in the dark, decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on germination medium. Control A explants were not exposed to any cryoprotectant or flash drying treatments, while control B explants were flash dried only. Neither control A nor B explants were cooled. Values represent the mean ± standard deviation. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for germination and 5 for water content).

Cryoprotectant (np and/or p)*	Cooling method	WC ($g\ g^{-1}$)	% axes producing roots	% axes producing shoots
Control A	–	0.90 ± 0.17^{a}	100^{a}	100^{a}
B	–	0.46 ± 0.06^{b}	93^{ac}	90^{a}
None	cryovial	0.49 ± 0.09^{b}	23^{b}	0^{b}
	slush		20^{b}	0^{b}
Sucrose (np)	cryovial	0.50 ± 0.09^{b}	7^{b}	0^{b}
	slush		73^{c}	7^{b}
Glycerol (p)	cryovial	0.42 ± 0.06^{b}	10^{b}	10^{b}
	slush		87^{ac}	7^{b}
Sucrose+glycerol (np + p)	cryovial	0.37 ± 0.08^{b}	30^{b}	0^{b}
	slush		67^{c}	0^{b}
DMSO (p)	cryovial	0.39 ± 0.06^{b}	30^{b}	0^{b}
	slush		87^{ac}	7^{b}

*np = non-penetrating, p = penetrating

Table 3.14 shows the results obtained after exposure to cryogenic temperatures. From this Table it can be seen that very limited shoot production was obtained after cooling, regardless of the rate. The highest percentage of axes producing shoots (10%) was obtained when explants were cryoprotected with glycerol, flash dried and then cooled within cryovials (Table 3.14). However, this was not significantly different from the 7%

of axes which produced shoots after cryoprotection with sucrose, glycerol or DMSO, flash dried and then cooled in nitrogen slush. In contrast to shoot production, the percentage of axes producing roots after cryogen exposure ranged from 87 – 7%, the latter being sucrose-cryoprotected and cooled within vials (Table 3.14). In all treatments, a significantly higher percentage of axes produced roots when they had been cooled in nitrogen slush (87 – 67%) than within cryovials (30 – 7%). The highest percentage of axes producing roots after exposure to cryogenic temperatures, 87% (Table 3.14), was obtained when explants were treated with the penetrating cryoprotectants glycerol or DMSO, flash dried and then cooled in nitrogen slush.

c) Application of the protocol to generate adventitious shoots from roots developed by cryopreserved axes

Seedling establishment (i.e. the production of functional plantlets with both roots and shoots) after axis cryopreservation was a prime objective at the outset of this investigation. However, irrespective of treatments, less than 10% of axes produced shoots, even though roots were produced by more than 70% of axes following cryoprotection and cooling in nitrogen slush, the only exception being 67%, when axes were cryoprotected with the sucrose/glycerol combination (Table 3.14). The present aspect of the study then assumed a novel departure from attempts to produce shoots directly (i.e. from the apical meristem), building on the earlier achievement of inducing adventitious shoot formation from intact *in vitro*-germinated roots (Chapter 2). Therefore, the protocol developed for adventitious shoot production from seedling roots, was applied to roots generated from axes after retrieval from liquid nitrogen.

Explants comprising the axis with attached cotyledonary segments were cryoprotected, flash dried and cooled in nitrogen slush, which afforded the best retention of rooting ability (refer to Table 3.14). After retrieval from liquid nitrogen, the roots formed by 67 – 87% of axes were used to generate adventitious shoots by placing them in the RITA® temporary immersion system for 24 h (as reported in Chapter 2). Apart from the control,

only those explants that were cryoprotected with glycerol alone or with DMSO produced roots capable of subsequent adventitious shoot formation (Table 3.15; Figure 3.3). Of the roots developed from glycerol-cryoprotected explants, 40% formed buds, yielding an average of 5 shoots per root. This was better, but not significantly different from roots produced by DMSO-cryoprotected explants, where 30% formed buds with an average of 3 shoots produced per root. Interestingly, the percentage of roots forming adventitious shoots was the same for axes which had been glycerol cryoprotected and cryopreserved, as for control axes which were placed in the RITA® temporary immersion system after elongation *in vitro* with no further manipulation after excision from the seeds. Furthermore, the number of shoots forming per root was identical (Table 3.15). Roots generated from explants that had been cryoprotected with sucrose or with a combination of sucrose and glycerol did not yield shoots over the experimental culture period (Table 3.15).

Table 3.15: Production of adventitious shoots from roots generated after cryostorage. Explants (axis with cotyledonary attachments) were excised and immersed in 5% and 10% solutions of cryoprotectants for 30 min in each. Following cryoprotection, explants were flash dried for 20 min and then cooled, warmed (at 40°C for 2 min) and rehydrated (at room temperature) in a solution containing 0.5 µM calcium and 0.5 mM magnesium chloride for 30 min in the dark. Explants were then surface decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and then cultured on germination medium. Elongated roots (20 – 40 mm) were placed in the RITA® system for 24 h and then on adventitious shoot induction medium. Control explants were not cryoprotected, dehydrated or cryostored. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 20 – 30 for survival).

Root sample	% roots producing shoots	Mean number of shoots per root
Control	40[a]	5[a]
Sucrose	0[b]	0[b]
Glycerol	40[a]	5[a]
Sucrose+glycerol	0[b]	0[b]
DMSO	30[a]	3[a]

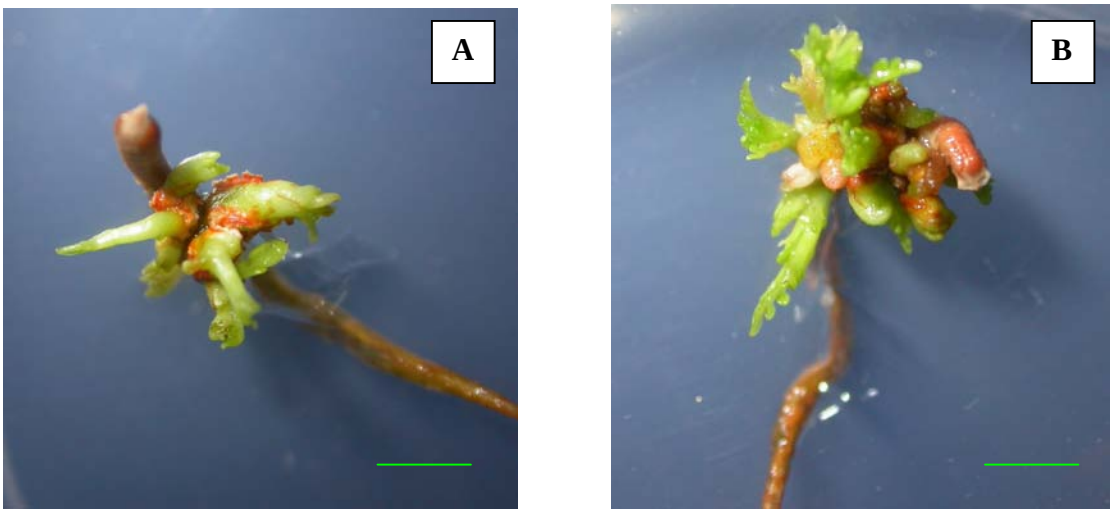

Figure 3.3: Adventitious shoot production on roots developed by glycerol-cryoprotected axes after retrieval from liquid nitrogen and following 12 weeks in culture. A: Adventitious bud induction, bar = 5 mm and B: shoot multiplication, bar = 5 mm.

The adventitious shoots produced from roots regenerated after cryostorage were successfully rooted (83% rooting) and 78% of the resultant plantlets were successfully acclimatised. Thus, the final goal of producing functional plants after cryostorage of seed-derived genetic resources of *E. capensis* was achieved by the application of an unconventional method to produce adventitious shoots from *in vitro*-germinated roots.

ii) Provenance 2: St Lucia

a) Cryopreparative procedures: dehydration and cryoprotection

The protocol that was developed to cryopreserve type 3 explants using seeds from Port Elizabeth was applied to type 3 explants from seeds of the sub-tropical provenance, St Lucia, which were first subjected to a flash drying trial to assess the response to

dehydration. Explants were sampled at 10-min intervals during flash drying for up to 60 min (refer to Table 3.11). Control explants that were not flash dried showed 100% root and shoot production (Table 3.16), providing evidence that the germination medium developed for Port Elizabeth material was suitable for explants from seeds collected at St Lucia. As the water content was reduced from 1.08 ± 0.11 g g^{-1} to 0.29 ± 0.07 g g^{-1} after 50 min flash drying, no significant decline in percentage root or shoot production was observed (Table 3.16). After dehydration for 50 min, the water content was significantly reduced (by 73%), but all axes retained the ability for both root and shoot production (Table 3.16). However, after 60 min flash drying, although water content did not decline further, shoot production declined significantly.

For seeds from the St Lucia provenance, it was decided to flash dry explants for 20 min before cooling since this resulted in a mean water content of 0.41 ± 0.09 g g^{-1} with 100% root and shoot production. It was conceivable that such explants would have tolerated further reductions in water content, as adverse effects of dehydration had not been manifested (in terms of germination) after flash drying for up to 50 min. However, it was considered that cryoprotection and cooling had the potential to impose additional stresses with cumulative damage possibly being too severe for explants to recover, as suggested by Reed *et al*. (2005).

Table 3.16: Effect of dehydration of explants (embryonic axis with cotyledonary attachments) by flash drying, on germination (root and shoot production) and water content from seeds of the St Lucia provenance. Explants were excised, flash dried for 0 – 60 min and rehydrated in a solution containing 0.5 µM calcium and 0.5 mM magnesium chloride for 30 min in the dark. Explants were then decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on germination medium. Values represent the mean ± standard deviation. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for germination and 5 for water content).

Time (min)	Water content (g g^{-1})	% axes producing roots	% axes producing shoots
0	1.08 ± 0.11^{a}	100^{a}	100^{a}
10	0.59 ± 0.11^{b}	90^{a}	90^{a}
20	0.41 ± 0.09^{bc}	100^{a}	100^{a}
30	0.38 ± 0.12^{bc}	100^{a}	100^{a}
40	0.36 ± 0.06^{c}	100^{a}	100^{a}
50	0.29 ± 0.07^{c}	100^{a}	100^{a}
60	0.29 ± 0.05^{c}	80^{a}	53^{b}

Considering that germplasm from different provenances may not display the same responses to similar procedures, a trial was performed to establish the effect of cryoprotectants on water content and germination of explants excised from seeds from St Lucia. Since exposure of explants for 30 min in each cryoprotectant solution was found to be best for seeds from Port Elizabeth (Table 3.12), the same treatment was applied (Table 3.17). Exposure of explants to the various cryoprotectant solutions did not affect survival as 100% roots and shoots were obtained irrespective of the cryoprotectant solution applied (Table 3.17).

Table 3.17: Effect of cryoprotectants on germination (root and shoot production) and water content of explants (embryonic axis with cotyledonary attachments) from seeds of the St Lucia provenance. Explants were excised and immersed in 5% and 10% solutions of cryoprotectants for 30 min in each. Following cryoprotection, explants were decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on germination medium. Control explants were not exposed to any cryoprotectants. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for germination and 5 for water content).

Cryoprotectant (np and/or p)*	WC ($g\ g^{-1}$)	% axes producing roots	% axes producing shoots
Control	1.35 ± 0.12^{a}	100	100
Sucrose (np)	1.41 ± 0.16^{a}	100	100
Glycerol (p)	0.96 ± 0.04^{b}	100	100
Sucrose+glycerol (np + p)	0.91 ± 0.04^{b}	100	100
DMSO (p)	1.26 ± 0.26^{ab}	100	100

*np = non-penetrating, p = penetrating

Since all explants produced both a root and a shoot irrespective of the cryoprotectant(s) to which they were exposed, all treatments were used in the subsequent experiment to assess the effect of cryoprotection combined with dehydration. This procedure was necessary since the water contents of explants after cryoprotection (Table 3.17) were all too high for cryopreservation to be attempted. Thus, explants excised from St Lucia seeds were cryoprotected and then flash dried for 20 min (Table 3.18), as had been done for those derived from seeds of the Port Elizabeth provenance (Table 3.13). In no case was the ability for root and shoot production significantly impaired (Table 3.18). The lowest percentage of axes showing shoot production, 90%, occurred when explants were cryoprotected with glycerol and then flash dried. However, this result was not significantly different from those of the other treatments.

Table 3.18: Effect of cryoprotection followed by flash drying on germination (root and shoot production) and water content of explants (embryonic axis with cotyledonary attachments) from seeds from St Lucia. Explants were excised and immersed in 5% and 10% solutions of cryoprotectants for 30 min in each. Following cryoprotection, explants were flash dried for 20 min, rehydrated in a solution containing 0.5 μM calcium chloride and 0.5 mM magnesium chloride for 30 min in the dark, decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on germination medium. Control explants were not exposed to any cryoprotectant or flash drying treatments. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for germination and 5 for water content).

Cryoprotectant (np and/or p)*	Flash dry	WC ($g\ g^{-1}$)	% axes producing roots	% axes producing shoots
Control	–	1.08 ± 0.11^{a}	100	100^{a}
None	+	0.48 ± 0.03^{b}	100	90^{a}
Sucrose (np)	+	0.52 ± 0.07^{b}	100	100^{a}
Glycerol (p)	+	0.47 ± 0.04^{b}	100	90^{a}
Sucrose+glycerol (np + p)	+	0.41 ± 0.04^{b}	100	100^{a}
DMSO (p)	+	0.44 ± 0.06^{b}	100	100^{a}

*np = non-penetrating, p = penetrating

b) Exposure of explants derived from St Lucia seeds to nitrogen slush

Explants from the St Lucia provenance were cooled using two methods, *viz.* cooling within cryovials or directly in nitrogen slush (Table 3.19). Two-step cooling was not attempted as no survival was obtained when this procedure was applied to explants from the Port Elizabeth seeds. No explants from St Lucia seeds survived cooling within cryovials (results not shown). When explants were cooled in nitrogen slush, shoot development was obviated in all, with at best, 10% retaining the ability for root production (Table 3.19). Root development by 10% of the explants occurred when they were flash dried without cryoprotection and then cooled, or following cryoprotection in glycerol or a combination of sucrose and glycerol, flash dried and then cooled (Table

3.19). No explants that were cryoprotected in sucrose survived cooling, while only 3% of DMSO-cryoprotected explants were able to form roots (Table 3.19).

Table 3.19: Effect of cooling in nitrogen slush on germination (root and shoot production) and water content of explants (embryonic axis with cotyledonary attachments) from seeds from St Lucia. Explants were excised and immersed in 5% and 10% solutions of cryoprotectants for 30 min in each. Following cryoprotection, explants were flash dried for 20 min and then cooled, warmed (at 40°C for 2 min) and rehydrated (at room temperature) in a solution containing 0.5 µM calcium chloride and 0.5 mM magnesium chloride for 30 min in the dark. Explants were then surface decontaminated in 1% (w/v) NaOCl for 10 min, rinsed and cultured on germination medium. Control explants were not exposed to any cryoprotectant or flash drying treatments. Values represent the mean ± standard deviation. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 for germination and 5 for water content).

Cryoprotectant (np and/or p)*	Flash dry	WC ($g\ g^{-1}$)	% axes producing roots	% axes producing shoots
Control	–	1.08 ± 0.11^{a}	100^{a}	100^{a}
None	+	0.40 ± 0.09^{b}	10^{b}	0^{b}
Sucrose (np)	+	0.48 ± 0.04^{b}	0^{b}	0^{b}
Glycerol (p)	+	0.45 ± 0.04^{b}	10^{b}	0^{b}
Sucrose+glycerol (np + p)	+	0.40 ± 0.03^{b}	10^{b}	0^{b}
DMSO (p)	+	0.41 ± 0.03^{b}	3^{b}	0^{b}

*np = non-penetrating, p = penetrating

Since explants from seeds of the St Lucia provenance showed a very low capacity for root production after exposure to cryogenic temperatures (Table 3.19), they were not subjected to the protocol to induce adventitious shoots as was done for the Port Elizabeth material (Table 3.15). It is only feasible to apply such a protocol if a sufficiently high percentage of roots are generated after cryopreservation. Thus, future work should focus on improving this aspect as, in the interest of genetic resources conservation of a species, material needs to be derived from as many geographically separated populations as is possible (Kramer and Havens, 2009).

3.3.4 Assessment of potential ROS-mediated damage caused by cryopreparative stages and exposure to cryogenic temperatures

In order to develop effective cryopreservation protocols, it is necessary to consider factors that may contribute to failure or success. Considering that ROS generation, exemplified by superoxide, appears to accompany several of the pivotal steps required for cryopreservation (Whitaker *et al*., 2010), in the present study, quantification of superoxide was carried out following each of the procedures used (Figures 3.4 – 3.6). Separate assays were performed following each step of the protocol: 1) after explant excision; 2) dehydration; 3) cryoprotection; 4) a combination of cryoprotection and dehydration; and 5) cryogenic exposure. Figure 3.4 shows root and shoot production in relation to the production of superoxide from axes during the cryopreparative stages, i.e. stages 1 to 4 mentioned above (control explants were excised with 2 mm^3 cotyledonary blocks and were not exposed to any cryopreparative treatments). As a comparison, results were included for the production of superoxide from intact seeds and isolated axes without cotyledonary segments. Minimal amounts of superoxide were liberated from intact seeds. However, upon explant excision, a burst of superoxide was apparent. The most superoxide was produced by isolated axes without any cotyledonary attachments. The relationship between superoxide production and germination was strongly influenced by two outliers, *viz*. for treatments relating to superoxide production from isolated axes and isolated axes that had been dehydrated. Including these outliers in the data set resulted in R^2 values of 0.0698 for roots and 0.7639 for shoots (reflected in Figure 3.4). It is important to point out that the outliers represented explants that were not normally processed for cryostorage, i.e. the explant used in all cryopreparative and cryostorage trials comprised the embryonic axis with 2mm^3 cotyledonary blocks while the outliers represented explants without any cotyledonary attachments. For these reasons, the outliers were not included in the analysis. Consequently, the results of the correlation analysis indicated that there was no correlation between superoxide production and shoot (Pearson correlation = -0.213, p = 0.194) or root production (Pearson correlation = -0.238, p = 0.145) from axes in response to the cryopreparative stages. Thus, the levels of

superoxide liberated at any of the cryopreparative stages presently employed, apparently did not compromise either root or shoot production.

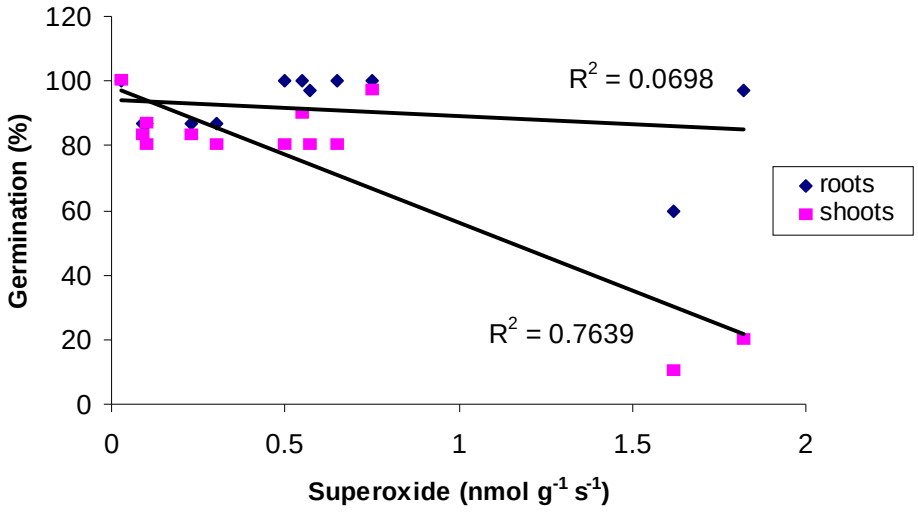

Figure 3.4: Relationship between germination of axes and superoxide production, all stages of cryopreparative treatments included (except exposure to cryogen).

Superoxide production was also assessed for axes following cooling at two rates – as achieved within cryovials and in nitrogen slush (Figure 3.5) – both of which had enabled axis survival in previous experiments (refer to Table 3.14). The results for both cooling rates were combined in Figure 3.5. Neither cooling rate preserved the potential for shoot production by more than 10% of axes. While cooling in cryovials preserved the ability for root production by 30% of axes at best, up to 87% retained the ability for root development following rapid cooling in nitrogen slush (Table 3.14). There was no correlation between superoxide liberation and shoot production (Pearson correlation = 0.255, p = 0.167) but there was a positive correlation between superoxide liberation and root production (Pearson correlation = 0.643, p = 0), but this latter relationship was not very strong. Irrespective of the actual levels of superoxide evolved, shoot production was

severely compromised in all cases, leading to the conclusion that either even where seemingly low levels of superoxide were produced, the consequences may have been lethal to the shoot apices or superoxide had no effect and damage was caused by another factor.

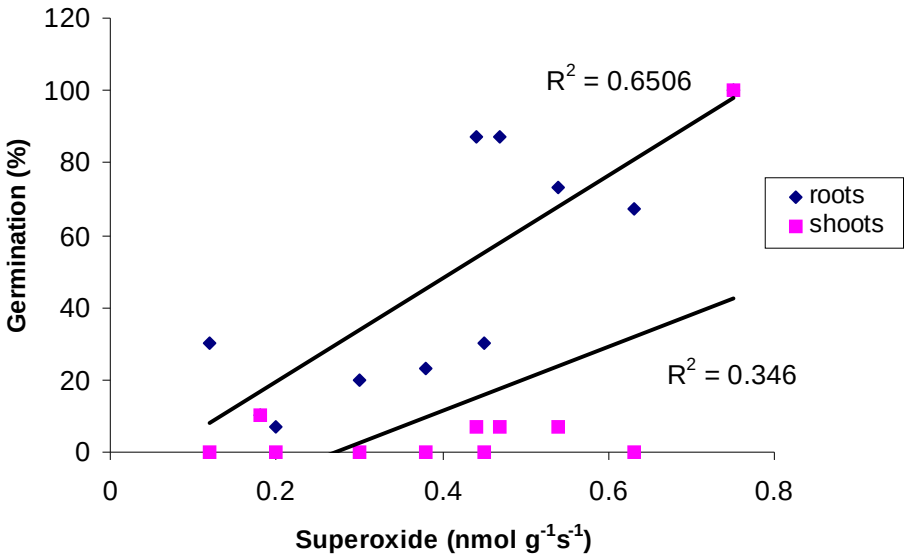

Figure 3.5: Relationship between germination and superoxide production for axes cooled at different rates.

The production of superoxide was also assessed for adventitious shoots during the cryopreparative stages and following cooling (Figure 3.6). The highest quantity of superoxide was produced upon excision of adventitious shoots from the original clump of shoots. However, as 100% of adventitious shoots tolerated this level of superoxide, it could not be considered as detrimental. The only treatments facilitating shoot survival after cooling were flash drying (7% survival) and cryoprotection with a combination of sucrose and glycerol prior to flash drying (20% survival). There was no correlation between superoxide production and survival (Pearson correlation = 0.307, p = 0.034),

however, the p value obtained indicated that the relationship was significant, but this was likely to have been determined by a single outlier. Therefore, it seems that evolution of superoxide cannot be considered to be the basis of ROS-mediated, lethal damage.

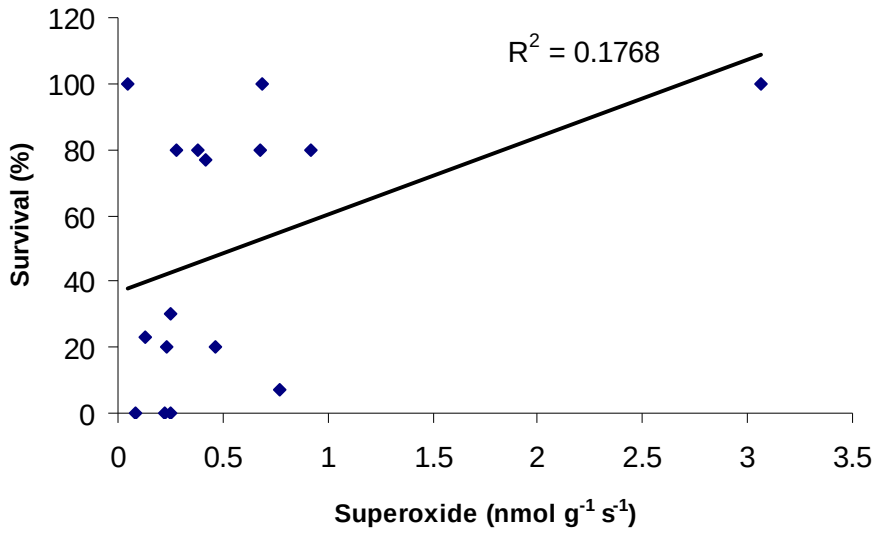

Figure 3.6: The relationship between superoxide production and survival of adventitious shoots that have been subjected to the cryopreparative stages and cooling in nitrogen slush.

3.3.5 Probing the germplasm for possible epigenetic changes following cryopreparative stages and cryopreservation

Digested and undigested DNA from all treatments was amplified using the PCR conditions described in section 3.2.9.e and the RAPD profiles were examined for two explant types, *viz*. axes with cotyledonary attachments (explant type 3; Table 3.20) and adventitious shoots (explant type 2; Table 3.21). This was done for explants that were not treated, and those that were: 1) dehydrated; 2) treated with cryoprotectants; 3) treated with cryoprotectants and then dehydrated; and 4) for explants that were cooled in slush following the cryopreparative stages.

When material from germinated axes was considered (explant type 3), a total of 1 731 bands was obtained with 11 primers. Most of the bands analysed were monomorphic (Fig 3.7A), indicating no change in DNA methylation status of treated explants (those exposed to the cryopreparative stages and/or cooled in sub-cooled nitrogen) compared with non-treated (control) explants. The percentage polymorphism ranged from 0% (in glycerol cryoprotected explants) to 0.46% (in explants that were germinated on filter paper in the laboratory). Figure 3.7B shows the presence of two additional bands in lanes 3 and 11, in the RAPD profiles of explants that were flash dried (lane 3) and flash dried and then cryopreserved (lane 11), respectively.

Table 3.21 illustrates the results obtained when adventitious shoots were considered. This table summarises two sets of results, *viz*. the first four columns (C, SG, SGD and SGDC) display the results obtained when isolated adventitious shoots were exposed to the cryopreparative stages and also after retrieval from sub-cooled nitrogen. Columns 5 and 6 (GDC and DMDC; Table 3.21) show the results obtained for adventitious shoots that developed on roots that germinated from cryopreserved axes (Table 3.15). For all analyses after the various treatments of adventitious shoots, a total of 558 bands were scored. Of these, most (94%) were monomorphic (Table 3.21), and the highest percentage polymorphism (1.79%) was obtained in adventitious shoots that had been cryoprotected.

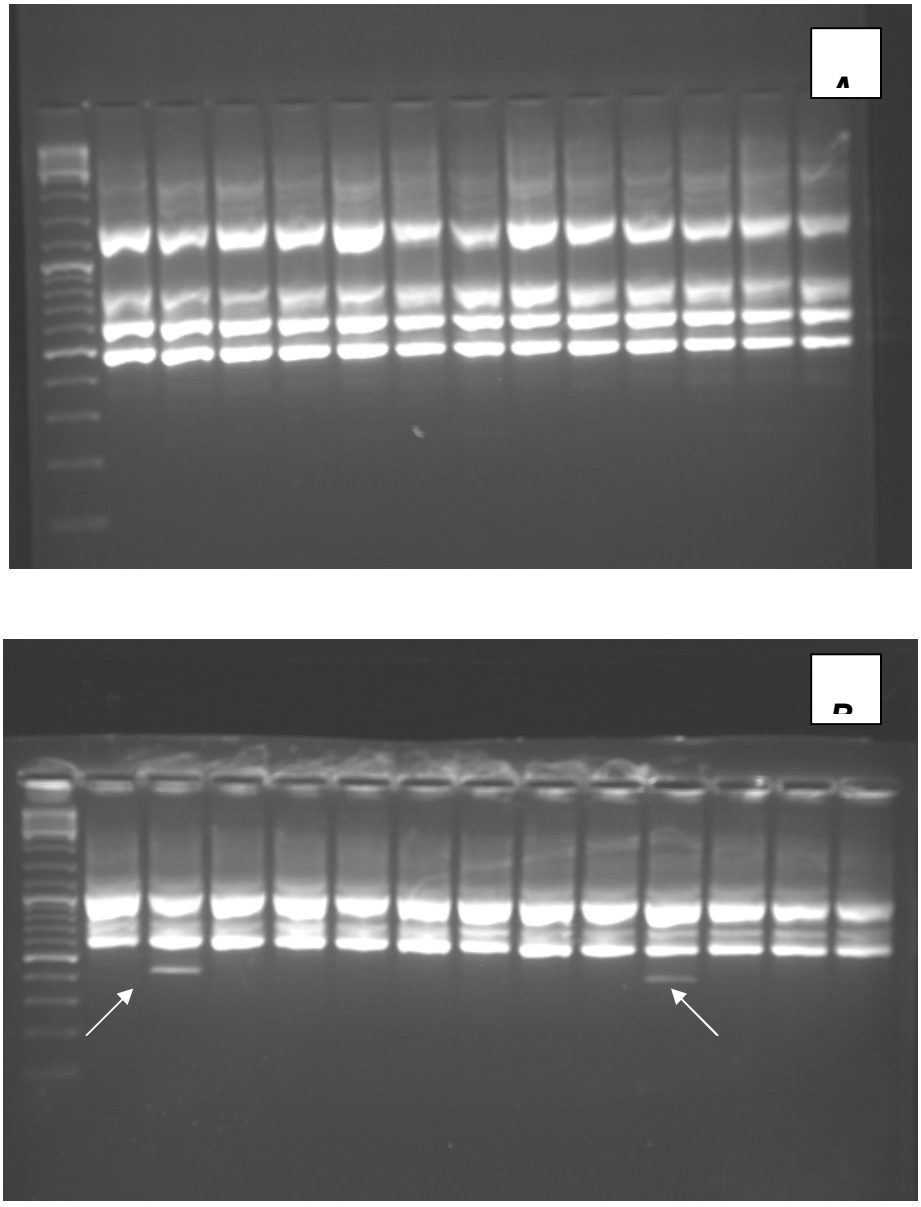

Figure 3.7: RAPD profiles generated using the CRED-RA method. A: Monomorphic RAPD profile using primer OPA19 and B: polymorphisms using primer GB7. Arrows indicate presence of additional bands. Axes with cotyledonary segments were cryoprotected and/or flash dried, cooled in slush, warmed and rehydrated in a CaMg solution, decontaminated and plated for survival. DNA was isolated from recovered plants (after 6 weeks *in vitro*) and subjected to CRED-RA. The additional bands correspond to explants that were initially flash dried (lane 3) and those that were flash dried and then cooled (lane 11).

Table 3.21: Number of polymorphisms identified using RAPD analysis by comparing changes in methylation profiles of isolated adventitious shoots exposed to the cryopreparative stages (C, SG and SGD) and following cryostorage (SGDC) and for adventitious shoots produced from roots regenerated from cryopreserved axes (GDC and DMDC). Survival of all explants was assessed by onwards development following each cryopreparative stage and cooling. DNA was then extracted from plant material and subjected to CRED-RA.

			No. polymorphisms Treatments					
Primer	Enzyme	No.monomorphic bands	C	SG	SGD	SGDC	GDC	DMDC
OPU5	*HpaII*	50	0	0	0	0	0	0
OPU8	*HpaII*	32	0	0	0	0	0	0
OPU14	*HpaII*	22	0	0	0	0	0	0
OPU15	*HpaII*	34	0	0	0	0	0	0
OPA19	*HpaII*	17	1	1	1	0	0	0
OPA22	*HpaII*	35	0	0	0	1	0	0
OPB22	*HpaII*	18	1	1	1	0	0	0
OPC8	*HpaII*	28	0	0	0	0	0	0
OPC12	*HpaII*	34	0	0	0	1	0	0
GB7	*HpaII*	24	0	0	0	0	0	0
GB8	*HpaII*	14	0	0	0	0	0	0
OPU5	*MspI*	29	1	0	0	0	0	0
OPU8	*MspI*	22	0	2	0	0	0	0
OPU14	*MspI*	31	3	0	0	0	0	0
OPU15	*MspI*	32	1	0	0	0	0	0
OPA19	*MspI*	11	0	1	1	1	0	0
OPA22	*MspI*	26	0	0	0	0	0	0
OPB22	*MspI*	36	0	2	2	2	0	0
OPC8	*MspI*	15	0	1	1	1	0	0
OPC12	*MspI*	24	1	2	2	1	0	0
GB7	*MspI*	12	0	0	0	0	0	0
GB8	*MspI*	12	0	0	0	0	0	0
Totals		558	8	10	8	7	0	0
%polymorphism			1.43	1.79	1.43	1.25	0	0

C: control, SG: sucrose and glycerol cryoprotection, SGD: sucrose and glycerol cryoprotection+flash dry, SGDC: sucrose and glycerol cryoprotection+flash dry+slush, GDC: glycerol cryoprotection+flash dry+slush and DMDC: DMSO cryoprotection+flash dry+slush.

3.4 DISCUSSION

3.4.1 Cryopreparative procedures

a) Choice of explants and dehydration

In the present study, two types of explant were investigated for conservation of germplasm of *E. capensis*, i.e. vegetative *in-vitro*-derived explants ('broken' buds and adventitious shoots) and explants immediately derived from seeds (axes with cotyledonary blocks). In conventional micropropagation practices, the advantage of using 'broken' buds and shoot meristems is that these represent clonal material of known genotype. This enables the selection of superior genotypes for specific applications, while the advantage of using seeds is that they represent unknown genotypes that will facilitate conservation of the gene pool of a species (Reed, 2008). In general, two types of vegetative explants from *in-vitro*-germinated seedlings (which do offer the diversity afforded by seeds) are commonly used in cryopreservation studies, *viz.* nodal segments [the axillary bud subtended above and below by a stem segment (González-Benito *et al*., 1998; Martínez *et al*., 1999; Niino *et al*., 2000; Suzuki *et al*., 2006)] and meristem explants [comprising the shoot apical dome generally with a few pairs of leaf primordia (Wu *et al*., 1999; Abdelnour-Esquivel and Engelmann, 2002; Schocchi *et al*., 2004; Schoenweiss *et al*., 2005; Sanayaima *et al*., 2006; Varghese *et al*., 2009)]. Nodal segments are larger (2 – 4 mm) than meristem explants (<1 mm), but both types are recommended for cryopreservation, as they are reported to maintain the genetic stability of cryopreserved material, since plantlets will be produced from already-differentiated meristems (Turner *et al*., 2001a; Gagliardi *et al*., 2003). There are advantages and disadvantages associated with the use of both types of explants: the size of nodal segments could be a disadvantage, as it may limit the rate of dehydration and cooling, but an advantage is that such explants can be easily and quickly excised. Meristems, on the other hand, are very small, but their excision is extremely time-consuming and the explants are easily damaged during excision, which is detrimental to the success of protocols (Clavero-Ramírez *et al*., 2005). Considering these factors, and that a

regeneration medium had already been established for *in vitro* nodal segments of *E. capensis* (Chapter 2), this explant type was used for the present cryopreservation studies.

For successful cryopreservation, it is necessary first to dehydrate explants to limit ice formation during cooling as this has potentially lethal consequences (Suzuki *et al.*, 1998; Hitmi *et al.*, 1999; Volk and Walters, 2006; Gonzalez-Arnao *et al.*, 2008). If explants can survive the rigours of dehydration stress, then they can be considered for cryopreservation (Pence, 1990). This implies that selected explants must tolerate dehydration to a level that is low enough to facilitate cryostorage but the imposed dehydration stress must not compromise viability (Wesley-Smith *et al.*, 1992). Thus, for successful cryostorage a balance must be reached to avoid, or at least to minimise, both dehydration and cryo-injury. This, however, is often not easy to achieve. For example, Padaychee *et al.* (2008) emphasised the difficulties encountered in dehydrating axillary bud explants (of *Eucalyptus grandis*) whilst still maintaining acceptable levels of survival. A key step in successful cryostorage of *in vitro* buds is to attempt to induce some degree of dehydration tolerance in explants (Uragami *et al.*, 1990) by the application of suitable additives or by manipulation of the dehydration rate. However, regardless of the procedure employed (see section 3.3.1.a), nodal segments of *E. capensis* did not tolerate dehydration.

It was therefore decided to use slightly more developed nodal segments (described as 'broken' buds in the present studies) for cryopreparative trials. Explants were dehydrated by flash drying, since Padaychee *et al.* (2008) highlighted the advantage of using a fast dehydration rate to lower the water content of *Eucalyptus* axillary buds. Also, flash drying is the method of choice for the dehydration of germplasm from recalcitrant seeds (Berjak *et al.*, 1989; 1990; Pammenter *et al.*, 2002a). Thus, in the present study, all three explant types were flash dried, particularly since previous efforts to dehydrate explants (nodal segments) slowly in silica gel or over a saturated salt solution proved unsuccessful (results not shown).

Examination of the results presented in Table 3.3 showed that 'broken' buds tolerated dehydration to 0.45 ± 0.13 g g^{-1} water content with no decline in the ability for onwards development. This result represented the first instance for *E. capensis* where *in-vitro*-derived explants could be dehydrated to low water contents with good survival. In all previous trials with nodal segments, viability was lost at considerably higher water contents (in the range of 1.83 – 3.06 g g^{-1} [results not shown]). Therefore, for *in vitro* germplasm of *E. capensis*, it appeared that 'broken' buds were more tolerant to dehydration than in the 'unbroken', dormant, condition. Adventitious shoots were also dehydrated with periodic sampling over 80 min (Table 3.6) and the water content after 60 min dehydration (0.46 ± 0.17 g g^{-1}) was within the range for cryopreservation with acceptable levels of survival (Perán *et al.*, 2006).

For axes, explants were sampled at 10-min intervals up to 60 min (Tables 3.11 and 3.16). The water content of freshly excised explants from St Lucia seeds was considerably higher than that of Port Elizabeth seeds (Tables 3.16 and 3.11; 1.08 ± 0.11 *vs.* 0.82 ± 0.19 g g^{-1}), thus demonstrating an *a priori* difference in seed characteristics from these two provenances. However, the water contents of explants from both provenances were similar after 20 min flash drying.

Following dehydration of axes, they were rehydrated for 30 min in a solution containing 0.5 μM calcium chloride and 0.5 mM magnesium chloride (Mycock, 1999), which has been shown to promote vigorous and normal seedling establishment from embryos/axes excised from recalcitrant seeds and then dehydrated (Berjak *et al.*, 1999; Berjak and Mycock, 2004). In contrast, the vegetative explants, i.e. 'broken' buds and adventitious shoots, were not rehydrated by immersion after flash drying as this had been shown earlier to affect survival adversely (results not shown).

A common trend that emerged upon examination of the dehydration profiles of adventitious shoots (Table 3.6) and axes from Port Elizabeth (Table 3.11) and St Lucia (Table 3.16) was that a point was reached where further dehydration no longer reduced explant water content significantly, but explant survival was compromised. These

observations are best explained in terms of the duration of the applied stress (Pammenter *et al.*, 2002b). According to those authors, even if the water stress imposed on desiccation-sensitive specimens is relatively mild, extending the duration for which it is applied will have markedly adverse effects on survival.

An observation apparent from examination of the results in Table 3.11, is that the shoot meristems of axes appeared to be consistently more sensitive to dehydration than those of the roots. Berjak *et al*. (1998; 1999) and Kim *et al*. (2002) also reported that the shoot meristem was more easily damaged by dehydration than the root meristem in *Q. robur* and *C. sinensis* axes, respectively. Berjak *et al*. (1998; 1999) suggested that a possible reason for this could be because the root apex cells are protected by the root cap. Therefore, even if the outermost cells of the root cap are damaged by dehydration or cryogen exposure, the root apical meristem cells could effectively remain protected, thus retaining the ability for root formation upon rehydration. In contrast, the shoot apex does not have an equivalent protective structure. The shoot apical meristem is composed of the outer tunica and inner corpus regions (Cutter, 1971; Jiang and Feldman, 2003; Evert, 2006) and the only potentially protective coverings are the leaf pairs that surround the apical meristem. However, as these do not form a continuous cover, they are not likely to be as effective in affording protection as the root cap is suggested to be. Thus, cells of the shoot meristem are likely to be more readily damaged by imposed stresses (such as dehydration) and therefore less likely to retain the ability for onwards development upon rehydration (Berjak *et al*., 1998; 1999).

As a further pertinent factor, Kioko *et al*. (2006) showed that cells of the shoot pole of *Trichilia emetica* axes lost water far more rapidly than did cells of the root pole. As a consequence, the duration for which shoot meristems were exposed to water stress was considerably longer than for the root meristems. It is likely that this is generally the case across species, when excised axes are subjected to dehydration, and it must be emphasised that overall explant water content gives no information about water content of component tissues. In intact seeds, the orientation of the embryonic axis is such that the shoot tip is concealed and surrounded by cotyledonary or endosperm tissues, while

the root apex is relatively superficial. Thus, when intact recalcitrant seeds lose water to the environment after shedding, presumably the shoot apex is the last of the embryo tissues to be subjected to dehydration stress and its constituent cells may well remain viable after those of the root tip have sustained lethal injury. This conjectured situation would be reversed once axes are excised and the shoot apex exposed, which leads to the suggestion of a modification to the flash drying technique, *viz.* that axes are 'half-excised' initially, to expose the hypocotyl for flash drying, while leaving the epicotyl enclosed, and only later removing the surrounding seed tissues.

In the present study, all explant-types were successfully dehydrated by flash drying but it is must be emphasised that flash drying does not induce desiccation tolerance in recalcitrant material (Pammenter *et al.*, 1998). Rather, if explants are rapidly dehydrated, there is less time for unbalanced, aqueous-based metabolism to occur (at intermediate water contents) and damage to accumulate. Explants can therefore be dehydrated to a lower water content before viability is lost (Pammenter and Berjak, 1999; Wesley-Smith *et al*., 2001b; Pammenter *et al.*, 2002a). If embryos are rapidly dehydrated, much of the freezable (solution) water can be removed without adversely affecting viability (Pammenter *et al*., 1991; 1993). However, further removal of water (at or near the level of non-freezable water) underlies desiccation damage *sensu stricto* and results in the destruction of cellular components (Pammenter and Berjak, 1999; Walters *et al*., 2001). It is of pivotal importance that this is avoided when dehydrating explants in preparation for cryopreservation. Thus, ascertaining the water content range avoiding such desiccation damage, but facilitating survival of cryogenic temperatures is of paramount importance.

b) Cryoprotection of explants

Pretreatment of explants with cryoprotectants prior to dehydration and cooling has been reported to promote survival (Dumet *et al.*, 1997; Volk and Walters, 2006). In this study, sucrose, glycerol, a combination of sucrose and glycerol and DMSO were investigated as potential cryoprotectants (Tables 3.4, 3.7, 3.12 and 3.17) as this allowed assessment of

the efficacy of both penetrating (glycerol and DMSO) and non-penetrating (sucrose) compounds. To ameliorate possible cytotoxic effects of cryoprotectants (Volk *et al*., 2006; Nadarajan *et al.*, 2008), they were applied at the lower (5%) and then at the higher (10%) concentration. Cryoprotection by preculturing explants on medium supplemented with the cryoprotectant (Pence, 1991; Thierry *et al*., 1997; Panis *et al*., 2000) was avoided, as this is time-consuming, involves an additional decontamination step, and is not practical when a large number of explants have to be processed in a short time, as is the case with recalcitrant seeds.

The use of DMSO as a cryoprotectant can be controversial since it has been reported to be toxic to plant cells, depending on the concentration and duration of exposure (Bajaj *et al*., 1970; Yu and Quinn, 1994; Wolfe and Bryant, 2001) and there are concerns that DMSO may affect the genetic (and epigenetic) integrity of cells (Takagi, 2000). The latter may relate to the activity of DMSO in inducing cell differentiation, where it apparently can affect gene regulation (Yu and Quinn, 1994) and may have a stimulatory effect on cell division (Benson, 1990). Nevertheless, despite such concerns, DMSO has been widely used as a cryoprotectant for many years (Haskins and Kartha, 1980; Henshaw *et al*., 1985; Chen and Kartha, 1987; Mix-Wagner and Schumacher, 2003). However, when DMSO has been used prior to cryopreservation of plant specimens, it was applied at a relatively low concentration, e.g. at 5% for 60 min (Haskins and Kartha, 1980), 10% for 45 min (Pence, 1991) and 7.5 – 10% for 120 min (Mix-Wagner and Schumacher, 2003). These factors were therefore considered in the present study, when DMSO was used explants were cryoprotected in 5% (v/v) and 10% (v/v) solutions for a maximum of 30 or 60 min at each concentration. Considering the reporting of successful cryopreservation using DMSO (Henshaw *et al*., 1985; Chen and Kartha, 1987; Mix-Wagner and Schumacher, 2003), this cryoprotectant could not be disregarded in the present study. A further advantage in using DMSO as a cryoprotectant is that it has been reported to have free radical scavenging activity (Benson, 1990) converting hydroxyl radicals into less reactive products (Yu and Quinn, 1994). Since a burst of reactive oxygen species in response to wounding has been demonstrated upon axis excision from recalcitrant seeds (Roach *et al*., 2008), the activity of DMSO as a free radical/ROS

scavenger could be beneficial. It has been recommended that if there are persistent concerns regarding the use of DMSO, then it may be replaced by glycerol (Meryman and Williams, 1985; Panis and Lambardi, 2005), which was used in parallel in the present investigations.

Since vegetative tissues have been reported to be easily damaged by cryoprotectant solutions (Maruyama *et al*., 1998), 'broken' buds and adventitious shoots were not exposed to cryoprotectants for longer than 15 and 30 min in each solution, respectively. 'Broken' buds tolerated exposure to all cryoprotectants applied with no decline in onwards development (Table 3.4), while adventitious shoots showed a non-significant decline in survival after application of cryoprotectants for 15 min at each concentration (Table 3.7).

When cryoprotected axes from the St Lucia and Port Elizabeth provenances were compared, the former appeared less sensitive to the applied cryoprotectant solutions, since all explants formed both a root and shoot (Table 3.17; 100%) compared with explants derived from seeds from Port Elizabeth (Table 3.12; 80 – 93% of axes developing roots and 80 – 87% producing shoots). The water content reached after cryoprotection of explants from the St Lucia provenance was higher (Table 3.17; 0.91 ± 0.04 to 1.41 ± 0.16 g g^{-1}) than those attained after cryoprotection of explants from seeds of the Port Elizabeth provenance (Table 3.12; 0.66 ± 0.09 to 1.12 ± 0.24 g g^{-1}). A similar trend was observed when the water contents after flash drying were compared (Tables 3.11 and 3.16). This was probably because, at the outset, explants from Port Elizabeth seeds were at a lower water content (0.82 ± 0.19 to 0.90 ± 0.17 g g^{-1}) than those from St Lucia seeds (1.08 ± 0.11 to 1.35 ± 0.12 g g^{-1}). Consequently, explants from seeds from Port Elizabeth were consistently at lower water contents at each sampling point than those excised from St Lucia seeds. It is possible that, at the lower end of the water content ranges, although these were too high for desiccation damage *sensu stricto*, less vigorous specimens from Port Elizabeth seeds were unable to withstand deleterious, aqueous-based events.

c) Cryoprotection and dehydration of explants

Since the water content of all cryoprotected explants was considered to have remained too high for non-injurious cooling, they were subsequently flash dried. For 'broken' buds, all explants that were cryoprotected and dehydrated showed high survival (Table 3.5; 80 – 100%), and this procedure was therefore used in subsequent cooling trials. In contrast, for adventitious shoots, only one treatment yielded acceptable levels of survival (80%), i.e. when explants were cryoprotected with a combination of sucrose and glycerol and then flash dried (Table 3.8). This indicated that for adventitious shoots, although explants could survive the individual stresses of cryoprotection (Table 3.7) and flash drying (Table 3.6), when these stresses were applied sequentially, the damage imposed by the combined stresses compromised their survival (Table 3.8). Even the best of these cryoprotective treatments, using a combination of sucrose and glycerol, could have imposed damage on some of the shoots, as survival was reduced from 100% after cryoprotection (Table 3.7) to 80% after cryoprotection and flash drying (Table 3.8). This treatment effected a substantial decline in water content, from 3.67 ± 0.47 g g^{-1} after cryoprotection (Table 3.7) to 0.46 ± 0.29 g g^{-1} after flash drying (Table 3.8). In view of the relatively severe dehydration and considering the standard deviation about the mean, it is suggested that shoots which did not survive were adversely affected by desiccation, rather than by direct effects of the cryoprotectant solution.

For axes excised from seeds obtained from Port Elizabeth and St Lucia, the survival obtained after cryoprotection and flash drying (Tables 3.13 and 3.18), and the water contents reached, were deemed suitable to subject such explants to cooling. That is, no cryopreparative treatments precluded the ability for seedling production, and all reduced the water content of explants to a level that theoretically could facilitate non-injurious cooling. The ability for root and shoot production by cryoprotected and flash dried explants from the St Lucia provenance (Table 3.18) was superior to that of the explants derived from seeds from Port Elizabeth (Table 3.13), suggesting a differential, provenance-related tolerance to the stresses of dehydration and cryoprotection. Another trend that emerged was that when glycerol was used, there were some adverse effects on

the potential for shoot and/or root production after flash drying, although not significant for axes from seeds derived from St Lucia (Tables 3.13 and 3.18). Hence, it appeared that when applied as the sole cryoprotectant, glycerol had some cytotoxic effect, particularly on axes excised from seeds of the Port Elizabeth provenance.

3.4.2. Exposure of explants to cryogenic temperatures

a) Survival of explant types 1 – 3 after cooling

According to Benson and Noronha-Dutra (1988) retrieval of plant material from liquid nitrogen involves further events that can culminate in two possible situations: either damaged cells may undergo further degradative events leading to explant death; or cells that are not as extensively damaged can stabilise, engage repair processes and eventually resume normal metabolic and physiological activity. A third, and probably more likely explanation in terms of onwards development (not simply callus formation or elongation/greening), is that a critical proportion of cells of the explant survive, allowing for mitosis and normal differentiation (Volk and Caspersen, 2007; Volk, 2010), e.g. from axes by root production (Kim *et al.*, 2008) or seedling establishment (e.g. Sershen *et al.*, 2007; Ngobese *et al.*, 2010). In the present investigation, efforts were made to limit exacerbation of damage after retrieval of explants from the cryogen by, for example, ensuring that rehydration (when carried out) and incubation of cultures (in all cases) were performed in the dark, as all the explants of *E. capensis* are green and may well therefore be subject to photo-oxidative damage (Benson, 1990).

Maintenance of cultures in the dark is common practice for *in vitro*-derived explants from tropical (González-Arnao *et al.*, 1998; Pennycooke and Towill, 2001; Abdelnour-Esquivel and Engelmann, 2002; Panis *et al.*, 2002) and temperate species (Wilkinson *et al.*, 1998; Blakesley and Kiernan, 2001; Turner *et al.*, 2001b). This is because the various steps of a cryopreservation protocol impose sequential stresses on explants, all of which may be accompanied by the production of free radicals (Benson, 1990). Touchell and Walters (2000) showed that embryos of *Zizania palustris* could be cultured in the light

after dehydration, but after retrieval from liquid nitrogen, culturing such explants under light conditions was associated with an increase in lipid peroxidation (indicative of free radical/ROS damage) and a significant decline in survival. A possible explanation for this could be that when explants were exposed to stresses (dehydration or cryogenic temperatures), the mitochondrial and photosynthetic electron transport chains may have become disrupted and in the light essentially uncontrolled generation and escape of ROS could occur (Nishioka *et al*., 2010), triggering a cascade that ultimately leads to lipid peroxidation, the production of other toxic byproducts and damage to subcellular constituents, including DNA (Ojima *et al*., 2009). Evidence suggests that light accelerates such processes (Touchell and Walters, 2000; Nishioka *et al*., 2010).

The rate of cooling is another critical factor that can affect the success of cryopreservation (Chandel and Pandey, 1995; Normah and Makeen, 2008). This, in conjunction with explant water content (Touchell and Walters, 2000; Wesley-Smith *et al*., 2001a), will play a vital role in influencing the nucleation and growth of ice crystals and subsequent survival of germplasm (Nashatul Zaimah *et al*., 2007; Sershen *et al*., 2007; Normah and Makeen, 2008). Sershen *et al*. (2007) highlighted that it is of utmost importance to determine the optimal water content and cooling rate for each explant type on a species basis, even when relationships are familial or genetic. Thus, three cooling rates were investigated in the present study, *viz*. two-step cooling at 1°C min^{-1} to -40°C after which the cryovials were plunged into liquid nitrogen, cooling explants within cryovials at approximately 3 – 10°C s^{-1}, and tumbling explants within nitrogen slush at -210°C at a cooling rate of approximately 1 000°C s^{-1} (Vertucci, 1989; Wesley-Smith, 2002). In the present investigation, explants were cryoprotected, flash dried and then cooled using these three procedures. No explants survived the two-step cooling process (results not shown). This outcome is not uncommon when complex tissues are cryopreserved (Engelmann, 2004; Benson, 2008b).

An examination of the possible consequences of each cooling rate may help to explain the results observed in the present study. Slow cooling results in the efflux of water from cells, so that ice crystals form in the extracellular spaces and their propagation to the

interior of cells (i.e. as intracellular ice crystals) is suggested (when all parameters are ideal) to be prevented by the presence of the plasma membrane (Muldrew *et al*., 2004). However, in the present study it is possible that sub-optimal cooling rates may have caused freeze-induced dehydration injury (i.e. the accumulation of toxic levels of solutes as ice crystals developed) (Rajashekar and Burke, 1996; Muldrew *et al*., 2004) or, if insufficient water was removed from cells during the freeze-induced dehydration step (as a result of the complex nature of tissues), this could have led to the formation of large lethal ice crystals when explants were plunged into liquid nitrogen (Gonzalez-Arnao *et al*., 2008). Such phenomena may account for the lack of survival observed in the present study. Alternatively, cells may have incurred damage due to osmotic contraction and plasmolysis injury (Wolfe and Bryant, 2001; Muldrew *et al*., 2004) or even complete cell rupture as a result of sudden volume changes during rehydration (Wolfe and Bryant, 2001; Uemura *et al*., 2006). Further, it is conceivable that intracellular ice crystals may have formed and, as ions accumulated, an electrical gradient could have resulted, thus destabilising the plasma membrane, and permitting intracellular ice seeding (Steponkus, 1984).

When explants were cooled within cryovials, some survival was obtained only for axes from seeds from Port Elizabeth (Table 3.14; 7 – 30% roots and 0 – 10% shoots). It is possible that the cooling rate achieved when vegetative explants were cooled in this manner was too slow to avoid injurious ice crystallisation. While it is most probable that the size (approximately 1.5 – 2 mm) and surface:volume ratio of these vegetative explants was the major factor in the lethal effects of cryogen exposure (Wolfe and Bryant, 2001; Wesley-Smith *et al*., 2004), the unfavourably high water content may have exacerbated the potential for damage. It should be noted, however, that explant size and cell heterogeneity could be inextricably bound up with cooling rate effects in critical tissues.

In the present study, the maximum shoot production from axes of Port Elizabeth seeds was just 10%, from glycerol-cryoprotected explants that were flash dried and then cooled within cryovials (Table 3.14), noting that this is difficult to explain, in view of the

adverse effects after flash drying when glycerol was the sole cryoprotectant (Table 3.13). A comparable level of shoot survival (15% of axes) was reported by González-Benito *et al*. (2002) for *Quercus ilex* while Berjak *et al*. (1999) found that no axes of *Q. robur* survived cooling within cryovials. The observation of limited shoot production by cryopreserved axes after relatively slow cooling is not uncommon, with many authors reporting similar results, e.g. Pritchard and Prendergast (1986); Kioko *et al*. (1998); Berjak *et al*. (1999); González-Benito *et al*. (2002); Kim *et al*. (2002); Engelmann (2004); Goveia *et al*. (2004) and Walters *et al*. (2008). Thus, it can be suggested that slow cooling rates are not appropriate to cryopreserve germplasm from recalcitrant seeds successfully.

In contrast, when explants were cooled within nitrogen slush, better survival was obtained, except in the case of 'broken' buds, where no explants survived. During rapid cooling (> 100°C min^{-1}), the cytoplasm can become supercooled thereby promoting the formation of small, potentially non-lethal (intracellular and extracellular) ice crystals (Muldrew *et al*., 2004). Although cooling rates of hundreds of degrees per second are achieved when nitrogen slush is used (Echlin, 1992), in the case of explants that did not survive rapid cooling it is possible that the location of even small intracellular ice crystals could have physically damaged membranes such as the nuclear envelope (Helliot *et al*., 2003) and plasmalemma (Fujikawa and Jitsuyama, 2000; Kaczmarczyk *et al*., 2008) and in the most extreme cases, loss of cellular integrity may have occurred (Suzuki *et al*., 1997; Padaychee, 2007).

It must also be considered that in the present study, the water content of 'broken' buds (Table 3.5; 0.34 ± 0.25 to 0.53 ± 0.09 g g^{-1}), in combination with the relatively large explant size is likely to have resulted in slower and uneven cooling upon immersion in the cryogen, with lethal intracellular ice crystal formation in particular regions or tissues (González-Arnao *et al*., 1998). For adventitious shoots, using this means of rapid cooling gave 7 – 20% survival, when explants were flash dried, or cryoprotected in a solution of sucrose and glycerol and then flash dried before tumbling in nitrogen slush. The beneficial effects of this cryoprotectant solution may be attributed to the combined action

of the low molecular weight compound glycerol entering cells thereby decreasing the concentration of solutes (colligative action), while the high molecular weight sucrose acting osmotically, could prevent or minimise intracellular ice formation (Finkle *et al*., 1985; Dumet *et al.*, 1993; 2000). Further, Brison *et al*. (1995) suggested that the cryoprotective properties of the two compounds are additive when they are used together. These factors may explain why 20% of adventitious shoots cryoprotected with sucrose and glycerol survived cryogenic temperatures compared with 7% of those not cryoprotected. Other workers have reported levels of survival similar to the 20% obtained in the present study after warming, for cryoprotected adventitious shoots, *viz.* 25% shoot regeneration in bud clusters of *Guazuma crinita* (Maruyama *et al.*, 1998) and 23 – 32% survival of adventitious buds of rice (Zhang and Hu, 1999). Higher levels of survival after cooling were reported for shoot primordia of *Armoracia rusticana* (46%; Hirata *et al*., 1995), adventitious shoots of *Begonia* x *erythrophylla* (50%; Burritt, 2008), and in meristem clumps of *Musa* species (60%; Panis *et al.*, 2000).

For axes from Port Elizabeth seeds, the percentage of explants producing roots after cooling in slush ranged from 20 – 87% compared with the 7% of axes producing shoots (Table 3.14). These results suggest that the shoot and root meristem of *E. capensis* show a differential sensitivity to the stresses imposed by the cryopreservation protocol, with the shoot meristem displaying a greater sensitivity to the applied stresses post-warming, a phenomenon that was also observed after dehydration. In addition, more axes produced roots after cooling in nitrogen slush (67 – 87%) than within cryovials (7 – 30%). Explants within cryovials cool at a much slower rate than those exposed directly to nitrogen slush (Sershen *et al*., 2007). As pointed out by those authors this, in turn, necessitates lower water contents, if explants relatively slowly cooled within cryovials are to survive (Sershen *et al*., 2007) without injurious ice crystallisation. Since recalcitrant seeds are desiccation sensitive, the water content to which explants can be dehydrated before viability is lost, is a limiting factor. Thus, it can be speculated that the relationship between water content and cooling rate (for explants cooled within cryovials), which resulted in lowered capacity even for root production, was far from optimal.

A number of investigators have reported higher survival when explants were cooled more rapidly (Wesley-Smith *et al*., 1992; Berjak *et al*., 1998; Goveia, 2007; Sershen *et al*., 2007). Although there are cases where embryonic axes within cryovials can be cooled without catastrophic effects on viability, for example, *Araucaria hunsteinii* (Pritchard and Prendergast, 1986), *Corylus avellana* (González-Benito and Perez, 1994), *Camellia sinensis* and *Artocarpus heterophyllus* (Chandel *et al*., 1995), more recent reports have highlighted the advantages of cooling in nitrogen slush (Volk and Walters, 2006; Goveia, 2007; Sershen *et al*., 2007; Reed, 2008).

Some of the success achieved in cooling axes in nitrogen slush may be contributed by the use of particular cryoprotectants. In this study, the highest percentage of axes producing roots from seeds from Port Elizabeth after exposure to the cryogen, 87% (Table 3.14), was obtained when explants were treated with the penetrating cryoprotectants glycerol or DMSO, flash dried and then cooled in nitrogen slush. Dashnau *et al*. (2006) described how glycerol (at sufficiently high concentrations) cryoprotected cells by preventing protein unfolding, thus stabilising protein structure. Such a protective effect of glycerol may account for the higher percentage of axes producing roots when glycerol was used as a cryoprotectant in the present study. Suzuki *et al*. (2005) attributed the success of DMSO as a cryoprotectant to its ability to minimise volume expansion during slow cooling. Those authors suggested that the formation of extracellular ice crystals can compress cells as a result of the mechanical stress that occurs when the volume of the extracellular solution expands during freezing. Cryoprotectants are suggested to minimise this volume expansion thereby protecting cells, and DMSO was identified as the best cryoprotectant for this purpose (Suzuki *et al*., 2005). In contrast, the non-penetrating cryoprotectant, sucrose, exerts a cryoprotective effect by osmotic dehydration (Wolfe and Bryant, 2001).

A difficulty that was encountered in the present study was that it was often not possible to make direct comparisons with the published records because of uncertainty as to what constituted the survival recorded after cryopreservation of axes. Many authors report a percentage survival or viability but this does not mean that those explants developed further to form shoots from axes. Hence, the reported survival/viability may often be

higher than the percentage of explants that actually regenerate functional shoots. For example, González-Benito *et al*. (2002) reported 80% survival of *Q. ilex* axes after cryopreservation, but only 15% of these progressed to shoot development and Kioko (2003) reported 69% survival of axes of *Tricihilia emetica* after cooling, of which just 37% developed shoots. This point was also noted by González-Benito *et al*. (2004) who cautioned that careful interpretation is required when assessing results in published protocols that report any 'non-death' (i.e. organised/unorganised growth, greening, swelling of explants) as survival. In the present study, adventitious shoots were judged to have survived cryogenic exposure only if the shoot greened, increased in size and thereafter developed into an apparently normal and functional shoot, while for axes, survival was reported as that percentage forming roots and shoots. In all cases, the onwards development of explants after cryostorage occurred in the absence of any callus formation, indicating that the medium used was appropriate for germination after cryostorage (Verleysen *et al.*, 2005) and also that a significant proportion of meristem cells survived the cryogenic temperature (Brison *et al*., 1995).

b) Comparison of cryo-tolerance of germplasm (axes) from different provenances

It has been reported that seeds from different provenances do not necessarily display similar responses to cryopreservation (Wen *et al.*, 2010). This is important in conservation programmes where the aim is to cryopreserve germplasm from a range of provenances (González-Benito *et al.*, 2004). Thus, germplasm from two provenances, Port Elizabeth and St Lucia, were exposed to the same cooling conditions. Survival after cooling explants excised from St Lucia seeds was much lower (Table 3.19; no shoot production and only 10% of axes forming roots) than that obtained from Port Elizabeth seeds (Table 3.14; 7% of axes forming shoots and 20 – 87% developing roots), when explants were cooled using sub-cooled nitrogen. These results contrast with the trends observed in the cryopreparative stages where explants from the St Lucia provenance consistently retained the ability for onwards development, compared with lowered potential exhibited by those from the Port Elizabeth provenance. These findings not only

emphasise that similar explants from seeds from different provenances responded differently to the same imposed stresses, but also that the ability of explants to tolerate the cryopreparative stages (dehydration and cryoprotection) may not translate into a high percentage survival, post-cryopreservation.

A contributing factor might well have been the environmental parameters of the different provenances. Mondoni *et al*. (2009) suggested that seeds are adapted to their climate and this can subsequently affect their germination potential. It is possible that because seeds from Port Elizabeth were exposed to lower temperatures during development, this may have predisposed them to tolerate the stresses of cryopreservation better. Supporting evidence for this is provided by the work of Bharuth *et al*. (2007) who demonstrated that hydrated *E. capensis* seeds from sub-tropical St Lucia, were chilling sensitive, most losing viability within 9 days at 3°C, while those from temperate Port Elizabeth survived low temperatures (1 and 3°C) for almost 9 weeks without viability loss. Furthermore, Bharuth (2011) has reported genetic differences between the two *E. capensis* populations, suggesting that each forms a distinct clade.

The results reported (Bharuth *et al*., 2007; Bharuth, 2011) and those emerging from the present investigation, emphasise the difficulties in making any assumptions about cryopreservation procedures, even for explants of a purportedly single species.

c) Comparison of protocol presently developed for cryopreservation of *E. capensis* axes with other procedures for cryopreservation of meliaceous species

In 2006, Perán *et al*. reported cryopreservation of isolated embryonic axes of *E. capensis*. Those authors found that the addition of BAP to the culture medium used after retrieval of the explants from liquid nitrogen, promoted adventitious shoot formation at the wound sites where the cotyledons had been severed, by 50% of the axes. However, presently, when the procedure described by those authors was followed, the same results could not be achieved, even for control (non-dehydrated, non-cryostored explants). A maximum of

10% of axes produced shoots after being isolated from the cotyledons and cultured on medium supplemented with BAP (results not shown). Even so, this result could not be replicated with any consistency. A possible explanation may reside in the often marked intraspecific variability displayed by seeds harvested from the same source but in different seasons. In this regard, González-Benito *et al.* (2004) have warned that for any cryopreservation protocol to be adopted by a germplasm conservation centre, the results of that protocol must be reproducible.

In the present study, some of the adventitious shoots produced from non-cryopreserved axes using the protocol of Perán *et al.* (2006) were observed to develop from callus produced by the axes. Although the formation of callus can be viewed as an indicator of cell survival after cryopreservation, this is not ideal when the goal is to preserve the genetic integrity of germplasm (Mycock, 1999; Takagi, 2000). This is because the processes of de-differentiation to generate callus and subsequent re-differentiation to produce shoots are associated with a high risk of producing somaclonal variants (Larkin and Scowcroft, 1981). When Goveia (2007) added BAP to culture medium, callus, rather than shoot production was promoted after cooling axes of *Trichilia dregeana* and *T. emetica*. Because the production of callus rather than adventitious shoots (or shoot survival) was observed for *E. capensis* axes (see above) and for *Trichilia* spp. (Goveia, 2007), which are all meliaceous, there was a need to investigate alternative methodology – and explants – for germplasm cryopreservation.

Another observation made by Perán *et al.* (2006) was that when *E. capensis* axes with attached cotyledonary segments were cooled in liquid nitrogen, the cotyledons became detached during the procedure and these explants did not form shoots either from the apical meristem or at the lesion sites. This was not observed in the present study when the same explant-type was cryopreserved. This is likely to have been because of physical injury incurred in the study by Perán *et al.* (2006), as the explants were placed in mesh envelopes for cooling in liquid nitrogen. That particular procedure required considerable handling, and contact between the explants and mesh could well have been injurious, thus causing breakage of the axis-cotyledon connections upon cooling and warming. In the

present study, there was considerably less handling of explants, which were either placed into cryovials or directly immersed in nitrogen slush.

The present results (Table 3.14) also represented an improvement on those of Goveia (2007), who obtained only callus, from which neither shoots nor roots developed after cooling axes of *Trichilia* spp. in nitrogen slush. However, in that study slightly different combinations of cryoprotectants were used (combinations of DMSO, glycerol, sucrose, PVP and dextran) and explants were maintained for 60 min at each of the concentrations (5 and 10%). In the present study, cryoprotectants were applied for 30 min at each concentration to achieve the beneficial effects of cryoprotection without compromising explant survival as a consequence of potential toxicity.

In a study by Kioko *et al*. (1998), survival after cryopreservation of axes of *T. dregeana* was reported in the form of callus (49% of axes producing callus), as similarly noted by Goveia (2007). In contrast, for axes of *T. emetica*, Kioko (2003) reported better survival of 69% germination (of which 37% developed shoots). In both those studies, axes were cryoprotected in DMSO and glycerol and were cooled within cryovials. In contrast, in the present study, when DMSO-cryoprotected *E. capensis* axes were cooled within cryovials, only 30% of the axes formed roots, and none developed shoots (Table 3.14). These contrasting results for two meliaceous species further emphasise the present need to evaluate all procedures on a species (and provenance) basis.

Goveia (2007) also showed that no plant growth regulator was required for seedling establishment of control explants if cotyledonary segments were left attached to the axes. In the present study, therefore, BAP was not provided in the germination medium. It was, however, presently found that pyridoxine was a beneficial additive, possibly because of its proposed activity as a free radical scavenger (Bilski *et al*., 2000; Denslow *et al*., 2005; Dolatabadian *et al*., 2008). These factors in combination may account for the greater success obtained in this study when using embryonic axes of *E. capensis*, compared with the outcomes for the *Trichilia* species (Kioko *et al*., 1998; Kioko, 2003; Goveia, 2007).

In the present study, methods of cooling explants such as encapsulation-dehydration and vitrification were not carried out. Although some authors reported success when axes were cooled by encapsulation-dehydration (Cho *et al*., 2002b; Perán *et al*., 2006; Kaviani, 2007; Engelmann *et al*., 2008), this procedure was decided against, as encapsulation was found to reduce the rates of both dehydration and cooling of *E. capensis* axes, which compromised viability retention (results not shown). In addition, the use of the vitrification solutions (Sakai *et al*., 2008) was avoided since, in a trial preceding this study, embryonic axes of *E. capensis* were found not to survive exposure to the vitrification solutions, even when modifications were made.

d) Consideration of factors that contribute to lack of success in cryostorage of vegetative explants

A number of factors may account for the lack of success in the cryopreservation of explants, particularly in the case of 'broken' buds. *Ekebergia capensis* is a sub-tropical species and it has been noted that germplasm from tropical and sub-tropical regions is often difficult to cryopreserve, perhaps because such species are not intrinsically tolerant to low temperatures (Takagi, 2000). Further, the fact that *in vitro* explants were used for cryopreservation meant that tissues were highly hydrated as described by Padaychee *et al*. (2008; 2009) and Quain *et al*. (2009) as a consequence of the high humidity within the culture vessels, and were metabolically active (George, 1993). Since successful cryopreservation is dependent on the removal of as much water as possible from cells (without compromising viability) to minimise lethal ice crystallisation during cooling and warming, non-injurious dehydration of explants prior to cooling is a vital step (Mazur, 2004). In the early phase of this study, *in vitro* nodal segments of *E. capensis* were found to be very sensitive to water loss (as none survived, these results have not been detailed). This observation is not uncommon, as the vegetative tissues of many angiosperm species cannot tolerate excessive dehydration without a significant loss in viability (Reed, 2000). In the present study, this obstacle was overcome by using slightly more developed explants (i.e. 'broken' buds), which displayed greater tolerance to dehydration (Table

3.3). However, although these explants survived the cryopreparative stages of cryoprotection and dehydration, they did not tolerate cooling and/or warming.

Even the applied cryoprotectants could not induce cryogen tolerance in 'broken' buds and facilitated only limited survival of adventitious shoots. For cryoprotectants to protect explants against cryo-injury, they must sufficiently permeate tissues (Halmagyi and Pinker, 2006). In the present study it is possible that cryoprotectants did not sufficiently permeate tissues and cells of these relatively large explants and were therefore unable to afford protection against cryo-injury (Lane, 2004; Halmagyi and Pinker, 2006). In addition, growth by enlargement generally involves a substantial increase in vacuolation (Vidal *et al*., 2005). Such cells have a higher risk of incurring lethal damage upon cooling (Halmagyi and Pinker, 2006) than meristematic cells. It is also possible that the duration of exposure of explants to cryoprotectants was insufficient or the concentrations of applied cryoprotectants may not have been appropriate to afford protection from freezing damage. It may be that by focusing on avoiding cryoprotectant toxicity in the present study, the efficacy of cryoprotection was compromised.

From the results obtained, it was apparent that 'broken' buds of *E. capensis* incurred fatal damage upon cooling and warming. A possible solution despite the practical difficulties (see above) could be to use meristems alone as explants for cryopreservation. It has been reported that such explants from some tropical species are amenable to cryopreservation (e.g. *Manihot esculenta* [Charoensub *et al*., 1999]; *Solemostemon rotundifolius* [Niino *et al*., 2000]; *Ipomoea batatas* [Pennycooke and Towill, 2001]; *Sechium edule* [Abdelnour-Esquivel and Engelmann, 2002]; *Musa* species [Panis *et al*., 2005]; *Trichilia emetica* [Varghese *et al*., 2009]). Meristem explants are suitable for cryopreservation since the cells are small and minimally differentiated (Kartha *et al*., 1979), being relatively homogenous and with few vacuoles (Charoensub *et al*., 1999; Vidal *et al*., 2005) and, in fact, rooted plantlets have been produced after cryopreservation of the meristems of the meliaceous species, *Trichilia emetica* (Varghese *et al*., 2009).

e) Procedures to promote explant survival following exposure to cryogenic temperatures

i) Sucrose preculture of adventitious shoots

Preculture of explants on media supplemented with elevated levels of sucrose has been shown to promote freeze tolerance of some explants, e.g. shoot primordia (Hirata *et al*., 1995), adventitious shoots (Burritt, 2008), proliferating meristem cultures (Panis *et al*., 2000) and shoot tips (Halmagyi *et al.*, 2010). In the present study, adventitious shoots were exposed to sucrose using a stepwise procedure (Table 3.9) in order to mitigate against cytotoxicity and osmotic shock in sucrose-sensitive explants (Blakesley and Kiernan, 2001). Explants tolerated this treatment with no obvious adverse effects on survival (Table 3.9). An explanation of the success achieved by provision of a stepwise increase in sucrose concentration in a preculture medium to induce dehydration and cryo-tolerance was provided by Suzuki *et al*. (2006). Those authors suggested that the first preculture step affords cells the capacity to respond to the sucrose stimulus, so that during the second preculture step, cells can better withstand partial dehydration. The extent to which cells can tolerate dehydration depends on the final concentration of sucrose provided. Suzuki *et al*. (2006) further demonstrated for gentian axillary buds, that this response was accompanied by a transient increase in ABA, proline and soluble sugars during the first preculture step, which provided a mild osmotic shock, and by an increase in intracellular sucrose content during the second preculture step.

Adventitious shoots that were precultured on sucrose-supplemented media were subsequently cryoprotected and flash dried before cooling. The rationale for the three-step procedure (sucrose exposure during preculture, cryoprotection and flash drying) was that freeze tolerance may be promoted in adventitious shoots by the first step. Sucrose from the medium is hydrolysed by invertases to yield glucose and fructose, which then enter cells by active or passive transport (George, 2008). Upon its intracellular accumulation, sucrose has been reported to maintain the integrity of cell membranes and protein structure during dehydration and freezing (Panis *et al.*, 2005; Burritt, 2008). Dumet and Benson (2000) hypothesised that sucrose can afford further protection by

minimising the growth of ice crystals by causing many microcrystallisation events (which are suggested to be less injurious than the formation of large ice crystals). However, none of the precultured adventitious shoots survived cryogen exposure, and a number of factors may have contributed towards this. Successful cryopreservation is dependent on the sensitivity and tolerance of explants to the stresses cumulatively incurred at each stage of the cryopreservation protocol (Berjak *et al*., 1999; Reed *et al*., 2005). Adventitious shoots survived all the cryopreparative stages (Table 3.10), but it is not possible to say at this juncture, whether or not sub-lethal injury occurred. Consequently, at present, it is suggested that sucrose precultured explants were lethally damaged by cooling or warming injury, but were adversely preconditioned by the stresses preceding exposure to and/or retrieval from, the cryogen.

Takagi (2000) cautioned that sucrose preculture may not always promote survival of cryostored germplasm of tropical species, and in the present instance, sucrose preculture proved to be an ineffective procedure to promote cryotolerance of adventitious shoots of *E. capensis*. It could be that the duration of exposure of explants to the sucrose preculture medium was too short to afford protection against freezing injury. In the present study, adventitious shoots were precultured for a total of six days while other tropical species have been precultured for longer, e.g. 14 days for meristem explants of *Musa* species (Panis *et al*., 2002) and 22 days for shoot tips of *Sechium edule* (Abdelnour-Esquivel and Engelmann, 2002). Since it is not possible to increase the concentration of sucrose in preculture medium (because of the demonstrated sensitivity of *in vitro* vegetative explants of *E. capensis* to elevated sucrose concentrations), another option may be to prolong exposure of explants to the osmotica.

It is also possible that alternative procedures may promote tolerance to freezing, one of these being preculture in the presence of ABA (Na and Kondo, 1996; Suzuki *et al*., 2006; Padaychee *et al*., 2008) or proline (Burritt, 2008) or possibly a combination of these treatments. Abscisic acid is a stress-induced hormone (Gazzarrini and McCourt, 2001; Shinozaki *et al*., 2003) that, in turn, plays a role in initiating adaptive responses to stresses (Himmelbach *et al*., 2003; Xiong and Zhu, 2003) such as water stress (Sauter *et*

al., 2001; Jiang and Zhang, 2002). Furthermore, ABA is reported to play a role as an intermediate signalling molecule that participates in a complex cascade of events beginning with the perception of a stress and culminating in an appropriate response to the stress (Verslues and Zhu, 2005). The stress response elicited is suggested to occur as a result of crosstalk with other signalling networks involving sugars and reactive oxygen species (discussed in Verslues and Zhu, 2005). However, for exogenously supplied ABA to be effective in counteracting stress, the tissue concerned must be responsive. Current studies in our laboratory have shown that application of exogenous ABA had no effect in modulating desiccation sensitivity (nor of inducing any orthodox-seed-specific LEAs) in recalcitrant seeds of two unrelated species. Although neither of those species was meliaceous, the possible efficacy of ABA in a preculture medium for *E. capensis* explants must, at present, remain conjectural.

Proline is an amino acid that has been shown to increase in response to drought stress, apparently playing a role in stabilising DNA and membranes and protecting cells against oxidative damage from free radicals (Clifford *et al.*, 1998; Alia and Matysik, 2001). Thus, it may be possible to harness potentially beneficial effects of ABA and/or proline by preculturing explants on media incorporating appropriate concentrations of these compounds in attempts to induce freeze tolerance in *E. capensis* adventitious shoots. Despite the *caveat* above, such procedures are recommended for future work.

ii) Application of the protocol to generate adventitious shoots from roots developed by cryopreserved axes

The protocol developed to generate adventitious shoots from seedling roots (Chapter 2), was applied to roots generated from axes after retrieval from liquid nitrogen. The results (Table 3.15) indicated that in cells of 30 – 40% of axes that were cryoprotected with glycerol or DMSO, meristematic capacity was sufficiently unimpaired for root production *in vitro* after explants had been cooled in nitrogen slush. These roots also retained the morphogenic capacity to regenerate adventitious shoots directly by the

procedures previously developed (Chapter 2). Further, the morphogenic capacity of the roots that developed after cryopreservation was comparable to that of control roots formed by explants that were not cryopreserved, as illustrated by the similar number of adventitious shoots produced (Table 3.15). This was an encouraging result in terms of the development of a successful protocol for cryopreservation of *E. capensis* germplasm and suggests that roots developed by explants that were cryoprotected in glycerol or DMSO and then cryopreserved, retained similar physiological functions as control (non-cryopreserved) explants at least in terms of the potential to respond by adventitious shoot production.

In contrast, axes that were cryoprotected with either sucrose or a combination of sucrose and glycerol appeared to be incapable of adventitious shoot production (Table 3.15). Although not apparent in axes that were dehydrated after exposure to cryoprotectants (Table 3.13), adverse effects were exhibited already following dehydration and cooling, as evidenced by the significant reduction in proportion of axes capable of root production (Table 3.14), relative to those cryoprotected with glycerol or DMSO. This indicates the additive nature of the damage to axes, through the sequential steps of a cryopreservation protocol.

By applying the micropropagation protocol developed (Chapter 2) to roots generated after cryopreservation, the problem of lack of shoot development by type 3 explants (axes with attached cotyledonary segments) after cooling could be overcome. Although this necessitated extra culture stages, the end result was adventitious shoot formation from seed-derived specimens after cryogenic cooling. The need for additional time in culture may increase the risk of generating somaclonal variants (Karp, 1995; Pontaroli and Camadro, 2005); however, using the method developed in this study, there was no callus stage and explants were exposed to plant growth regulators (BAP) only at the adventitious shoot induction stage and not during the culture stage immediately after cooling. Nevertheless, there still remained the need to assess the genetic integrity of regenerated germplasm (see later).

3.4.3 Assessment of potential ROS-mediated damage caused by the cryopreparative stages and exposure to cryogenic temperatures

a) Superoxide production as a consequence of excision injury

The development of successful cryopreservation protocols for germplasm of recalcitrant-seeded species is complicated by a number of factors that have been discussed in previous sections. Once such factor highlighted by Pammenter *et al.* (2011), is that in order to cryopreserve explants from many dicotyledonous recalcitrant species that have fleshy cotyledons (including the species under investigation in this study, and other meliaceous species such as *Trichilia dregeana* and *T. emetica*), it is necessary to excise the embryonic axis completely from the cotyledons (Perán *et al.*, 2006) or to leave small blocks of each cotyledon attached to the axis (Goveia, 2007). Current thinking is that the excision of the embryonic axes from the cotyledons induces a wounding response implicating a burst of ROS. Evidence for this has been provided for recalcitrant seeds of *T. dregeana* (Goveia, 2007; Whitaker *et al*., 2010), *Strychnos gerrardii* (Berjak *et al.*, 2011b) and *Castanea sativa* (Roach *et al.*, 2008). Further, those studies also demonstrated that an oxidative burst occurred in response to dehydration. In all those studies, the indicator ROS investigated was extracellular superoxide and, following those examples, so it was in the present investigation on *E. capensis* (see later).

Reactive oxygen species can be produced intracellularly as a byproduct of metabolism or extracellularly by enzymes in the apoplast. Their levels are controlled by intracellular antioxidants and apoplastic enzymes (Foyer and Noctor, 2005; Minibayeva *et al*., 2009). The production of apoplastic ROS results in a redox difference across the plasmalemma (Atunes and Cadenas, 2000; Foyer and Noctor, 2005) with H_2O_2 suggested to be the only extracellularly-generated ROS that can cross the plasmalemma (Bhattacharajee, 2005; Pitzschke *et al*., 2006). Under normal conditions, superoxide is quenched by superoxide dismutase, which results in the production of H_2O_2 and $^3O_2$ (Kranner and Birtić, 2005; Gill and Tuteja, 2010). This dismutation is also possible under non-enzymic conditions (Kranner and Birtić, 2005) in the absence of superoxide dismutase. However, if the H_2O_2

generated is not rapidly quenched, this can lead to the formation of the hydroxyl radical (Hendry, 1993), which is the most strongly oxidising radical species (Benson and Bremner, 2004).

Following the procedure used by Roach *et al*. (2008; 2010) and Whitaker *et al*. (2010), extracellular production of superoxide was measured in response to the cryopreparative stages and cooling, in two explant types, *viz.* embryonic axes with cotyledonary attachments and adventitious shoots (Figures 3.4 – 3.6). For both explant types, the greatest amount of superoxide was liberated in response to excision. Similarly, Roach *et al*. (2008) working on *Castanea sativa*, and Whitaker *et al*. (2010) and Pammenter *et al*. (2011) in work on *Trichilia dregeana*, also reported an extracellular burst of superoxide in response to wounding upon excision of axes. In all three studies, good survival was obtained from explants with cotyledonary blocks attached despite the levels of superoxide evolved.

Pammenter *et al*. (2011) reported that the effect of the superoxide burst appeared to be related to the distance between lesions left after severing of the cotyledon(s) and the shoot apex of the embryonic axis. In this regard, Pammenter *et al*. (2011) reported that excision of explants involving complete removal of cotyledons by cutting the connections flush with the axis resulted in an elevated burst of superoxide while excision of explants by leaving basal or 2 mm^3 cotyledonary segments attached produced less superoxide. These trends were in agreement with the results reported in the present investigation. Pammenter *et al.* (2011) suggested that the observed lack of shoot development following complete severing of the cotyledons could be caused by the proximity of the shoot meristem to the burst of ROS.

b) Superoxide production in response to the cryopreparative stages and cooling

Good levels of survival (80 – 100%) were obtained for explants subjected to the cryopreparative stages, despite extracellular superoxide production. The correlation

analysis suggested that in most cases, there was no relationship between superoxide production and onwards development (except for a weak positive correlation between superoxide production and root production following cooling; Figure 3.5). However, it must be considered that although ROS are short lived, they are highly reactive (Shulaev and Oliver, 2006). Therefore, each burst of ROS can result in damage although this may be sub-lethal. Considering that the sequential steps of a cryopreservation procedure follow each other rapidly, there is insufficient time for incurred damage to be ameliorated. Thus, damage as a consequence of consecutive (repeated) oxidative bursts will be cumulative (Whitaker *et al*., 2010). Hence, the magnitude and duration of repeated ROS bursts could well be too much for the finite antioxidant system of the explants under consideration. This may result in damage (which could be extensive) that accumulates and ultimately contributes to loss of viability.

3.4.4 Probing the germplasm for possible epigenetic changes following cryopreparative stages and cryopreservation

Isolation of DNA of good quality is an essential step in any molecular biological application (Jobes *et al*., 1995), and this remains an important issue in the field of plant molecular biology (Ribeiro and Lovato, 2007; Zain Hasan *et al*., 2008). In the present study, DNA was initially extracted from shoots using the CTAB method. This extraction method produced low yields of DNA that were heavily contaminated, as has been similarly reported by other researchers (Meijjad *et al*., 1994; Friar, 2005; Zain Hasan *et al*., 2008). Attempts to remove phenolics using DTT and PVP (Rout *et al*., 2002) were unsuccessful. Such contamination of DNA is a significant problem which can affect downstream PCR applications by suppressing amplification (Pikaart and Villeponteau, 1993) and influencing the binding of random primers (Meijjad *et al*., 1994). In addition, protocols such as the CTAB method involve a number of time-consuming steps necessitating organic solvent extraction and ethanol precipitation (Bashalkhanov and Rajora, 2008). In view of all these factors, further refinement of the CTAB method was not undertaken for the isolation of DNA from germplasm of *E. capensis*.

Subsequent efforts were, therefore, directed towards assessment of the efficiency of the DNeasy Plant Mini Kit in extracting DNA from germplasm. This appears to be the most commonly-used commercially available kit, described in work on a variety of plant species by a number of researchers: for example, *Melia azedarach* (Olmos *et al*., 2002), *Solanum tuberosum* (Joyce and Cassells, 2002), *Dioscorea bulbifera* (Dixit *et al*., 2003), *Arabidopsis* species (Ruiz-García *et al*., 2005), *Cedrus atlantica* and *C. libani* (Renau-Morata *et al*., 2005), *Phoenix dactylifera* (Fang and Chao, 2007), *Quercus robur* (Sánchez *et al*., 2008), *Humulus lupulus* (Peredo *et al*., 2009), *Castanea sativa* (Viejo *et al*., 2010), *Vitis* species (Baránek *et al*., 2010) and *Gentiana cruciata* (Mikuła *et al*., 2011). In the present study, when the DNeasy Plant Mini Kit was used, good yields of DNA were obtained from both shoots and roots. Isolation of DNA from roots was an essential component of the present study since it had been found that survival of axes after cooling was predominantly manifested by root production (Table 3.14). Thus, for any molecular analyses that considered survival after cooling a prerequisite was a suitable method to extract DNA from roots. Although DNA extraction from roots is not often done, there are some studies, including those from roots of cacti (Tel-Zur *et al*., 1999), barberry (Kumar *et al*., 2003), ginseng (Hong *et al*., 2005), rhubarb (Hu *et al*., 2009), asparagus and winter cherry (Khan *et al*., 2007).

The method used in the present study to assess the methylation status of DNA involved the use of the RAPD technique coupled with the application of the methylation-sensitive restriction enzymes *HpaII* and *MspI*. These are the most frequently used methylation-sensitive isoschizomers (Baránek *et al*., 2010). Both these enzymes recognise the same nucleotide sequence (5'-CCGG-3') but differ in their sensitivity to DNA methylation. *HpaII* is inactive if one or both of the cytosines are methylated (both strands methylated) but it will cleave hemi-methylated DNA (one strand methylated), while *MspI* is inactive if the outer cytosine is methylated, but will cleave DNA if the internal cytosine is methylated (McClelland *et al*., 1994).

In the present study, most of the bands analysed were monomorphic (Tables 3.20 and 3.21). For explants generated from germinated axes (explant type 3), the overall highest

percentage polymorphism (0.46%) was found in material that was germinated in the laboratory under ambient conditions (i.e. those that were not cultured *in vitro*). When the cryoprotection stage was considered, the treatment that produced the most polymorphisms (0.23%) followed the use of sucrose. However, following cooling, the highest percentage of polymorphism was produced by explants that were cryoprotected in a combination of sucrose and glycerol, flash dried and then cooled (0.23% polymorphism).

Adventitious shoots (that were originally excised and treated for exposure to cryogenic temperatures) showed a higher percentage of polymorphisms (maximum of 1.79% after cryoprotection with a combination of sucrose and glycerol) compared with axes (maximum of 0.46% after germination *ex vitro*). Adventitious shoots produced on roots generated from cryopreserved axes (Table 3.21; GDC and DMDC) exhibited no DNA methylation changes compared with adventitious shoots that were originally excised and treated for exposure to cryogenic temperatures. Overall, very low levels of DNA methylation changes were detected during the cryopreparative stages and following cooling axes or adventitious shoots of *E. capensis* germplasm. These observations are in agreement with those of Kaczmarczyk *et al.* (2010) for *Solanum tuberosum*. Further, Kaity *et al.* (2008) reported low levels of DNA methylation changes following cryopreservation of *in vitro* shoot tips of papaya (0.52 – 0.62%) while Peredo *et al.* (2008) reported 2.6 – 9.8% changes in methylation sensitive loci following cryopreservation of *in vitro* shoot tips of hops.

The observation of some DNA methylation changes following cryopreservation is not uncommon (refer to Table 3.1), and has been attributed to different stages, *viz.* the *in vitro* culture process (Peredo *et al*., 2009), cryoprotection (Kaity *et al.*, 2008) or the cryopreservation process (Peredo *et al.*, 2008). However, the low levels of variation presently detected were considered acceptable, as the procedures used had not resulted in any significant manifestation of epigenetic change. In this respect, it has been suggested that DNA methylation changes (in response to cryopreservation procedures) may be

transient and could play a role in initiating adaptive responses to stress (Johnson *et al*., 2009).

In the present study, a total of 2 289 bands were assessed. However, it must be noted that a disadvantage of the method used was that it only assessed cytosines that were present within the recognition sites of the restriction enzymes (Portis *et al*., 2004). Therefore, such techniques analyse only a small portion of the genome (Lakshmanan *et al*., 2005). Further, the broad assessment of genome-wide levels of DNA methylation may be deceptive as there may be areas of significant local changes while other areas may remain relatively stable (Smulders and de Klerk, 2011). Thus, it has been suggested that the results from molecular biological analyses should be supplemented by phenotypic assessments to determine if observed polymorphisms will be displayed as heritable or stable phenotypic traits (Watt *et al*., 2009; Snyman *et al*., 2011).

The results obtained in the present study, suggest low levels of DNA methylation changes during the different stages of a cryopreservation protocol. Future studies should be directed towards gaining a complete picture of the fidelity of cryopreserved germplasm by assessing the genetic integrity (to detect any changes in DNA sequence) as well as the epigenetic situation (to assess changes in methylation status) of germplasm. In studies that have done this, few (if any) DNA sequence changes were reported but there were some changes in the methylation levels of DNA, as reported by Hao *et al*. (2001) and Peredo *et al.* (2008). It is also important to ascertain if any epigenetic changes detected are transient or if they persist and may possibly be expressed as altered phenotypes (Peredo *et al*., 2009). Thus, the present investigation serves as a preliminary assessment of the DNA methylation changes during cryopreservation but further studies are required.

3.5 CONCLUDING COMMENTS

The aim of the present work was to develop suitable protocols for the cryopreservation of germplasm of *E. capensis* by investigating the responses of different types of explants.

The first explant-type investigated, *viz.* nodal segments, proved to be intolerant to dehydration, but more developed explants ('broken' buds) tolerated the cryopreparative stages of dehydration and cryoprotection although they incurred lethal damage upon cryogenic exposure. In contrast, relatively low proportions of the second vegetative explant-type investigated, adventitious shoots, tolerated cryogenic exposure with 7 – 20% survival of explants that were flash dried and then cooled or those that were cryoprotected in sucrose and glycerol, flash dried and then cooled.

Seed-derived explants (from two provenances) comprising the embryonic axis with 2 mm^3 attached cotyledonary segments were also targeted for cryopreservation. Such explants from the first provenance tested (Port Elizabeth) tolerated cryogen exposure better if faster cooling rates were applied, using exposure to sub-cooled nitrogen. However, survival was predominantly demonstrated by root formation by the axes. A protocol established for roots that had not been exposed to cryogenic temperatures during the earlier phase of the study was used to induce adventitious shoots from roots generated from cryopreserved axes. This procedure proved successful, with 30 – 40 % of roots producing adventitious shoots which were subsequently rooted and the plantlets acclimatised. Thus, the goal of plant production from seed-derived explants of *E. capensis* was achieved. However, explants from the St Lucia provenance were less tolerant to cooling with, at best, 10% of axes producing roots, thus demonstrating differences in the response of germplasm from differing provenances.

In the present study, low levels of DNA methylation changes were detected during the different stages of a cryopreservation protocol. Considering the limitations of the technique used, the present study is informative as a preliminary assessment, but additional work is required to make more substantial conclusions.

The results of this study have contributed towards cryopreservation of seed-derived germplasm of *E. capensis*. Future work could be directed towards the use of meristems of seedlings as vegetative explants since they may be more resilient to the cumulative effects involved prior to, including, and after, cryostorage (Panis *et al.*, 2005; Varghese *et*

al., 2009). For adventitious shoots, the application of alternative preculture methods incorporating different additives such as ABA (Reed, 1993), proline (Burritt, 2008), antioxidants and anti-stress compounds such as vitamins C and E, lipoic acid, glutathione and glycine betaine (Uchendu *et al*., 2010a and b) may be considered in attempts to promote survival after cryostorage. In the case of axes, it is important to assess the antioxidant capacity of explants at each stage of the cryopreservation protocol. In this regard, possible quenching of ROS in conjunction with activity of enzymic and non-enzymic antioxidants, could provide useful information. In addition, preculture of explants on media supplemented with compounds that have free radical scavenging activity such as DMSO or ascorbic acid may also be considered (Cassandra Naidoo *et al*., 2011), as must the potential of amelioration of ROS bursts and consequent damage, by the use of cathodic water, which has strong reductant properties (Berjak *et al*., 2011b).

CHAPTER 4: OVERVIEW DISCUSSION, RECOMMENDATIONS FOR FUTURE RESEARCH AND CONCLUDING REMARKS

Biodiversity loss is a significant global problem that necessitates urgent attention, as the current rate of loss is proceeding at an unprecedented rate (Sarasan *et al*., 2006). A multidisciplinary approach to curb such high levels of biodiversity loss is needed (Reed *et al*., 2011), a view which is embodied in conservation biology (Primack, 1993). One of the fields of conservation biology, *viz*. conservation biotechnology, and in particular cryoconservation, is the focus of the present study. These techniques involve *ex situ* conservation methods to conserve plant germplasm for future use and afford an important research area with the potential to contribute towards the conservation of biodiversity in regions experiencing high levels of loss (Berjak *et al*., 2011a; Bunn *et al*., 2011).

South Africa is rich in biodiversity with many endemic plant species (Thuiller *et al*., 2006), the conservation of which should be a priority. Stern (2006) noted that countries on the African continent are likely to be greatly affected by climate change. This highlights the importance of *ex situ* conservation efforts that could potentially play a vital role in efforts to rehabilitate and restore habitats affected by changing climatic conditions. Aside from the obvious importance of biodiversity preservation *per se*, conservation of indigenous species is a necessity within the southern African context, as a large percentage of the population relies on traditional medicine using many representative species to treat a wide range of disorders (Mander *et al*., 1996). However, indiscriminate harvesting of indigenous species has led to the depletion of natural populations of targeted plant species. This problem is exacerbated by the fact that many indigenous tropical and sub-tropical species produce recalcitrant (non-orthodox) seeds (Tweddle *et al*., 2002; Baxter *et al*., 2004; Erdey *et al*., 2004; Wang *et al*., 2004) that cannot be stored using conventional methods of low temperature and low relative humidity (Chin and Roberts, 1980; reviewed by Pammenter and Berjak, 1999). There is an undisputed need for conservation of germplasm of indigenous species that produce recalcitrant seeds. In the present investigation, the subject of study was the indigenous

species *Ekebergia capensis* which produces recalcitrant seeds. The goal of this study was to develop strategies for the long-term conservation of germplasm of *E. capensis*.

The first aspect of this study focused on the development of micropropagation protocols for selected explants of *E. capensis* (Chapter 2; Hajari *et al*., 2009). Micropropagation protocols (for each species under investigation) need to be established *a prioi* (Krishnapillay, 2000), before cryopreservation trials can be attempted. The explant-types selected were nodal segments (excised from *in vitro*-germinated seedlings or from adult plants maintained in the greenhouse), adventitious shoots generated from intact *in vitro*-germinated roots and embryonic axes with small attached cotyledonary segments. Micropropagation procedures were developed for each explant type up to the acclimatisation stage, except for nodal segments excised from adult plants which proved difficult to root. This latter observation is in keeping with those of others reported when explants of mature origin are used for micropropagation (Chalupa, 2002; Kartsonas and Papafotiou, 2007). Micropropagation protocols can be used not only to retrieve material after cryostorage, but also to propagate the species under investigation, for example, for the propagation of superior genotypes.

A novel aspect of the present work was the development of the protocol to produce adventitious shoots from intact root explants (Table 2.4; Hajari *et al*., 2009). Examination of the available literature showed that there are a few reports on the use of intact roots as a source of explants for micropropagation (refer to Table 2.1), in most cases excised root segments being used for this purpose. The use of excised root segments was avoided in the present study to guard against the risks of generating somaclonal variants from callus which was thought to be likely to be induced in response to wounding. This was considered essential as the goal of the present work was to preserve the intrinsic genetic diversity of stored germplasm without artificially inducing variation. This factor was considered throughout this study and any treatments that were likely to induce genetic variation were avoided. Figure 4.1 provides a schematic representation of the various explant sources used and the micropropagation protocols developed.

The three explant types chosen were all sufficiently small to be considered for subsequent cryopreservation (Chapter 3). There were, however, additional reasons for selecting these explants. These included the advantages inherent in using vegetative *in vitro*-derived explants which afford a ready supply of contaminant-free material throughout the year (Engelmann, 1991; Liao *et al*., 2006; Mehrotra *et al*., 2007) unlike seeds which are only seasonally available. This strategy still allows for conservation of genetic diversity by using seeds to generate seedlings and saplings from which the vegetative explants are directly derived. A further application of the micropropagation protocol successfully developed, is that it could prove useful to multiply selected superior genotypes of interest for re-introduction or planting programmes (Krishnan *et al*., 2011; Reed *et al*., 2011).

The explant types selected were subjected to the procedures which, if successful, should have enabled their cryopreservation (Chapter 3). However, it was found that *in vitro* nodal segments could not tolerate dehydration to the levels required for subsequent cryopreservation. While slightly more developed explants, i.e. 'broken' buds, did tolerate the cryopreparative stages of dehydration and cryoprotection, they did not survive cryogen exposure, irrespective of the cooling rate employed. It was therefore suggested that the large size of these explants and unfavourable surface:volume ratio together with their originally highly hydrated state were not conducive to survival of exposure to cryogenic temperatures (Wolfe and Bryant, 2001; Wesley-Smith *et al*., 2004).

For future investigations, it is suggested that meristem explants (comprising the meristematic dome with one or two pairs of leaf primordia) may provide more suitable explants for cryostorage. Apart from their much smaller size, meristem explants are comprised of relatively homogenous, minimally differentiated cells (Kartha *et al*., 1979; Vidal *et al*., 2005) which are least likely to be damaged by the cryopreservation process. The use of meristem explants has proved successful for cryopreservation of a number of tropical species, e.g. *Manihot esculenta* (Charoensub *et al*., 1999), *Ipomoea batatas* (Pennycooke and Towill, 2001), *Musa* species (Panis *et al*., 2005), *Colocasia esculenta* (Sant *et al*., 2008) and *Trichilia emetica* (Varghese *et al*., 2009).

Use of the second explant-type, i.e. adventitious shoots, was marginally successful, in that 7 – 20% survival was obtained following cooling in sub-cooled nitrogen (refer to Figure 3.2). To our knowledge, this represented the first instance of successful cryopreservation of vegetative explants of *E. capensis*. However, survival was low, thus additional or amended procedures recommended for future work, include preculturing explants on media supplemented with additives such as ABA (Suzuki *et al*., 2006), proline (Burritt, 2008), vitamins C and E (Uchendu *et al*., 2010a), or anti-stress compounds such as lipoic acid, glutathione and glycine betaine (Uchendu *et al*., 2010b). Such procedures may precondition explants (particularly those that prove difficult to cryopreserve) such that they better tolerate the stresses incurred during the cryopreservation process and following warming (Lane, 2004).

When developing protocols for explants that do not readily tolerate exposure to cryogenic temperatures, consideration of wider aspects relating to the problem might provide insight. For example, research into the interaction of cryoprotectants with biomolecules (Pereira and Hünenberger, 2008) and investigation of the vitrification properties of cryoprotectants (Kreck *et al*., 2011) could well assist in developing more effective cryopreservation protocols.

The final explant type investigated for cryopreservation comprised the embryonic axis with attached cotyledonary segments. Small blocks (2 mm^3) of cotyledonary segments were left attached to axes to mitigate against direct ROS-mediated damage to the shoot apical meristem of the axis which has been reported to occur upon excision of explants (Whitaker *et al*., 2010; Berjak *et al*., 2011b; Pammenter *et al*., 2011). When these explants were exposed to cryogenic temperatures, shoot production from axes was effectively precluded (Table 3.14). In contrast, root production from axes was successful with up to 87% of axes developing roots following retrieval from cryostorage, for which they had been cooled in nitrogen slush. However, the goal of the present study was to generate established seedlings post-cryopreservation and the production of roots only does not achieve this. Therefore, the protocol that was developed to produce adventitious shoots from *in vitro*-germinated roots (Chapter 2; Hajari *et al*., 2009) was applied to roots

generated by axes after cryostorage. After twelve weeks in culture, adventitious shoots were produced from these roots (Table 3.15; Figure 3.3). These adventitious shoots were successfully rooted and the resultant plantlets acclimatised. This result highlights the need for flexibility and research adaptability when developing protocols (Bornman *et al*., 2007; Bunn *et al*., 2011). By following this principle, the goal of plantlet establishment from seed-derived explants following cryopreservation was achieved, albeit indirectly, by the application of the protocol to generate adventitious shoots from roots. This protocol was originally developed to use roots as a source of explants to generate adventitious shoots which could then be cryopreserved. Its application to roots generated from cryopreserved axes was a novel departure that achieved the goals of the investigation, although an additional 6 weeks in culture on semi-solid adventitious shoot production medium was required. Figure 4.2 shows a schematic representation of the stages followed to cryopreserve germplasm of *E. capensis*.

A finding that was apparent in the present study was the influence of seed provenance on explant response. For example, a clear provenance-related response was evident when adventitious shoots were generated on *in vitro*-germinated roots (Table 2.6), with explants from the Mtunzini provenance generating better yields than explants from Port Elizabeth or St Lucia. Similarly, following axis cryopreservation, only 3 – 10% of the explants excised from St Lucia seeds survived cryogen exposure (Table 3.19), while a higher percentage of explants excised from Port Elizabeth seeds survived (Table 3.14; 7 – 10% shoots and 10 – 87% roots from axes). Examination of the genetic characteristics between seeds of different provenances could help to explain these results. Work done in our laboratory has indicated that genetic differences exist between these two different populations of *E. capensis*, to the extent that each population apparently forms a distinct clade (Bharuth, 2011). The genetic variability was suggested to underlie differences in chilling sensitivity of germplasm from the different provenances with seeds from St Lucia reported to be chilling sensitive while those from Port Elizabeth tolerated cold storage for a relatively extended period (Bharuth *et al*., 2007). Similarly, genetic variability could underlie the differences in responses to cryogen exposure of the adventitious shoots developed from roots of material from the different provenances.

These findings have important implications in conservation programmes, emphasising that seed provenance needs to be taken into consideration when cryostoring germplasm. This may necessitate differential optimisation of protocols to ensure that acceptable levels of survival are obtained when germplasm from different provenances is used. A further complicating factor is that seeds harvested in different seasons or even at different times during the same season, may display variable responses (inter- and intra-specific variation; Berjak and Pammenter, 1997; 2004a). Such unpredictable variation makes it extremely difficult to recommend uniform procedures for cryopreservation of axes or axis-derived explants excised from recalcitrant seeds, even within a species.

In the present study, lack of shoot production from axes following exposure to cryogenic temperatures was observed. This is not uncommon when explants originate from recalcitrant seeds (Engelmann, 1998; Goveia *et al*., 2004; Walters *et al*., 2008), therefore attention was directed to the possible basis of damage precluding shoot formation. In this context, the extracellular production of the ROS, the superoxide anion, was assessed as an indicator of damage (Figures 3.4 – 3.6). The results revealed little or no correlation between superoxide production and onwards development of explants (axes with cotyledonary attachments and adventitious shoots) during the cryopreparative stages and after exposure to cryogenic temperatures. It was found that there was a burst of superoxide upon excision of explants (Figures 3.4 and 3.6), in line with other findings (Roach *et al.*, 2008; Whitaker *et al*., 2010; Berjak *et al.*, 2011b), but in the present case this was not detrimental to survival. Thus, the extracellular production of superoxide was not helpful in identifying the cause of damage of the *E. capensis* explants examined.

It must be noted that the assay used in the present study assessed the extracellular production of superoxide, which gave no information about consequent intracellular effects. Furthermore, only one ROS (superoxide) was assessed, but deleterious effects on onwards development could well have been associated with other ROS, particularly the hydroxyl radical (Benson and Bremner, 2004; Halliwell, 2006). In addition, consideration must be given to the role of antioxidant systems, as a suite of antioxidants operate to quench harmful ROS, therefore their operation – or deficiencies – may influence onwards

development of explants (Apel and Hirt, 2004; Van Breusegem and Dat, 2006; Varghese *et al*., 2011). Thus, future studies should consider the implication of a wide spectrum of ROS, and qualitative and quantitative indications of antioxidant activity, as well as attempting to discriminate between events that occur in the intracellular and extracellular environments, to elucidate the basis of damage that may account for the lack of shoot production when axes are used as explants for cryopreservation.

The final aspect of this study assessed possible epigenetic effects of the various procedures involved in germplasm cryoconservation by using the method of coupled restriction enzyme digestion and random amplification (Cai *et al*., 1996). The goal of conservation programmes is to maintain the integrity (genetic and epigenetic) of germplasm retrieved from cryostorage (Bunn *et al*., 2011). In the present study, the occurrence of epigenetic changes in terms of DNA methylation, was examined. If such epigenetic changes do occur, these could affect levels of gene expression (Finnegan *et al*., 2000; Zhang *et al*., 2010). Although epigenetic changes do not imply DNA sequence changes, there are concerns that they may persist and be displayed as altered phenotypes (Peredo *et al*., 2008) or may even be heritable (Brettell and Dennis, 1991; Saze, 2008). However, it has also been reported that transient DNA methylation changes may serve as an adaptive response to stresses incurred (Johnston *et al*., 2009).

The first challenge to overcome was to ensure that good quality, intact, high molecular weight DNA could be isolated from the plant material for subsequent analysis, as this remains a problem in current molecular biological research particularly on plant tissues (Ribeiro and Lovato, 2007; Zain Hasan *et al*., 2008). In the present study, survival of axes following cryostorage was manifested predominantly by root production (Table 3.14), which is not uncommon when embryonic axes from recalcitrant seeds are cryopreserved (as discussed above). Thus, it was vital to ensure that good quality DNA could be isolated from *in-vitro*-germinated roots, although DNA from plants is most commonly isolated from shoots (Jobes *et al*., 1995). The methodology presently used resulted in successful isolation of DNA from roots at even higher concentrations than that isolated from the shoots, *viz*. 83 – 480 ng μl^{-1} from roots compared with 61 ng μl^{-1} from shoots. This

enabled comparison of the DNA methylation status of explants recovered from the cryopreparative stages and also following cryogen exposure.

The results obtained suggested that few, if any, DNA methylation changes occurred in germplasm recovered from the cryopreparative stages or cooling (Tables 3.20 and 3.21). However, it must be considered that there were a number of constraints in the present study. As there was limited molecular biological information available on the species under investigation, commercially available random primers had to be used and only a limited number of primers could be tested as this was a preliminary study. Furthermore, the analysis was subject to the limitations inherent in the technique: specifically, the whole genome was randomly sampled (which could not give information about any methylation changes which may have occurred in specific areas [of the genome]) as only areas within the recognition sequences of the methylation-sensitive restriction enzymes (*HpaII* and *MspI*) were analysed. Considering these constraints, the method used in the present study was suitable for a preliminary assessment, but more in-depth studies are required to make more definitive conclusions.

Future work should be directed towards the use of more informative and specific techniques to assess the DNA methylation status of the germplasm of this species. This would necessitate the development of suitable primers for this purpose, but the advantage would be that the analysis could then answer more specific and directed research questions. In addition, it would be useful to assess the genetic status of the germplasm (in conjunction with the epigenetic status) to determine if there were any changes in sequences in the DNA. In this way, a complete picture could be gained of the genetic integrity and epigenetic situation of germplasm in response to the preparative procedures for, and after, cryogenic exposure. This would allow for identification and adjustment of procedures that are likely to induce genetic or epigenetic variation. This should ensure that the methods finally adopted for cryoconservation would not compromise the genetic integrity of stored germplasm.

Overall the present study contributes to the body of knowledge on the cryopreservation of plant germplasm. The results showed that slow cooling rates cannot be recommended for cryostorage of explants of *E. capensis*. It was confirmed that 'broken' buds of *E. capensis* were not amenable to cryostorage, the primary limitations being explant size and hydration status. Adventitious shoots were slightly better suited to tolerate cryogenic temperatures, but only low levels of survival were obtained. Shoot production was adversely affected when axes with cotyledonary segments were exposed to cryogenic temperatures, but this problem was overcome by the application of the protocol developed to generate adventitious shoots on roots. In this way, plantlet establishment, following cryostorage was possible and was representative of the genetic diversity offered by seeds. The observed lack of shoot production following cryogenic exposure of axes and lack of survival of adventitious shoots could not be correlated with superoxide production. The epigenetic status of germplasm revealed little, if any, change in the methylation status of DNA following the cryopreparative stages and cooling. A number of recommendations have been made from the present findings that may contribute to further success in the long-term conservation of germplasm of *E. capensis*.

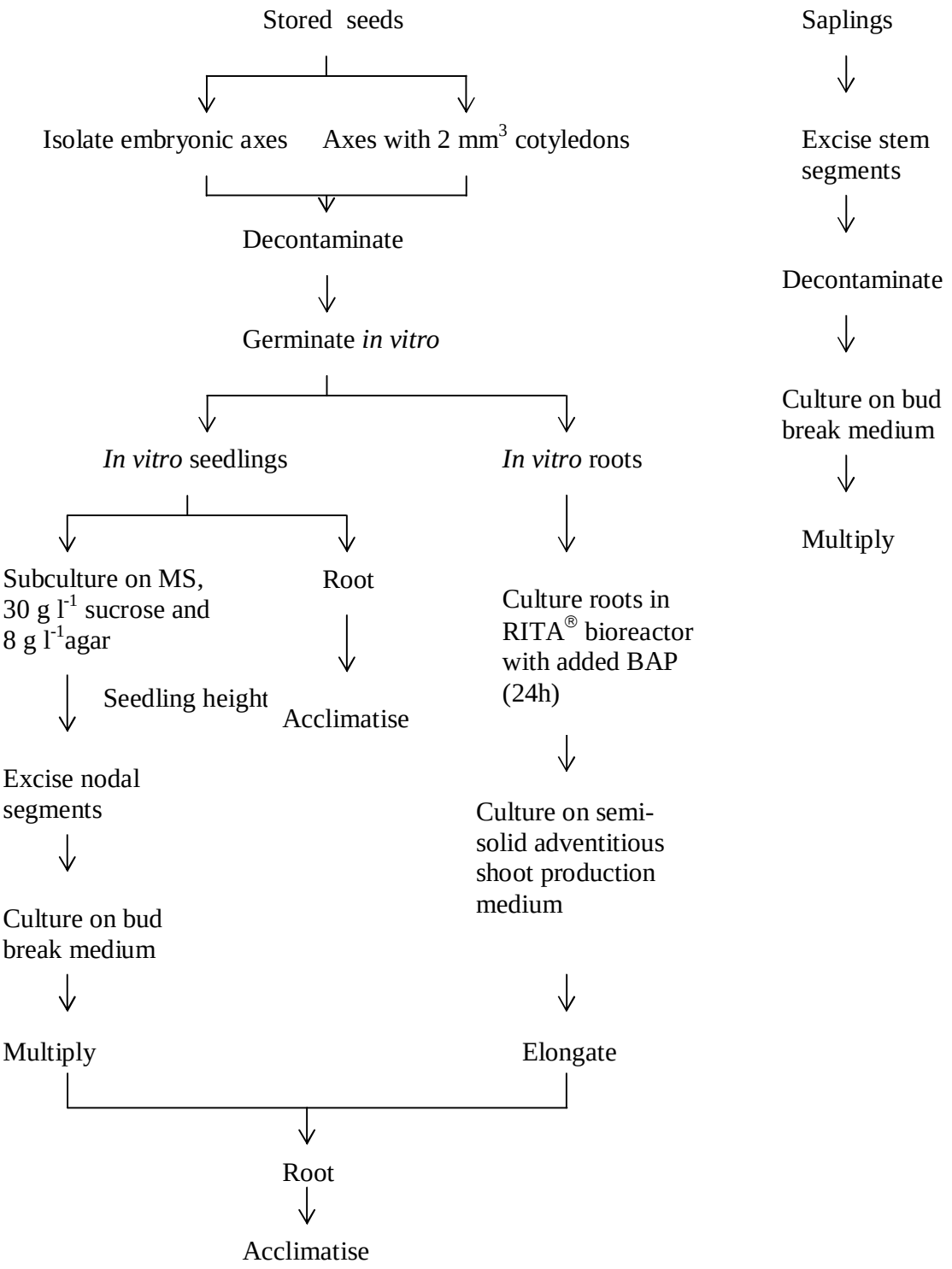

Figure 4.1: Schematic diagram summarising explant source and protocol tested for the micropropagation of *E. capensis*.

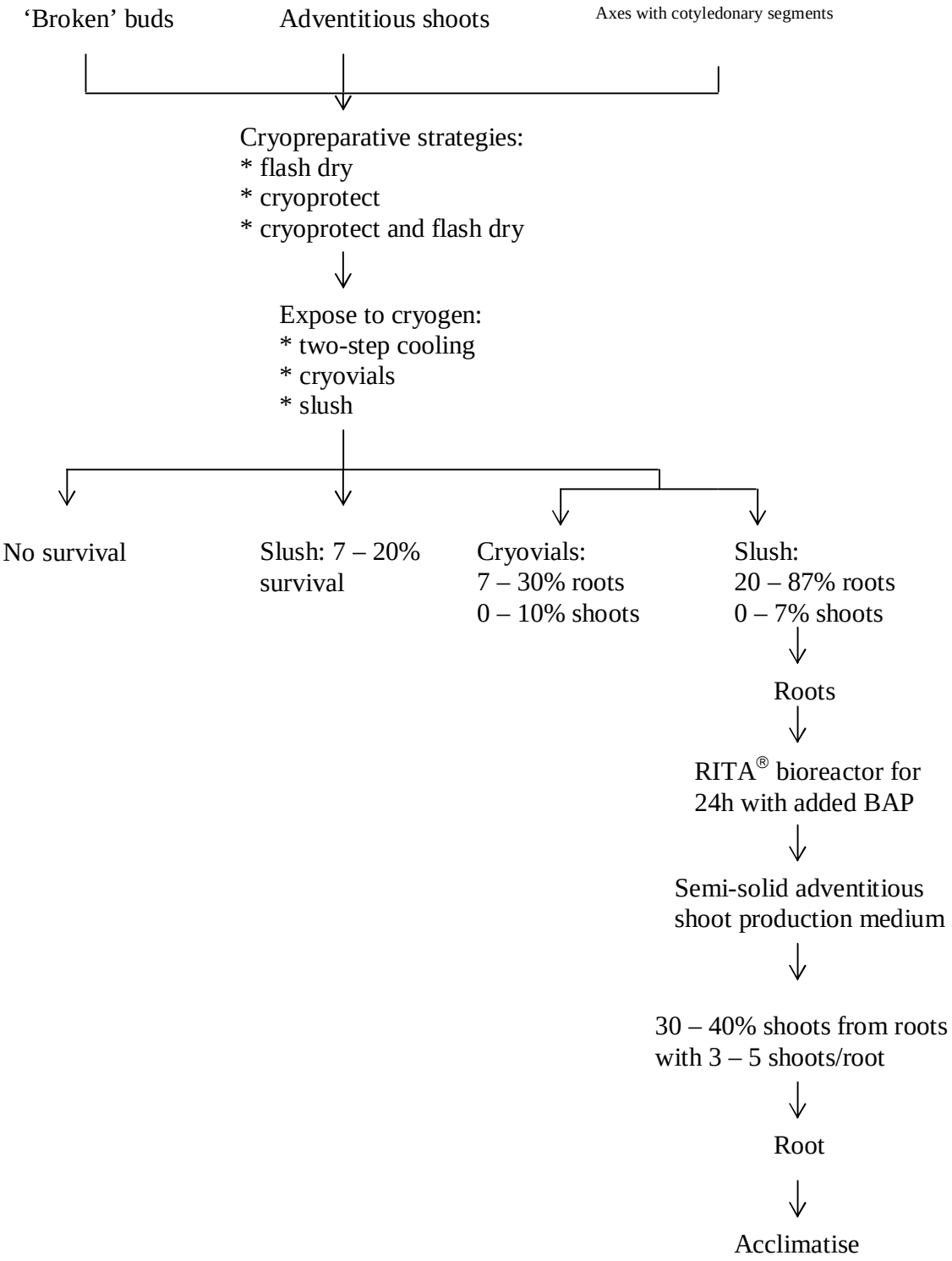

Figure 4.2: Schematic diagram summarising the strategies followed to cryostore germplasm of *E. capensis*.

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