Oxidative status and stress associated with cryopreservation of germplasm of recalcitrant-seeded species

by

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Abstract

Genetic diversity of cultivated species and their wild relatives, as well as of wild species encompasses plant genetic resources or germplasm, the *ex situ* preservation of which embodies a critical aspect of biological conservation. While seed storage affords an efficient *ex situ* conservation method, recalcitrant seeds are intolerant of desiccation and cannot be stored conventionally in seed banks. Seeds of the three indigenous tree species investigated in this study, *viz. Trichilia emetica, T. dregeana* and *Protorhus longifolia* are recalcitrant, with the species considered to be endangered. Cryopreservation, which involves storage at ultra-low temperatures of selected tissue(s) from which plants are subsequently able to be generated, is currently the only method available for long-term *ex situ* conservation of recalcitrant-seeded species and affords significant potential for the future. Many protocols that have been applied for the cryopreservation of the germplasm of recalcitrant zygotic embryonic axes excised from seeds of tropical/sub-tropical species have resulted in survival post-cryo which has been recorded only as root development or callus formation, with shoot formation seldom occurring. Successful cryostorage of genetic resources cannot be achieved until post-cryopreservation recovery facilitates normal seedling development, i.e. the formation of both a fully functional root and a shoot.

Cryopreservation requires the utilisation of the smallest explant possible (greatest surface area to volume ratio), the most suitable for recalcitrant seeds in general being the zygotic embryonic axis. Based on preliminary studies it was demonstrated that shoot production by axes is inhibited in association with a burst of reactive oxygen species (ROS), produced in response to wounding upon excision of the axis from the cotyledons, when these are attached close to the shoot apical meristem. It was postulated that a combination of the oxidative burst at the site of excision coupled with inadequate antioxidant machinery within the recalcitrant axis tissue, precludes shoot production. It was further considered highly probable that each subsequent stressful manipulation throughout the cryopreservation process would be accompanied by a surge of uncontrolled oxidative activity within the tissue, in response to the stress. Therefore, the primary aim of the study was to investigate the underlying causes of failure of shoot production after procedures associated with cryopreservation and to focus on ways to ameliorate the consequences of unbalanced oxidative metabolism. Additionally, studies were carried out to optimise each step of the cryopreservation procedure, *viz.* cryoprotection, dehydration, rehydration and cooling, and subsequent recovery, in conjunction with assessment of oxidative responses, ultimately to

achieve successful cryopreservation of the embryonic axes of these species. The experimental work conducted to achieve this aim assessed changes in various biomarkers of injury, those selected for this study being three ROS, *viz.* superoxide, the hydroxyl radical and hydrogen peroxide, after axes were exposed to various pre-treatments, cryopreservation and recovery.

Concomitantly, the elicited responses of endogenous antioxidant systems accompanying these steps were assessed. Changes in the levels of ROS and antioxidant activity were determined using various biochemical assays, and these parametres, together with assessment of shoot development, were investigated after each step of the cryopreservation process. The effect of stress on oxidative metabolism was tested after exposure to pre-treatments with and without the provision of various antioxidants, *viz.* DMSO, ascorbic acid and cathodic water, so as to determine the efficacy of selected ROS scavengers and, in general, to develop the best protocol for cryopreservation of embryonic axes of the three species. Significant results, in terms of shoot development and regulated ROS generation, were obtained after three major processes of the cryopreservation procedure. The production of roots and shoots by excised axes of *T. emetica, T. dregeana and P. longifolia* after excision (75%, 80% and 75%, respectively), and by 40% of excised axes of *T. dregeana* after each of the two further stages, cryoprotection and desiccation, were major achievements towards cryopreservation of the recalcitrant germplasm. The modulation of ROS by ascorbic acid and cathodic protection significantly improved survival of axes of both *Trichilia* species. In its entirety, the present study made significant advancements towards cryopreservation of recalcitrant germplasm and also towards understanding oxidative events associated with cryogenic processing and exposure to cryogenic conditions.

This study concludes that unregulated metabolism is one of the underlying causes of failure of recalcitrant germplasm represented by zygotic axes, to survive cryopreservation. The application of antioxidants and cathodic protection during cryopreservation facilitated survival that has been previously unattainable. The outcomes of this study provide an informative platform for further optimising cryopreservation procedures for the germplasm of the species investigated, and extending the work to other recalcitrant-seeded species, especially those of tropical/sub-tropical provenances.

Preface

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

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

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Cassandra Naidoo

November 2012

Declaration 1 - Plagiarism

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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: Naidoo, C., Benson, E., Berjak, P., Goveia, M., Pammenter, N.W., 2011. Exploring the use of DMSO and ascorbic acid to promote shoot development by excised embryonic axes of recalcitrant seeds. CryoLetters 32, 166-174.

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

Signed: .....

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List of Abbreviations and Symbols

ABTS•1	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	analysis of variance
APX	ascorbate peroxidase
AsA	ascorbic acid
AsC	ascorbate
BAP	6-Benzylaminopurine
BCGI	Botanic Gardens Conservation International
CAT	catalase
Ca^{2+}	calcium cation
CaMg	calcium magnesium
°C	degrees celcius
°C min^{-1}	degrees celcius per minute
cm	centimetre
$CaCl_2$	calcium chloride
CaOCl	calcium hypochlorite
conc	concentration
CGA	5-*O*-caffeoylquinic acid
CH_3	methyl radical
CW	cathodic water
d	days
dmb	dry mass basis

DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dm	dry mass
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
EPOXC	apoplastic peroxidase
ER	endoplasmic reticulum
FAO	Food and Agriculture Organisation of the United Nations
FD	flash dried
Fe^{3+}	iron cation
g	gram
g g^{-1} dm	gram of water per gram of dry mass
g l^{-1}	gram per litre
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidised glutathione
h	hours
H_2	hydrogen
H_2O	water
H_2O_2	hydrogen peroxide

HCl	hydrochloric acid
$HgCl_2$	mercuric chloride
IBPGR	International Board for Plant Genetic Resources
IUCN	International Union for Conservation of Nature
l	litre
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LEA	late embryogenic abundant/accumulating
LPI	living planet index
LN	liquid nitrogen
LSD	least significant difference
m	metre
mg	milligram
mm	millimetre
ml	millilitre
min	minute/s
ml l^{-1}	millilitre per litre
mM	millimolar
M	molar
MAPK	Mitogen-Activated Protein Kinase
MDA	malondialdehyde
MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase

mg l^{-1}	milligram per litre
$m^{-1}s^{-1}$	metre per second
Mg^{2+}	magnesium cation
$MgCl_2$	magnesium chloride
MS	Murashige and Skoog medium (1965)
MSA	methane sulphinic acid
MPa	mega pascals
mV	millivolt
nm	nanometre
nmol g^{-1} DW s^{-1}	nanomols per gram dry weight per second
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
O_3	activated/triplet state oxygen
$^1O_2$	singlet oxygen
$^{\cdot}$OH	hydroxyl radical
pH	hydrogen ion concentration
pKa	acid dissociation constant
PBS	phosphate buffer saline
PGR	plant growth regulator
PS 1	photosystem 1
PS 2	photosystem 2
PVP	polyvinylpyrrolidone
PVS	plant vitrification solution

%	percentage
rER	rough endoplasmic reticulum
RFU	relative fluorescence unit
RH	relative humidity
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
rpm	revolutions per minute
TAA	total antioxidant activity
T_n	ice-nucleation temperature
TTZ	triphenyl tetrazolium chloride
Tween 20/80	polyoxyethylene sorbitan monolaurate
USDA	United States Department of Agriculture
µM	micromolar
µm	micrometre
µmol m^{-2} s^{-1}	micromolar per metre per second
µmol g^{-1} DW s^{-1}	micromols per gram dry weight per second
v/v	volume/volume
w/v	weight/volume
wc	water content
wmb	wet mass basis
ψ	water potential

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CHAPTER 1: General Introduction

1.1 The need to conserve: sustainable preservation of global biodiversity

Pinchot (1910), one of the first American conservationists and foresters once said that unless conservation is actively practised, those who come after us will pay the price of misery, degradation and failure for the progress and prosperity of our day. The inability to conserve progressively in a sustainable manner would be directly linked to the exacerbation of the current biodiversity crisis we face today. Biodiversity, coined as a contraction of 'biological diversity' in 1985, was defined as "the full range of variety and variability within and among living organisms and the ecological complexes in which they occur, encompassing ecosystem or community diversity, species diversity, and genetic diversity" (US Congressional Biodiversity Act, 1990). Conservation biology remains one of the most fundamental pillars of global sustainable development, creating interconnected ecosystems on which we rely for goods and services such as provision of food, purification of air and water, detoxification and decomposition of wastes and stabilisation of global climatic conditions among many others. However, as exemplified by the Living Planet Report in 2010, the Living Planet Index (LPI) which measured the health of 8,000 populations of over 2,500 species, showed biodiversity to be on an unprecedented downwards spiral, with global biodiversity down by 30% since 1970. In addition, the IUCN Red List, which stands as one of the most comprehensive inventories of the global conservation status of plant and animal species, shows the current extinction rate to be between 1,000 and 10,000 times higher than it has been in the recent geological past. If current trends continue in terms of degradation of natural resources and an increasing extinction rate, the capacity of two planets will be required to support the demands on natural resources and carbon dioxide waste absorption by 2030 (Living Planet Report, 2010).

According to recent statistics released by the World Conservation Union (2007), the Red List shows 20,000 out of 60,000 species assessed to be threatened. Specifically, one in four mammal, one in eight bird, one third of all amphibian and 70% of globally assessed plant species are at risk of extinction. In South Africa, 97 out of 444 threatened species on the IUCN Red List are plant species. In the light of all these statistics, the urgency to preserve genetic resources has emerged as being a chief priority and has set in motion numerous conservation strategies by both the public and private sectors on a global scale, with the aim of preserving biodiversity, especially plant genetic resources, which contribute towards food security, stable agricultural systems, ecosystem services and traditional medicine (FAO,

2010). Efforts towards the sustainable conservation of plant germplasm, must meet the needs of the present without reducing the ability of future generations, to meet their needs as well.

1.2 Preservation of plant genetic resources

According to the United States Department of Agriculture (USDA) Forest Service (1993), the extinction of a single plant species can result in the disappearance of up to 30 other plant and wildlife species, with the Botanic Gardens Conservation International (BGCI) reporting that one in every five plant species is currently endangered. Loss of plant diversity jeopardises resilience in ecosystems, e.g. the ability to respond to the stresses brought about by climate change (Abbas & Quaiser, 2011), thereby diminishing possible re-establishment of species after disasters. 'Threatened' plant species are deemed those that are on the verge of disappearing due to extensive exploitation and/or severe habitat threat to their natural habitat (IUCN, 2011). In recent years the pressures on forest and land resources have increased due to an exponentially increasing population and this, coupled with the increasing concentration of greenhouse gases which influences climatic conditions, have led to the decline in medicinally and commercially important plant species (Bakkenes *et al*., 2002; Jackson & Kennedy, 2009). The current awareness of the impact of climate change on natural systems has given rise to the prediction that the potential loss of plant diversity by the year 2050 will be anything between 60,000 – 100,000 species (Bramwell, 2002; Hawkins *et al*., 2008). Apart from the many anthropogenic stresses that negatively affect plant diversity (urbanisation, pollution, habitat destruction and fragmentation and over exploitation; Pitman *et al*., 2002), the impact of climate change poses further challenges (Catarious & Espach, 2009). Factors associated with climate change include temperature increases, changes in precipitation patterns, an increase in extreme weather conditions ranging from severe drought to storms and floods, desertification, rising sea levels, shifts in growing seasons of plants, and loss of pollinators and seed dispersers (Hawkins *et al*., 2008). Importantly, the rapid loss of biodiversity is irrevocably linked to provision of food security to a rapidly growing population. As such, the pressing need for conservation of plant genetic resources is two-fold, illustrated by two statistics: i.e. one billion malnourished people (FAO, 2006) and between 1-10% of plant biodiversity predicted to be lost by 2030 (Hanski *et al*., 1995).

Future survival of humanity – and of many other animal species – is based on the conservation of plant genetic resources. The repercussions of plant diversity loss, the associated decrease in ecosystem services together with the numerous challenges facing conservation today are widely acknowledged. Global concern has spurred on numerous

attempts to protect and preserve plant species, both commercially propagated and uncultivated, by way of their germplasm (Fraleigh, 2006; Dodds *et al*., 2007). In this context, plant germplasm refers to the genetic information encoded in the seed (Kloppenburg & Kleinman, 1987) or can be more broadly defined as the total genetic diversity encompassed by cultivated species and their wild relatives (Ford-Lloyd & Jackson, 1991). Germplasm can be extracted from various sources of plant material and the type of germplasm collected determines the method of conservation, which is categorised as either *in situ* or *ex situ* (Fig. 1.1).

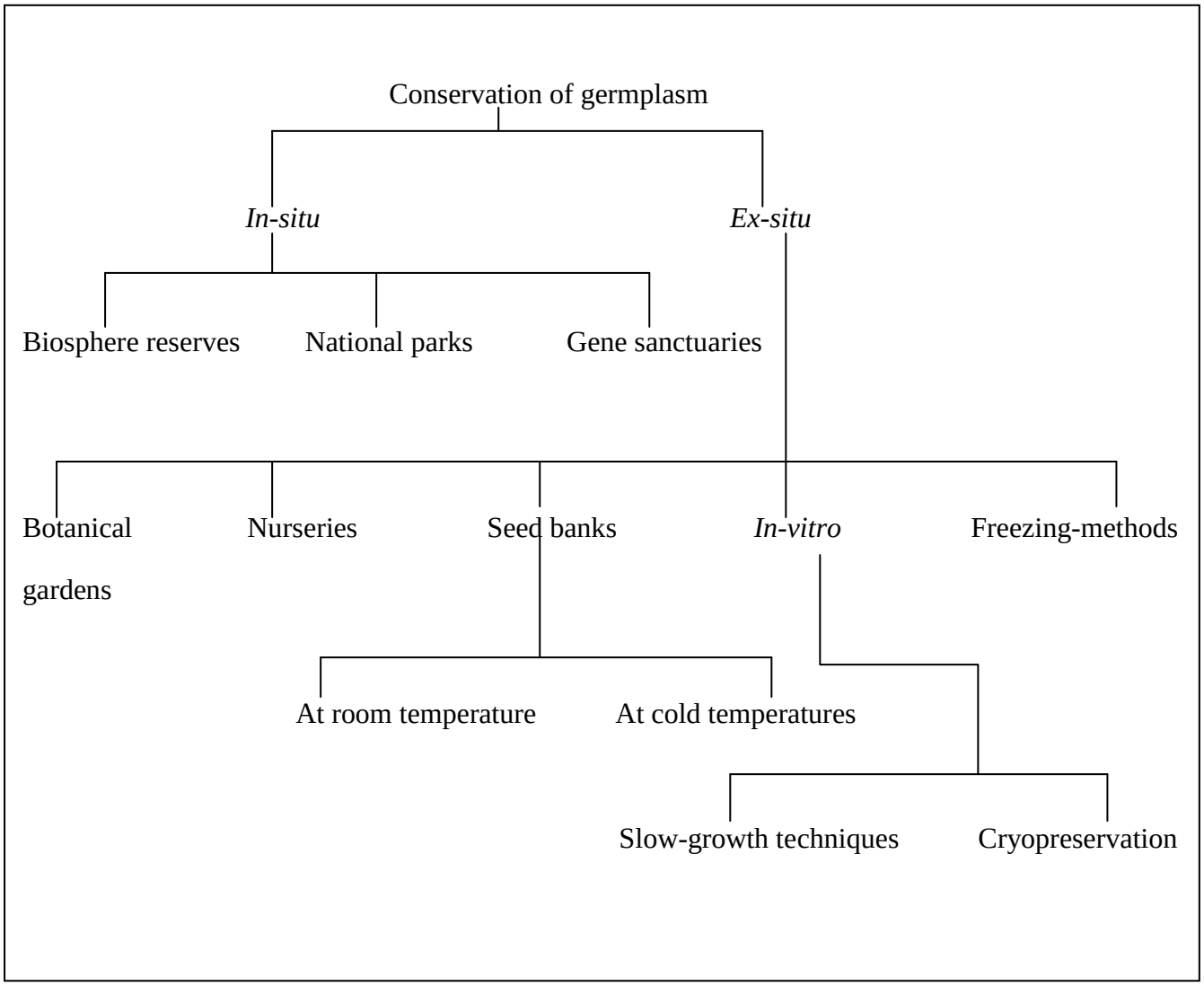


Fig. 1.1: Diagram representing various approaches to conservation of plant genetic resources.

Conservation of germplasm *in situ* usually refers to wildlife conservation or preservation of genetic material in natural reserves, where populations continue to evolve in

the environments which have influenced the development of their distinctive traits, i.e. perpetuation of the natural progression of evolutionary processes (Benson, 2008). Also referred to as Genetic Reserve Conservation, this approach involves the location, management and monitoring of genetic diversity in natural wild populations within defined areas designated for active, long-term conservation (Maxted & Bennet, 2001). *In situ* conservation offers the distinct advantages of dynamic evolution of species where genes are subject to change as a result of both natural and artificial selection (Hummer, 1999), as well as crop/pathogen interaction and an understanding of evolution and diversity (Hawkes, 1991*).* However, *in situ* conservation is limited by the risk of significant decline of preserved species in the face of natural and man-made disasters (Li & Pritchard, 2009; Paunescu, 2009), and by the restricted spectrum of diversity that can be contained within natural reserves (Hawkes, 1991). Of great concern is the fate of long-lived species, e.g. cycads and many temperate tree species, as renewal–replacement cycles take decades to centuries to complete (Queensland Herbarium, 2007). *Ex situ* conservation is considered to be a principal mode of plant germplasm conservation and involves the preservation and maintenance of samples of living organisms outside their natural environment in the form of whole plants, seeds, pollen, vegetative propagules, tissue or cell cultures (FAO, 1993; Paunescu, 2009).While many conservation efforts advocate a combination of both *in situ* and *ex situ* strategies, with difficult-to-conserve species such as those producing recalcitrant or asexually propagated seeds, *ex situ* approaches are the only feasible option to conserve germplasm and is also often considered to be a safer and more prudent alternative (Bioversity International, 2011).

1.3 *Ex situ* conservation

For effective longevity, availability and viability of germplasm, this approach includes storage of seed or plant material under artificial conditions, i.e. removal from the natural habitat for cultivation in botanic gardens, arboreta and nurseries, and by seed storage or cryopreservation in genebanks and *in vitro* cultivation, depending on the type of germplasm (Ford-Lloyd & Jackson, 1991; Engels & Visser, 2003; Paunescu, 2009; Bioversity International, 2011). *Ex situ* conservation involving maintenance of clonal crops in field gene banks and *in vitro* banks, certain tree species in conservation stands, numerous seed-bearing species in botanic gardens and conventional and cryogenic seed banks (Li & Pritchard, 2009) serves as a safeguard for species that are vulnerable to depletion in nature, and in ecosystems dominated by anthropogenic activities (Li & Pritchard, 2009). It is generally accepted that botanic gardens serve as an efficient way to conserve species *ex situ*

as is illustrated by more than one third of the world's flowering plant population being cultivated in 2204 botanic gardens around the globe (BGCI, 2006). However, this facet of *ex situ* conservation is both land and labour-intensive (Panis & Lambardi, 2005) and has the disadvantage of necessitating acclimatisation and accommodation (Hawkes, 1991; Paunescu, 2009). Therefore, before a conservation strategy can be undertaken, it is important that the diversity within each species is thoroughly investigated in order to obtain an appropriate sample size and a genetically diverse representation of material (Kramer & Havens, 2009). This is a challenging task with many wild species as the amount of material is often limited and difficult to access. Two types of storage are generally recognised within *ex situ* conservation, i.e. active collections which refer to available short- to medium-term stored samples, and base collections, which refer to material preserved for long term genetic security (Withers, 2001; Engels & Visser, 2003).

1.3.1 Active collections: seed banks and *in vitro* cultivation

Storing genetic diversity as seeds that are obtained – commonly from various cultivars – is considered to be the most convenient, researched, widely-used and cost-effective means of *ex situ* conservation (Justice & Bass, 1978; Engels & Visser, 2003). The main responsibility of facilities preserving genetic stock in the form of seeds is to ensure that they are maintained for an indefinite period while retaining the highest possible viability (Vertucci & Roos, 1990). To achieve this, it is crucial to optimise the conditions for seed storage. While there is the requirement to consider factors such as the chemical composition, physiology and physical status of water within seeds of each species, the wide practice is to maintain seeds away from excessive heat, relative humidity (RH) and oxygen to prolong longevity (Justice & Bass, 1978; Vertucci & Roos, 1993). As such, two major requirements for storage of orthodox (desiccation-tolerant) seeds are: sufficient drying - i.e. seed moisture content (fresh mass basis) at 3% for oily seeds and 5% or higher for starchy seeds; and appropriate storage temperature (-18°C to -20°C recommended for long-term storage [Bonner, 2008]). A major shortcoming of this approach is its applicability to plant species propagated by orthodox seeds only. There remains a large number of species that are vegetatively propagated (to preserve unique genomic constitution of certain cultivars); or produce seeds which are recalcitrant (desiccation-sensitive), immature or sterile (Engels & Visser, 2003; Panis & Lambardi, 2005; Paunescu, 2009). Conventional seed storage is also constrained by the limited duration for which seeds will retain vigour and remain viable; their heterozygous

nature rendering them unsuitable for maintenance of true-to-type genotypes and their potentially rapid deterioration due to seed-borne pathogens (Taha *et al*., 2007).

Active collections can also involve *in vitro* conservation via the maintenance of cultures in the growing state, *viz*. actively growing cultures or minimal growth cultures. Actively growing cultures involve the ongoing transfer of material on to fresh medium at regular intervals while minimum growth involves manipulating certain physical environmental conditions and components of the tissue culture environment in order to slow down growth *in vitro*. Both have the advantages of preserving large amounts of material in small areas and provision of bulk material for multiplication and distribution of species (Withers, 2001). However, there is the possibility of losing accessions due to contamination; human errors and somaclonal variation (spontaneous mutations that take place during tissue culture with an increased occurrence upon frequent sub-culturing) when working with actively growing cultures (Larkin & Scowcroft, 1981; Withers, 2001). With minimal growth cultures, the stress imposed to retard growth may lead to a selective loss of viability within the tissue, translating into genetic drift (Withers, 2001). Hence, cryopreservation or freeze-preservation is considered the only sound option for the long-term conservation of plant genetic resources (Withers, 2001; Engels & Visser, 2003; Panis & Lambardi, 2005; Normah & Makeen, 2008; Paunescu, 2009).

1.3.2 Base collection: cryopreservation

Cryopreservation refers to the use of ultra-low temperatures, usually in liquid nitrogen (-196°C) or the vapour above liquid nitrogen (minimally at -140°C), to preserve viable and structurally intact cells, tissues, organs and organisms (Pegg, 1995; Walters *et al*., 2004; Benson, 2008). It is considered to be a form of *in vitro* conservation which has a primarily biotechnological basis, and aims to establish stable conditions to preserve life in perpetuity (Pegg, 1995; Benson, 2008). Cryopreservation has been strongly advocated as the most promising method for long-term storage of plant germplasm (Reed, 2002; 2008; Panis & Lambardi, 2005; Kaviani, 2011) to protect and preserve economically important, endangered and difficult-to-conserve plant genetic resources (Pearce, 2004; Sakai, 2004; Benson, 2008). Preservation in this manner is based on three theoretical principles, the first being the suspension of biological ageing at the cellular, molecular and physiological levels in active cells; the second being the non-injurious interruption and subsequent arrest of molecular mobility and metabolic function within cells, upon exposure to and storage at, cryogenic temperature (Sakai, 2004; Benson, 2008), and the third involving the retrieval of viable,

genetically sound, unaltered material that is able to resume normal function (Taha *et al*., 2007; Benson, 2008). On this basis, potentially indefinite preservation of an array of germplasm types including meristems, shoot tips, embryos (both zygotic and somatic), embryonic axes, protoplasts, cell suspension culture, seeds, pollen and buds has been achieved cryogenically.

The development of robust cryopreservation protocols calls for a broad understanding of the biophysics underlying the freezing process, including: (1) the behaviour of water; (2) cryo-induced injury; and (3) cryoprotection (Day *et al*., 2008) (aspects of which will be detailed in Chapter 2). For now, it is necessary to highlight the influence of temperature on the transition between the four phases of water i.e. liquid, glass (vitrified), solid (ice) and vapour; and the importance in maintaining stability of the vitrified state to avoid lethal intra-cellular ice formation. Plant species adapted to habitats at sub-zero temperatures possess avoidance mechanisms to bypass ice-crystal formation via synthesis of substances (sugars, proline and certain anti-freeze proteins) which lower the freezing point in cells, leading to a 'supercooled' state (Wisniewski *et al*., 2002). However, when dealing with the ultra-low temperatures of cryopreservation, avoidance of ice-crystallisation while still maintaining a water content required for survival is difficult to attain. Hence, both classic and modern approaches to cryopreservation (to be discussed in Chapter 2) aim to attain a 'vitrified' state within cells, i.e. the phase achieved by the transition of an aqueous solution to an amorphous and glassy (non-crystalline) state to avoid intracellular crystal formation (Day *et al*., 2008).

As mentioned above, seeds afford the most amenable plant propagules for *ex situ* preservation of genetically diverse germplasm, and, in cases where seeds display non-orthodox or recalcitrant behaviour (see section on post-harvest behaviour below), cryopreservation is the only feasible means of germplasm storage. While there are documented cases of freeze preserving whole non-orthodox (but probably not recalcitrant) seeds, in most recalcitrant-seeded plant species, desiccation and/or chilling sensitivity combined with the large size of the seeds hinders successful cryopreservation. Hence, a suitable alternative representing the requisite genetic diversity needs to be chosen. For the purpose of this study, cryopreservation of embryonic axes excised from seeds was investigated. However, regardless of germplasm type, cryopreservation of explants of recalcitrant-seeded species and other desiccation-sensitive material usually involves the following processes, as shown in Figure 1.2.

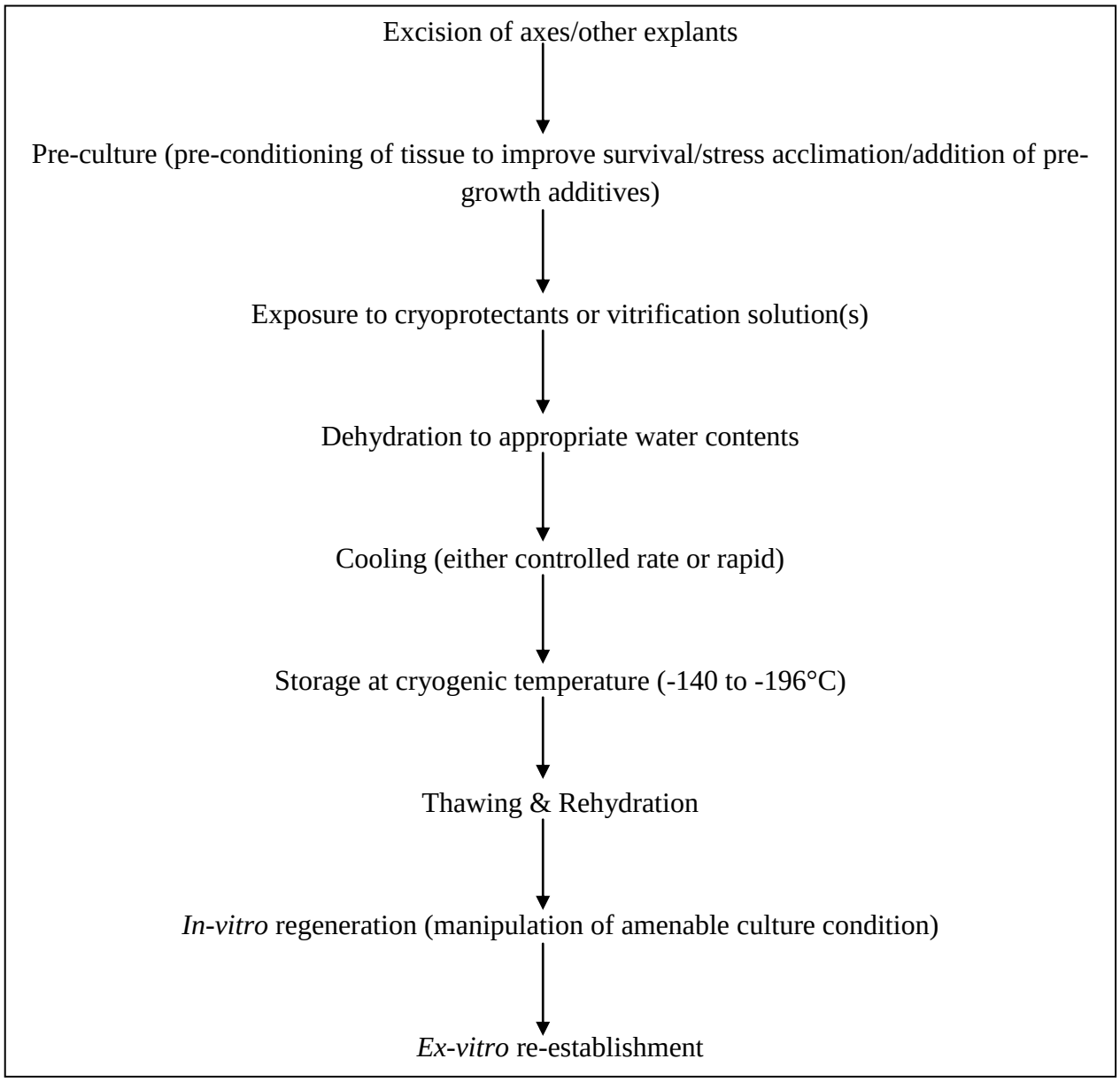


Fig. 1.2: Diagram representing the stages of a cryopreservation protocol.

Successful cryopreservation of germplasm calls for the optimisation of each of the above-mentioned steps, involving a number of different techniques. The function of each manipulation of the cryo-protocol in preparing tissue for survival after cryopreservation will be discussed in detail in Chapter 2. It is first necessary to present a basic overview of post-harvest seed behaviour, as this is a major determinant for selecting the most appropriate method of germplasm conservation.

1.4 Post-harvest seed behaviour and seed storage

The responses of seeds in any given storage regime differs from species to species, even amongst those closely related within a genus (Hong & Ellis, 1996). During the early

1900s seed longevity and storage methods were not considered in the context of responses of the seeds to the environment (Altman & Dittmer, 1972; Ewart, 1908; Harrington, 1972). Later, categorisation of seeds was based on desiccation tolerance and survival in response to environmental factors. In 1973, Roberts classified seeds into two discrete categories according to their response to water loss; he coined the terms, orthodox for desiccation-tolerant types and recalcitrant to describe those that were desiccation sensitive. Upon experimentation using four cultivars of Arabica coffee (*Coffea arabica*), Ellis *et al*. (1990) proposed a third category which he described as showing intermediate seed storage behaviour – i.e. characteristics between the extremes of orthodoxy and recalcitrance. With further studies on a variety of species, it was advocated that as there appears to be extensive variability in post-harvest responses of seeds, their categorisation should not be rigidly confined (Berjak & Pammenter, 1994, 2008; Pritchard, 2004). Those authors suggested an 'open-ended continuum' of seed behaviour with extreme orthodoxy at one end and acute recalcitrance at the other. This continuum of categorisation accommodates the marked variability in post-harvest seed behaviour between, and even within, species, emphasising the necessity of species-specific procedures (for germplasm storage) according to the degree of dehydration tolerated. Although the response to desiccation is a major characteristic differentiating behaviour between categories, within categories there are factors such as provenance and developmental status that influence these responses, so not all seeds – even those of the same species – within a category can be assumed to be equally desiccation tolerant/sensitive (Daws *et al*., 2004; 2006; Bharuth, 2012). When considering seed behaviour and the contrasting response patterns between orthodoxy and recalcitrance, there are two practical aspects to note, i.e. the effect of desiccation on seed viability and the response of seed longevity to storage conditions (Hong & Ellis, 1996).

1.4.1 Orthodoxy

Orthodox seeds are those that can be dried to low moisture contents of 10% (wet mass basis; wmb) and below, and stored successfully at sub-zero temperatures, where their longevity increases with a decrease in moisture content and storage temperature in a predictable and quantifiable manner (Roberts, 1973; Bonner, 1990; Hong & Ellis, 1990). Bonner (1990) further classified orthodoxy into two sub-groups i.e. True orthodox seeds, which can be stored at sub-freezing temperatures for a relatively extended period provided their moisture content is lowered to ± 5-10% (wmb); and sub-orthodox seeds, which can be maintained under the same conditions as true orthodox seeds but for a shorter time frame.

Seed development and maturation can be divided into different stages, the early stages being the least tolerant to desiccation. During development, orthodox seeds entrain a suite of mechanisms that together have been implicated in the acquisition and maintenance of desiccation tolerance (Vertucci & Farrant, 1995; Pammenter & Berjak, 1999; Berjak, 2006), although the extent to which desiccation is tolerated and the rate at which water is lost differs substantially between species (Hong & Ellis, 1996; Pammenter & Berjak, 1999). These tolerance mechanisms include physical characteristics of cells and intracellular constituents; insoluble reserve accumulation; intracellular de-differentiation; metabolic 'switching off'; presence and effective operation of putatively protective substances including late embryogenic accumulating proteins (LEAs), sucrose and other oligosaccharides; effective anti-oxidant systems; and the presence and operation of repair systems during rehydration (Pammenter & Berjak, 1999; Berjak, 2006).

Orthodoxy is based on the relationship between the organisation and function of cell components, and hydration levels in seeds (Vertucci & Leopold, 1986), which are explored in more detail in Section 1.5.1, below. Briefly, however, specific water potentials (ψ) subtend each hydration level within seeds, in each of which water has distinct properties. Full, unimpaired metabolism proceeds at $\psi \sim\geq$ -1 MPa (hydration level V), but as ψ declines with increasing dehydration, metabolism becomes unregulated: for example, in hydration level III ($\psi \sim\leq$-10 MPa), while respiration continues, accompanying free radical production occurs (Vertucci & Farrant, 1995; Walters *et al.*, 2005a; [Figure 1.3, Berjak, 2006]). In orthodox systems, the appropriate tolerance mechanisms are entrained, thus avoiding this and related metabolism-linked desiccation damage. In desiccation-sensitive seeds, it is the absence or incomplete expression of these mechanisms that is presumed to underlie the different responses to dehydration, with dehydration-induced damage incurred by recalcitrant seeds (especially when water is lost slowly [Pammenter *et al*., 1998]) translating into cell death (Vertucci & Leopold, 1986; Pammenter & Berjak, 1999; Walters *et al.*, 2001). The sequence of major processes enabling the acquisition of desiccation tolerance when orthodox seeds are dried, and events upon subsequent rehydration, will be reviewed later (Section 1.5.2).

In terms of post-harvest behaviour, orthodox seeds undergo drying during maturation and are shed at a low water content which is in equilibrium with the prevailing atmospheric RH. All orthodox seeds can generally withstand dehydration to water contents ± 5% (wmb) even if maturation drying is not complete upon shedding. Any seed behaving differently from this cannot be considered orthodox. Based on this behaviour, it is accepted that orthodox seeds possess the full spectrum of mechanisms that confer desiccation tolerance and, as such,

can be stored for predictable periods under defined conditions of low temperature and RH with minimal deterioration (Ellis & Roberts, 1980; Walters & Engels, 1998; Walters *et al*., 2005b).

Unless debilitated by xerotolerant storage fungi, orthodox seeds usually maintain vigour and viability from harvest to the next growing season (Berjak *et al*., 1989) or for decades if stored at -18°C (IBPGR, 1976). However, given the most amenable conditions for seed storage, orthodox seeds do not have an infinite lifespan and will ultimately undergo the ageing process due to activities such as free radical-mediated lipid peroxidation, enzyme inactivation, disruption of cellular membranes and degradation of DNA integrity thus losing their ability to germinate with time (Murthy *et al*., 2003; Walters *et al*., 2005b; Berjak & Pammenter 2008). Many genetically and economically valuable accessions of orthodox-seeded species have been conserved in genebanks around the world, where seeds are banked conventionally i.e. at -18°C ± 3°C or lower temperatures (FAO, 2011). Such practices provide a reliable source of seeds in the absence of annual crops, and have been endorsed by many international institutions concerned with plant germplasm conservation (KEW, FAO, Bioversity International).

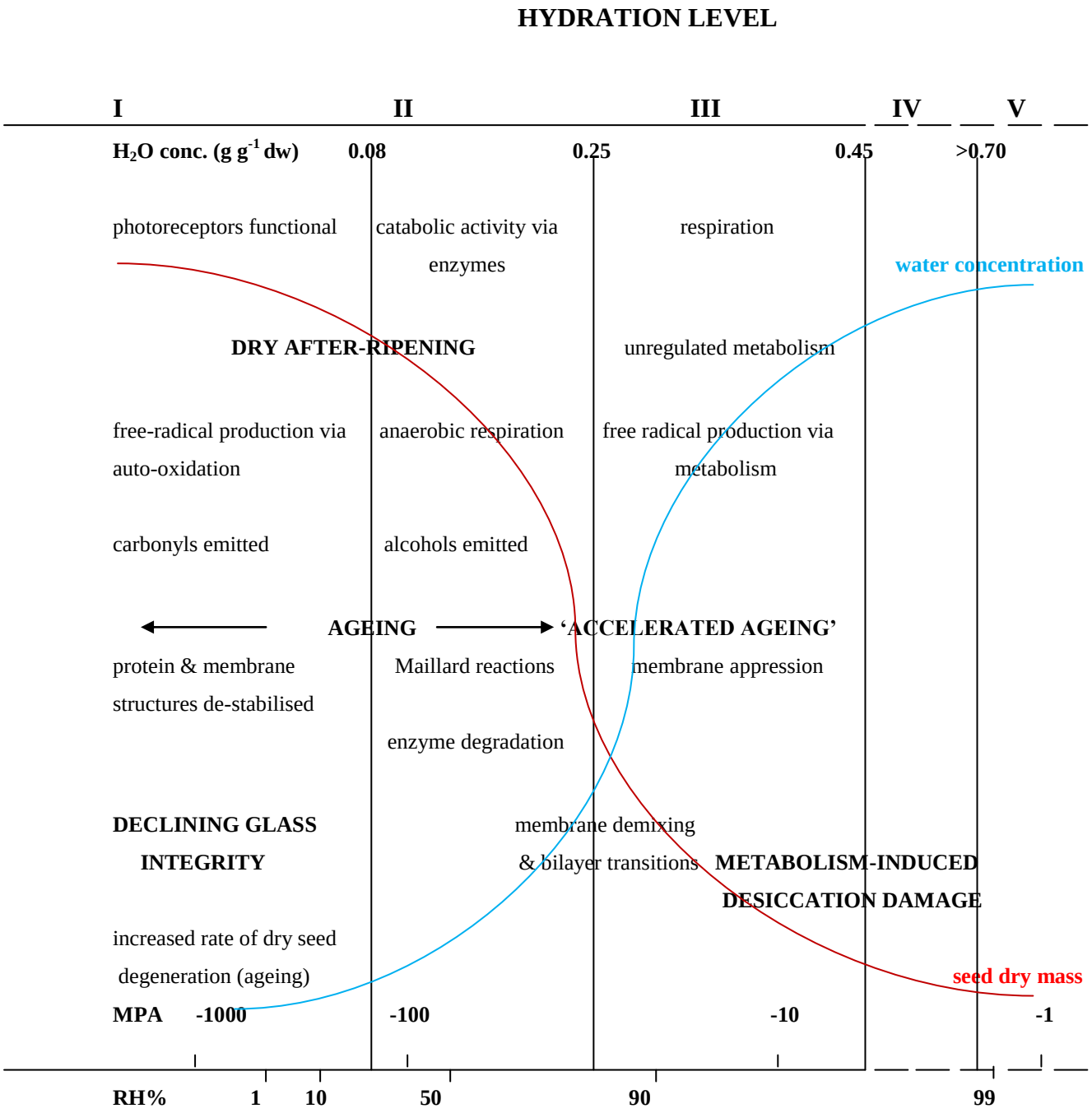


Fig. 1.3: A schematic representation of processes that are associated with hydration levels III to I. Water content ranges (dry mass basis) are reflected at the top of the figure, and equivalent water potentials (ψ) and relative humidities (%) are shown at the bottom (after Berjak, 2006; Berjak *et al.*, 2007). Red and blue lines represent seed dry mass and water concentration respectively.

1.4.2 Recalcitrance

Recalcitrant seeds are those characterised by relatively high water contents and their inability to tolerate reduction in contained water below a relatively high level (Roberts, 1973;

Grout *et al*., 1983). Unlike orthodox seeds that acquire desiccation tolerance while developing on the mother plant, recalcitrant seeds undergo little or no maturation drying. As they cannot tolerate much water loss at any stage without incurring injury, such seeds are desiccation sensitive throughout development, and after being shed (Hong & Ellis, 1990; Pammenter & Berjak, 1999; Berjak & Pammenter, 2008). Recalcitrant seeds may also display chilling-sensitivity and are impossible to conserve using conventional methods applied to orthodox seeds (Roberts, 1973; Chin & Roberts, 1980).

Further categorised by Bonner (1990), such seeds may be divided into temperate-recalcitrant which refers to those that are sensitive to desiccation but may be stored at refrigeration temperatures, or tropical-recalcitrant which display similar desiccation sensitivity to the temperate variety but are additionally sensitive to low temperatures. For many tropical species even very short periods of exposure to temperatures below 10-15°C lead to loss of viability (Chin & Roberts, 1980; Berjak & Pammenter, 1996). Certain temperate-recalcitrant species have been stored for ± 3-5 years at low temperatures, whereas the longevity of their tropical counterparts (even at moderate storage temperatures) is usually measured in weeks to months (Bonner, 1990). Additionally, Vertucci & Farrant (1995) reviewed the variable behaviour in the extent of dehydration tolerated by mature recalcitrant seeds amongst different species. The differences in these characteristics highlight the erratic nature of post-harvest responses of seeds between and within individual species falling within the same category of behaviour (Berjak *et al*., 1989; Pammenter & Berjak, 1999), and reinforces the idea of a continuum of seed responses (Berjak & Pammenter, 2004; 2008).

Upon shedding, recalcitrant seeds begin to dry resulting first in a slight stimulation of germinative metabolism (Eggers *et al.*, 2007), followed by the onset of loss of vigour and viability. However, once seeds undergo further dehydration to low water contents (the levels of which are drying-rate dependent), associated with lower hydration levels, the ability to survive may be lost if the necessary protective and adaptive mechanisms are not present (Sun, 2000; Berjak & Pammenter, 2002). When considered in relation to orthodox seeds, desiccation sensitivity may be attributed to the absence or dysfunction of one or more of the processes that confer the ability to withstand dehydration (Pammenter & Berjak, 1999). As water content and ψ continue to drop through hydration level III, essential intracellular events become increasingly curtailed, leading to unregulated metabolism (Vertucci & Farrant, 1995; Walters *et al*., 2005a; Berjak, 2006). If the seed tissues do not possess the mechanism(s) to counteract the stress incurred by the removal of water in this hydration level – which is the case with recalcitrant material – either of the following two categories of damage may be

endured: (1) mechanical damage due to a reduction in cell volume; (2) 'metabolism-induced damage', which involves aqueous-based degradative processes, and is dependent on both metabolic processes and rate of dehydration. In hydration level II (water contents generally ≤ 0.25 g g^{-1}; ψ approaching -100 MPa) desiccation damage *sensu stricto* is held to occur. This refers to damage incurred when structure-associated (bound) water that is required to maintain intracellular integrity, is removed (Pammenter *et al.*, 1994; 1998; Pammenter & Berjak, 1999; Walters *et al.*, 2005a; Berjak, 2006; Berjak *et al.*, 2007; Fig.1.3).

The response to desiccation of recalcitrant seeds is influenced not only by the inherent characteristics of individual species but also by their developmental status and the method and rate of drying (Berjak & Pammenter, 2002; Song *et al*., 2003). The variability shown by recalcitrant seeds among species and within species extends to water content at shedding, the extent to which dehydration can be tolerated, the response to drying rate, storage lifespan under hydrated conditions and response to low temperature (Pammenter & Berjak, 2000). Therefore it is an over-simplification to categorise seeds simply as being recalcitrant, only on the general phenomenon of desiccation sensitivity. Based on the wide range of post-harvest responses, it is not possible to generalise about common critical water contents below which viability will be lost for seeds of any species. This makes the development of any storage protocol (whether by cryopreservation or not) a time-consuming and difficult task.

There are no successful means for storage of intact recalcitrant seeds for long-term preservation of germplasm (FAO, 2006). They cannot be even moderately dried, as this has been shown to diminish storage longevity (Eggers *et al.*, 2007), neither can they be stored at sub-zero temperatures without lethal intracellular ice formation. Because of their large size (small surface area:volume ratio), intact recalcitrant seeds cannot be dried sufficiently fast to avoid lethal metabolism-linked damage by water contents around 0.8 g g^{-1} on a dry mass basis (Pammenter *et al.*, 1994; 1998). However, the embryonic axes (and embryos, in endospermous types) of recalcitrant seeds of most species constitute only an insignificant fraction of the mass and volume of the entire seed (Berjak & Pammenter, 2004). Since axes/embryos can be rapidly dehydrated, metabolism-linked damage is curtailed (Pammenter *et al.*, 2002), these explants can be dried to sufficiently low water contents for cryopreservation, facilitating the opportunity for *in vitro* conservation (Hong & Ellis, 1990; Pammenter & Berjak, 1999; Berjak & Pammenter 2004; 2008). Excised embryonic axes were thus the explants of choice for the three recalcitrant-seeded species presently explored in this investigation.

Embryonic axes, like other explants (e.g. meristems) to be cryopreserved, contain considerable free solution water intracellularly, which would normally form ice crystals upon exposure to cryogenic temperatures. To avoid lethal intracellular ice formation it is, therefore, crucial for explants to be dehydrated to appropriate water contents for cryopreservation. The following section considers dehydration in the context of desiccation sensitivity, and the implications for cryopreservation procedures.

1.5 Dehydration and associated phenomena implicated in desiccation tolerance: relevance to cryopreservation of recalcitrant-seeded species

Two considerations are necessary in the context of desiccation-tolerant systems, the first being the processes affected when water is removed through the various hydration levels, and the second being the interplay of mechanisms that counteract stress caused by water loss, thus bypassing dehydration damage. These considerations have implications for the development of protocols for cryopreservation of the germplasm of species having desiccation sensitive seeds.

Desiccation tolerance can be defined as the "ability of an organism to equilibrate its internal water potential with that of moderately dry air, resuming normal functioning upon rehydration" (Alpert, 2000). This necessitates an efficient suite of mechanisms to counteract stress induced by extreme water deficits, and within the Kingdom Plantae, this characteristic is not inherent in vegetative tissues of any gymnosperms or tree species thus far studied (Alpert, 2000). However, this is not invariably the case for the seeds, which are variously desiccation-tolerant or -sensitive, depending on the species. Seeds of the three tree species investigated in this study have been shown to be desiccation sensitive (Kioko *et al*., 1998, 2005; Goveia *et al*., 2004; Naidoo *et al*., 2011). It is therefore appropriate to consider the physiological events associated with sequential removal of water from specific hydration levels when tissue is dehydrated, as well as the means by which imposed water stresses are counteracted in desiccation-tolerant tissues. For the purpose of this introduction, a brief overview of the major mechanisms entrained during drying of tolerant tissue is presented, while the presence and efficient operation of repair mechanisms will be more fully discussed in Chapter 3.

1.5.1 Hydration levels and the corresponding properties of water in seed tissue

Vertucci (1990) originally defined the five hydration levels of seed tissues, each one associated with a specific water content/concentration range (the amount of water present)

and range of water potential. At high water contents (ranges of water content and activities presented below) the properties of water are characteristic of a dilute solution, thus permitting normal metabolism and germinative events to occur. As tissue is dehydrated, there is an increasingly strong interaction between water and solutes, causing the water to take on properties characteristic of a concentrated solution, at which point the system deviates from 'ideal' behaviour. Upon further dehydration, water takes on the properties of a viscous or glassy solution until eventually, extensive dehydration translates into very low water contents and potentials (as in orthodox seeds), where remaining water is now intimately associated with macromolecules and is now referred to as 'bound' or structure-associated water. A summary of water content (g g^{-1} dry mass basis), water ψ and normal and abnormal metabolic activities concomitant with each hydration level is given below and illustrated in Figure 1.3 (Berjak, 2006; Berjak *et al.*, 2007):

- *Hydration level V (wc:>0.70 g g^{-1}; ψ ≥-1 MPa): Full metabolism; cell division; germination*
- *Hydration level IV (wc:0.45-0.70 g g^{-1}; ψ ≤-1 MPa):Continuing protein and nucleic acid synthesis* (Pammenter & Berjak, 1999)
- *Hydration level III (wc:0.25-0.45 g g^{-1}; ψ ≤~-10 MPa):Metabolism-induced desiccation damage; respiration; unregulated metabolism; free radical production via metabolism; membrane appression*
- *Hydration level II (wc:0.08-0.25 g .g^{-1}; ψ ~-100 MPa): Catabolic activity via enzymes; anaerobic respiration; alcohols emitted; Maillard reactions; enzyme degradation*
- *Hydration level I (wc:>0.08 g g^{-1}; ψ down to -1000 MPa): photoreceptors functional; free radical production via auto-oxidation; carbonyls emitted; protein and membrane structure de-stabilised; increased rate of dry seed degeneration (ageing); declining glass integrity*

Embryonic axes excised from recalcitrant seeds need to be dehydrated rapidly to curtail metabolism-linked damage (Pammenter *et al.*, 1998; 2002) if they are to be amenable to exposure to cryogenic temperatures with no, or minimal, ice-crystallisation occurring. In the absence of protective mechanisms, removal of water through specific hydration levels will induce the associated degradative processes detailed above. The mechanisms entrained in orthodox seeds which mitigate against dehydration damage will be reviewed below, with the

intention of highlighting why the absence or deficiency of one or more of these events can lead to cell death at the dehydration step of cryogenic procedure.

1.5.2 Mechanisms which enable maturation drying and survival in the desiccated state, of orthodox seeds

Intracellular physical characteristics: Iljin (1957) was the first to suggest that a major requirement involved in desiccation tolerance is the ability to withstand mechanical stress that accompanies volume reduction upon dehydration. Thereafter, others (Bewley, 1979; Farrant *et al*., 1997; Farrant & Walters, 1998; Pammenter & Berjak, 1999; Berjak & Pammenter, 2002) provided evidence to support this when they showed decreased vacuolation to correspond with an increased tolerance to desiccation. Studies on an orthodox, and two recalcitrant-seeded species differing in desiccation sensitivity showed that in the orthodox seeds, vacuolar volume was reduced to an insignificant proportion by shrinkage of space that is usually occupied by fluid-filled organelles, or by insoluble reserve accumulation (Farrant *et al*., 1997; Berjak & Pammenter, 2002), which counteracted the physical stress incurred by cells due to shrinkage (Pérez *et al*., 2012). In the recalcitrant species, vacuolation was directly correlated and insoluble reserve accumulation inversely correlated to the degree of desiccation sensitivity as initially suggested by Iljin (1957).

The reaction of the cytoskeleton to dehydration plays an integral role in the structural maintenance of cells. Among other functions, the cytoskeleton, which is composed of extensive microfilamentous and microtubular systems, imposes organisation on the cytoplasm and provides external support for the nucleus (Gehringer, 2012). Upon dehydration of recalcitrant seeds or axes, the cytoskeletal network dismantles and is unable to reassociate upon rehydration as shown in embryonic axes of *Quercus robur* by Mycock *et al*. (2000) and *Trichilia dregeana* (Gumede *et al*., 2003). In orthodox systems there is an orderly reassembly of the cytoskeleton and normal intracellular support and structural organisation is resumed upon rehydration e.g. displayed in *Medicago truncatula* seeds (Faria *et al*., 2005).

A third fundamental mechanism that orthodox seeds must possess is the ability to maintain the integrity and stability of the chromatin network throughout dehydration and rehydration of the tissue (Osborne & Boubriak, 1994). In orthodox seeds, chromatin is highly condensed which is a state that is maintained during early rehydration (Pammenter & Berjak, 1999). Upon germination, DNA is decondensed and replicated, coinciding with the time that cells become susceptible to desiccation damage (Deltour & Jacqmard, 1974; Crèvecoeur *et al*., 1988). Therefore, orderly reversible chromatin compaction and the

maintenance of the integrity of genetic material are associated with the desiccation-tolerant state and the inability to entrain this reversible process is presumably associated with the desiccation-sensitive state.

Intracellular de-differentiation: De-differentiation is a necessary process occurring in desiccation tolerant seeds preceding maturation drying (Pammenter & Berjak, 2000). In this process, intracellular membranous structures are minimised (e.g. the ER), or reduced to the most basic form (e.g. mitochondria), as membranes are susceptible to dehydration damage (Bewley, 1979; Bergtrom *et al*., 1982; Senaratna *et al*., 1984; Vertucci & Farrant, 1995; Pearce, 2001). In orthodox seeds, these processes were found to be completely reversible upon water uptake during early germination (Bewley, 1979). A consideration of mitochondria across an orthodox-seeded species (*Phaseolus vulgaris*) and two recalcitrant types, one temperate (*Aesculus hippocastanum*) and the other tropical (*Avicennia marina*), showed two trends (Farrant *et al*., 1997). Firstly, the mitochondria occupied a significantly small proportion of the entire cell volume in embryo cells of *P. vulgaris*, compared with the situation in both *A. hippocastanum* and *A. marina*. Secondly, the mitochondria present in the orthodox seed embryo tissue were highly de-differentiated prior to the onset of maturation drying, when tissue water content was still high, whereas the recalcitrant embryos in mature recalcitrant seeds of both species showed mitochondria that had well-structured cristae, typical of metabolically-active, hydrated tissue. Based on this it seems that the ability for orderly de-differentiation is necessary for survival in a state of dehydration, while the differentiated, well-developed state of these organelles is able to be correlated with a heightened state of desiccation sensitivity (Pammenter & Berjak, 2000).

Metabolic 'switch off': In the intermediate water content range consistent with the third hydration level, unregulated generation of metabolism-induced free radicals occurs (Leprince *et al*., 1996; 1999; 2000; Fig. 1.3). In desiccation-tolerant seeds, metabolic switch off and the operation of antioxidant systems during the maturation drying phase allows for the tissues to pass through these intermediate water contents with minimal damage. An early study showed that one of the ways in which orthodox seeds can tolerate water loss is a decline in respiration rate before maturation drying, associated with a decline in respiratory substrates (Rogerson & Matthews, 1977), and Farrant *et al*. (1997) correlated the decrease in respiration rate with de-differentiation of mitochondria, also prior to maturation drying in embryo tissues of orthodox *P. vulgaris* seeds.

It was later shown that if recalcitrant material is rapidly dehydrated, the metabolism-induced damage presumably occurring predominantly in the intermediate water content range

(third hydration level) can be minimised, and survival at low water contents can be achieved. The outcome of air-drying excised axes of recalcitrant *Hevea brasiliensis* in a laminar air-flow was first reported by Normah *et al*., (1986), and was followed by development of the more efficient flash drying technique (Berjak *et al*., 1990; Pammenter *et al.*, 2002) for rapid dehydration of recalcitrant embryos/embryonic axes. A by-product of continued respiration and consequently active metabolism during dehydration, which is characteristic of most recalcitrant material (Berjak & Pammenter, 2008), is the accumulation of high-energy intermediates that leak out of mitochondria and plastids forming reactive oxygen species (ROS) (Benson, 1990; Hendry, 1993; Smirnoff, 1993). While rapid dehydration may allow for cryopreservation of axes/embryos of recalcitrant-seeded species by minimising the effects of ongoing metabolism, some free radical-mediated damage still occurs in relation to unbalanced metabolism (Walters *et al*., 2002). The importance of drying rate and desiccation-induced injury will be discussed in Chapter 2 while the consequences of unregulated ROS production and metabolism-derived damage will be detailed in Chapter 3.

Accumulation and roles of putatively protective molecules: Late Embryogenic Accumulating/Abundant Proteins (LEAs) are hydrophilic, heat-resistant proteins that have been strongly associated with abiotic stress tolerance, and specifically desiccation and cold stress (Tunnacliffe & Wise, 2007). The precise function of LEAs in the acquisition of desiccation tolerance, however, remains unclear: nevertheless, their characteristic nature and particular set of conditions under which they appear have strongly suggested them to have protective functions. In particular, it has been suggested that LEAs possibly stabilise subcellular structures by inhibiting denaturation of a range of macromolecules in the desiccated state and buffering water loss during maturation drying (Bray, 1993; Farrant *et al*., 1996; Tunnacliffe & Wise, 2007; Battaglia *et al*., 2008).Various investigations on species across the continuum of post-harvest behaviour have not been conclusive about the presence or absence of LEAs in the context of seed desiccation-sensitivity or –tolerance of particular species, e.g. *A. marina* (Farrant *et al*., 1992; 1993); *Arabidopsis thaliana* (Illing *et al*., 2005) and *M. truncatula* (Boudet *et al*., 2006). However, studies have shown that while the presence of LEAs (often from studies on dehydrins, which do not typify LEAs in general (Kosová *et al*., 2010) cannot alone confer desiccation tolerance; their presence, does appear to contribute to desiccation tolerance when implicated in relation to other processes and mechanisms (Close, 1996; Farrant *et al*., 1996; Cumming, 1999; Farrant & Moore, 2011). In this regard, it has further been suggested that LEAs act synergistically with sucrose during the formation of the glassy matrix in cells of desiccation-tolerant tissues, and this association

might form the 'backbones' of intracellular glasses at water contents ≤0.3 g g^{-1} (Wolkers *et al*., 2001; Goyal *et al*., 2003; Berjak, 2006).

The presence of non-reducing sugars, in the form of sucrose, oligosaccharides or galactosyl cyclitols and other cytomatrical constituent molecules (Walters, 1998) was originally implicated in the maintenance of desiccation-tolerant state in orthodox seeds in two major ways, *viz*. their role in replacing water (Clegg, 1986), and in the formation of the glassy state (Koster & Leopold, 1988). In terms of metastable glass formation which occurs at water contents ≤ 0.3 g g^{-1} in orthodox seeds, these sugars contribute to the high-viscosity, vitrified amorphous solutions (Obendorf, 1997). Bryant *et al*. (2001) described two effects on membrane lipid phase transition during dehydration. First, those authors proposed that the small solutes such as sugars between contiguous membranes can act by reducing the closeness of membranes (acting as a physical buffer) and thereby reduce the compressive stresses incurred during dehydration. The related increase in fluid-to-gel transition temperature that occurs in the absence of sugars is not as high when these small solutes are present (Bryant *et al*., 2001). Secondly, in the event of solutes vitrifying while they are between bilayers, the mechanical resistance offered by the glass will deter further transitions between the fluid to gel phase. Tentative suggestions by Buitink & Leprince (2004) viewed the function of sugars in a different light, those authors proposing that sugars increase the density of glasses by filling in free volume between large molecules. What has received prominence recently, however, is the implication of the ratio of sucrose:oligosaccharides/galactosyl cyclitols in maintaining viability in the dry state (Obendorf, 1997; Hoekstra *et al*., 2001; Peterbauer & Richter, 2001; Mello *et al*., 2010). The vitrified or glassy state was originally suggested to confer protection against denaturation of macromolecules and phase changes of the lipid bilayers of membranes by Leopold *et al*. (1994). It was later suggested that the incorporation of LEAs in the intracellular glasses state might play a role in the control and optimisation of the rate of water loss during dehydration of orthodox seeds (Manfre *et al*., 2009), and, as mentioned above, there is evidence that the concentration of sugars may function to avoid lateral appression of membranes (Bryant & Wolfe, 1992; Bryant *et al*., 2001). However, regardless of the role of these sugars in desiccation-tolerant systems, it is inconsequential to draw parallels with recalcitrant systems as vitrification will not occur until water contents are ≤ 0.3. g g^{-1}, which is significantly below the lethal water content (~0.8 g g^{-1} [Pammenter *et al.,* 1994]) for slowly-dried recalcitrant seeds.

Presence and optimal functioning of antioxidant systems: As illustrated in Fig. 1.3, free radical production via unregulated metabolism occurs within the intermediate water content range (hydration level III; 0.25-0.45 g g^{-1}), and must be counteracted by appropriate antioxidant systems in desiccation-tolerant tissues. This must also be the case when orthodox seeds are exposed to water and germination proceeds, as there is a physiological switch from tolerance to sensitivity (Senaratna & McKersie, 1986; Vertucci & Farrant, 1995). On a gene expression level, studies conducted on *M. truncatula* seeds, showed common changes in the transcriptome associated with the acquisition, and re-establishment, of desiccation tolerance (re-establishment occurring upon osmotic treatment of germinated radicals): the changes included up-regulation of stress defence genes; the down-regulation of DNA processing and primary metabolism (Hoekstra *et al*., 2001; Buitink *et al*., 2006) and the immobilisation of the cytoplasm in a stable glassy matrix (Hoekstra *et al*., 2001). In the context of desiccation tolerance, it is during the phases of maturation drying on the one hand, and rehydration on the other, that the presence and optimum functioning of antioxidant or free-radical scavenging systems is imperative (Kranner & Grill, 1993; Kranner *et al*., 2002; Bailly, 2004; Kranner & Birtić, 2005). There are, however, many suggestions that loss of viability accompanying dehydration, and upon rehydration, of desiccation-sensitive (recalcitrant) material occurs as a consequence of an increase in free-radical/ROS production associated with oxidative metabolic processes which are essentially unregulated (Benson, 1990; Hendry, 1993; Smirnoff, 1993; Hoekstra *et al*., 2001; Varghese & Naithani, 2002; Bailly, 2004; Apel & Hirt, 2004; Kranner & Birtić, 2005; Berjak & Pammenter, 2008; Varghese *et al.*, 2011). Since ROS-associated damage does occur upon dehydration of recalcitrant material (Benson, 1990; Bailly, 2004; Kranner & Birtić, 2005; Whitaker *et al*., 2010; Varghese *et al.*, 2011), it seems that the combined effect of free radical generation and the inadequacy of antioxidant mechanisms during both dehydration and rehydration, are major factors in the desiccation sensitivity of recalcitrant seeds. Free radical/ROS generation and antioxidant responses to the stresses imposed by the cryopreservation process will be detailed in Chapter 3.

1.6 Species investigated

Three species indigenous to South Africa were the focus of this study.

1.6.1 *Trichila emetica* Vahl.

This is the first of two species studied belonging to the family Meliaceae and is typically located in southern and eastern parts of Africa, with many trees usually occurring in

the same vicinity. The genus name *Trichilia* has its roots in the Greek "*tricho*" meaning "in three parts", referring to the often 3-celled fruits. The specific epithet, *emetica*, refers to the traditional use of the powdered bark as an emetic (Schmidt *et al*., 2002; Pooley, 1993). In South Africa it is commonly known as the Natal Mahogany and bears a striking resemblance to its sister species, *Trichilia dregeana* (see below). *Trichilia emetica* trees are evergreen to briefly deciduous and medium to large, reaching heights of 5-10 m (Pooley, 1993). They are single-trunked with a dark brown bark and have low branches forming a rounded canopy of dark green foliage. The leaves are compound (imparripinate) with a terminal leaflet and rounded tips, alternate or opposite and are dull green with hairs protruding from the under-surface. Flowers are usually borne from August to November and are creamy to silvery-green and velvety. The characteristic fruits, which usually occur in dense clusters, are 25-30 mm in diameter and creamy green turning pale brown when ripe, with each fruit having a long stipe connecting it to the stalk. When mature, fruits usually split into three segments to reveal 3-6 large, black seeds (depending on the size of the capsule) each incompletely covered by a fleshy, brilliant scarlet aril (Fig. 1.4). The fruiting season of *T. emetica* is relatively short, usually occurring between mid-December and the latter part of January in Kwa Zulu-Natal, but availability varies from season to season.

Fruits of this species are eaten by baboons and some antelopes (e.g. nyala) while seeds are consumed by various species of birds. In terms of domestic uses, wood of *T. emetica* is utilised in the carving industry in rural southern Africa. Many traditional items such as bowls (*iziMbenge*), dishes for meat (*uGqoko*), spoons (*iziNkhezo*) and head rests (*iziGqiki*) have been carved from the wood of the Natal Mahogany (Grant & Thomas, 1998). Seeds can be pounded and boiled in water which is then skimmed to obtain an odourless oil used to manufacture soap and cosmetic products, and the kernel remains can be used in the production of candles (van Wyk & Gericke, 2000). The seed aril is used for cooking and to make a milky suspension often eaten with spinach (Pooley, 1993). Parts of *T. emetica* are also extensively harvested for traditional medicine. The bark is mainly employed as a common cure for stomach and intestinal complaints. Other complaints reportedly remediated by parts of the plant include dysentry, kidney problems, indigestion and parasites, and leaf or fruit poultices are applied to soothe bruises and eczema (van Wyk & Gericke, 2000).

Trichilia emetica typifies many tropical species producing recalcitrant seeds in that it has exceedingly high exploitation and extinction rates (Palmer & Pitman, 1972). Seeds are generally shed at an average axis water concentration of 2.8 g g^{-1} and lose viability upon dehydration (Kioko *et al*., 2005). Many attempts have been made in our laboratory to

cryopreserve the excised embryonic axes of this species, but hitherto without success. However, based on reviewed studies, there is some insight about the responses of the embryonic axis to various cryogenic stresses (Kioko, 2003) as well as the viability and ultrastructural responses of seeds and embryonic axes to different dehydration and storage conditions (Kioko *et al*., 2005). While the species is not considered threatened, wild populations have rarely been encountered.

Fig. 1.4: Freshly harvested seeds of *Trichila emetica*, covered by fleshy aril. Scale bar = 10 mm.

1.6.2 *Trichilia dregeana* Sond.

Trichilia dregeana, is commonly known as the Forest Mahogany in KwaZulu-Natal and is distributed in the same locality as *T. emetica*. The specific name, *dregeana*, honours the German collector and botanical explorer, Johan Franz Dregè who collected specimens of this species when visiting South Africa. Its overall appearance is very similar to that of *T. emetica* but there is a difference in height (10-40m) and seed size, which is noticeably bigger in *T. dregeana*. A further distinguishing feature of *T. dregeana* is the leaves, which are shiny, have an almost hairless under-surface and pointed, rather than rounded, tips. The fruit and seeds of this species (Fig. 1.5) are consumed by many birds while the bright scarlet aril is eaten by baboons (Schmidt *et al*., 2002).

The wood of *T. dregeana* is also used in the rural carving industry and the seeds can be cooked as a vegetable once the aril has been removed (van Wyk & Gericke, 2000). Like *T. emetica*, the oil contained in the seeds can be used for industrial purposes (Mulholland & Taylor, 1980; Schmidt *et al*., 2002). Five limonoids have been isolated from the seeds, which include trichilin A and dregeanin, both of which are known to have insect repellent properties

(Hutchings *et al*., 1996) and may contribute to the medicinal nature of this species. With regard to its medicinal properties, the bark is exploited mainly for its purgative effects and is also used in concoctions to treat backache and kidney ailments (Hutchings *et al*., 1996). The use of leaves for the treatment of syphilis is quite common in Nigeria, and aqueous leaf extracts have been shown to have some antimicrobial properties (Hutchings *et al*., 1996).

In 1990, Choinski reported that the seeds of *T. dregeana* were recalcitrant and chilling-sensitive. Since then many investigations have been conducted resulting in in-depth characterisation of the seeds, as well as attempts to develop a protocol for their cryopreservation (Kioko, 2003; Goveia, 2007). Some of the published work on this species includes strategies for their field collection (e.g. Berjak *et al*., 2004), the potential for sub-imbibed storage (Drew *et al*., 2000), the effect of dehydration on the nucleoskeleton (Anguelova-Merhar *et al*., 2003), the possible association of dehydrin type molecules on their desiccation sensitivity (e.g. Han *et al*., 1997), the effect of developmental status on the explant choice for cryopreservation (e.g. Goveia *et al*., 2004), the effect of desiccation and cooling on survival of *T. dregeana* embryonic axes (Kioko *et al*., 1998); the production of reactive oxygen species by excised, desiccated and cryopreserved axes (Whitaker *et al*., 2010) and the effect of differential drying rates of embryonic axes on survival and oxidative stress metabolism (Varghese *et al*., 2011). Present work is focused on promoting shoot development after each step in the cryo-procedure, as well as assessing free radical generation and the antioxidant systems at each stage.

Fig. 1.5: Freshly harvested fruit and seeds of *Trichilia dregeana*. Scale bar = 10mm.

1.6.3 *Protorhus longifolia* (Bernh.) Engl.

Protorhus longifolia is a medium sized, evergreen tree commonly known as Red Beech, of the family, Anacardiaceae. There is little information available on *P. longifolia*, with only a few studies having being conducted, mainly on the morphological structure and

taxanomic significance (Teichman, 1991). The tree is identifiable by its simple opposite leaves, creamy-green very small flowers comprising much-branched inflorescences up to 120 mm long. Seeds (Fig. 1.7) are pale green during early development becoming mauve to purple when ripe. The pericarp is fleshy and the embryonic axis, unlike the other two species investigated, is not enclosed within the cotyledons. Fruits usually occur clustered on short branches and the season is short – from mid November to late December. These seeds are very short-lived in hydrated storage as they germinate rapidly and are generally contaminated, hence the need to be used for experimentation immediately after collection.

The fruits are eaten by birds and monkeys, while black rhino are recorded as eating bark and leaves of this species (Pooley, 1993). That author also notes that the 'milky sap' is used to remove hair and that, although the powdered bark is reputedly poisonous, it is also used medicinally, the preparation being known as *umuthi embomvu* in isiZulu. The wood of *P. longifolia*, described as being fairly hard and fine-grained, is used for furniture and planking (Pooley, 1993).

Work in our laboratory has shown the seeds of *P. longifolia* to display the typical recalcitrance characteristics of germination in hydrated storage without provision of any extraneous water, and desiccation- and chilling-sensitivity. The main aim of the study on the seeds of this species was to optimise drying and cooling methodology for cryopreservation of the embryonic axes, with the objective of obtaining shoot development (i.e. seedlings developing both a root *and* a shoot) after exposure to cryogenic stresses.

Fig. 1.6: Mature seeds of *Protorhus longifolia*. Scale bar = 10 mm.

1.7 Rationale for the present study

With the objective of conserving genetic resources of recalcitrant-seeded species, many attempts and successes from work thus far in our laboratory have shown that protocols devised to preserve such germplasm are firstly, species-specific. In the second place, these ventures have sought to explain the underlying causes of failure of axes to produce seedlings (or plantlets in the case of vegetative explants) after cryopreservation. The current investigation aimed primarily not only to optimise protocols to cryopreserve the zygotic germplasm of the three species of interest, but also to examine the underlying causes of reduced viability and/or failure of shoot production accompanying steps in the cryopreservation procedure, particularly in the context of associated oxidative stresses. It was also anticipated that the studies would contribute further understanding of seed recalcitrance.

Chapter 2 details investigations on pre-conditioning, desiccation and cooling procedures on axes of the three species. The main objective was to obtain shoot development or, at least, survival in the form of root development after manipulations preceding, and following, cryopreservation. This chapter involved the provision of exogenous antioxidants before cryogenic cooling, in the context of ameliorating unregulated free radical (ROS) production.

Chapter 3 addresses one of the persistent inadequacies of much of the work on cryopreservation, i.e. lack of analysis of parameters that may underlie successes and/or failures of particular protocols. This Chapter, therefore, reports on free radical-/ROS-associated injury in the context of stresses associated with the cryo-protocol, as well on as the related endogenous antioxidant activity. The production of three ROS is reported, as is total antioxidant activity in the presence and absence of various exogenously applied antioxidants, in the context of the various steps of the cryopreservation procedure. This Chapter therefore provides an overview of oxidative metabolism in relation to viability retention and cryo-induced damage.

Chapter 4 draws together critical findings of the study, and provides recommendations for taking the investigations further.

CHAPTER 2: Cryopreservation

2.1 Introduction

2.1.1 The freezing process

Prior to developing a cryopreservation protocol, it is vital to understand the principles upon which freezing of living matter is based. Once reviewed, one can better conceptualise the processes of cryoprotection, dehydration and cryo-induced injury. Water possesses unique physico-chemical properties that play a fundamental role in cell functioning. As manipulations necessitated by cryopreservation largely influence the states of water, it is necessary to emphasise the relationship between these states, with a focus on the behaviour of water at low temperatures.

Phase changes refer to discrete changes in the properties of water at specific chemical potentials (Atkins, 1982). Importantly, the processes of cryopreservation involve manipulation of water contents of the explants and of temperature to which explants are exposed, and both of these variables influence the phases of water (Meryman, 1966; Mazur, 1970; Ozkavakcu & Erdemli, 2002). Three phases of water are possible in a system where solutes, including sugars, and water co-occur, *viz*. water in the liquid state – i.e. solution water, ice and glass (super-viscous liquid; Mazur, 1970). While liquid water and ice represent equilibrium phases, glasses, which are metastable revert **–** albeit slowly **–** back to water or ice depending on temperature (Mazur, 1970). When considering the interaction between solutes and water, it is important to note that as solute concentration increases, the temperature at which water freezes declines, a phenomenon known as freezing point depression. During the water to ice phase change, what is important in cryobiology is ice nucleation (either by heterogeneous seeding or homogeneous nucleation), and cooling rate. It is popularly believed that water freezes at 0°C; however, water can be 'supercooled' to far lower temperatures and then manipulated to freeze via ice-nucleating agents (Benson *et al*., 2005). As long as cell contents remain supercooled, a driving force is provided for water to leave the cell and freeze at nucleation sites externally: effectively, therefore, the cells become dehydrated during cooling (Mazur, 1984). The rate and extent of dehydration depends on the inherent permeability of the cell membrane to water, and the cooling rate (Mazur, 2004).

During the freezing process at -40°C, homogenous ice nucleation occurs as a consequence of molecular fluctuations in supercooled liquid, which leads to the initiation of intracellular ice (ice embryo) formation (Benson *et al*., 2005; Gonzalez-Arnao *et al*., 2008). Once ice embryos reach a critical size, they form ice crystals (Mazur, 1970) and ice will

continue to form as temperature declines until a eutectic point is reached (Muldrew *et al*., 2004). In all biological systems, heterogeneous ice-nucleation occurs. This refers to the nucleation of ice in a supercooled aqueous solution, catalysed by nucleating agents (surfaces or particles) and this process occurs above the temperature of homogenous ice-nucleation (Meryman, 1966; Wilson *et al*., 2003; Benson *et al*., 2005; Mullen & Critser, 2007).

Unlike the crystalline phase, glasses do not have a regular structure, being described as amorphous solids (Walters, 1998). Nevertheless, as described by that author, molecules comprising a glass are interconnected, which results in limited mobility compared with a liquid. Glasses can be formed in two ways: (1) By increasing the concentration of a solution so that the glass transition temperature, T_g, is higher than the freezing temperature. Under these circumstances, when the concentrated solution is cooled a stable glass is formed. (2) A glass may be formed by cooling a dilute solution below the glass transition temperature at an ultra-rapid rate, so that ice crystals do not have adequate time to grow (Franks, 1982; Vertucci, 1993). According to Mazur (2004), a major determinant of cell survival at sub-zero temperatures is the cooling rate, and plots of survival usually take the form of an 'inverted u'. This translates as maximum survival of cells occurring at an intermediate cooling rate, few cells surviving when cooling rate is too low and even fewer surviving when cooling rate is too high.

The formation and stability of glasses during the cooling process has been implicated in the prevention of desiccation and freezing damage (Dumet & Benson, 2000). Ideally, a successful cryopreservation protocol should aim at achieving intracellular vitrification – i.e. glass formation.

2.1.2 Cryo-induced injury

Processes involved in cryopreservation impose severe stress on embryonic axes, with numerous studies showing cell death to occur frequently during dehydration and cooling of the tissue (Normah *et al*., 1986; Wesley-Smith *et al*., 1992; Kioko *et al*., 1998). However, because of the high water contents of axes of recalcitrant seeds, dehydration is imperative for survival of cooling (Engelmann, 1992; 2004; 2011; Kaviani, 2011). Life processes necessitate the presence of water in the internal environment of all living organisms; however, the amount of water required to sustain structure, and by implication, resumption of function, differs across biological systems (Aguilera & Karel, 1997). Therefore, in preparation for cryogen exposure, the proportion of water removed from any explant tissue requires careful optimisation, such that water content is high enough for intracellular

structures to remain intact but low enough to avoid intracellular ice crystallisation upon cooling of the tissue.

In terms of dehydration-induced damage, Pammenter & Berjak (2000) envisaged three categories of damage to occur upon dehydration of recalcitrant embryonic axes. The first involves mechanical or physical damage to cells that is associated with a reduction in cellular volume; the second concerns aqueous-based oxidative degradation consequent upon unregulated oxidative metabolism (occurring at intermediate water contents); and the third, biophysical damage to macromolecules occurring at low water contents once structure-associated water is perturbed. An important point made, is that the response to dehydration is influenced largely by metabolic activity and drying rate (Pammenter & Berjak, 2000).

Aqueous-based metabolic reactions associated with intermediate water contents are seen as becoming deranged, with consequent free-radical-mediated oxidation processes occurring when tissues remain at these water contents for extended periods (Vertucci & Farrant, 1995; Walters *et al*., 2005a; Berjak, 2006 [refer to Fig. 1.3]). In orthodox seed tissues, such oxidative damage is curbed by protective mechanisms such as the switching-off of metabolism, de-differentiation of membranous organelles and adequate antioxidant systems, all of which are present and functional (Pammenter & Berjak, 1999). However, this is not the case for embryos of recalcitrant seeds, as discussed by those authors and others (reviewed in Chapter 1).

To appreciate the influence of drying rate on damage incurred by tissue, one must note that many reactions require the medium of water in which to occur (Simione, 1998). Therefore, the slower the drying rate, the longer the time in which aqueous-based degradative processes within the cell can occur and their products accumulate (Pammenter *et al.*, 1998; Pammenter & Berjak, 1999). Based on this, many cryopreservation protocols aim to dry tissue rapidly to minimise the time spent at intermediate water contents thereby reducing the occurrence of damage (Pammenter & Berjak, 1999; Pammenter & Berjak, 2000; Pammenter *et al.*, 2002). When embryos are dried, water molecules diffuse from the tissue into the surrounding atmosphere, with a number of factors influencing this rate of diffusion (to be discussed under dehydration; Pammenter *et al*., 2002). However, regardless of the drying rate, there does exist a lower water content limit below which desiccation-sensitive tissue will not survive. Dehydrating such material to water contents near, or below, this limit leads to damage incurred as a result of biophysical disruptions, probably when water is removed from macromolecular or membrane surfaces (Pammenter & Berjak, 2000), such damage being termed desiccation damage *sensu stricto* (Pammenter *et al*., 1998; Walters *et al.*, 2001).

Removal of a considerable proportion of structure-associated water occurs naturally in orthodox seed tissues, where protective mechanisms must mitigate against this type of damage. It is possible (although not established) that stress-associated proteins and LEAs serve this function, and vitrification of cell contents at such low water contents certainly does occur (Vertucci & Farrant, 1995; Farrant, 2000; Pammenter & Berjak, 2000; Illing *et al*., 2005).

Rate of cooling is a primary variable affecting physico-chemical events in cells, thus ultimately influencing cell survival (Mazur, 1970; Farrant *et al*., 1977). Cooling damage is a two-component process, involving both solution effects and intracellular freezing (Mazur, 1970). Solution effects comprise (at minimum) four discrete events that occur during freezing: removal of water as ice; concentration of high and low molecular weight solutes; decrease in cell volume; and solute precipitation, the first three occurring simultaneously during freezing (Mazur, 1970). Damage upon intracellular freezing has been suggested to be manifested mainly as disruption or rupturing of plasma membranes and the membranes of macro-organelles (Mazur, 1970). In summary, it is suggested that if cells are cooled too slowly, they are killed by the combined effects of excessive shrinkage, exposure to hypertonic solutions and the decline in temperature, and when cooled too rapidly death is the consequence of the formation of intracellular ice rather than shrinkage (Morris & Farrant, 1972; Farrant *et al*., 1977; Mazur, 2004). Therefore, it has been suggested that the ideal cooling rate is that which is rapid enough to minimise the length of exposure to solution effects and avoid excessive shrinkage, but not so rapid that extensive intracellular ice crystallisation occurs (Mazur, 1970; Farrant *et al*., 1977). In parallel, it has been suggested that it is the growth of intracellular ice crystals upon slow re-warming, rather than their initial formation that is lethally injurious to cells (Mazur, 1970). It should also be noted that optimum cooling rates will differ between species and tissue/cell type, mainly due to differences in sensitivity to shrinkage, permeability of the cells to water, and their surface to volume ratios (Farrant *et al*., 1977). In this regard, work in our laboratory has shown that for recalcitrant axes across species, ultra-rapid cooling, achieved by the use of nitrogen slush (-210°C) has given the best survival after retrieval from the cryogen (liquid nitrogen) and rapid re-warming (reviewed by Berjak & Pammenter, 2008; Sershen *et al.*, 2012 a; b). However, for some species we have also found that ultra-rapid cooling is not invariably the best procedure (e.g. for *Acer saccharinum* [Wesley-Smith, 2003]; and *Landolphia kirkii* [Kistnasamy *et al*., 2011])

Theories of freezing injury suggest that one of two things may occur during cooling: either ice crystals may pierce or tear apart the cellular membranes (mechanical damage); or there may be secondary effects of changes in the composition of the liquid phase (occurring when water freezes resulting in the concentration of solutes in the residual liquid phase; [Pegg, 1995]). The use of cryoprotectants has implications for the latter type of damage and will be discussed later (see pre-conditioning). Intracellular and extracellular freezing impose damage by different mechanisms, with the latter creating mechanical stress on densely packed cells causing damage to their external structure (Pegg, 1995). However, as *caveat*, it must be realised that many of the generalisations cited here resulted from work on animal cells and the presence of plant cell walls may make a difference.

While every step in cryopreservation procedure has possible injurious repercussions, stress-induced injury incurred as a consequence of dehydration and cooling of tissue is possibly the most damaging. The development of a protocol for each species requires individual optimisation of various steps. Each of these, together with problems generally associated with them, will be detailed below.

2.1.3 Development of a cryopreservation protocol: factors influencing successful cryopreservation

2.1.3.1 Explant of choice

It is generally accepted that the most suitable explant small enough to avoid freezing injury and adequately representing the genetic diversity of a species, is the excised zygotic embryonic axis (Engelmann, 1992). Zygotic embryos are widely utilised, being representative of the genetic diversity afforded by seeds, and because of their relatively lower water content (compared with somatic embryos) and their well organised morphological structure (cotyledon/s, and embryonic axis having a clearly defined shoot and root pole). The ideal developmental stage at which zygotic embryos should be used would afford axes with an adequate number of meristematic cells to ensure survival of a functional proportion after cryopreservation, and obviously, a stage which permits ongoing development/germination upon cultivation *in vitro*. Immature zygotic embryos have the advantage of being comprised mostly of meristematic tissue and being smaller than mature embryos, but may be at a physiological stage precluding germination; also, mature embryos are generally easier to manipulate for culture. Therefore, it is common practice to use mature zygotic embryos but to reduce their size and structural complexity by severing the cotyledons, with the remaining

embryonic axis being the explant of choice. Reduction in size of the explant has direct implications on drying, cooling and re-warming rates, where a smaller explant translates into a shorter drying period when the tissue is maintained at detrimental intermediate water contents. Small explants are also more rapidly and evenly cooled. Rapid cooling of small specimens minimises the time spent in the temperature range at which nucleation (intracellular ice crystallisation) can occur, and faster re-warming similarly minimises the time for growth of ice crystals on thawing. The zygotic axes excised from a number of recalcitrant species have been successfully preserved in liquid nitrogen, for example; *Hevea brasiliensis* (Normah *et al*., 1986); *Cocos nucifera* (Chin *et al*., 1989; Assy-Bah & Engelmann, 1992); *Poncirus trifoliata* (Wesley-Smith *et al.*, 2004a;b; several *amaryllid* species (Sershen *et al*., 2007); *Landolphia kirkii* (Kistnasamy *et al*., 2011) and *Ekebergia capensis* (Hajari *et al*., 2011).

While there are definite advantages to reducing the size of the explants, excision of the fleshy cotyledons from axes of sub-tropical/tropical species often leads to failure of shoot development, as is exemplified by previous work on both *Trichilia* species (Kioko, 2003; Goveia, 2007) and *Ekebergia capensis* (Perán *et al.*, 2006). Lethal necrosis of the shoot tip results upon excision and has been correlated with the proximity of the shoot apical meristem to the site of excision in these species (Kioko *et al*., 1998; Perán *et al*., 2006; Pammenter *et al*., 2011). Several studies have demonstrated damage-associated ROS production (Wojtaszek, 1997; Grunweld *et al*., 2002; Stobbe *et al*., 2002; Kusumoto & Suzuki, 2003), and in the present investigation it was postulated that shoot development would be precluded as a consequence of an initial oxidative burst of reactive oxygen species (ROS) in response to excision wounding.

This presents a fundamental problem when cryopreserving embryonic axes, since the entire structure has to be intact in order to regenerate a seedling developing both a functional root and shoot. While studies have shown that roots can be generated from axes that have been cryogenically processed (Hajari *et al.*, 2011), shoots have not been obtained for *T. dregeana* (Kioko *et al*., 1998), and the development of a fully functional seedling/plant from the excised embryonic axes of several other species has remained elusive (Kioko *et al*., 1998; Goveia *et al*., 2004; Perán *et al*., 2006; Hajari *et al*., 2009). Shoot development fails to occur after severing of cotyledons, possibly as a consequence of unregulated free radical or reactive oxygen species (ROS) generation in response to wounding (Wojtaszek, 1997). However, other procedures involved in cryogenic processing have the potential to lead to uncontrolled ROS generation (Benson & Bremner, 2004; Roach *et al*., 2008; Whitaker *et al*., 2010), which

may also be the basis of failure of shoot development. The consequences of unregulated metabolism accompanying procedures necessary for cryopreservation will be detailed in Chapter 3. For the purpose of the initial study, which focused on the optimisation of a cryopreservation protocol, survival after cryopreservation was assessed as the regeneration of a functional plant, making it imperative that a shoot was developed after the initial step of excision.

2.1.3.2 Pre-conditioning

Pre-conditioning of embryonic axes refers to regimes applied prior to cooling, to increase survival after additive stresses (Reed, 1996). There are three categories of pre-treatment, *viz*. cultural, chemical, and dehydrative, for a range of tissue types such as shoot tips, meristems, and somatic and zygotic embryos. However, only selected pre-treatments that are applicable and have promoted growth of zygotic embryos will be discussed here.

Cultural pre-treatments involve manipulation of the growth medium on which explants are cultured for determined periods, with various osmotically-active chemicals (e.g. mannitol) growth regulators (e.g. abscisic acid) and higher concentrations of sucrose being added to the medium to enhance survival of stress (Reed, 1996). Depending on the additive, they act by dehydrating tissue or triggering resistance mechanisms associated with natural cold acclimation, rendering tissue more likely to survive freezing (Benson, 1990). Acclimation is considered as the physiological changes that occur in an organism in response to a change in a specific environmental condition(s), particularly under laboratory conditions (Nemali & Iersel, 2004). Within the context of this study, DMSO was used in the medium as a pre-conditioning antioxidant treatment prior to cotyledon excision in an attempt to acclimate tissue for the exposure to DMSO during the post-excision soaking phase. Other antioxidants were also incorporated in the cryoprotectant solutions to prepare the tissue to 'cope' metabolically with the additive stresses of drying, cooling and rehydration.

Chemical pre-treatments were explored by Nag & Street (1975) and refer to the inclusion of chemicals in media or use of chemicals immediately prior to cooling. Those authors found that pre-treating plant tissue and cells with 5% DMSO and or a 10% DMSO and glycerol solution for an hour improved survival after exposure to cryogenic conditions. Chemical pre-treatments often include the use of cryoprotectants, comprising DMSO, sugars and/or sugar alcohols, as these confer protection against freezing and thawing injury. Primarily, their mode of action is to lower the point of initial freezing, and to diminish the size of ice-crystals, while their colligative properties reduce the detrimental effects of

electrolyte concentration upon loss of liquid water when ice forms (Pegg, 1995). Cryoprotection will be discussed in detail below.

Drying or desiccation pre-treatments serve to enable recalcitrant tissue to overcome (transiently) their natural lack of resistance to drying (Pegg, 1995). Embryonic axes are usually pre-treated by drying in a laminar air-flow or dried as rapidly as possible using a flash dryer (to be discussed later). Both methods require the determination of the water content window facilitating safe cryogen exposure, while avoiding desiccation damage *sensu stricto*, which differs among species and dying rates.

The use of single or combined pre-treatments is usually necessary to facilitate survival of tissue upon exposure to the stress imposed by exposure to liquid nitrogen.

2.1.3.3 Cryoprotection

Cryoprotection is often essential to obviate injurious solute concentration effects and ice formation incurred upon cooling (Benson, 2008). In general the employment of a cryoprotective strategy has been shown to modulate the physical effects of cooling on the survival of cryopreserved germplasm (Benson, 2008). Cells, tissues and other biological structures are extremely temperature dependent and the process of freezing produces conditions that are far from those associated with normal physiological activity (Pegg, 1995). In 1949, Polge *et al.* showed the benefits of including a 10-20% glycerol pre-treatment in a cryopreservation protocol for avian spermatozoa, which resulted in prolonged survival of the cells at -80°C. Glycerol, like DMSO, enters cells – i.e. is a penetrating cryoprotectant – while sugars and sugar alcohols do not (Reed, 1996). The principal function of both penetrating and non-penetrating cryoprotectants is the reduction or prevention of intracellular ice formation via the induction of the vitrified state in cellular water (Muldrew *et al*., 2004). Colligative or penetrating cryoprotectants are able to ameliorate the detrimental effects of extensive cell volume changes (mechanical damage) and harmful toxic solution effects (chemical and osmotic effects of concentrated solutes in the residual unfrozen water between ice-crystals) upon cooling, and depress the freezing point so that nucleation is no longer injurious (Pegg, 1995; Benson, 2008). This kind of cryoprotection has three requirements, *viz*. the cryoprotective compound must remain highly soluble in water at low temperatures in order to depress the freezing temperature sufficiently; cryoprotectants must have penetrative properties; and must be non-toxic at their efficacious concentration (Pegg, 1995; Benson, 2008). Non-penetrating cryoprotective agents are also used to dehydrate cells osmotically by removing potentially freezable water (Panis & Lambardi, 2005; Benson, 2008). However

colligative cryoprotection has been shown to be more beneficial in terms of improving survival after freezing of embryonic axes. It is common practice to combine non-penetrating and penetrating cryoprotectant agents and to carry out cryoprotection through exposure of embryos to cryoprotectant-containing solid media and/or liquid solutions.

Despite the benefits of colligative cryoprotection, irreversible injury can be caused, rather than prevented, by cryoprotectants, either due directly to chemical toxicity or osmotic swelling of cells accompanying the dilution of the cryoprotective additive during and after thawing (Farrant *et al*., 1977).

An alternative approach to induce a vitrified state entails extremely rapid cooling of partially dehydrated axes such that tissue is almost instantaneously cooled to below -40°C (the temperature promoting homogenous ice nucleation), thus passing extremely quickly through the range where intracellular ice formation and growth occurs. If the use of 'traditional' cryoprotectants is employed, one should note that permeability and toxicity of cryoprotectants can be species- and cell-type specific, therefore concentration, time, temperature, and the rate of the addition and dilution of the selected cryoprotectants/s must be carefully optimised (Farrant *et al*., 1977; Benson, 2008). This notwithstanding, work in our laboratory has shown that for axes of certain tropical/sub-tropical species, cryoprotection alone, or when followed by dehydration and/or cryogen exposure, is lethal (Kioko, 2003); Kistnasamy *et al.*, 2011).

2.1.3.4 Rate of desiccation

Initial water content varies across species, seed developmental status and explant type (Engelmann, 1992) and season and provenance of collection (Berjak & Pammenter, 2008). However, regardless of water content when seeds are harvested, there is a limit to which recalcitrant germplasm will tolerate drying without dying regardless of the desiccation rate (Normah & Makeen, 2008). Often, but not invariably, the embryonic axis constitutes a very small proportion of a recalcitrant seed and when excised, can be dehydrated relatively more slowly or very rapidly in a laminar-air flow or flash dryer, respectively, until water contents amenable to cryopreservation have been reached. The concept of uneven drying must be considered here: measured axis water content is the bulk average of a variety of tissue types located at different depths from the surface, and the cells within the explant have been shown to dry at different rates (Wesley-Smith *et al*., 2001). This translates to less intensive drying and hence less damage incurred by the meristematic cells than by cortical cells during rapid drying. However, upon slow drying, there is adequate time for water content to reach

equilibrium across the various tissue types, resulting in intense, potentially injurious, drying stress also being imposed on meristematic cells (Wesley-Smith *et al*., 2001). Slower drying of axes in a laminar-flow air current is, therefore, generally not the preferred choice for embryonic axes. Additionally dehydration conditions are not always precisely controlled due to variations in ambient temperature and humidity, and are therefore often not reproducible (Engelmann, 1992; Panis & Lambardi, 2005). Cells may be more susceptible to the rate of water loss rather than to water loss *per se* (Pritchard, 1991; Berjak *et al*., 1993) and prolonged desiccation periods translate to rapid deterioration of samples due to desiccation induced injury (Engelmann, 1992; Panis & Lambardi, 2005) which is probably metabolism-linked (Pammenter *et al.*, 1998; 2002; Walters *et al.*, 2001).

Transient survival, associated with rapid rates of dehydration, can be attributed to the minimal time of exposure of explants to intermediate water contents where aqueous based metabolically-linked degradative processes occur (Pammmenter *et al*., 1991; 1998; 2002; Normah & Makeen, 2008). While this assists in curtailing the unbalanced metabolism associated with the failing ability to control the ROS generated, it does not confer the ability of explants to survive at ambient – or even refrigerator – temperature while held at low water contents (Walters *et al*., 2001). Ultra-rapid or flash drying has been shown to allow recalcitrant embryonic axes to retain viability well into hydration level 3 (Fig. 1.3), and below the water content where decline in survival is generally associated with slow water loss (Vertucci & Farrant, 1995). It is therefore considered to be an essential tool to yield explants at appropriate water contents for cryostorage.

When a cryopreservation protocol has been developed to the point where survival of germplasm can be attained after the necessary pre-conditioning treatments have been applied such that the tissue is appropriately prepared for freezing, the method and rate of cooling can be investigated.

2.1.3.5 Cooling rates and methods

The method of cooling chosen when developing a specific protocol and the mechanisms upon which it is based will depend on whether a ‘classic’ or ‘new’ mode (Engelmann, 2011) of cryopreservation is adopted. As mentioned earlier, the classic approach to cryopreservation involves freeze-induced dehydration whereas new techniques are based on vitrification.

With classic techniques, explants are slowly cooled to a determined pre-freezing temperature prior to being plunged directly into liquid nitrogen. Under optimised conditions,

almost all freezable water is removed from the cells due to differences in vapour pressure and concentration of solutes between the internal and external environment accompanying the reduction in temperature. This should serve to reduce, or avoid, lethal intracellular ice formation upon exposure to liquid nitrogen (Engelmann, 2009). This approach often involves preliminary processing, such as pre-growth treatments, cryoprotection, slow-cooling (0.5 – 2.0 °C min^{-1}) to a -40°C (or lower), immersion and storage in liquid nitrogen, followed by rapid thawing and recovery (Engelmann, 2009). While this method has been successfully applied to undifferentiated culture systems, e.g. cell suspension and calli, it is a less-used option, as slow freezing ideally requires the use of expensive programmable freezers (Engelmann, 2009).

In comparison with rapid cooling rates which may precipitate intracellular ice nucleation, slow cooling has the advantage of the formation of ice-crystals in the extracellular solution while water is removed from the intracellular one, leading to cellular dehydration and therefore avoiding intracellular ice formation (Meryman & Williams, 1985). Nevertheless, rapid cooling has been favoured in our laboratory, as it theoretically limits the formation of ice crystals and the associated damaging effects by facilitating a rapid passage through the temperature range, -2°C to -80°C. Often employed as an alternative approach to slow-cooling, rapid cooling involves the direct immersion of explants into liquid or sub-cooled nitrogen ('nitrogen slush', at -210°C) and allows an extended water content range at which they will survive freezing (Wesley-Smith *et al*., 1992; Berjak *et al*., 1996).

New cryopreservation protocols are based primarily on vitrification, where cell dehydration occurs via exposure to highly concentrated cryoprotective media – i.e. plant vitrification solutions (PVS), prior to freezing (rapid cooling). This should allow for the avoidance of intracellular ice formation as there is a direct transition from the liquid phase to amorphous glass via dehydration of tissue to low water contents using highly concentrated vitrification solutions (Volk & Walters, 2006). Vitrification-based procedures offer practical advantages such as simplicity, the preclusion of ice formation, and far broader applicability, since only minor manipulations are required to render the process appropriate for the conservation of a range of different cell types (Engelmann, 2009). There are several vitrification-based procedures, most of which include processing steps of cryoprotective treatment, dehydration via vitrification solutions, rapid cooling and re-warming, removal of cryoprotectants/s and recovery. While this approach has also been applied to a range of cell types and species (Sakai *et al*., 1990; Yoshimatsu *et al*., 1996; Takagi, 2000; Fang *et al*., 2004; Kaviani *et al*., 2010) it was not the technique chosen for cryopreservation of embryonic

axes for this study, particularly in view of the potentially cytotoxic concentrations of dimethyl sulfoxide (DMSO) in vitrification solutions.

Successful cryopreservation of embryonic axes necessitates a balance between the minimum extent of desiccation and the appropriate cooling rate (Benech-Arnold & Sánchez, 2004; Normah & Makeen, 2008), regardless of the chosen techniques. Sakai (1986) demonstrated that ultra-rapid cooling in the absence of vitrification medium prevented or minimised intracellular ice crystal formation, and such cooling rates have also been found to facilitate the survival of adequately desiccated embryonic axes after cryoexposure at moderate and high water contents (Berjak *et al*., 1996). In view of preceding work in our laboratory, rapid cooling was selected for explants throughout this study.

2.1.3.6 Plant tissue culture environment: *in vitro* regeneration and culture conditions

Cryopreservation can be considered as successful only if whole seedlings are able to develop in culture from post-cryo embryonic axes, thus efficient, repeatable and rapid *in vitro* regeneration systems are pre-requisites for any protocol (Mohamed *et al*., 1992). Plant material requires specialised media to grow *in vitro* (George, 2008), therefore the optimisation of a recovery/germination medium is of paramount importance. Prior to the application of a cryopreservation protocol, a medium on which excised embryonic axes will develop into seedlings must be optimised. The medium developed must be such that untreated or 'control' axes will produce both a root and shoot after cotyledon excision. However, axis excision and the treatments applied to the explants may inhibit or stunt the growth of either the root or shoot pole, or the entire embryonic axis, as exemplified by work on *Trichilia* spp. (Kioko *et al*., 1998; Goveia, 2007; Whitaker *et al*., 2010); *E. capensis* (Perán *et al*., 2006; Hajari, 2012) and *Podocarpus henkelii* (Essack, 2012). It is therefore necessary to manipulate media using appropriate plant growth regulators (PGRs) as well as appropriate nutrients, vitamins and minerals, to develop the most suitable medium for whole seedling regeneration (Gaspar *et al*., 1996; George, 2008). Components of the growth medium, as well as external growth conditions such as light intensity, photoperiod and temperature, also depend largely on the morphogenic result required, explant type and species (George & Davies, 2008; Preece, 2008).

If manipulated correctly, the *in vitro* environment can be used as a tool to overcome developmental problems associated with tissue culture recalcitrance (Benson, 2000a). (This term used in the context of tissue culture, alludes to material which is not amenable to growth/development under *in vitro* conditions). Key medium components that should be

adjusted include inorganic compounds (macro and micronutrients); organic compounds (vitamins, amino acids, carbon source); phytohormones (auxins, cytokinins, gibberellic acid, abscisic acid [Gaspar *et al*., 1996]); gelling agent (Gelrite®, agar, agarose, Phytagel™, Alginate) and other factors and additives (pH, activated charcoal, antioxidants, ethylene, oligosaccahrides [Benson, 2000a; George & de Klerk, 2008; Iliev *et al*., 2010]). The addition of growth hormones (PGRs) is a particular manipulation and certain influential factors to consider are the potency of the PGR; optimising a PGR regime specific to the recalcitrant genotype and avoiding any inhibitory interactive effects between exogenous and endogenous hormones.

Physical factors such as the size of the culture vessel, aeration/ventilation and quality of light also have an influence on culture responses (Engelmann, 1991) and will have to optimised accordingly. Whatever the many specific manipulations required for the *in vitro* regeneration step, a common requirement for tissue culture across species and explant type are decontaminated explants and an aseptic culture environment – i.e. free from fungi, bacteria and viruses (Iliev *et al*., 2010).

Once seedlings have been established in a suitable *in vitro* environment, it is important to consider the effect of the microclimate change from an *in vitro* to an *ex vitro* environment, for transfer of seedlings for hardening off (Thiart, 2003). Poor acclimation to *ex vitro* conditions can negatively affect plant development as a consequence of changes in humidity and light intensity levels as well as the adjustment from heterotrophic to fully autotrophic growth, hence caution is advised during the transfer of cultures from one environment to another (Benson, 2000a).

2.1.4 Objectives

The objectives for this part of the study were focused on the optimisation of parameters to develop a successful cryopreservation protocol for excised embryonic axes of *P. longifolia* and both *Trichilia* species.

In this regard, previous studies conducted on both *T. dregeana* and *T. emetica* made progress with certain desiccation and cooling regimes; however, success in terms of a fully functional plant after either of these stages in the cryo-protocol has remained elusive once cotyledons have been excised. While there was success in terms of root development after certain treatments, shoots were never obtained, and this was conjectured to occur as a consequence of injurious oxidative events which occurred in response to stress. In the light of those results, preliminary experimentation with certain antioxidants was conducted, in

parallel with the optimisation of parameters required for successful cryopreservation, to investigate the connection, if any, between inhibited shoot development after stresses associated with cryopreservation and unregulated free radical generation.

2.2 Materials and methods

2.2.1 Seed procurement

Opened fruits were harvested directly from *Trichilia emetica*, *T. dregeana* and *Protorhus longifolia* trees and, only if newly-fallen, from the ground. Seeds of *T. emetica* were collected from trees located in coastal LaLucia, just North of Durban (between January – February) and seeds for *T. dregeana* were collected from trees located in Shallcross, Glenwood and Westville, all suburbs of Durban (between April – June). During collection for both *Trichila* species, the only seeds collected from the ground were those that were similar in all respects to those in from newly-split fruits i.e. seeds having intact, smooth and shiny scarlet arils almost enclosing the entire surface of the black coat (Figs. 1.4 & 1.5). Subsequent to collection, seeds from both *Trichilia* species were removed from the fruit, with those contaminated by fungi, damaged in any way, or harbouring insects/insect larvae being discarded. The seed coat and aril were removed manually, after which seeds were surface decontaminated and stored as described below.

Seeds of *P. longifolia* were collected from trees located on the Westville campus of the University of KwaZulu-Natal, throughout December during both harvesting seasons. Bunches of mature fruits harvested from the trees and the ground displayed a bright mauve to purple, fleshy, slightly ridged pericarp (Fig. 1.6). Following collection, intact fruits were clipped off the branches and the pericarp together with the papery cream coloured testa, were removed. Seeds of this species were predisposed to rapid germination and fungal proliferation occurred readily in storage, therefore the embryonic axes were excised from seeds on the day of collection, decontaminated and further processed for experimentation immediately.

2.2.2 Seed processing

2.2.2.1 Surface decontamination

Cleaned seeds of *T. emetica* and *T. dregeana* were decontaminated by treatment with a 1% NaOCl solution containing a few drops of the wetting agent, Tween 20/80®, for 10 and 20 min, respectively. Seeds were subsequently treated with an anti-fungal 'cocktail' of 0.5 ml l^{-1} Early Impact (active ingredients, triazole and benzimidizole; Zeneca Argrochemicals, S.

Africa) and 2.5 ml l^{-1} Previcur N (active ingredient, propamocarb-HC; AgrEvo, S. Africa) for 60 min (*T. emetica*) and 240 min (*T. dregeana*). The active ingredients contained in the decontamination treatment have previously been shown to be effective in curtailing fungal proliferation during storage of recalcitrant seeds (Calistru *et al*., 2000; Berjak *et al*., 2004). Seeds were finally rinsed 3 times with sterile distilled water before being placed on paper towel to be dried back to their original batch fresh weight. They were then used immediately or prepared for storage as described below.

2.2.2.2 Hydrated seed storage

Decontaminated seeds of both *Trichila* species were lightly dusted with Benomyl 500 WP (active ingredient, benzimidizole; Villa Protection, S. Africa) and placed as a monolayer on a plastic mesh suspended 200 mm above paper towel saturated with water to which a few drops of commercial domestic bleach had been added, in white, translucent 5 l plastic buckets. Bucket lids were lined with paper towel (to prevent condensate dripping back onto the seeds) before the buckets were sealed and stored in a 16°C constant temperature room. Both plastic mesh and buckets had been previously decontaminated by soaking them in a 1% NaOlC solution for 1 h, and subsequently wiped with 70% ethanol prior to use.

2.2.3 Primary excision of explant

Whole seeds of both *Trichilia* species and *P. longifolia* are too large and, as work in our laboratory (unpublished) has shown, lose viability at axis water contents that are far too high, for cryostorage to be contemplated. Previous investigators have therefore opted to use the excised embryonic axis as the explant of choice. In terms of *Trichilia* spp., Goveia (2007) showed that once cotyledons had been excised from the embryonic axis, shoot development failed to occur at any stage of the cryopreservation process. The current study continued the work on germplasm cryopreservation of *T. dregeana*, *T. emetica* and *P. longifolia* using the excised axis as the selected explant, with a particular focus on developing procedures enabling shoot development.

For all experimental work, embryonic axes were excised using an 11 pt sterile blade, from seeds considered to be at the same stage of development. The primary explant, which was exposed to antioxidant pre-conditioning (described further below), consisted of the embryonic axis with a 2 mm attached portion of the basal segment of each cotyledon. The final explant, which was exposed to all cryogenic steps subsequent to pre-culture, consisted of the embryonic axis only. The conditions under which this was carried out and the manner

of excision will be discussed later on. The controls for germination after each treatment consisted of untreated embryonic axes with and without cotyledonary blocks (as described above) attached.

2.2.4 Gravimetric determination of water content

Water content of treated and freshly excised axes was established gravimetrically and expressed on a dry mass basis, i.e. g H_2O g^{-1} dry mass (g g^{-1}). This was done also after the cryoprotection and desiccation treatments, both of which influenced the water content of the tissue. Using a six-place micro-balance (Mettler MT5; Germany), embryonic axes (n=10) were weighed individually in aluminium foil boats, after which they were dried to constant weight (at 80°C for 48 h). Axes were then brought to ambient temperature in the weighing boats over activated silica gel in closed glass Petri dishes, and reweighed to ascertain the dry mass.

2.2.5 Surface decontamination of embryonic axes (in all cases for this procedure with cotyledonary blocks attached)

The least injurious, but effective, surface-sterilants and decontamination procedures for *T. emetica* and *T. dregeana* were optimised by Kioko (2003). Axes of *T. emetica* were decontaminated by immersion in 0.02% (w/v) $HgCl_2$ for 2 min and 2% (w/v) CaOCl for 5 min, before three rinses with a sterile solution containing 1 µM $CaCl_2$ and 1 mM $MgCl_2$ (CaMg solution; Mycock, 1999). Axes of *T. dregeana* were decontaminated by serial immersion in 2% (v/v) Hibertane® for 2 min, 70% (v/v) ethanol for 2 min and 1% (v/v) NaOCl for 5 min. Axes were subsequently rinsed three times with sterile distilled water before being immersed again for 5 min in 1% (w/v) NaOCl, followed by three rinses with a sterile CaMg solution. Axes of *P. longifolia* were decontaminated according to a protocol determined by Goveia (pers comm.[1]) as follows: The embryos were immersed in 0.1% (w/v) $HgCl_2$ for 5 min followed by 3 rinses with CaMg solution. Embryos were then immersed in 0.01% (w/v) aqueous suspension of Cicatrin, an antibiotic preparation containing neomycin and bacitracin (Pfizer, South Africa) for 5 min followed by 5 rinses with CaMg solution.

In all cases, axes were decontaminated in a laminar air-flow cabinet immediately prior to *in vitro* regeneration.

[1] Goveia, M.J.T. School of Biological and Conservation Sciences (now Life Sciences), University of KwaZulu-Natal (Westville Campus), Durban

2.2.6 Antioxidant pre-conditioning: pre-culture of axes

Unless stated otherwise, all procedural steps from pre-conditioning onwards were conducted in a darkened laminar air-flow cabinet. Subsequent to primary excision, embryonic axes (with the attached portion of cotyledonary segments) were cultured on medium containing an antioxidant for 6 h in the dark (n=20). Axes were orientated such that the region of the shoot meristem was immersed in the semi-solid medium. The pre-culture medium consisted of full strength (4.4 g l^{-1}) MS medium (Murashige & Skoog, 1962), 30 g l^{-1} sucrose and 10 g l^{-1} agar at a pH of 5.6 – 5.8. Filter-sterilised DMSO (1 ml l^{-1}, Sigma, ≥ 99.5% [GC]) was added to the medium after autoclaving. Preparation of medium was done in a darkened laminar air-flow cabinet.

2.2.7 Final excision of explants and antioxidant post-excision soaking

After pre-culture treatment of the primary explants, they were immediately processed for final excision and post-excision soaking. The cotyledonary remnants were excised from the embryonic axes under a sterile solution of 1% (v/v) DMSO made up in distilled water using two hypodermic needles (Naidoo *et al*., 2011). The axes (n=20) were then immersed in one of the following post-excision soaking solutions, (1) 1% (v/v) DMSO, (2) 1% (w/v) ascorbic acid (AsA; AR grade) solution, (3) sterile distilled water (as controls for antioxidant soaks), or (4) a no-soak treatment (control for post-excision soaking) for 30 min. Aqueous solutions were prepared in autoclaved distilled water and individually filter-sterilised in a laminar air-flow. Final excision of explants and post-excision soaking was the end of the first stage of the cryopreservation procedure. In preliminary trials, *T. dregeana* explants were from freshly harvested seeds and from those stored under hydrated conditions for a month. Once the optimised pre-culture period and post-excision antioxidant soaking treatment were established, the protocol was tested on freshly harvested *T. emetica* and *P. longifolia* seeds. Repeat experiments on *T. dregeana* and *P. longifolia* were conducted on material harvested in two consecutive fruiting seasons, but this could not be done for *T.emetica* due to unavailability of seeds in the second season.

2.2.8 Cryoprotection

Embryonic axes (n=20) were treated with the following penetrating cryoprotectants subsequent to post-excision soaking: DMSO and glycerol (5 and 10% (v/v) each) individually and in combination, according to Goveia (2007). A lower concentration of cryoprotectants (1

and 2% (v/v) was also tested, to reduce possible toxic effects of cryoprotectants. Axes were immersed in the cryoprotectant solutions at the lower of the two concentrations (5% or 1%, respectively) for 60 min, followed by exposure to the higher concentrations (10% or 2%, respectively) for a further 60 min.

2.2.9 Desiccation

Based on work by Kioko (2003) and Goveia (2007) on *T. emetica* and *T. dregeana,* flash-drying (Berjak *et al.*, 1990; Pammenter *et al.*, 2002) of axes was chosen as the optimal mode of desiccation. According to this method, axes placed on a mesh were exposed to a stream of silica-gel-dried air circulated from below at a rate of approximately 10 l min^{-1} by a computer CPU fan within a closed glass jar. In order to establish the drying period required to reduce tissue water contents to 0.35-0.4 g g^{-1} (i.e. the range facilitating cryogenic cooling with no or minimal ice crystallisation), a flash-drying curve was generated for each species. Axes of *T. emetica* and *T. dregeana* were desiccated over periods of 120 and 180 min, respectively, with sampling for water content (n=10) and germinability (n=20) at 30 min intervals. Axes of *P. longifolia* were desiccated over a period of 120 min and sampled at 20 min intervals.

Flash-drying curves were similarly generated for axes after cryoprotection treatments, which were expected to bring about some dehydration.

Axes assessed for *in vitro* germination after cryoprotection and drying were initially soaked for 30 min in the CaMg solution described above.

2.2.10 Cryopreservation

Rapid cooling in nitrogen slush (-210°C) immediately after cryoprotection and/or drying treatments was the method of choice for cooling axes of *T. emetica*, *T. dregeana* and *P. longifolia*, based on the optimised cooling treatment selected by Goveia (2007) for both *Trichilia* species. Axes (n=20) were plunged directly into nitrogen slush in a polystyrene cup where they were tumble-mixed with swirling for 5 min, during which the slush reverted to the liquid form (-196°C). The axes were then thawed by immersion in CaMg solution at 40°C for 2 min and thereafter transferred to the same solution at 25°C (room temperature) for 30 min (Berjak *et al*., 1999) for rehydration.

In addition to rapid cooling, axes of *P. longifolia* were exposed to a slow cooling/two-step cooling treatment. This involved cooling axes (n=20) at 1°C min^{-1} in a Nalgene cryo-freezing container (Mr Frosty™) to -70°C and maintaining axes at this temperature for 4 h

before transferral to liquid nitrogen. As for rapid cooling, thawing and rehydration was effected immediately after exposure to the cryogen.

2.2.11 *In vitro* germination medium and viability assessment

The *in vitro* medium established for *T. dregeana* and *T. emetica* by Kioko (2003) and subsequently selected by Goveia (2007) was full strength (4.4 g l^{-1}) MS basal medium (Murashige & Skoog, 1962) containing sucrose (30 g l^{-1}) and agar (10 g l^{-1}) at a pH of 5.6-5.8. This medium was also chosen for *in vitro* germination for axes of *P. longifolia*.

However, those previous studies showed that for both *Trichilia* species, excised axes without any vestiges of cotyledons cultured on this medium did not produce shoots. Thus for additional investigations, the basic nutrient medium described above was supplemented with the growth hormone, 6-benzylaminopurine (BAP; 0.1 mg l^{-1} [Perán *et al*., 2006]) in an attempt to stimulate shoot production. Germination medium supplemented with BAP and soluble polyvinylpyrrolidone (PVP; 0.5 g l^{-1}) was used for axes of *P. longifolia*, the latter being introduced to curtail phenolics exudation (Narayanaswamy, 1994). Control explants (those with attached segments of cotyledons) for each treatment were cultured on the basic *in vitro* medium. Consequently, as a control for the modified germination medium, axes treated with a pre-culture and post-excision soaking treatment were cultured on both the basic germination medium and the modified media (this was aimed to establish the effect of BAP before continuing with its use at the *in vitro* stage throughout the cryopreservation process.) All explants were cultured in 65-mm petri dishes (5 explants per dish) and set to germinate in a growth room at 27°C with a 16 h photoperiod (light intensity approximately 50 μmol m^{-2} s^{1}). For axes after desiccation alone or followed by cooling, petri dishes were initially maintained in a decontaminated dark cupboard for 7 d, or until radical extension growth was observed, before being transferred to the growth room.

Viability was assessed *in vitro* for all three species after each step in the cryo-procedure. Axes were monitored for signs of root and shoot development on a weekly basis, with production of the latter considered as the primary indicator of success. Assessment was continued for 20 weeks, with sub-culturing onto fresh germination media at 6-week intervals.

2.2.12 Imaging

Digital images of whole seeds and fruit were captured using a Nikon Coolpix® digital camera, while images of *in vitro* explants were captured using the camera mounted on attached to a Wild M4A (Heerbrugg, Switzerland) stereo microscope, all surfaces of which

were thoroughly sprayed with 70% ethanol and wiped down before it was placed in the laminar air-flow cabinet.

2.3 Results and Discussion

Results pertaining to *T. emetica*, *T. dregeana* and *P. longifolia* and *P. longifolia* are presented for each procedural stage of cryopreservation unless otherwise stated. A sample size of 10 and 20 axes was used for water content assessment after selected treatments and viability assessment after each treatment, respectively.

2.3.1 Responses after excision, pre-culture and post-excision soaking

Results appearing in this section were published during the course of the present study in Naidoo *et al*. (2011).

Selected treatments showing higher efficacy in the first fruiting season were repeated in the next fruiting season. Shoots were produced only by axes cultured on modified media, i.e. MS medium supplemented with BAP and with BAP and PVP, for both *Trichila* species and *P. longifolia*. Therefore results presented in Table 2.1 represent shoot development occurring on these media only. For each species, between 30 -78% of axes treated with a DMSO pre-culture and a subsequent post-excision soak produced shoots, in contrast to total lack of shoot production in axes not receiving any exogenous antioxidant treatment (controls [No pre-culture, Table 2.1]). In all cases the effect of treatment was significant ($p < 0.05$, null-model chi-squared analyses).

Table 2.1: Percentage of axes producing shoots when cultured on medium incorporating BAP and BAP with PVP, after a 6 h DMSO pre-culture followed by a DMSO, AsA or distilled water soak, or no soak after pre-culture. (n=20)

Species	**Weeks in culture**	**No pre-culture**	**Treatment – 6 h pre-culture on a medium containing 0.1% DMSO, followed by:**			
			No soak (pre-culture only)	**Distilled water**	**1% AsA**	**1% DMSO**
***T. dregeana* (2009)**	20	0	0	0	50	70
***T. dregeana* (2010)**	12	0	0	20	Not done*	65
***T. dregeana* (2009, stored for 1 month)**	20	0	0	0	50	78
***T. emetica* (2010)**	6	0	55	50	Not done*	30
T. emetica* *(2011)	6	0	60	60	Not done*	45
***P. longifolia* (Jan 2009)**	28	0	65	75	45	70
***P. longifolia* (Dec 2009)**	20	0	50	20	Not done*	60

*AsA post-excision treatment was discontinued, having proved to be less efficacious than application of DMSO

Differences among species were noted in terms of the required regimes to promote shoot development; however these differences were consistent across fruiting seasons. Axes of *T. dregeana* needed both a pre-culture treatment and a post-excision soak to induce shoot production while a pre-culture treatment alone was sufficient to promote shoot development in excised axes of *T. emetica* and *P. longifolia*. There was a lower percentage shoot development by axes of *P. longifolia* seeds harvested in the second fruiting season across treatments. The reason for this is unknown, although it does demonstrate the inter-seasonal variability in recalcitrant germplasm (reviewed by Berjak & Pammenter, 2004). Figure 2.1

illustrates the effect of treatment on excised axes of *T. emetica* after 6 weeks *in vitro*. In these images (a) represents an excised axis not afforded any treatment, where cotyledons were severed flush with the surface; (b) shows shoot development by an axis excised with segments of cotyledons left attached and (c) illustrates the situation where the cotyledons were completely severed and axes exposed to treatment (pre-culture and post-excision soak in DMSO) which promoted normal shoot development.

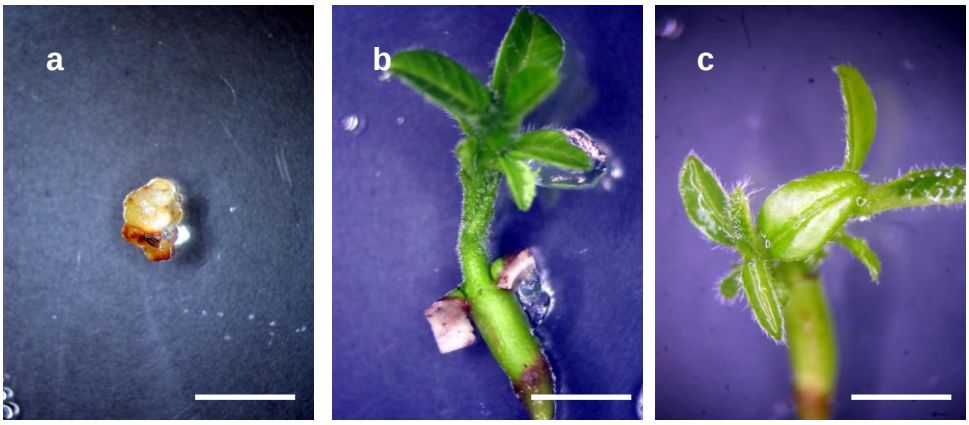


Fig. 2.1: Embryonic axes of *Trichilia emetica* after six weeks in culture showing (a) necrosis following complete excision of the cotyledons from the axis in the absence of any treatment; (b) shoot development from an axis excised with attached cotyledonary blocks; and (c) shoot development by an axis with no cotyledonary attachments, exposed to 6 h preculture on DMSO-containing medium and post excision soak in 1% DMSO. Bar = 1 mm.

A batch of *T. dregeana* seeds was stored for a month under hydrated conditions, during which epicotyl extension occurred (data not shown), increasing the distance between the shoot meristem and the site of excision. It was anticipated that this could potentially decrease damage incurred by the shoot tip as a result of the oxidative burst (Pammenter *et al.*, 2011). However, there was no observed long-term effect on shoot production (Table 2.1), but it was noted that axes from stored seeds produced shoots substantially faster after 8 weeks in culture than those axes excised from fresh seeds (Fig. 2.2). However, the difference became negligible after a total of 20 weeks.

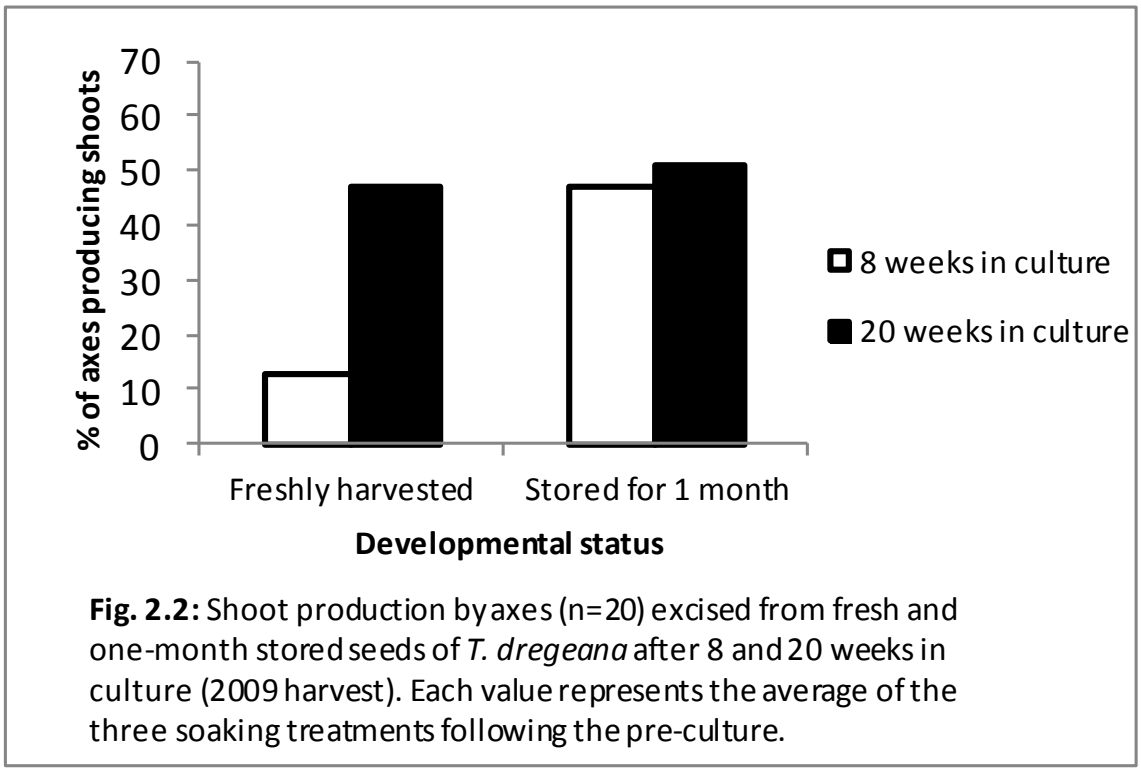


Fig. 2.2: Shoot production by axes (n=20) excised from fresh and one-month stored seeds of *T. dregeana* after 8 and 20 weeks in culture (2009 harvest). Each value represents the average of the three soaking treatments following the pre-culture.

2.3.2 Discussion: Pre-culture and post-excision soaking

Results presented thus far show that excised axes did not produce shoots unless cultured on medium modified with BAP. Excised axes that were untreated (controls) did not produce shoots despite being cultured on medium containing BAP, therefore shoot production could not be attributed to the action of BAP alone, but rather as a consequence of treatment with DMSO or AsA. It is suggested that the BAP enhances shoot development once oxidative damage caused by excision is circumvented by the ROS scavenging activities of exogenously applied antioxidants.

The topography of the axes of the species investigated here is such that the point of excision lies in very close proximity to the shoot meristem. In such cases it has been suggested that the oxidative burst typical of excision injury results in shoot tip necrosis, precluding shoot development (Whitaker *et al.*, 2010' Berjak *et al.*, 2011; Pammenter *et al.*, 2011). Furthermore, from the present observations, it is suggested that such damage - or the consequences of necrotic degeneration of the shoot tip – could be propagated throughout small axes, causing death of the entire explant (Fig. 2.1.a). This was observed to be the case in excised axes from both *Trichilia* species. However, in certain species such as *P. longifolia* and *Ekebergia capensis* (Perán *et al*., 2006; Hajari *et al.*, 2011), while shoot development is

precluded, root production continues normally, highlighting the need for the development of species-specific protocols.

Several studies have shown that physical wounding of the axis associated with the process of excision gives rise to an oxidative ROS burst across species (Beckett & Minibayeva, 2003; Minibayeva *et al*., 2003; Whitaker *et al*., 2010). This suggests a link between an oxidative burst and events that subsequently lead to the preclusion of shoot development probably as a consequence of death of all, or most, of the apical meristem cells (Goveia *et al*., 2004; Roach *et al*., 2008; Pammenter *et al*., 2011; Whitaker *et al*., 2010). The optimisation of any further cryogenic steps is futile if oxidative stress inhibits the development of fully functional seedlings after axis excision, therefore the results obtained here represent a crucial step forward in the development of cryopreservation protocols for these species. Amelioration of oxidative stress throughout cryopreservation is paramount, especially in desiccation-sensitive, recalcitrant germplasm, which may well lack the necessary antioxidant capacity to counteract free radical damage (Benson, 1990; Berjak *et al*., 2011). Additionally, it is essential to develop the means to combat free-radical-mediated injury at each stage of the cryopreservation process as it is probable that every step is accompanied by ROS production (Benson, 1990). In this regard, the first progressive step was made by achieving the primary aim of this study which was to relieve oxidative stress by pre-conditioning axes upon excision with DMSO and AsA and thereby facilitating shoot development.

While the application of both DMSO and AsA were efficacious in promoting shoot development, axes exposed to AsA after pre-culture were observed to produce leaflets that were lighter in colour than those treated with DMSO, suggesting a bleaching effect of AsA. This observation could implicate AsA as a pro-oxidant rather than an antioxidant in this instance, as bleaching of woody plant germplasm in culture is recorded as typical of pro-oxidant action (Kanner *et al*., 1977). Ascorbic acid also has the propensity to deplete natural defences occurring via the glutathione-ascorbate (GSH-AsC) cycle unless the redox balance is maintained by GSH reduction (Bailly, 2004; Kranner *et al*., 2006; detailed in Chapter 3). Further to this exogenous provision of DMSO was far more effective in promoting shoot development than was AsA. Therefore, it was decided to continue using DMSO soaking following complete removal of the cotyleonary tissue as the antioxidant treatment of choice for the remainder of the experiments detailed in this Chapter.

There are certain properties of DMSO that allows for postulation on its mode of action in facilitating shoot development. Firstly, its ability to augment certain growth

regulators upon mutual interaction may have enhanced the action of BAP in promoting shoots by axes cultured on medium containing this PGR (Sciuchetti, 1967). Secondly, DMSO is known to influence cell division positively, possibly by promoting cytoskeletal activity, which would be essential to counteract retarded growth induced by wounding/injury (Benson, 1990). Thirdly, DMSO is a potent hydroxyl radical scavenger (Yu & Quinn, 1994), thus its quenching action – especially of hydroxyl radicals – may be the basis of mitigating oxidative stress consequent upon wounding.

The facilitation of shoot production following treatment with DMSO/AsA was also achieved for axes from seeds stored under hydrated conditions for a month. However, it was observed that shoot production by axes from the same stored material that did not receive an antioxidant treatment, remained precluded. Goveia *et al*. (2004) demonstrated that axes from seeds stored under hydrated conditions for 5 months produced shoots without an antioxidant treatment. The difference observed between the responses of axes from seeds stored for 1 and 5 month/s could simply be due to greater extension of the shoot meristem away from the points of cotyledon removal (and therefore the oxidative burst) upon elongation of the epicotyl in storage. After 5 months in storage (as compared to 1 month), this extension could have resulted in the shoot meristem being sufficiently physically removed from the injury sites to avoid extensive oxidative damage and retain the ability for normal growth. The rapidity with which the axes from stored seeds produced shoots compared with those excised from newly-harvested seeds, can be accounted for by ongoing development which grades into germinative metabolism during hydrated storage of recalcitrant seeds (Berjak & Pammenter, 2008).

In conclusion, the use of DMSO and in some cases AsA, promoted shoot development by excised axes of *T. emetica*, *T. dregeana* and *P. longifolia* , which has never before been achieved despite extensive experimentation (Goveia *et al*., 2004; Kioko *et al*., 2006; Perán *et al*., 2006). This progress was consistent for the three recalcitrant-seeded species across two fruiting seasons, validating the use of the protocol to ameliorate wounding injury in excised axes prior to further cryogenic manipulation (Benson *et al*., 2007).

While oxidative damage as a consequence of unregulated free radical/ROS production, was curtailed during the first crucial stage of excision, there are numerous subsequent stages which are accompanied by ROS generation in a cryopreservation protocol – including the extremely stressful process of desiccation (Whitaker *et al*., 2010). Further investigations therefore, firstly assessed the use of DMSO and other antioxidant treatments during later steps required for axis cryopreservation and secondly, examined the

consequences of exogenously applied antioxidants on ROS activity. Importantly, the studies aimed to profile changes in endogenous antioxidant activity concurrently, with the objective of developing a protocol that to quench ROS at every step. The development of cryopreservation protocols is currently species-specific but problems usually have a facet of commonality across species. Oxidative profiling in relation to whole seedling regeneration after all cryogenic manipulations required to be investigated. Thus work in this regard, in addition to in-depth biochemical studies on oxidative stresses associated with procedures throughout the cryopreservation protocol, was carried out and is reported in Chapter 3.

2.3.3 Responses after pre-culture, soaking and cryoprotection

After successful seedling development from axes following a pre-culture and soaking treatment, a variety of cryoprotectants at different concentrations were tested. Cryoprotection was applied to axes following the DMSO pre-culture treatment and post-excision soak in 1% DMSO.

Table 2.2: Survival of excised embryonic axes of *T. emetica*, *T. dregeana* and *P. longifolia* after exposure to different cryoprotectants

Species	Cryprotectant/s	Root	Shoot	Greening/Callus
T. emetica	5% + 10% DMSO	-	-	-
	5% + 10% glycerol	-	-	-
	5% + 10% DMSO + glycerol	+	-	-
T. dregeana	1% + 2% DMSO	-	-	-
	1% + 2% glycerol	-	-	-
	1% + 2% DMSO + glycerol	-	-	-
	5% + 10% DMSO	-	-	-
	5% + 10% glycerol	-	-	-
	5% + 10% DMSO + glycerol	+	-	-
P. longifolia	5% + 10% DMSO	-	-	-
	5% + 10% glycerol	-	-	-
	5% + 10% DMSO + glycerol	+	-	-

Table 2.3.3.1 shows results obtained when various cryoprotectants/s treatments were applied to excised zygotic axes of *T. emetica*, *T. dregeana* and *P. longifolia*. In all cases, axes were exposed to cryoprotectants subsequent to the established optimum pre-culture and post-excision soaking regime. However, no shoot development was obtained after cryoprotection, and 10% of axes of each species developed a root after exposure to 5% and 10% DMSO mixed with 5% and 10% glycerol, respectively. Note that as no axes of *T. dregeana*, the first species tested, had survived to produce either a root or a shoot after exposure to the lower concentrations of DMSO + glycerol (1% + 2%), this treatment was not attempted for axes of *T. emetica* and *P. longifolia*.

Goveia (2007) showed that the application of cryoprotectants to axes of *Trichilia emetica* and *T. dregeana* did not improve survival after cooling, and since no substantial positive results in terms of germination (root alone or root and shoot) were achieved, this step

was omitted during further processing of axes, i.e. desiccation and cooling were carried out on axes that had not undergone cryoprotection.

2.3.4 Discussion: cryoprotection

The retrieval of both prokaryotic and eukaryotic cells, from temperatures approximately two hundred degrees below the freezing point of water, is in most cases largely attributed to the presence of cryoprotectants which serve to modulate the lethal effects of intracellular salt concentration and ice crystallisation, as well as the occurrence of ice in the matrix of tissues (Fuller, 2004; Elmoazzen *et al*., 2005). The term, cryoprotectant, can be described as any additive supplied to cells prior to cooling that can potentially yield a higher post-thaw survival than that which can be obtained in its absence (Karlsson & Toner, 1996). In terms of cryopreservation of embryonic axes, the rationale behind the use of cryoprotectants was to promote conditions under which ice crystal formation could be inhibited down to the glass transition temperature, as detailed for successful preservation by Fuller (2004).

The cryoprotectants, glycerol and DMSO, confer protection during cryoprotection and ultimately the freezing process (Nash, 1962; Finkle *et al*., 1985; Fuller, 2004; Morris *et al*., 2006). These compounds were selected because previous work (Kioko *et al*., 1998; Kioko, 2003; Goveia, 2007) showed that although the response was minimal, it was better than other cryoprotectants tested by those investigators. In agreement with their findings, however, this aspect of the present study showed that excised axes with no vestiges of cotyledonary tissue did not survive exposure to cryoprotectants regardless of the concentrations applied following the optimised procedures used at excision. Survival in terms of successful axis cryopreservation, requires the development of both a root and a shoot. In terms of this requirement, there was no survival, and the only indication of any on-going functionality was root development by 10% of axes treated with combinations of 5% and 10% DMSO and glycerol.

Incomplete survival after cryoprotection cannot be attributed to toxicity of the chemicals since a few axes of all the species exposed to the combination of the two cryoprotectants at the higher concentration used, retained the ability for root formation, while those exposed to lower concentrations of cryoprotectant, did not. In addition, toxicity of both these cryoprotectant compounds, as well as any damage induced within the lipid bilayer as a consequence of their penetration, have been generally associated with the high concentrations

used in Plant Vitrification Solutions (PVS; Benson, 2008). Berjak *et al*. (1999) noted that axes from temperate and tropical recalcitrant-seeded species differ in their responses to cryoprotection, with the latter having a history of low survival as a consequence of positional derangement of intracellular organelles. In this respect, Sershen *et al*. (2007) demonstrated that embryos/axes of some monocotyledonous recalcitrant-seeded species respond favourably to cryoprotective treatments, as seen in an array of *amaryllid* species.

Trichilia emetica and *T. dregeana* and *P. longifolia* are tropical, dicotyledonous species, and, in common with other such species, appear to be adversely affected by exposure to cryoprotectants. In this regard, several reported studies aimed at cryopreservation of germplasm of recalcitrant-seeded species, reveal that cell death is common at the cryoprotection and desiccation stages (Kioko *et al*., 1998; Kioko, 2003; Goveia, 2007; Whitaker *et al*., 2010; Kistnasamy *et al*., 2011), which further reduces the possibility of survival post-cooling. While explants may survive individual stresses, it is likely that the cumulative stresses imposed upon the tissue during processing for, and retrieval from, cryopreservation, will culminate in a spectrum of degradative, and ultimately lethal, events. It is therefore necessary to investigate the possible causes of these events at each stage of the cryopreservation protocol, and after retrieval of explants from cryogenic conditions, to devise ameliorative treatments potentially leading to enhanced survival.

There are a number of possible reasons why the excised axes were unable to survive cryoprotection. Considering the explant type chosen for investigations, reasons should be discussed for death of the entire explant as well as the preclusion of shoot development. Dimethyl sulfoxide and glycerol have long since been recognised as cryoprotective additives that permit and improve survival after cryopreservation of both plant and animal tissues and cells (Polge *et al*., 1949; Lovelock & Bishop, 1959). Glycerol, a trihydric alcohol, and DMSO, a small and highly reactive dipolar molecule, are both penetrating agents and their direct interactions with cellular components and/or alterations of water distribution inside/outside the cell have marked implications for the cryo-stability of the plasma membrane (Finkle *et al*., 1985; Yu & Quinn, 1994; Dashnau *et al*., 2006). In the present study, combined use of these substances was chosen based on the possibility of an additive or synergistic augmentation of cell survival (Brockbank & Taylor, 2006). Cryoprotectants in factorial combination also have the advantage of facilitating decreases in concentration of components that may be toxic, while not decreasing the overall cryoprotective effect (Finkle *et al*., 1985). The mechanism by which DMSO and glycerol confer protection at low concentrations has been described through the ‘shrink-swell’ response of cells, i.e. the

initially high solute concentration outside the cell first causes cell shrinkage due to osmosis, but the cells subsequently swell as cryoprotectant molecules penetrate them and prevent excessive dehydration during the freezing process (Finkle *et al*., 1985; Wowk, 2007). Penetrating compounds influence the characteristics of the internal cell solution and prevent an extreme exodus of water from cells too early in the cooling process (Finkle *et al*., 1985). Taking cognisance of the mechanism of a glycerol/DMSO cryoprotective treatment, the death of cells prior to freezing cannot be attributed to a toxic build up of solutes or intracellular freezing, which are events known to occur as temperature declines to ultra-low levels. At this point, it is reasonable to suggest that the failure of explants to survive could be due the inability of the cryoprotectants to prevent against the physical/structural damage (e.g. collapse of cells) that occurs when water is removed from the cell during cryoprotection (Walters & Koster, 2007).

The employment of moderate concentrations of DMSO and glycerol in a cryoprotective capacity has also been known to inhibit photosynthesis and cause ultrastructural alterations, both events possibly contributing to cell death (Morris, 1976). Therefore, the detrimental effects of cryoprotection prior to cooling, could be a consequence of physical damage caused by cryoprotective additives (shrinkage of cells) and/or the direct and indirect effects such as the disruption of membranes, resulting in cell death throughout the embryonic tissue. Additionally, it is known that DMSO can cause changes in membrane permeability and enhance the permeability of low molecular weight solutes across biomembranes (Yu & Quinn, 1994). The increased permeability of membranes may give way to an uncontrolled egress of compounds and/or molecules from the cytomatrix.

An alternate (or additional) cause of death could be disruptive free radical activity associated with the process of cryoprotection itself. Prior to cryoprotection, the development of shoots after excision was promoted by treatment with the antioxidants, DMSO and AsA. While the inclusion of these antioxidants may be suggested to have counteracted deleterious events occurring in response to wounding, it was evident that this treatment was inadequate in facilitating shoot development after cryoprotection. If cell death is due to oxidative, rather than physical, damage, it could be suggested that uncontrolled ROS generation occurred during exposure to the cryoprotectants and could not be quenched by the DMSO present in the solution, thus ROS-induced cell death in the shoot meristem may have occurred. According to Benson & Bremner (2004), DMSO is a known, potently-reactive free radical scavenger and has been shown to confer preferential protection to essential macromolecules containing sulphur, and to repair unstable intermediates by the presence of its S atom and the

donation of its hydrogen atom, respectively. Dickinson *et al*. (1967) demonstrated the protective ability of DMSO when they reported that it shields mitochondria from freezing damage thereby indirectly preventing the exacerbation of free radical activity associated with damage to the electron transfer chain. Based on this, if lack of shoot development was a consequence of oxidative stress it would be expected that this should be overcome by treatment with DMSO. However, it is known that DMSO is a particularly potent scavenger of the hydroxyl radical. If oxidative stress is caused by unregulated production of other free radicals, it is likely that DMSO may be ineffective.

Therefore, from this point the study aimed to investigate further the connection between survival/shoot development, the incidence of ROS, of antioxidants and of cryoprotection. By establishing the possible cause of death and remediating unbalanced metabolism using various antioxidant treatments, if the damage proved to be oxidative in nature, it was hoped that seedling establishment could be achieved (Chapter 3).

The judicious use of cryoprotection can increase the chances of survival post-cryopreservation. The presence of DMSO at the stage of cryoprotection can be highly advantageous and useful for long term cryogenic storage of tissue if the postulated defects ascribed to the chemical can be counter-balanced by its many protective attributes. The benefits afforded by cryoprotective action are often seen at the cooling stage. The mechanism of colligative cryoprotection is lowering of the freezing point of the solution in contact with unmelted ice by the additive compounds (Benson *et al*., 2005). In the absence of a vitrified state upon freezing, growing ice compartmentalises cells into small pockets of liquid as temperature is lowered. Cell components are squashed between ice crystals (Wesley-Smith, 2003) and may be exposed to lethal concentrations of solutes. In the presence of cryoprotectants, the unfrozen fraction or 'pockets' of liquid are larger at any given temperature than they would be in their absence (Wowk, 2007). An increased volume of liquid and adequate protective compounds thus translate to reduced injury from both mechanical and solution effects (Finkle *et al*., 1985; Brockbank & Taylor, 2006; Wowk, 2007).

Studies on *Arabidopsis* shoot tips have shown that certain cryoprotectants are involved in the induction of gene expression and biochemical pathways that play a role in lipid transport and osmoregulation, thus cryoprotection has the potential to influence membrane composition, flexibility and permeability and may even reduce membrane fusion during cryopreservation (Brockbank & Taylor, 2006; Benson, 2008). Dimethyl sulfoxide particularly, has been known to generate genetic and epigenetic changes but at the same time

to decrease genetic damage from radiation and preserve interactions between membrane lipids and proteins at low temperatures (Finkle *et al*., 1985; Yu & Quinn, 1994). Another noteworthy characteristic of DMSO is its ability to retain the structure of macromolecules suggested to be by substituting for water in the hydration sheath of polysaccharides, proteins and nucleic acids, thus rendering them less vulnerable to radiation-induced damage: DMSO may confer a similar protective action during cryopreservation procedures (Brockbank & Taylor, 2006).

In the broader context, there are several benefits to explants that survive cryoprotection such as: the minimisation of cell damage associated with ice formation (Mazur, 1984); phenotypic adaptation to better cope with freeze-thaw stress (Panoff *et al*., 2000); increase in the viscosity of freeze concentrated material (especially with glycerol) which is important for the induction of the glassy state (Morris *et al*., 2006); modulation of hydrogen bonding (Yu & Quinn, 1994; Fuller, 2004; Dashnau *et al*., 2006); mild antibacterial action (Fuller, 2004); ability to act to scavenge free radicals, scavengers contributing to their protective efficacy as well as improving their colligative and osmotic properties (Benson & Bremner, 2004); and the propensity of solutes preferentially to exclude or bind proteins from the surface of macromolecules thereby acting to stabilise proteins under stressful conditions (Fuller, 2004). Final considerations in the present study in terms of cryoprotection and ensuing cryogenic manipulations aimed fundamentally to minimise the amount of water present in the tissue, incorporate a free radical scavenger in the cryoprotectant mixture and have an antioxidant present at each stage of cryopreservation. This approach progresses towards limiting potentially damaging effects of free radicals/ROS or disrupted metabolism on the functionality and long-term genetic and biochemical stability of cryopreserved tissue (Benson & Bremner, 2004).

2.3.5 Responses after desiccation

Figures 2.3, 2.4 & 2.5 show the water content of, and percentage root development by, excised fresh (first datum point) and flash dried axes (without cotyledonary attachments) of *T. emetica*, *T. dregeana* and *P. longifolia*, respectively, over 0 - 120 min. Flash drying curves were generated to ascertain the required time for axes to reach water contents between 0.4 - 0.5 g g^{-1} dw. Plant material within or below this water content range can usually survive exposure to cryogenic temperatures (Vertucci *et al*., 1991; Wesley-Smith *et al*., 1992). Initial axis water contents were high for axes of all the species, as is characteristic of recalcitrance.

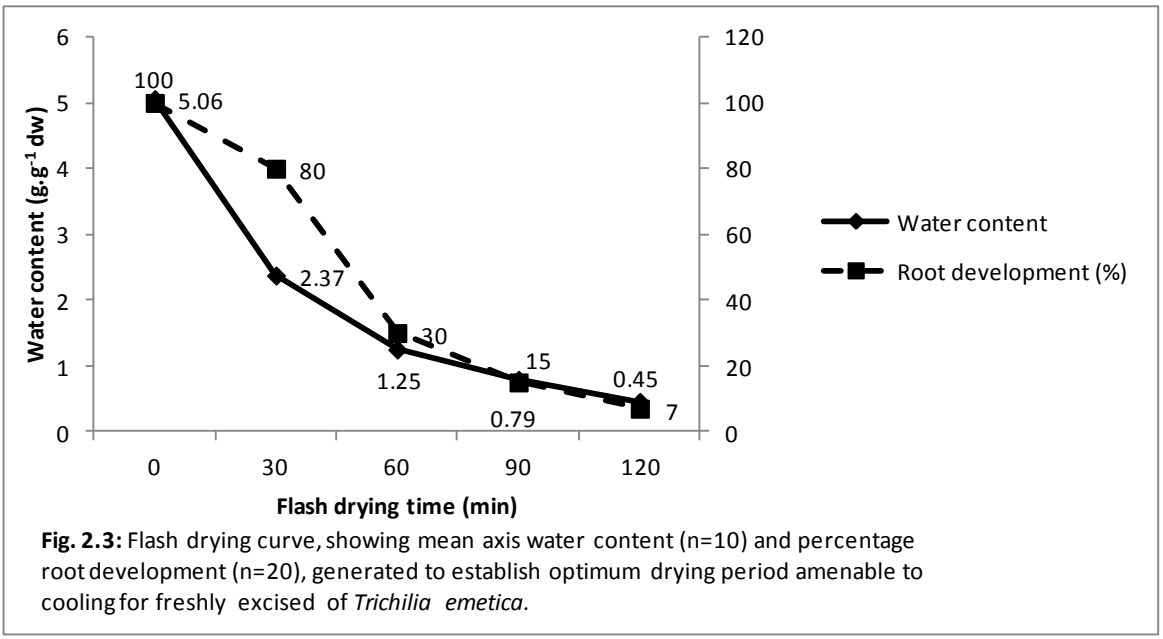


Fig. 2.3: Flash drying curve, showing mean axis water content (n=10) and percentage root development (n=20), generated to establish optimum drying period amenable to cooling for freshly excised of *Trichilia emetica*.

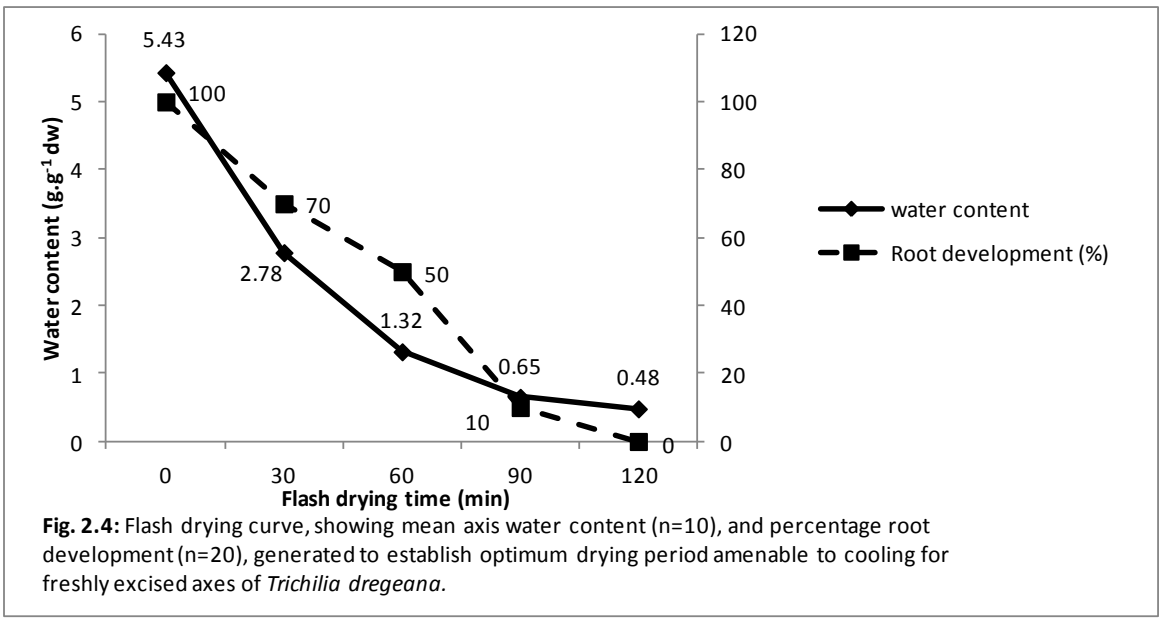


Fig. 2.4: Flash drying curve, showing mean axis water content (n=10), and percentage root development (n=20), generated to establish optimum drying period amenable to cooling for freshly excised axes of *Trichilia dregeana*.

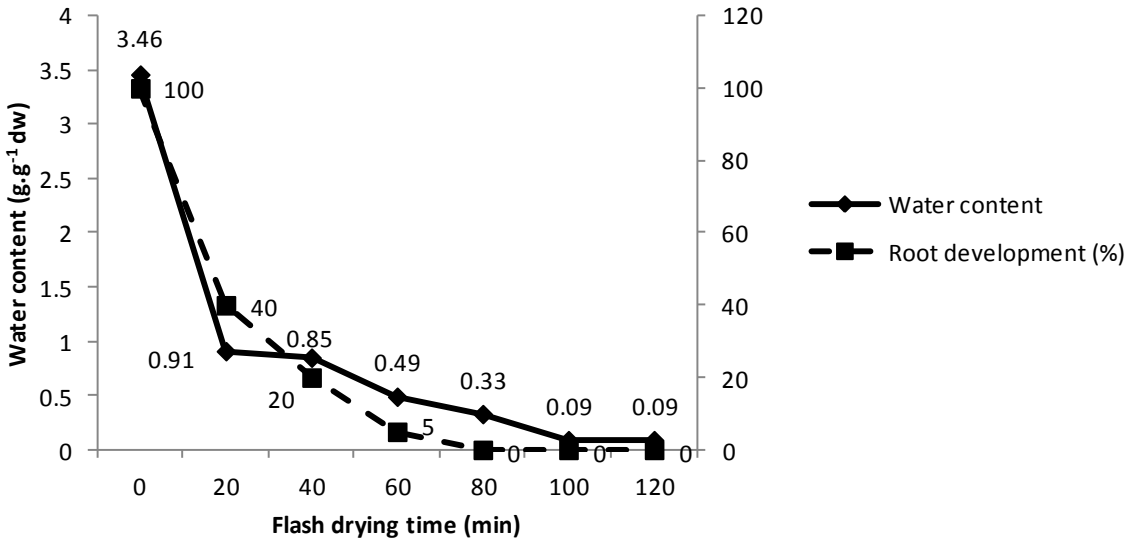


Fig. 2.5: Flash drying curve, showing mean axis water content (n=10), and percentage root development (n=20), generated to establish optimum drying period amenable to cooling for freshly excised axes of *P. longifolia.*

While axes excised from both *Trichilia* species required a drying period of 2 h to achieve the required water contents, axes of *P. longifolia* reached the requisite water content after an hour of drying. However, regardless of the time required to reach the desired water content, root development in the water content range of 0.4 – 0.5g g^{-1} was poor i.e. 7%, 0% & 5% for *T. emetica*, *T. dregeana* and *P. longifolia* respectively, and no shoots were developed by axes of any of the species even after the shortest duration of flash drying. This indicated that the shoot apices (apical meristems) are more sensitive to drying than those of the roots.

The poor root production when axes had been flash dried to the water content range appropriate for cryogenic cooling was not a promising result for successful cryopreservation. Axes were processed for cooling trials concurrently with the flash-drying trials, as seed availability was extremely limited at this stage of the investigation. However, not surprisingly in view of axis sensitivity to dehydration, none survived cryogenic exposure.

2.3.6 Discussion: desiccation

A key factor for successful cryopreservation is the removal of sufficient water from explants such that they are amenable to cooling with minimal intracellular ice formation, but have not lost so much water that they suffer desiccation damage by removal of structure-associated water – i.e. desiccation damage *sensu stricto* (Pammenter *et al.*, 1998; Walters *et al.*, 2001). When desiccation-sensitive embryos are subjected to dehydration, their responses in terms of survival and accumulated damage will differ depending on the means and rate of water loss, rehydration, explant developmental status, as well as the species (Pammenter *et al.*, 2002). The method, and therefore rate, of desiccation chosen in this study was flash

drying, a technique which has been applied successfully in our laboratory to axes of a variety of other recalcitrant-seeded species. All drying curves were generated from axes that were excised from freshly harvested mature seeds collected from the onset of the fruiting season and were therefore all could be considered to be at the same developmental stage. Axes from all species were highly hydrated when seeds were harvested although those of both *Trichilia* species had higher water contents (> 5 g g^{-1}) than did axes of *P. longifolia* (average of 3.46 g g^{-1}). These factors considered, one would presume that the inability to survive desiccation would have a common basis across the species. Once again, lack of survival upon dehydration could be explained by both physiological or mechanical damage, and oxidative damage (the latter especially related to the preclusion of shoot development in this study). Mechanical damage is directly related to the role of water in a structural capacity, and oxidative damage adversely impacts on metabolism regulation (Walters *et al*., 2002).

Pammenter & Berjak (2000) showed unequivocally, via ultrastructural evidence, that there is a direct correlation between drying rate and the extent of water loss tolerated by tissue, where different deteriorative events occur as a consequence of different drying rates. While rapid drying may allow tissue to pass rapidly through intermediate water contents at which aqueous-based injurious events occur, at low water contents damage is due to removal of structure-associated water which could cause biophysical disruptions within the membrane and to macromolecules (Bryant *et al*., 2001) ultimately having a detrimental influence on survival. It is, however, important to note that axes of both *Trichilia* species lost water relatively slowly (Figs 2.3 & 2.4), and, although those of *P. longifolia* dried rapidly over the first 20 min of flash drying, dehydration rate was considerably slower over the 30-60 min period (Fig. 2.5). In this context, it has been found that axes of *Amaryllis belladonna*, which reached the water content range appropriate for cooling in 15 min, survived both flash drying and cryogen exposure (Sershen *et al*., 2012a). In contrast, considerably lower survival was obtained for axes of another amaryllid, *Haemanthus montanus*, which required 3 h to reach the required water content range (Sershen *et al.*, 2012b). Those authors postulated that survival of both dehydration and cryogen exposure was adversely affected by the drying rate which was slow, despite the use of flash drying. Thus it is conjectured that metabolism-linked damage is a key event during protracted drying, which may well have been the case for axes of all the species presently under investigation. (In this regard attempts to increase the rate of *T. dregeana* axis water loss are presently being undertaken in our laboratory.)

The effect of desiccation on the subcellular matrix in *Quercus robur* embryonic axes was shown by Mycock *et al*. (2000), where those authors suggested that the reduction in

viability due to drying to low water contents could be associated with irreversible derangement of the cytoskeletal elements. Viability of recalcitrant axes would be negatively affected if the cytoskeleton, spatial relationships of organelles, and biochemical pathways are not reconstituted. Derangement of macromolecules and membranes could be a direct consequence of disruption of microtubules which are responsible for maintaining the position of certain organelles (Cole & Lippincott-Schwartz, 1995), while a slower growth rate could result from reduction in ATP synthesis due to inadequate functioning of the glycolysis pathway (microfilament-associated event; Masters, 1984). Walters *et al*. (2002) defined desiccation damage as the inability of tissue to recommence normal activity upon rehydration rather than a consequence of physical and physiological changes to the cell upon removal of water. This ties in with imbibitional damage, in which slow, or lack of, membrane reconstitution is held to give rise to excessive leakage from seeds during imbibition (Crowe *et al*., 1989). In this study, rehydration after drying was conducted rapidly in a CaMg solution as this has been suggested to be the preferred method of rehydration of partially-hydrated recalcitrant axes to avoid imbibitional damage (Mycock, 1999; Perán *et al*., 2006). Imbibitional damage, however, is a characteristic of direct immersion of very dry (orthodox) seeds. Nevertheless, the process of rehydration of recalcitrant axes after flash drying can still be considered as an avenue for degradative events to occur.

In the current study it was observed that shoot formation did not occur regardless of the axis water content, while root production decreased with decreasing water content until presumably the lowest water concentration limit was reached, beyond which root development was precluded. This trend is consistent with studies conducted by Kioko *et al*. (2006) and Whitaker *et al*. (2010) which showed that there was a differential response to drying between root and shoot tips in excised axes of both *T. emetica* and *T. dregeana*, respectively. The embryonic axis is comprised of various tissue types and does not dry evenly as demonstrated microscopically by Wesley-Smith *et al.* (2001), resulting in different root and shoot water contents (with the latter often being lower) by the end of the drying period (Kioko *et al*., 2006). This drying pattern is characteristic of axes of several recalcitrant seeded species and leads one to suggest that the shoot meristematic region and root region are not equally tolerant of individual and combined stresses (Walters *et al*., 2008). The shoot meristem, which appears to dry more rapidly (Kioko *et al.*, 2006) would incurs a more severe stress while held at perhaps damaging low water content while the rest of the axis tissues, including the root pole, continue to be dried to the (overall) required water content range.

Ultimately, however, the root tip too becomes damaged during protracted dehydration, as shown by microscopical analyses in early studies on *T. dregeana* (Kioko *et al*., 1998).

Desiccation damage involving structural changes accrued by cellular constituents is often linked to metabolically derived damage (Walters *et al*., 2002). Metabolic reactions respond to dehydration in different ways: e.g. protein synthesis is retarded at relatively high water potentials while respiratory pathways can maintain normal activity until much lower levels (Walters *et al*., 2002). As a consequence of differing responses among, and within, metabolic pathways, metabolic imbalances often ensue (Walters *et al*., 2002). In addition, continued respiration during drying stimulates the accumulation of high-energy intermediates that leak out from mitochondria, forming reactive oxygen species (Leprince *et al*., 1996). The subsequent interactions between ROS and macromolecules cause a slew of permanent damage that could very likely be a prominent cause of cell death: these events will be discussed in detail in Chapter 3. Suffice it to say that if free-radical damage at the stage of drying is indeed a major cause precluding shoot development, it could be inferred that the provision of antioxidants at the pre-culture and post-excision stage was inadequate in alleviating ROS-induced damage to the shoot meristem. When aiming to optimise a cryopreservation protocol, it is necessary that the causes of failure, as well as the basis of successes, can ultimately be explained. Therefore, upon death of axes at the desiccation stage, the direction of the study progressed towards establishing underlying causes of failure, particularly focusing on events related to oxidative metabolism (Chapter 3).

2.3.7 Responses after cooling

Rapid cooling was applied to embryonic axes as the final step (i.e. after excision, pre-culture, post-excision soaking and flash drying to appropriate water contents had been done). In addition, two-step cooling of excised axes of *P. longifolia* was applied at the same stage in the protocol. Survival after cooling was assessed as root and/or shoot development. No embryonic axes of any of the three species survived cooling. Preliminary studies showed that excised axes of *Trichilia* species survived rapid cooling in terms of the ability to produce callus, but only when a portion of the cotyledon remained attached (Naidoo, 2009). The incorporation of antioxidant treatments prior to and after excision (pre-culture and post-excision soaking respectively) promoted seedling development prior to cryogenic processing and it was therefore thought that this treatment would facilitate survival throughout the cryopreservation process. However, results have shown that this addition was not adequate to

promote survival after manipulation of axes subsequent to excision that are integral to cryopreservation i.e. cryoprotection, desiccation, cooling, thawing and rehydration.

2.3.8 Discussion: cooling

A major limitation to obtaining a functional root and shoot from recalcitrant cryopreserved embryonic axes is the cooling process itself. Publications over the past two decades indicating survival, have often used the criterion of swelling and greening of axes, rather than production of viable seedlings, and in some cases reports cite survival despite the lack of shoot (Perán *et al*., 2006; Sershen *et al*., 2007) or root production (Pence, 1992). Of the 52 species for which success has been reported, only 31 can be considered to have been successfully cryopreserved, based on at least 50% of axes/embryos forming both a root and a shoot post-cooling and thawing (Pritchard *et al*., 1995). However, the growth potential of many of those seedlings was retarded or delayed, showing the practical difficulties imposed by cryogenic treatments (Pritchard *et al*., 1995; Berjak *et al*., 2010).

Zygotic axes of the species on which this study focused have never been cryopreserved successfully. The present results from the entire cryopreservation process showed importantly, that growth was inhibited after both cryoprotection and drying, and so death of explants post-cooling could be a consequence of stress incurred during an individual process, or the combined effect of stresses imposed on tissue from the stage of excision. It should be noted that while embryonic tissue may be able to cope with individual stresses, the cumulative effect (both physical and biochemical) of serial manipulations prior to the stage of cooling could overwhelm the capabilities of endogenous mechanisms. Therein lies the rationale for the further investigations of possible mechanisms of damage throughout stages of the cryopreservation process, with particular focus on oxidative stress profiling (Chapter 3).

At this stage of experimentation though, one should consider the widely accepted tenet of intracellular ice formation being the cause of death. The basis of death by intracellular ice is elegantly detailed by Acker & Croteau (2004) who explained that intracellular ice formation during rapid cooling occurs when cells are unable to respond via exosmosis to extracellular ice formation and the concomitant concentration of extracellular solutes, translating to death by intracellular retention of supercooled water and the associated probability of intracellular ice nucleation. Furthermore, the amount, location and mechanism of ice formation are explicitly implicated in the extent of cellular damage (Farrant *et al*., 1977; Acker & McGann, 2002).

Imbibitional-effects upon thawing and rehydration after cooling could also be considered a contributing factor leading to cell death, as explained earlier. Rewarming or thawing of embryos is a crucial stage that could influence the ability of tissue to survive or not (Normah & Makeen, 2008). The re-warming procedure employed in current experiments was optimised on embryonic axes from various recalcitrant species including those of the species used in this study. However, it is possible that factors contributing to death could occur at the re-warming stage e.g. disruption of the plasma membrane by small ice crystals coalescing into larger ones i.e. recrystallisation (Mazur, 1984). This phenomenon usually occurs when axes have been cooled rapidly but thawed or re-warmed at a slow rate (Mazur, 1984). Nevertheless, in the current study axes were warmed as rapidly as could be achieved, therefore perhaps the composition of the thawing medium (the CaMg solution) might not afford the potential for adequate protection during this process.

Touchell & Walters (2000) showed that recovery of excised axes of *Zizania palustris* improved from 35% to 56% upon maintenance of cultures in the dark, suggestedly due to the limitation of free radical production in the absence of light-dependent reactions. However, in the current study, despite the entire cryopreservation process, including in-vitro recovery, being conducted under dark conditions, no survival was observed.

There are also degradative events that could play a role in cell deterioration when axes have been previously cryoprotected, such as the rate of deplasmolysis post-thawing (Finkle *et al*., 1985) and osmotic swelling during the dilution of the additive during thawing (Farrant *et al*., 1977). However, during this phase of the study, axes did not survive cryoprotection, and, from observations by Goveia (2007) showing that cryoprotection did not improve survival of tissue post-cooling, axes in this study taken through to cooling were flash dried only. Therefore, the reasons for cell death associated with cryoprotectant additives can be ruled out at this stage but will be discussed later.

It is pertinent to consider at this point whether or not free radical stress, perpetuated by free radical chemistry will cease to occur at low temperatures. If not, damage derived from, and driven by, unregulated free radical reactions could influence initial recovery and survival of germplasm as well as the long term stability of preserved material (Benson & Bremner, 2004). When considering the long-term study of genetic stability in plants recovered from cryogenic storage, primary and secondary oxidative stresses are implicated in both genetic and molecular damage (Benson, 1990; Harding, 1999), and understanding the role of oxidative stress in cryo-injury may offer insights into the future development of both ‘traditional’ and contemporary cryo-conservation protocols (e.g. see Fleck *et al*., 2000).

The work described in this chapter formed a framework within which investigations directed at possible underlying causes of failure to survive cryogenic manipulations were carried out, as detailed on Chapter 3.

CHAPTER 3: Oxidative Stress Profiling

3.1 Introduction

3.1.1 Reactive Oxygen Species (ROS)

A free radical may be defined as any atom or molecule capable of independent existence that contains one or more unpaired electrons (Halliwell & Gutteridge, 1984, Halliwell, 2006), and is consequently highly reactive (stability varying between species) and paramagnetic in chemical and biological reactions (Fridovich, 1998; Benson, 2000b; Benson & Bremner, 2004). Much debate has arisen over the term "free" in the context of radicals. For the purpose of our investigations it is considered to be those radicals that immediately participate in new chemical reactions once generated (Benson & Bremner, 2004). Common radicals existing in tissue include the hydrogen atom (one unpaired electron), most transition metals, hydrogen peroxide (H_2O_2), molecular (O_2), activated (O_3) and singlet ($^1O_2$) oxygen (Halliwell & Gutteridge, 1984; Benson, 2000b). These radicals are generally accepted to be produced and perpetuated by oxidative metabolic processes in all aerobic organisms, specifically occurring at sites of photosynthetic, respiratory and signalling events in chloroplasts, mitochondria and peroxisomes (Benson, 2000b; Benson & Bremner, 2004; Apel & Hirt, 2004; Bailey-Serres & Mittler, 2006; Halliwell & Gutteridge, 2007; Van Breusegem *et al*., 2008). Initially, reactive oxygen species (ROS) were recognised chiefly as toxic by-products of aerobic metabolism (Hendry *et al*., 1992; Hendry, 1993; Chaitanya & Naithani, 1994; Bailey-Serres & Mittler, 2006) and were only later discovered to be integral components of normal cellular metabolism in terms of their participation in redox reactions and transferral of single electrons, both frequently occurring events in a multitude of life processes (Schopfer *et al*., 2001; Halliwell & Gutteridge, 2007; Oracz *et al*. 2007; Kranner *et al*., 2010). Notwithstanding their cytotoxic capacity to damage proteins, lipids and nucleic acids (Chaitanya & Naithani, 1994; Varghese & Naithani, 2002; Halliwell & Gutteridge, 2007), increasing evidence suggests that ROS act as essential signalling molecules in plant cells with involvement in regulation of growth and development, hormone signalling, responses to biotic and abiotic stresses and programmed cell death (Schopfer *et al*., 2001; Apel & Hirt, 2004; Bailey-Serres & Mittler, 2006; Gechev *et al*., 2006; Bailly *et al*., 2008; Kranner *et al*., 2010; Varghese *et al*., 2011).

Oxygen plays a pivotal role in aerobic life and centrally participates in primary metabolism, electron transfer reactions and substrate level oxidations (Benson & Bremner, 2004). Consequently aerobes are consistently rendered susceptible to free-radical mediated

oxidative stress (Benson, 2000b; Benson & Bremner, 2004). The dual role of free radical species highlights the importance of the plant 'reactive oxygen species gene network' which modulates the cellular homeostasis of these compounds, and the array of antioxidants (enzymatic and non-enzymatic) in plant cells, which combat increased ROS levels (Neubauer & Yamamoto, 1992; Foyer, 1993; Scandalios, 1993; Apel & Hirt, 2004; Foyer & Noctor, 2005; Bailey-Serres & Mittler, 2006). Considering the numerous signalling cascades that can be triggered by the arsenal of ROS-generating enzymes recently found to initiate and amplify ROS production for the purpose of signalling (Bailly *et al*., 2008; Van Breusegem *et al*., 2008; Roach *et al*., 2010), an equilibrium between the production and scavenging of ROS is integral to normal cellular function (Bailey-Serres & Mittler, 2006; Kranner *et al*., 2006). To establish the relationship, if any, between ROS and cell death or dysfunction during the cryopreservation of recalcitrant embryos/embryonic axes, it is imperative to understand the chemistry of free radical production, the protective mechanisms required to regulate their steady-state existence tightly, their activities (both destructive and constructive) within the cell, and the role of abiotic stresses in initiating and perpetuating free-radical mediated oxidative stress.

3.1.2 Free Radical Chemistry: production and proliferation

It is generally accepted that most chemical or biochemical reactions occur as a consequence of breaking/forming of covalent bonds or activities involving ions (Benson & Bremner, 2004), both being dependent on the interaction between paired electrons (Benson, 1990). Electrons spin about their axes and, according to Pauli's principle, in order to confer bond stability a pair of electrons in an atomic or molecular orbital must have anti-parallel spins (Halliwell & Gutteridge, 1984; Benson, 1990). Free radicals and molecular oxygen have properties that prevent conformation to the usual requirements for bonding, and under specific circumstances, composite electrons will split homolytically rather than heterolytically: this is the fundamental basis of a free radical reaction (Benson, 1990). More specifically, free radicals are formed via the gain or loss of an electron from a neutral molecule or homolysis (cleavage of a bond between molecules such that each one receives an electron [Hermes-Lima, 2004]). This, in turn, creates another free radical, setting off a chain of self-propagating reactions (Halliwell & Gutteridge, 1984; Benson, 1990; Hermes-Lima, 2004). Notably, two factors contribute to free radical activity in biological systems, these being the dependence of metabolism on the movement of electrons and the involvement of molecular oxygen in oxidative events (Benson, 1990).

Ground state triplet molecular oxygen ($^3O_2$) is a bi-radical that has its two outermost valence electrons occupying separate orbitals of parallel spin (Halliwell & Gutteridge, 1984; Benson, 1990; Apel & Hirt, 2004). If oxygen attempts to oxidise a non-radical molecule by accepting a pair of electrons, these are required to be of parallel spin to occupy the vacant spaces in the free electron orbitals (Halliwell & Gutteridge, 1984; Apel & Hirt, 2004). This imposes a restriction on oxidations by triplet oxygen with non-radical species; nevertheless, many radical and non-radical species are derived from the addition of energy or electrons to ground triple state diatomic oxygen ($^3O_2$), which is then converted to the much more reactive singlet oxygen ($^1O_2$). The reduction of oxygen to water requires four electrons from NADH, therefore free radical intermediates (focused on in this study) encountered along this pathway are generated by energy transfer or sequential univalent reduction of ground state triplet oxygen, i.e. the addition of one electron to $^3O_2$ results in the superoxide radical, the addition of two electrons and two protons results in the species, hydrogen peroxide, and the possibly most volatile, reactive and oxidising species, *viz*. the hydroxyl radical, is usually generated from the Fenton or associated reactions (a reaction between hydrogen peroxide and a ferrous ion; Fridovich, 1998; Arora *et al*., 2002; Apel & Hirt, 2004; Benson & Bremner, 2004; Roach *et al*., 2008).

Oxy-radicals have the greatest potential to act as promoters of damage in electron-rich cellular domains, necessitating their tight regulation to minimise the subsequent cascade of free-radical-mediated secondary reactions (Benson, 1990; 2000b). These 'chain' reactions amplify the consequences of the initiating event by perpetuating the continuous propagation of intermediate free radical species and their toxic by products (Halliwell & Gutteridge, 1984; Fridovich, 1998). An important consideration in free radical chemistry is that a reaction between a radical and non-radical molecule always propagates another radical (Fridovich, 1998). This phenomenon, at times, may contribute to a rapid increase in ROS concentration and this is referred to as an 'oxidative burst' (Apostol *et al*., 1989), which is a universal plant response to the detection of biotic and abiotic stresses (Doke, 1983). Subsequently, if enhanced production of free radical species is unabated, i.e. unregulated by antioxidant systems, and these react with lipids, proteins or nucleic acids, the ensuing oxidative damage inflicted on cellular components are symptomatic of 'oxidative stress' (Foyer & Noctor, 2005).

Initial reactions that generate free radicals are termed primary reactions and define the processes leading to primary oxidative stress. Damage incurred due to such primary reactions often occurs as a result of disrupted electron-rich domains, e.g. polyunsaturated fatty acids

(Benson, 1990). The toxic by-products of initial reactions cause cellular damage by disrupting the normal functioning of macromolecules including DNA, these reactions being termed secondary reactions, which define the processes involved in secondary oxidative stress (Benson *et al*., 2005). Oxidative stress is usually curbed in the cell by a series of termination interactions with protective mechanisms which are termed scavenging reactions (Benson, 1990) and will be discussed later. In germplasm challenged by cold treatments, oxidative stress can increase (Benson & Bremner, 2004; Varghese and Naithani, 2008). The effect of oxidative stress on cell metabolism and stability can be elucidated by considering the mechanisms of reactions mediated by free radical species and the sources of their generation.

3.1.3 Mechanisms and consequences of superoxide, hydrogen peroxide and hydroxyl radical production

To grasp the potential of damage caused by reactive oxygen species one should take note of the estimation that in human DNA, 1000 oxidant-mediated degradative events occur per cell per day (Halliwell & Gutteridge, 1989), hence the association of accumulated free radicals with ageing, senescence and disease. Similarly, it is suggested that free-radical-associated damage is an important marker of injury and cause of death in recalcitrant germplasm (Varghese & Naithani, 2002; Chappell Jr, 2008; Roach, 2009; Whitaker *et al*., 2010). Of the three ROS investigated in this study, superoxide is possibly the least reactive, and oxidative damage to cellular components is likely to be a consequence of the subsequent formation of hydrogen peroxide and the hydroxyl radical (Halliwell & Gutteridge, 1989; Smirnoff, 1993). The formation of these radicals begins with the single electron reduction of $^3O_2$ to form the superoxide anion ($^{\cdot}O_2^-$ [Halliwell & Gutteridge, 1984]). Superoxide then dismutates to form the peroxide ion (O_2^{2-}) which is not a radical but immediately protonates at physiological pH to form hydrogen peroxide (H_2O_2 [Halliwell & Gutteridge, 1984; Smirnoff, 1993; Apel & Hirt, 2004]). The rate of this dismutation is higher at acidic pH (Halliwell & Gutteridge, 1984). Both radicals then react according to the Haber-Weiss reaction to form hydroxyl radicals ($^{\cdot}OH$; Smirnoff, 1993). Substantial oxidative damage is however, highly dependent on iron or other transition metals to catalyse the Haber-Weiss reaction; this is known as the Fenton reaction (Smirnoff, 1993).

Superoxide is short-lived and is known to be less selective in its reactivity than the hydroxyl radical, and is therefore likely to permeate throughout a wider radius in a cell before finding a suitable target (Fridovich, 1998), and consequently may cause damage to a wider

spectrum of organelles. The conjugate acid of superoxide is the hydroperoxyl radical which has a pKa [acid dissociation constant] of 4.88; therefore upon dissociation in solution at a neutral pH, the majority of superoxide exists in acid-base equilibrium with its protonated form and many spontaneous dismutations are initiated (Fridovich, 1998). Disrupted electron transport activities that cause electrons to divert off course are usually a source of superoxide generation (Smirnoff, 1993). In mitochondria, auto-oxidation of ubiquinone whilst in the presence of oxygen describes a minor pathway that results in superoxide production (Smirnoff, 1993). Chloroplasts too, become sites of superoxide generation upon oxygen reduction of PS1 and PS2 (Asada & Takashi, 1987). Superoxide production is also promoted by NADH that is associated with peroxisomal and glyoxysomal membranes (del Río *et al*., 1989; Sandalio *et al*., 1988; Roach, 2009). Broadly speaking, superoxide, like the other two free radical species investigated in this study, $^{\cdot}OH$ and H_2O_2, are frequently generated by cytoplasmic, membrane-bound or exocellular enzymes participating in redox reactions (Wojtaszek, 1997). Amongst its positive functions in the cell, superoxide is known to play a role in growth (Foreman *et al*., 2003); cellular organisation (Gapper & Dolan, 2006) and gravitropism (Joo *et al*., 2001); however, produced within the hydrophobic layer of biological membranes, it is also capable of extensive and acute damage to phospholipids (Niehaus, 1978; Benson, 1990).

Hydrogen peroxide can be produced as a by-product of numerous processes, possibly the most significant one being the dismutation of the superoxide radical via the enzyme superoxide dismutase (SOD [Benson *et al*., 2005]). It is considered to be a somewhat stable ROS that is electrically neutral and is able to pass through the cell membrane and disperse to locations far from its site of production (Wojtaszek, 1997). Hydrogen peroxide has a spectrum of suggested roles in plant cells including modulation of phosphates involved in abscisic acid signalling (Apel & Hirt, 2004), overall disease resistance mechanisms (Apostol *et al*., 1989), transcription of defence-related genes (Orozco-Cárdenas *et al*., 2001), antimicrobial activity and mediator of cell wall strengthening (Morkunas *et al*., 2004; Gapper & Dolan, 2006). The molecule itself has fairly low reactivity (Bestwick *et al*., 1997) compared with the hydroxyl radical, production of which is possibly the most damaging consequence of hydrogen peroxide participating in Fenton reactions with metal ions (Benson, 1990). The intracellular concentration of H_2O_2 is tightly controlled by various enzymatic and non-enzymatic antioxidant systems and is assumed to vary between 1 and 700 nM (Chance *et al*., 1979). However, intracellular steady-state concentrations of H_2O_2 above 1 μM are

considered to cause oxidative stress, inducing growth arrest and cell death (Stone & Yang, 2006).

The hydroxyl radical is the most highly reactive radical in nature and is believed to be a significant contributor to the irreversible damage and modifications incurred by cellular macromolecules during oxidative stress (Gutteridge, 1995; Wojtaszek, 1997). It is produced via the reactions involved in Fenton chemistry or *in vivo* via the Harber-Weiss reaction (Benson *et al*., 2005). Its reactions are of three main types, *viz.* abstraction of the hydrogen atom, addition onto aromatic ring structures and electron transfer (Cohen, 1986). There are no known endogenous scavengers for ˙OH, therefore the only way to avoid oxidative-related damage is for control of events leading to its generation (Apel & Hirt, 2004), especially the regulation of the co-occurrence of hydrogen peroxide and transition elements (Roach, 2009). Being a potent oxidant, the hydroxyl radical attacks organic compounds at diffusion-limited rates and is particularly destructive when oxidising or hydroxylating sugars, purines and pyramidines indiscriminately (Fridovich, 1998; Benson & Bremner, 2004; Benson *et al*., 2005). The resultant oxidative damage caused to DNA has significant implications for genetic stability that far surpass those of superoxide and hydrogen peroxide, neither of which react notably with DNA and RNA bases (Benson & Bremner, 2004). While most systems are equipped with mechanisms to deal adequately with lesions caused to DNA, excessive production of radical adducts can saturate and overwhelm repair systems, translating to extensive long-term damage to DNA (Benson & Bremner, 2004).

The generation of secondary products of oxidative stress is also of great significance in biological systems. Malondialdehyde (MDA) is a particularly toxic secondary reaction product generated by a series of reactions that are initiated by the ˙OH (Cheeseman *et al*., 1988), and the production of MDA is often (as in this study) used as a quantitative measure to assess hydroxyl radical content in tissue. Although direct interaction between proteins and the hydroxyl radical is possible, impairment to the structure and function of proteins (and hence unstable cellular metabolism) is more commonly brought about through the formation of Schiff's bases when MDA destructively reacts with proteins by cross-linking with amine groups (Benson & Bremner, 2004; Benson *et al*., 2005). Lipid peroxidation, a common symptom ascribed to oxidative damage, is initiated via the abstracation of hydrogen by a hydroxyl radical from a methylene group to form a conjugated diene (Halliwell & Gutteridge, 1984; Smirnoff, 1993; Benson & Bremner, 2004; Scott & Jackson, 2008). Polyunsaturated fatty acids are most predisposed to peroxidation, the consequences of which include

breakdown of lipids and membrane derangement (Smirnoff, 1993). Notwithstanding its extensively destructive role in cells, the hydroxyl radical has also been implicated in plant growth by clearing breakages of existing primary cell wall polysaccharide matrices (Fry, 1998; Müller *et al*., 2009).

Cognisance of antioxidant mechanisms tightly controlling, rather than completely eliminating, ROS from cells, emphasises the importance of free radicals in cell signalling. As mentioned above, each ROS investigated in this study plays a normal physiological role in cells. The ROS are involved in various signalling roles, for example, in cellular defence against pathogens (hydrogen peroxide [Prasad *et al*., 1994]), programmed cell death (hydrogen peroxide [Jabs, 1999]; superoxide [Doke *et al*., 1994]), response to wounding (hydrogen peroxide [Orozco-Cárdenas *et al*., 2001]) and ABA-mediated stomatal closure (hydrogen peroxide [Pei *et al*., 2000]). They also play an integral role in the activation of mitogen-activated protein kinase (MAPK) signalling pathways (Apel & Hirt, 2004; Bailly, 2004; Pitzschke & Hirt, 2006). This is especially noteworthy since MAPK signalling modules act both upstream and downstream of an oxidative burst and are actively involved in eliciting responses to numerous stresses, with hydrogen peroxide specifically activating several MAPKs (Apel & Hirt, 2004; Van Breusegem *et al*., 2008). This suggests that ROS-induced activation of MAPKs has a fundamental role in mediating cellular responses to an array of stresses (Apel & Hirt, 2004).

The normal cellular *milieu* is maintained by avoidance of oxidative stress or, in the event of this phenomenon, the rigorous functioning of antioxidant and repair mechanisms (Benson, 1990). The balance between production and scavenging of ROS can be upset by an array of conditions which may be broadly divided into two categories: (1) environmental and (2) physiological factors (Benson, 1990). The aims of this study call for an assessment of ROS in relation to abiotic stresses which is detailed below.

3.1.4 ROS and their responses to abiotic stresses in the context of cryopreservation

During cryopreservation, isolated embryonic axes are exposed to a variety of pre- and post-storage treatments consisting of individual and compounded stress-inducing procedures, *viz*. excision, cryoprotection, drying, rehydration, cooling and thawing (Berjak *et al*., 2011; Pammenter *et al*., 2011). Compared with other conservation regimes, cryopreservation is likely the most challenging in terms of biochemical stress, as the injuries (mechanical & physiological) incurred due to excision of explants compounded by the various steps of cryo-

preparation, cryopreservation and recovery may provide outlets for the initiation of free radical production (Benson, 1990; Varghese & Naithani, 2008; Sershen *et al*., 2012c).

One of the first and inevitable steps in cryopreservation of hydrated plant germplasm is excision of explants such as embryonic axes, shoot-tips, buds, etc. Excision-induced damage can result simply from lifting embryos from the endosperm (as for *Strychnos gerarrdii* [Berjak *et al.*, 2011]) and inevitably seems to occur upon the manual removal of cotyledons from the embryonic axes: these procedures are intricately associated with ROS production, elicited in response to wounding (Ruuhola & Yang, 2006; Goveia, 2007; Whitaker *et al*., 2009; Roach *et al*., 2010). The production of ROS during pathogenesis or wounding is mostly apoplastic (as opposed to symplastic sources such as mitochondrial, chloroplastic or peroxisomal generating systems [Bolwell *et al*., 2002]) and is an important component of plant defence responses (Wojtaszek, 1997; Van Breusegem *et al*., 2008). Previous studies have shown that there is indeed a complex interaction between excision, subsequent desiccation (two of the early stages in the cryopreservation protocol) and an oxidative burst of extracellular ROS production by excised embryonic axes from recalcitrant seeds (Wojtaszek, 1997; Goveia, 2007; Roach *et al*., 2008; Whitaker *et al*., 2010; Berjak *et al.*, 2011). While death of explants after each cryogenic manipulation could be a result of physical/mechanical damage; deranged metabolic activity or interplay of both, it is critical to establish cause of injury after these two stages since optimal survival pre-cooling is essential for any survival post-cooling.

Cryo-preparation of plant tissues for cryopreservation, especially from species producing recalcitrant seeds, most often involves physical or chemical dehydration to bring the material to water contents amenable for cryopreservation. Dehydration is inevitably associated with oxidative stress and is possibly the most documented stress associated with ROS generation by embryonic axes (Leprince *et al*., 1990; Smirnoff, 1993; Chaitanya & Naithani, 1994; Liang & Sun, 2002; Varghese & Naithani, 2002; Cheng & Song, 2008; Xin *et al*., 2010; Whitaker *et al*., 2010). Oxidative damage is very likely one of the most deleterious consequences of water depletion (França *et al*., 2007). According to Senaratna & McKersie (1986), desiccation induces many changes in the cellular *milieu* that could lead to ephemeral dysfunctions in specific enzymes and/or electron transport chains. Such changes include reduction in the hydration shells of macromolecules; reduced cytoplasmic and intracellular transport; shifts in cytoplasmic pH and concentration of organic and inorganic ions. The dysfunction emanating from such changes is more likely to occur in desiccated tissue rather than in a fully hydrated system, and often initiates chemical reactions that

promote the generation of ROS (Senaratna & McKersie, 1986; Carvalho, 2008). Reduced hydration within the tissue invariably results in the inability of enzymatic mechanisms to scavenge free radicals (Carvalho, 2008). As a consequence of inefficient scavenging systems, a chain of events is initiated, including membrane phospholipid degradation, reorganisation of the lipid bilayer to accommodate for by-products of degradation and increased permeability of the bilayer, causing loss of compartmentalisation during rehydration (Blokhina *et al*., 2003). These processes eventually culminate in cell death (Senaratna & Mckersie, 1986).

The colligative action of cryoprotection that allows cells to avoid osmotic and biochemical toxicity and aids in preventing intracellular ice formation upon cooling, also acts in a dehydrative capacity. This mode of action during the stage of cryoprotection can, in itself, potentially perpetuate ROS generation in the event of stress (Sershen *et al*., 2012c).

Oxidative stress has also been shown to occur as a consequence of cryogen exposure (Whitaker *et al*., 2010), the duration of cryopreservation (Varghese and Naithani, 2008) and in association with thawing and recovery of explants (Sershen *et al*., 2012c). The potential relationship between free radical generation and the water status of the cells of cryogenically-stored tissues may have implications for the long-term genetic stability of the cryopreserved material. Cooling embryonic axes involves several sequential stress-inducing steps (Berjak *et al*., 2011), with the freezing of axes possibly leading to the loss of physical compartmentalisation and metabolic uncoupling, both of which translate to metabolic disruption including compromised functionality of antioxidant systems and production of toxic free radicals and their by-products (Benson & Bremner, 2004). Tapell (1966) demonstrated mitochondrial enzymes and ATP synthesis to become uncoupled during freeze-thaw cycles of cold-sensitive germplasm. This ultimately could lead to derangement of a suite of enzymes that are key to the regulation of primary metabolic pathways. During freezing stress, both primary and secondary oxidative damage can cause irreversible destruction and permanent loss of cellular integrity (Benson & Bremner, 2004).

There has been evidence to show that an oxidative burst occurs during rehydration after desiccation in bryophytes and lichens (Minibayeva & Beckett, 2001; Mayaba *et al*., 2002). However there have been no definitive studies on ROS production in response to thawing and rehydration in recalcitrant embryonic axes. Perán *et al.* (2006) suggested that because rapid rehydration reduces time spent at damaging intermediate water contents (hydration level 3), this would minimise damage (possibly ROS-induced) accumulated when water is made available to partially-dehydrated recalcitrant embryonic axes, as demonstrated

in *Artocarpus heterophyllus*, *Podocarpus henkelii* and *Ekebergia capensis*. Whitaker *et al*. (2010) later showed that two loosely-bound cell wall peroxidises were responsible for an oxidative burst with production of extracellular superoxide at every stage of the cryopreservation procedure, including rehydration and thawing. These two stages are procedures conducted after severe stress-inducing steps of desiccation and cooling. According to Levitt (1962), frost injury occurs in four phases: instantaneous point of freezing; while frozen; upon thawing and during post-thawing. Each of these stages upset the water status of cells which in turn has an effect on cellular metabolism (Benson & Bremner, 2004) in terms of the generation of ROS and impaired antioxidant activities. While ROS produced during rehydration and thawing may have positive signalling roles as demonstrated by Minibayeva & Beckett (2001) production that is inconsistent with signalling at this point may contribute to cell death. This is especially so since the tissue would be more likely to succumb to metabolic injury induced by oxidative imbalances after exposure to the cumulative stresses imposed by prior cryogenic steps (Berjak *et al*., 2010).

Each cryogenic procedure imposes a factor of abiotic stress to the conservation protocol. These stresses can have a two-fold influence on survival, i.e. individual stresses can cause cell death due to mechanical damage or through oxidative imbalances. Often, it seems that physical injury induces an oxidative response which generates free radicals that the system is unable to cope with as a result of inadequate antioxidant and repair mechanisms. Hence, the focus of work described in this chapter was to establish the underlying oxidative events in embryonic axes in response to abiotic stresses incurred during the cryopreservation protocol. To understand oxidative metabolism holistically, there was a need to investigate antioxidant systems concurrently with ROS production, and the following sections detail both endogenous antioxidants and those provided exogenously in the context of to this study.

3.1.5 ROS Detoxification: antioxidant and repair mechanisms

An antioxidant has been described as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell & Gutteridge, 2007). The evolution of antioxidant defences is solely responsible for the survival of aerobes contending with oxygen toxicity (Benson & Bremner, 2004; Halliwell & Gutteridge, 2007). Antioxidant mechanisms are required to inactivate or remove damaging ROS and to maintain an equilibrated state of the oxidative *milieu*. An array of antioxidant defences come in to play for these purposes, and afford protection as follows: enzymatic compounds such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT) that

catalytically remove ROS; agents that reduce ROS formation e.g. albumin, haem oxygenases, mitochondrial uncoupling proteins as well as proteins that minimise the availability of pro-oxidants; chaperones i.e. proteins that confer protection to biomolecules against oxidative damage via mechanisms other than reduction of ROS formation; physical quenching of ROS, e.g. carotenoid quenching of singlet oxygen; substitution of molecules susceptible to oxidative damage by those that are more resistant or provision of 'sacrificial agents' that are favoured for oxidation by ROS to conserve more vital biomolecules, e.g. GSH (reduced glutathione), α-tocopherol (vitamin E), ascorbate etc. (Apel & Hirt, 2004; Hermes-Lima, 2004; Halliwell & Gutteridge, 2007).

The extent of oxidative stress in a cell is determined by the amounts of superoxide, hydrogen peroxide and hydroxyl radical ions present relative to the concentration of antioxidants to scavenge free radicals (Benson, 1990; Apel & Hirt, 2004). To lend focus to the broad topic of antioxidants, this section specifically considers antioxidants with two questions in mind, *viz.* what they do and why are they important *in vivo* when cryopreserving recalcitrant germplasm.

When considering enzymatic detoxification, superoxide dismutase acts as the initial line of defence against ROS by dismutating superoxide to hydrogen peroxide (Benson, 1990; Apel & Hirt, 2004; Benson *et al*., 2005; Halliwell & Gutteridge, 2007). The reaction sequence in which dismutation occurs accommodates both for scavenging of radicals and regeneration of the oxidised catalyst (Benson, 1990). Superoxide dismutases, do occur apoplastically (where SOD dismutates superoxide as described above; Bogdanović *et al*., 2006) and are generally located in the chloroplasts of photosynthetic tissue since these are the organelles most susceptible to oxygen toxicity (Benson, 1990). Hydrogen peroxide is subsequently detoxified by CAT, APX and GPX (Benson, 1990). Ascorbate peroxidase requires the ascorbate-glutathione cycle (which reduces oxidised glutathione–(GSSG), monodehydroascorbate (MDHA) and dehydroascorbate (DHA) to form GSH and ascorbate) as a regeneration system in order to detoxify hydrogen peroxide to water via oxidation of ascorbate to MDA. Like APX, GPX also detoxifies hydrogen peroxide to water but uses the glutathione-peroxidase cycle involving regeneration of GSH from GSSG by glutathione reductase, GR (Benson, 1990; Apel & Hirt, 2004).

Glutathione is a thiol which has a pivotal protective role against oxidative stress and structural maintenance of proteins in plant tissues (Benson & Bremner, 2004). Glutathione occurs in cells in its reduced form (GSH) and is oxidised to its disulphide form (GSSG) during oxidative stress. Glutathione reductase and NADPH are important for the regeneration

of GSH from GSSG in order for glutathione to exist principally in its reduced form in cells. Under severe oxidative stress, GSSG content may increase if the oxidation of GSH occurs faster than GSSG reduction by GR, and so the relative content of GSH to GSSG in cells is often used as a marker of oxidative stress (Benson & Bremner, 2004).

The ascorbate-glutathione antioxidant coupling pathway occurs in the mitochondria, cytoplasm and peroxisomes and is responsible for the regeneration of ascorbate from its oxidised form dehydroascorbate, which is generated under oxidative stress (Potters *et al*., 2002). Ascorbate is a powerful endogenous antioxidant and has a spectrum of protective roles (Benson & Bremner, 2004). Ascorbate or ascorbic acid (AsA) was one of the key exogenous antioxidants applied and tested in this study, the other two being dimethyl sulfoxide (DMSO) and cathodic water. Exogenously applied antioxidants will be detailed further below as will be their relation to endogenous antioxidant pathways within the cell.

3.1.6 Ascorbate (ascorbic acid)

L-Ascorbic acid (L-AsA) or vitamin C is the common name for the organic molecule, L-*threo*-hexenon-1,4-lactone (Potters *et al*., 2002). This compound is one of the principal weak acids in plant cells and at physiological pH (5-7) dissociates into the ascorbate anion (ASC; Potters *et al*., 2002). The ascorbate anion is frequently involved in the cell as an electron donor and one of its initial oxidation products is the free radical monodehydroascorbate (MDHA) which is fairly harmless as it contains delocalised electrons which prevents its frequent reactivity with other molecules (Potters *et al*., 2002). In the chloroplast, AsA is regenerated via the Halliwell-Asada cycle (Foyer & Halliwell, 1976; Potters *et al*., 2002). Events involved in AsA regeneration begin with the oxidation of ASC to MDHA and dehydrascorbate (DHA) by the enzymes monodehydroascorbate reductase (MDHAR) and ferredoxin. MDHAR uses electrons from NADPH to reduce MDHA, while ferredoxin reduces MDHA directly. Dehydroascorbate reductase (DHAR) is the link between ascorbate and glutathione, where DHAR uses an electron from GSH to convert DHA to ASC. GSH itself is then oxidised to GSSG (oxidised glutathione). Glutathione reductase then re-reduces GSSG to GSH via two electrons from NADPH. These reactions (Fig. 3.1) together with the activity of APX comprise the cycle responsible for ASC regeneration in the chloroplast (Potters *et al*., 2002).

Ascorbate is a crucial element of oxidative-mediated metabolic control and is a known antioxidant of multiple functions (Halliwell & Gutteridge, 2007). Amongst these functions, ascorbate directly interacts with and scavenges superoxide and the hydroperoxyl

radical (rate constant > 10^5 M^{-1} s^{-1}); the hydroxyl radical (rate constant > 10^9 M^{-1} s^{-1}); thiyl (sulfenyl) and oxysulphur radicals; singlet oxygen and nitroxide radicals (Halliwell & Gutteridge, 2007). Ascorbate also serves as a substrate for ascorbate peroxidases which are essential enzymes required for the detoxification of hydrogen peroxide in chloroplasts. Importantly, ascorbate co-operates with α-tocopherol and regenerates this lipid-soluble anti-oxidant from α-tocopheryl radicals in membranes and lipoproteins (Halliwell & Gutteridge, 2007).

Generation of free radicals and the inability of antioxidant systems to modulate them are consistent with water-stress-induced damage in recalcitrant germplasm (Berjak & Pammenter, 2008). The failure of antioxidant systems to regulate ROS adequately in recalcitrant germplasm could be due either to the absence of certain crucial pathways, or dysfunctional expression of fundamental genes coding for the functioning of essential enzymes and protective molecules (Berjak & Pammenter, 2008). In the present investigation, embryonic axes were exposed to solutions of DMSO and/or ascorbic acid made up in cathodic water (cathodic water being suggested to be an enhancer of antioxidant activity [Berjak *et al.*, 2011]) after each stage of the cryopreservation protocol and the levels of extracellular ROS production and antioxidant activity measured to establish if treatments inclusive of a free radical scavenger would reduce ROS generation and promote survival of explants.

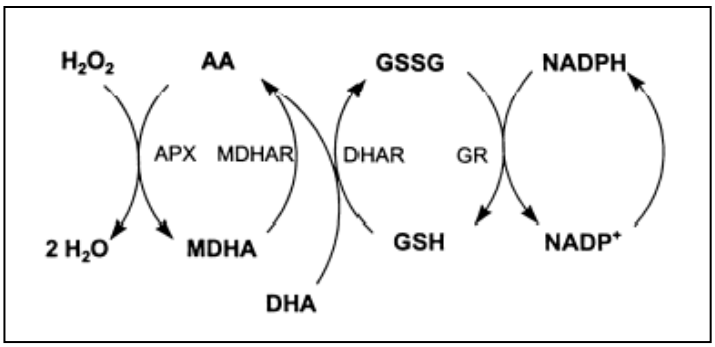


Fig. 3.1: Illustrating the reactions of the cycle by which AsA is regenerated in the chloroplast (Ascorbic acid denoted by the abbreviation AA [May *et al*., 1998]).

3.1.7 Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide was initially synthesised via the oxidation of dimethyl oxide in 1867 (Yu & Quinn, 1994). This compound has widespread applications in many fields, most particularly in cell biology as a solvent for drugs (Miller, 1992), for its cryoprotective properties (Rall & Fahy, 1985; Karlsson *et al.*, 1993), as a bio-membrane penetrator (Hsieh, 1994), a free radical scavenger (Simic, 1988), as a cell fusogen (Ahkong *et al.*, 1975; Tilcock & Fisher, 1982; Anchordogny *et al.*, 1991), and initiator of cell differentiation (Maclean & Hall, 1987). Unlike ascorbic acid, DMSO is not a naturally occurring molecule in cells and its exogenous application in this study was based on both its cryoprotective (see Chapter 2) and radical scavenging properties, the latter being the focus in the work described in this chapter. Nevertheless, it is pertinent in the context of ROS being suggested to be produced at low and ultra-low temperatures, a compound such as DMSO that has both free radical scavenging capacities as well as colligative and osmotic properties could contribute significantly to protection in freezing protocols if modulated appropriately (Benson & Bremner, 2004).

The hydroxyl radical will react with a spectrum of cellular constituents: however, it can be scavenged by DMSO which captures the radical and upon interaction forms methane sulphinic acid (MSA) and a much less reactive methyl radical (CH3). This methyl radical can then be further converted to methane, ethylene or formaldehyde (Klein *et al*., 1981). Dickinson *et al*. (1967) demonstrated another useful function of DMSO when they showed its ability to protect tightly coupled mitochondria from freezing damage. According to Dickinson et al. (1967) the protection of mitochondria indirectly reduces ROS generation that occurs in response to primary damage of the electron transport chain. Many known hydroxyl radical scavengers are efficacious at concentrations toxic to cells and, by contrast, DMSO at an effective concentration appears to be reasonably endured by cells while acting as a powerful hydroxyl radical scavenger (Yu & Quinn, 1994). However, DMSO toxicity has been documented in biological systems and hence the duration of exposure to, and concentration of, DMSO has to be carefully optimised before implementation in a cryopreservation procedure. Toxicity is closely related to properties of DMSO: e.g. when mixed with water (68% v/v), the strong heat of mixing can increase the temperature of the system by 30°C (Yu & Quinn, 1994), translating to possible – or even probable – thermal damage to macromolecules. In addition, the ability of DMSO to enhance permeability of membranes may promote membrane damage at physiological temperatures (Yu & Quinn, 1994). Its ability to cause protein denaturation was demonstrated by Jacobson & Turner (1980).

In the present study, as was the procedure with ascorbic acid, DMSO was exogenously provided at various stages of the cryopreservation protocol to assess its ability to act as an antioxidant, thereby regulating oxidative stress generated by cryogenic procedures. A third exogenous and novel treatment in the field of cryobiology that pertained to the provision of a scavenger of a potentially broad spectrum of ROS during the stages of cryopreservation was examined. This involved the use of cathodic protection (see 3.1.8), consisting of cathodic water (electrolysed solution of calcium and magnesium chloride) and cathodic flash-drying (rapid drying on a grid on which a static field was generated via the cathode of a power-pack) at appropriate stages associated with processing of embryonic axes for cryopreservation, and upon retrieval from cryogenic conditions.

3.1.8 Cathodic Protection

Conventional attempts to counteract stress induced by unbalanced ROS activity include the provision of exogenously applied synthetic or naturally-occurring antioxidants and transition-metal-chelating agents. However, the use of such compounds can have cytotoxic side-effects (as discussed for DMSO above); naturally occurring antioxidants (like glutathione) could possibly upset the balance of endogenously existing compounds and chelating transition metal cations could induce nutrient deficiencies (Berjak *et al.*, 2011). Therefore, a novel alternate strategy in the form of cathodic protection has been proposed to ameliorate destructive consequences of unregulated ROS generation (Pammenter *et al*., 1974; Berjak *et al*., 2011).

The first recorded attempt of (dry) cathodic protection of (orthodox) seeds was that by Pammenter *et al*. (1974) who placed the seeds on the extended cathode (aluminium foil sheet) in a static electric field with a potential difference between cathode and anode of 300 volts. This approach can be extended to embryos or embryonic axes of desiccation-sensitive seeds by applying a similar field whilst drying, with the grid on which the tissue is dried being the cathode. The premise here is that a highly reducing cathode could bring about reduction of ROS, possibly ameliorating oxidative damage during desiccation. Embryos that are cathodically protected in this manner are in direct contact with the cathode therefore the direct cathodic charge would be applied to the surface of the explant and subsequently transmitted throughout the tissue as cell walls essentially constitute a continuum from the surface throughout the tissue. Provision of electrons to free radicals generated during desiccation would possibly quench ROS by pairing with them instead of those abstracted from biomolecules of the explant.

The second mechanism of cathodic protection applied was by means of electrolysed, reduced "cathodic" water (wet cathodic protection). Cathodic water is generated by electrolysis, with the cathode and anode in separate chambers, of a solution containing dilute electrolyte(s). Cathodic water can be distinguished from anodic water (produced at the anode) by its high pH (10.00 – 11.50); high dissolved hydrogen content and low oxidation-reduction potential (-800 to -900mV) (Huang *et al*., 2008). It has been shown to have antioxidant properties (mainly *in vitro*) in terms of its free radical scavenging ability (superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen) and its role in protecting DNA, RNA and proteins from oxidative damage (Shirahata *et al*., 1997). Hanaoka (2001) reported an increase in superoxide dismutation by antioxidants in the presence of cathodic water as a consequence of increased dissociation activity of water. While cathodic water itself did not show superoxide dismutation activity on its own, it was able to reduce hydrogen peroxide levels due to the activity of its constituent – activated, dissolved H_2. Berjak *et al.* (2011) provided evidence of the efficacy of cathodic water provided at appropriate stages during cryo-processing and upon retrieval of recalcitrant axes of *Strychnos gerarrdii* after cryopreservation, and also showed its positive effects on total antioxidant activity of axes of a second species, *Boophane disticha*. The use of cathodic water in the present investigation is based on its proposed antioxidant abilities either as a powerful reductant and/or as an enhancer of endogenous antioxidant activity. Based on the mode/s of action of cathodic water, its use throughout appropriate stages of the cryopreservation process was tested with and without provision of the known antioxidant, AsA, primarily to establish its free radical scavenging capabilities and its effect on endogenous antioxidants after each step during the cryo-procedure.

3.1.9 Oxidative metabolism investigations on embryonic axes from species specific to this study

The rationale behind investigating oxidative metabolism in this study was based on the success achieved in terms of regeneration of functional seedlings (with roots and shoots) from embryonic axes after excision followed by treatment with antioxidants (Naidoo *et al*., 2011). Earlier work on both *Trichilia* species (Whitaker *et al*., 2010; Varghese *et al*., 2011) that has focused on oxidative metabolism had provided the basis for experimentation in this context.

Goveia *et al*. (2004) first proposed a link between an oxidative burst of superoxide accompanying excision of the fleshy cotyledons of *Trichilia* spp. and necrosis of the shoot

tip, particularly when the shoot meristem is in close proximity to the wound sites (Pammenter *et al*., 2011). It was then shown by Whitaker *et al*. (2010) that the steps of excision, desiccation, cooling, thawing and rehydration in the cryopreservation of *T. dregeana* axes were accompanied by excessive generation of superoxide and those authors proposed that this superoxide radical production was probably the cause of no axis survival after cryopreservation. Song *et al*. (2004) undertook a study on *T. dregeana* axes and reported a strong correlation between their desiccation tolerance, an increase in antioxidant enzyme activities and a decrease in lipid peroxidation in association with an ascorbic acid treatment. Those authors showed ascorbic acid to reduce electrolyte leakage from dehydrated axes, which corresponds to reduced membrane damage. A recent study on *T. dregeana* axes showed that the greater retention of viability at lower water contents in rapidly-dried axes compared with those that were slowly dried could – at least partly – be explained by the retention of functional antioxidant status (Varghese *et al*., 2011) upon rapid dehydration. As part of the present study, Naidoo *et al*. (2011) showed death of the shoot meristem to be obviated in excised axes of both *Trichila* species and *P. longifolia* by using exogenously applied treatments of DMSO and ascorbic acid pre- and post-excision. The implications of that part of the study were that the damaging effects of the oxidative burst accompanying excision of axes were ameliorated via applied antioxidant treatments (as described in Chapter 2).

Following from this, the current study was directed at investigating the link between cryogenic procedures, the accompanying ROS production and endogenous antioxidant activity in the presence and absence of selected exogenously-applied (potential) antioxidants. Due to restricted seed availability, certain experiments described in this chapter had to be conducted either on *T. emetica or T. dregeana.*

3.1.10 Objectives

The underlying problem of poor survival after cryopreservation across species may be uncontrolled ROS generation perpetuated by additive stresses and further exacerbated by dysfunctional endogenous antioxidant systems. The aim of the work detailed in this chapter was firstly, to quantify the production of superoxide, hydrogen peroxide and the hydroxyl radical after each stage of the cryopreservation process as markers of stress injury. Secondly, the work aimed concurrently to assess endogenous antioxidant activity after the various procedures to establish the relationship between ROS production and the inherent abilities of scavenging mechanisms in recalcitrant embryonic axes in response to stress. In terms of

assessment of antioxidant activity, the work also tested the effect of pH of exogenously applied antioxidants on endogenous antioxidants. Lastly, the experimentation was aimed at elucidating the effect of exogenously applied antioxidants on both ROS activity and endogenous antioxidants at each step of the cryopreservation protocol. During each of these undertakings, oxidative profiling was assessed in parallel with the effect of treatments on viability of embryos.

The over-arching objective of the research presented in this chapter was to profile oxidative metabolism throughout the various steps of cryopreservation so as to make advances towards solving the problems associated with production of viable explants and subsequent seedling establishment after cryostorage of axes of tropical/sub-tropical recalcitrant-seeded species. Essentially, the studies conducted and described in this chapter aimed to ascertain the cause of death during cryopreservation in terms of oxidative events, while continuing to optimise procedure in terms of provision of external antioxidant treatments.

3.2 Materials and methods

The work detailed in this chapter was split into two major experiments. The first consisted of trials to assess quantitatively and (in some cases) qualitatively: (1) the effect of selected antioxidants on superoxide generation and viability of excised axes; (2) the effect of pH of the selected exogenously applied antioxidants on endogenous antioxidant activity and viability of excised axes; and (3) the effect of cathodic water on the efficacy of ascorbic acid and DMSO in terms of promoting endogenous antioxidant activity. These trials were conducted on *Trichilia emetica* at all stages of cryopreservation prior to desiccation as illustrated in Fig. 3.2. The second set of experiments quantitatively assessed: (1) endogenous antioxidant activity; (2) superoxide production; (3) hydrogen peroxide production; (4) hydroxyl radical production; and (5) viability/germination using excised axes of *T. dregeana* after each of the procedures outlined in the cryopreservation procedure as illustrated in Fig. 3.3. Each of these parameters was measured after axes were exposed to treatments that included selected antioxidant/s and to the same treatments in which these were not incorporated. The broad question addressed by this line of experimentation was aimed at establishing the effect of cryo-preparation and cryogenic stresses on ROS production, the corresponding endogenous antioxidant activity and viability, and secondarily to assess whether the application of exogenous antioxidants at stages of cryopreservation would influence the effect/s of stress on these parameters.

3.2.1 General overview: steps of cryopreservation carried out for assessment of the parameters listed above

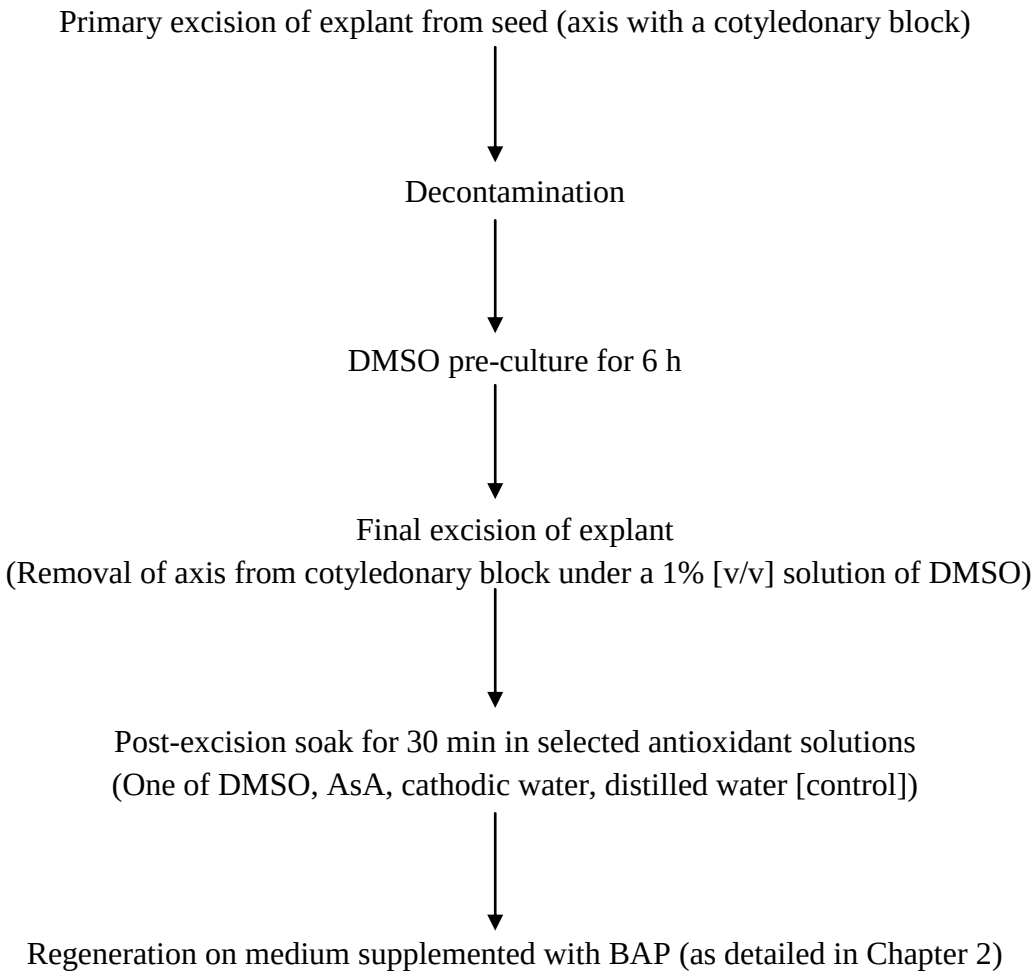


Fig. 3.2: Flow diagram showing successive steps prior to desiccation, after each of which selected parameters were assessed.

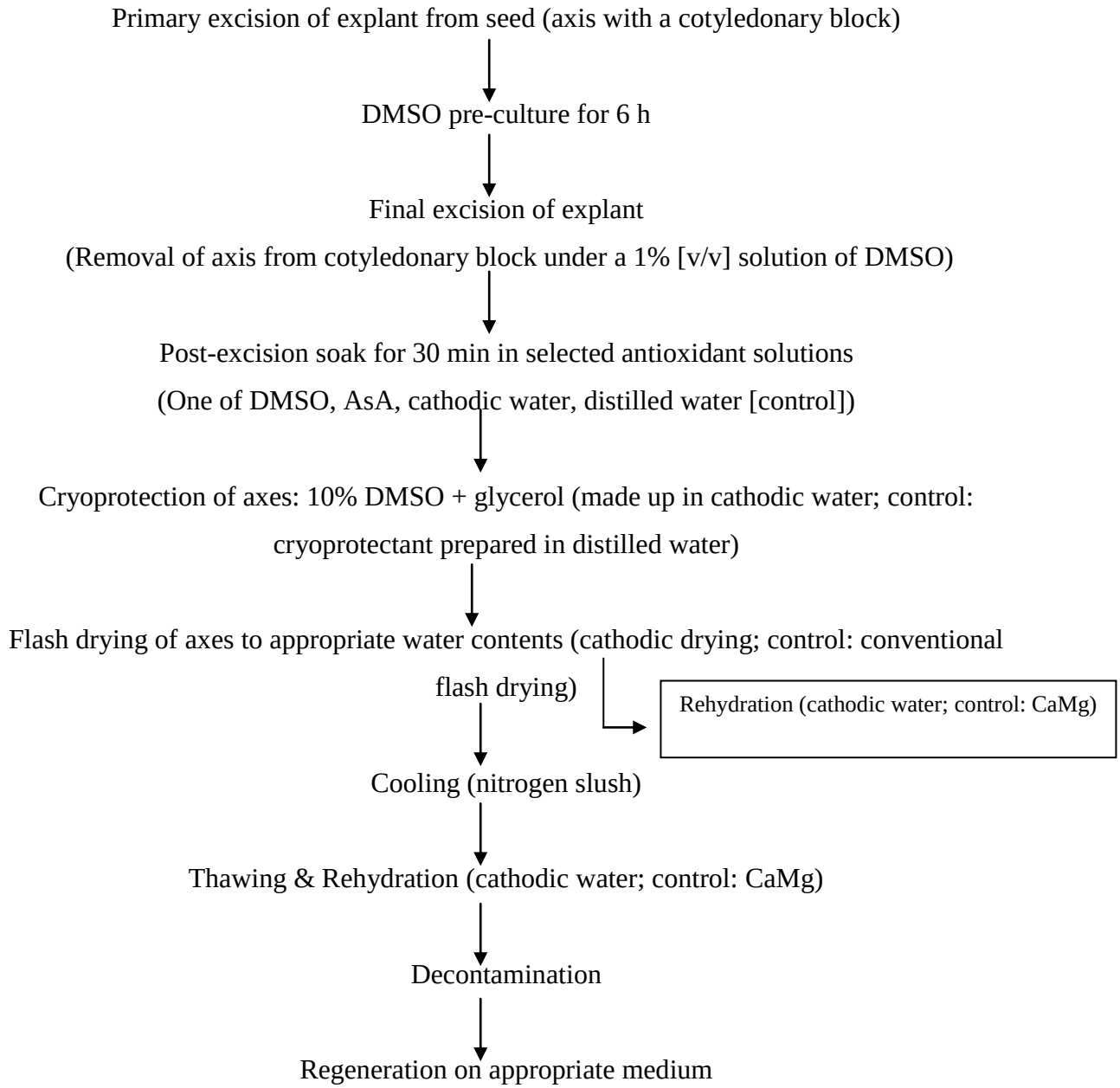


Fig. 3.3: Flow diagram illustrating successive steps of the cryopreservation protocol after each of which selected parameters were assessed.

The details of each of these steps were exactly as previously described (Chapter 2). Thus the methods presented here are confined to the procedures used to assess free radical/ROS levels and endogenous antioxidant activity, as well as details concerning cathodic protection applied at each stage. For every parameter tested, a sample size of 20 excised axes was used (5 axes per replicate). Furthermore, every step of the cryogenic procedure subsequent to primary excision of the explant was carried out in the dark.

3.2.2 The effect of DMSO during stages of pre-culture, final excision and post-excision soaking on superoxide production

Extracellular superoxide production was quantitatively measured using *T. dregeana* axes (n=20) during stages of excision. Measurement of superoxide production was taken after exposure of axes to treatments that included and omitted DMSO. Primary axes (n=20) were cultured for 6 h on full strength MS medium (Murashige & Skoog, 1962) incorporating 0.1% DMSO (preparation as in Chapter 2) and for the same period on unmodified full-strength MS medium. Final excision of axes was conducted under a solution of 1% DMSO prepared in distilled water and under distilled water as the control treatment. In addition to assessing superoxide production after these two treatments, generation of this ROS was also measured in both treatments over a period of 30 min at 5 min intervals to assess the effect of DMSO on the initial oxidative burst upon excision and the generation of superoxide over time. Axes were exposed to a post-excision soaking treatment in 1% DMSO made up in distilled water, in comparison with axes exposed to a non-antioxidant solution, i.e. distilled water.

3.2.3 The effect of soaking solutions on endogenous antioxidant activity and shoot development

To assess the most efficacious post-excision soaking solution, a 1% solution of AsA (w/v) and a 1% solution of DMSO (v/v) were prepared in distilled water or cathodic water. Thereafter, axes (n=20) were processed according to the procedure outlined in Fig. 3.2 and the effect of each of the following post-excision solutions on total antioxidant activity and shoot development were tested: 1% AsA in distilled water; 1% AsA in cathodic water; 1% DMSO in distilled water; 1% DMSO in cathodic water; cathodic water and distilled water alone (controls).

Electrolysed water was produced by provision of a 60V potential difference, using a BioRad™ Powerpac (BioRad, Hercules, California, USA) to which platinum electrodes were attached, through water containing the electrolytes 0.5mM $CaCl_2.2H_2O$ and 0.5µM $MgCl_2.6H_2O$ (CaMg [Mycock, 1999] – which has long been used in the non-electrolysed state for rehydration and rinsing throughout the cryopreservation procedure in our laboratory) as a control for cathodic water). Electrolysis of the CaMg solution was conducted over 1 h at room temperature and yielded cathodic (reducing) water at pH *c.* 11.2 and anodic (acidic) water at pH *c.* 2.4. The setup included two glass beakers, each containing 200 ml autoclaved CaMg solution, with the anode being immersed in one and the cathode in the other. The circuit was completed via a salt bridge which was agar-based and contained saturated

potassium chloride. In all cases, cathodic water was used within an hour of preparation. Axes were soaked for 30 min in the dark in each of the prepared solutions, after which shoot development and total antioxidant activity were assessed.

3.2.4 The effect of: (1) stages of excision and (2) pH of exogenously applied antioxidant solutions, on endogenous total antioxidant activity (TAA), viability and shoot development

Table 3.1: Illustrating the treatments after which TAA, viability and shoot development were assessed (n=20)

TREATMENT		
Primary excision		
Primary excision; decontamination		
Primary excision; decontamination; Pre-culture on medium supplemented with DMSO		
Primary excision; decontamination; Pre-culture on medium supplemented with DMSO; final excision under a solution of DMSO		
POST-EXCISION SOAKING SOLUTION		
	ORIGINAL pH	**ADJUSTED pH (prior to use of solution**
→ Distilled water	8.2	11.2
→ Cathodic water	11.2	8.2
→ 1% DMSO (distilled water)	7.6	11.4
→ 1% DMSO (cathodic water)	11.4	7.6
→ 1% AsA (distilled water)	2.8	3.0
→ 1% AsA (cathodic water)	3.0	2.8

Each of the treatments listed in Table 3.1 was carried out as outlined in Fig. 3.2. To test the effect of pH of the post-excision soaking solutions on selected parameters, the original pH of each of the soaking solutions was recorded. The pH of each of the solutions prepared in distilled water (and distilled water itself) was adjusted, using appropriate solutions of NaOH and HCl, to the pH of the same solution prepared in cathodic water and

vice versa, as shown in Table 3.1. After each of the 16 treatments, TAA was measured (as detailed below), shoot production was recorded and viability was qualitatively assessed using 2,3,5-Triphenyltetrazolium chloride (tetrazolium; TTC/TTZ) staining. A 2% tetrazolium (w/v) solution was prepared in 0.05 M Tris-HCl buffer and adjusted to pH 7.5. Treated axes were individually placed in a 2 ml Eppendorf® tube into which 300 µl of TTZ-Tris HCl buffer and 1.2 ml of Tris-HCL buffer were dispensed. Material was incubated in the dark for 24 h and axes were thereafter hand-sectioned under a dissecting microscope (Bausch & Lomb, ASZ 4SE, USA) and viewed for viability assessment.

3.2.5 The effect of cathodic water on the efficacy of DMSO and AsA in promoting shoot development and TAA

Solutions of DMSO and AsA were prepared in distilled and cathodic water respectively (at their original pH) as described in Chapter 2 (Section 2.7). Shoot production and TAA (n=20 for each) were then assessed after post-excision soaking in each solution, including treatments exposed to distilled water or cathodic water alone.

3.2.6 Total Antioxidant Activity (TAA)

Total antioxidant activity (enzymatic and non-enzymatic) was measured in treated and control axes (n=20) using a decolorisation assay, which is applicable to lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants (Re *et al*., 1999). The radical monocation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS•1) is pre-formed (i.e., not continually generated in the presence of an antioxidant), being generated by oxidation of ABTS with potassium persulfate and is thereafter reduced by hydrogen-donating antioxidants. The protocol for TAA assessment considers both the concentration of the antioxidant and the duration of the reaction on the inhibition of radical cation absorption. The estimation of TAA is described here in two stages: extraction and spectrophotometric analysis.

Extraction: Twenty axes, separated into four batches of 5 replicates, were pre-weighed and homogenised in liquid nitrogen (LN) and 0.1g insoluble polyvinylpyrrolidone (PVP), using pre-cooled pestles and mortars. Axes that were treated with antioxidant solutions were briefly rinsed 3 times with distilled water (to remove traces of treatment substances e.g. AsA, DMSO, cathodic water) and blotted with filter paper prior to extraction. Each replicate contained axes weighing approximately 20 mg. Extraction buffer (50 mM KH_2PO_4 buffer; pH 7.0) containing 1mM $CaCl_2$, 1mM KCl and 1mM EDTA, was prepared

in advance and kept in a 4°C constant temperature room, Using a micropipette, 1 ml of extraction buffer was added to the homogenised axes and mixed well. Each extract was dispensed into pre-chilled 2 ml Eppendorf® tubes held on ice. Each mortar was then rinsed twice with 0.5 ml of extraction buffer, bringing the total volume of extract to 2 ml. Each Eppendorf tube was then immersed in LN to snap-freeze the contents, and stored in Nalgene® vial storage boxes in a -70°C freezer until further use.

Once samples were removed from the -70°C freezer, centrifugation of extract samples and spectrophotometric analysis for TAA was conducted on the same day. The Eppendorf tubes containing the extracts were thawed and left on ice before centrifugation. The centrifuge (Hermle Labortechnik, Germany) was programmed to 14 000 rpm at 4°C. Eppendorf tubes were distributed evenly in the centrifuge and samples were centrifuged for 10 min. The supernatant after centrifugation (1 ml) was dispensed, on ice, into pre-chilled Eppendorf tubes and spun for a further 5 min. Thereafter, 600 µl of extract was dispensed into another set of pre-chilled Eppendorf tubes and kept on ice for the ABTS assay.

Spectrophotometrical analysis (ABTS cation decolorization assay): All spectrophotometric estimations, were carried out using an ultra-violet visible (UV-Vis) spectrophotometer (Cary 50 Conc UV Vis spectrophotometer, Varian, Palo Alto, CA), at a constant temperature of 25°C, in the dark. The spectrophotometer was zeroed with phosphate buffer saline (PBS; 5 mM Na_2HPO_4 and 37.5 mM NaCl, pH 7.4). Prior to assessment of TAA in the extracts, a working solution of ABTS was prepared. This involved the dilution of the ABTS radical solution (7 mM ABTS and 2.45 mM $K_2S_2O_8$ in 1 ml of distilled water and incubated in the dark 12-16 h before use) with PBS until an absorbance of 0.70 ± 0.02 at a wavelength of 732 nm was attained. Once the working solution was optimised, 1 ml was pipetted into a plastic cuvette and absorbance read at 0 min before the reaction was initialised. Immediately thereafter, 100 µl of extract from each sample was added to a cuvette and the reaction propagated by inverting the cuvette three times using a piece of Parafilm® to close it. The decline in absorbance was subsequently recorded at 0.5, 1 and 2 min. The assay was performed twice for each replicate and hence the values are the mean values of eight estimations.

Standard curve for ABTS: For each set of experiments a standard curve for the ABTS assay was generated for the calculation of TAA. For this purpose, a 5 mM Trolox® (6-hydroxy-2.5.7.8-tetramethylchromeane-2-carboxylic acid, 97%) solution was prepared using extraction buffer as the solvent. From the stock solution, 1 ml of the following standard mM concentrations was prepared: 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 mM, using the

extraction buffer for dilution. To generate a standard curve, the assay for Trolox was conducted exactly as the ABTS assay for extracts from tissues (described above), except that 100 µl of each standard solution was added to the ABTS working solution in place of 100 µl of extract from each treatment. Absorbance readings were taken from each of the standard solutions three times.

The ABTS radical decolorisation assay was conducted on axes after each step as outlined in Fig. 3.2 (*T. emetica*) and Fig. 3.3 (*T. dregeana*). The standard assay was conducted after both experiments (Figs 3.2 and 3.3). All calculations for TAA activity were expressed on a fresh mass basis as the extraction aspect of the assay was invasive.

3.2.7 Superoxide assessment

To assess extracellular production of superoxide, the oxidation of epinephrine to adrenochrome was measured spectrophotometrically at a wavelength of 490 nm (Misra & Frodovich, 1972), using the UV-Vis spectrophotometer (described above). A 1 mM epinephrine solution (pH 7.0) in 1 M HCl was prepared in dark prior to the assay. From this, the incubation medium consisting of 0.5 ml epinephrine solution and 1.5 ml distilled water was dispensed into 5 x 2-ml Eppendorf tubes containing 4 axes (per treatment, n=20). These were then placed on an orbital shaker (Labcon, Instrulab CC, Maraisburg) at 70 rpm, in the dark at room temperature, for 15 min (after Roach *et al*., 2008). After incubation, the solutions from each Eppendorf tube were pipetted into plastic cuvettes and the absorbance for each sample was recorded at 490 nm. Axes from each sample were then placed in an oven at 80°C for 48 h to obtain dry mass. The extinction co-efficient of adrenochrome at $A_{490,}$ 4.47 mM^{-1} cm^{-1} was used to calculate superoxide production and this was expressed in µmol min^{-1} g^{-1} on a dry mass basis. The absorbance was also measured for an internal control for each treatment in which embryonic axes were incubated (as described above) in distilled water to account for any leakage from the tissues during incubation. This absorbance was deducted from all estimations prior to calculations.

Validation of superoxide assay: It is possible for epinephrine to be oxidized non-specifically via certain enzymes; therefore the validity of the assay for the detection of extracellular superoxide production was assessed. Horseradish SOD was added to the incubation medium described above to give a final concentration of 0.1 µg ml^{-1} before this solution was dispensed into tubes containing axes (both treated and control). In both cases, the addition of SOD to the incubation medium inhibited the oxidation of epinephrine by more

than 50%, validating the use of this assay for extracellular superoxide detection. This assay was performed using the UV-Vis spectrophotometer described above.

3.2.8 Hydroxyl radical assessment

Five replicates of 4 axes each (n=20) were placed in 2-ml Eppendorf tubes and incubated in 1.5 ml of potassium phosphate buffer (prepared using 20 mM K_2HPO_4 and 20 mM KH_2PO_4, pH 6.0), containing 20 mM 2-deoxy-d-ribose on an orbital shaker (Labcon, Instrulab CC, Maraisburg) at 70 rpm in the dark, at room temperature, for 45 min (as described by Schopfer *et al*., 2001). Dèbris originating from the axes was cleared by centrifugation at 9 000 rpm for 2 min. After centrifugation, 0.5 ml of incubation medium (from each replicate), 0.5 ml of 2-thiobarbituric acid (TBA; 10 $g.l^{-1}$ in 50 mM NaOH) and 0.5 ml tricholoroacetic acid (TCA; 28 $g.l^{-1}$ in distilled water) were dispensed into 2 ml Eppendorf tubes to estimate the formation of the breakdown product malondialdehyde. The Eppendorf tubes were then placed on a floatation device and heated in a water bath at 95°C for 10 min, and then cooled on ice for 5 min. Dèbris was once again cleared by gentle spinning as above. The reaction product was measured by dispensing 300 µl of solution into each well of black Elisa® plates, and subsequently reading the absorbance using a fluorescence spectrophotometer (FLx 800, Bio-Tek Instruments Inc.; excitation: 530 nm and emission: 590 nm). The dry mass of axes from each replicate was recorded as detailed for superoxide assessment.

3.2.9 Hydrogen peroxide assessment

Hydrogen peroxide levels were measured using the xylenol orange assay (Gay & Gebicki, 2000). Five replicates of 4 axes per treatment (n=20) were placed in 2 ml Eppendorf tubes and incubated in 2.0 ml working reagent containing 25 mM$FeSO_4.7H_2O$; 25 mM$(NH_4)_2SO_4$; 2.5 M H_2SO_4; 125 mm xylenol orange (w/v) and 100 mM sorbitol (w/v). Tubes were placed on an orbital shaker (Labcon, Instrulab CC, Maraisburg) and rotated at 70 rpm, at room temperature, in the dark for 30 min. The working solution was used as a blank, after which optical densities of treatment samples were read at 560 nm. The extinction coefficient of Fe^{3+} xylenol orange complex at 560 nm is 267 mM^{-1} cm^{-1}, which was used in calculations to estimate hydrogen peroxide content in axes. Dry mass of axes was measured as described above.

All experimentation subsequent to primary excision of the explant was conducted under dark conditions to limit free radical activity perpetuated by possible photo reactions.

3.2.10 Cathodic drying

Cathodic drying was conducted on axes with and without the prior steps of post-excision soaking, cryoprotection and a combination of these treatments (Table 3.2). This form of cathodic protection is applied during dehydration (as carried out exactly as for conventional flash-drying [described in Chapter 2]) with the modification of the provision of a static electric field with the grid supporting the axes being the cathode. A potential difference of 300 V was applied throughout the duration of drying. Three sets of conditions were tested: (1) excision of axes, pre-culture, post-excision soak and flash-drying (90 min); (2) excision of axes, pre-culture, post-excision soak, cryoprotection and flash-drying (120 min); (3) excision of axes and flash-drying (75 min). Each of these regimes was tested using both cathodic and conventional flash-drying.

3.2.11 Statistical Analysis

Free radical production (superoxide, hydroxyl radical and hydrogen peroxide) and TAA were tested for significant inter-treatment differences across treatments by analysis of variance (ANOVA). Multiple comparisons were then made by use of least significant difference (LSD) or Scheffe's mean separation test for TAA. Correlations between free radical levels, TAA and viability were tested using a Pearson's correlation test. Viability data were tested for significant inter-treatment differences using a one-sample T-test. Percentages for viability data were arcsine transformed to conform data to parametric test assumptions. A Mann-Whitney-U test was performed in instances where the assumptions of normality or equal variance were not met. All statistical analyses were performed at the 0.05 level of significance using SPSS statistical package (Version 19; SPSS Inc. Chicago, Illinois, USA).

Table 3.2: Description of all the treatments, controls and parameters tested for the studies described in this chapter

TREATMENT	CONTROL	OXIDATIVE PARAMETERS					VIABILITY PARAMETERS		
		O_2^-	O_2^- OVER TIME (tested in intervals over 30 min)	OH^-	H_2O_2	TAA	TTZ	ROOT	SHOOT
EXCISION TREATMENTS									
Primary excision of explants	-	x		x	x	x		x	x
Primary excision, **Pre-culture on medium containing DMSO**	*Culture on unmodified medium	x		x	x	x		x	x
Primary excision, Pre-culture on medium containing DMSO, **final excision under DMSO solution**	*Final excision under water	x	x	x	x	x		x	x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); **post-excision soak: DMSO (distilled water)**	Post-excision soak: distilled water	x		x	x	x		x	x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); **post-excision soak: DMSO (cathodic water)**	Post-excision soak: distilled water	x		x	x	x		x	x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); **post-excision soak: AsA (distilled water)**	Post-excision soak: distilled water	x		x	x	x		x	x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO**); post-excision soak: AsA (cathodic water)**	Post-excision soak: distilled water	x		x	x	x		x	x

Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO**); post-excision soak: Cathodic water**	Post-excision soak: distilled water	x		x	x	x		x	x
pH OF POST-EXCISION SOAKING TREATMENTS									
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: DMSO (distilled water) **@ pH 7.64**	@ pH 11.45					x	x		x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: DMSO (cathodic water) **@ pH 11.45**	@ pH 7.64					x	x		x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: AsA (distilled water) **@ pH 2.84**	@ pH 3.04					x	x		x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: AsA (cathodic water) **@ pH 3.04**	@ pH 2.84					x	x		x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: Cathodic water **@ pH 11.2**	@ pH 8.2					x	x		x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: Distilled water **@ pH 8.2**	@ pH 11.2					x	x		x
CRYOPROTECTION									
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: AsA (cathodic water); **cryoprotection in DMSO+Gly (cathodic water)**	cryoprotection in DMSO+Gly (distilled water)	x		x	x	x		x	x
FLASH DRYING									
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: AsA (cathodic water); cryoprotection in DMSO+Glycerol (cathodic water); **Flash drying 120 min (Cathodic FD)**	Non-Cathodic FD	x		x	x	x		x	x

Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: AsA (cathodic water); **Flash drying 90 min (Cathodic FD)**	Non-Cathodic FD	x		x	x	x		x	x
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); **Flash drying 75 min (Cathodic FD)**	Non-Cathodic FD	x		x	x	x		x	x
POST-DRYING REHYDRATION									
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: AsA (cathodic water); cryoprotection in DMSO+Glycerol (cathodic water); Flash drying 150 min (Cathodic FD); **Rehydration 30 min (Cathodic water)**	Rehydration (CaMg)	x		x	x	x		x	x
POST- COOLING THAWING + REHYDRATION									
Primary excision, Pre-culture on medium containing DMSO, final excision (DMSO); post-excision soak: AsA (cathodic water); cryoprotection in DMSO+Glycerol (cathodic water); Flash drying 150 min (Cathodic FD); Rapid cooling; **Thawing + Rehydration (Cathodic water)**	Thawing + Rehydration (CaMg)	x		x	x	x		x	x

*Oxidative parameters were assessed for all treatments and controls with the exception of hydroxyl radical, hydrogen peroxide and TAA assessment for controls denoted with an asterisk. Bold text in each treatment denotes the stage of cryopreservation being assessed.

3.3 Results and Discussion

3.3.1 Responses: the effect of DMSO during stages of pre-culture, final excision and post-excision soaking on superoxide production

Figure 3.4 illustrates the effect of DMSO in terms of reducing superoxide production during stages of the excision process, which are likely to be accompanied by an oxidative burst (Goveia, 2007; Roach *et al*., 2008; 2010; Whitaker *et al*., 2010; Berjak *et al*., 2011; Varghese *et al*., 2011).

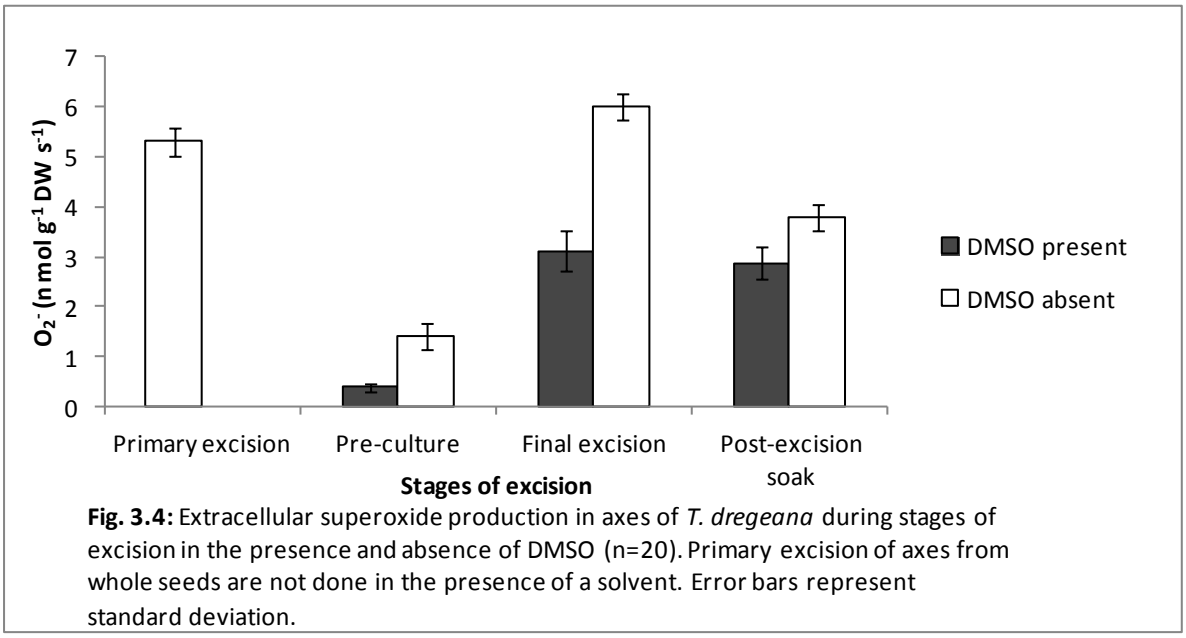


Fig. 3.4: Extracellular superoxide production in axes of *T. dregeana* during stages of excision in the presence and absence of DMSO (n=20). Primary excision of axes from whole seeds are not done in the presence of a solvent. Error bars represent standard deviation.

There were significant differences within each stage between treatments, i.e. those incorporating DMSO and those that did not (Two-way ANOVA, $p < 0.05$) as well as between the control (primary excision) and all treatments from each stage of excision (Two-way ANOVA, $p < 0.05$). The difference between treatments, within each stage, establishes that the presence of DMSO significantly lowers the production of superoxide during every step of excision. These differences further show that a significantly higher superoxide burst occurs within the explant during final excision especially in the absence of DMSO, compared with the other stages (Two-way ANOVA, $p < 0.05$). Primary excision and final excision (excluding DMSO) of axes generated similar quantities of extracellular superoxide, the important difference, however, between these stages being the explant type (i.e. with and without cotyledonary remnants, respectively) and proximity of the shoot apical meristem to the oxidative burst, which would ultimately affect shoot production (described later). Finally, Fig. 3.4 shows that the highest superoxide production across stages and treatments occurred

during the stage of final excision in the absence of DMSO and subsequently, the presence of DMSO during this stage was highly significant in lowering the production of superoxide (Two-way ANOVA, $p < 0.01$).

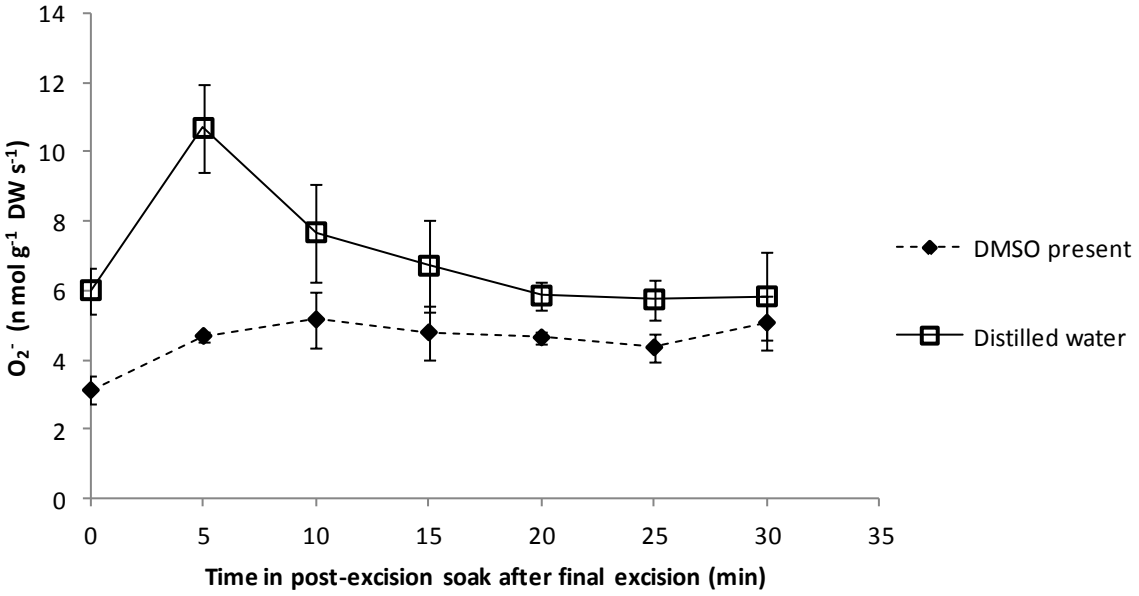


Fig. 3.5: Extracellular superoxide production in axes of *T. dregeana* over time in a post-excision soak (n=20). Error bars represent standard deviation.

Fig 3.5 shows the changes in extracellular superoxide production during the post-excision soak in the absence and presence of DMSO for *T. dregeana*. In the absence of the antioxidant there was an initial sharp rise followed by a gradual decline in superoxide production, with final values being similar to the initial values. In the presence of DMSO this initial burst was not present, and there was a gradual but slight rise over the first ten minutes with little subsequent change. Throughout, superoxide production was lower in the presence of DMSO.

3.3.2 Discussion: the effect of DMSO on superoxide production throughout stages of excision

In addition to the generation of the superoxide radical via normal cellular metabolic activity, there are a number of abiotic stimuli that induce considerable production of this radical, one of them being injury or wounding of tissue, specifically caused by excision of the explants for storage, propagation or cryopreservation etc. (Scandalios, 1993; Wojtaszek, 1997; Whitaker *et al*., 2010; Varghese *et al*., 2011) as seen in Fig. 3.4. It is known that in any

system generating superoxide, the accompanying production of hydrogen peroxide and the hydroxyl radical frequently occurs, especially where cells are subjected to mechanical stress (Wojtaszek, 1997) as occurs after excision. Germplasm that has optimally functioning antioxidant defences is able to eliminate toxic ROS species effectively via action of enzymic antioxidant defences such as interaction of SOD and CAT which serves to convert the superoxide radical to water and molecular oxygen (Scandalios, 1993). However, it is postulated that in recalcitrant germplasm, such as the embryonic axes used here, the necessary protective mechanisms against oxidative stress may be inadequate or are compromised and explants may therefore need an external source of antioxidants to regulate the levels of ROS production. Hence, results presented in Fig. 3.4 show that DMSO plays a definite role in reducing superoxide production stimulated by wounding injury, and this action is conjectured to promote the initiation of shoot development directly as shown by results presented in Chapter 2 (also see Naidoo *et al*., 2011).

Importantly, it is necessary to revisit the signalling role of ROS (Schopfer *et al*., 2001) at the onset of oxidative investigations. In this regard, ROS have been reported to affect gene expression by targeting and modifying the activity of transcription factors, as well as playing a prominent role in the activation of MAPK pathways that are central to mediating cellular responses to numerous stresses (Apel & Hirt, 2004). It is assumed that any given ROS species can selectively interact with a target molecule that senses an increase in ROS production resulting from metabolic disturbances, and translates this information into a change of gene expression important in coping with injury-induced stress in constituent cells of the tissue (Laloi *et al*., 2004). It is therefore crucial that any external scavenger of ROS be applied at a concentration, and for a duration, that permits a regulatory effect on the system, rather than imposing one of total inhibition.

Dimethyl sulfoxide has a known influence on membrane permeability and metabolism, with a specifically inhibitory effect on the latter by way of arresting respiration and RNA metabolism (Bajaj *et al*., 1970). In terms of its regulatory effect on superoxide, it is conjectured that DMSO acts by arresting certain respiratory pathways responsible for the generation of this radical and in so doing, indirectly inhibits the over-production of superoxide. However, the role of this compound as a ROS scavenger by direct interaction with a free radical species has been noted to be the case with its quenching action on the hydroxyl radical (Benson, 1990; Yu & Quinn, 1994). Nevertheless, the decrease in superoxide production leads to a decrease in other radical species which are formed by

interactions with superoxide, so translating to an overall reduction in ROS levels during various stress inducing stages pre- and post cryopreservation.

Moreover, results from this study also showed the efficacy of DMSO in significantly reducing the post-excision superoxide burst over the 30 min period after excision whereas an oxidative burst was observed to occur between 0-5 min after excision in the control (Fig. 3.5). This indicates the necessity of an antioxidant being present during post-excision soaking as ROS scavenging (superoxide here) at this stage could be critical to survival in culture, and during subsequent stages of cryopreservation. From evidence provided in Chapter 2, where it was postulated that shoot production after excision of the cotyledons flush with the axis was facilitated by the presence of an antioxidant prior to and at the onset of damage, it is likely that the control of excessive superoxide production by DMSO may inhibit the necrosis of the shoot tip (Goveia, 2007; Goveia *et al*., 2004) and allow for normal shoot development. This is supported by evidence presented by Whitaker *et al.* (2010) where those authors showed an oxidative burst of superoxide to occur in excised axes of *T. dregeana* which declined slowly over 50 min with poor survival of the axes. Results presented in this study show that the presence of DMSO post-excision ameliorates the effect of prolonged superoxide production and accumulation. By increasing viability retention prior to desiccation, treatment with DMSO increases the possibility of survival post-desiccation and post-cooling, if, in fact, death occurs as a result of oxidative damage at these stages.

The findings presented on the relationship between stages of excision, DMSO and superoxide production led to investigations on the relationship between these stages and endogenous antioxidant responses within the cell, as well as studies on other externally applied antioxidants which are less toxic than DMSO and their effect on shoot development. One of the treatments tested was wet cathodic protection, in the form of treating the explants with cathodic water, and its effect on known antioxidants, as well as its singular effect on endogenous antioxidant activity and shoot development was investigated.

3.3.3 Responses: the effect of soaking solutions containing antioxidants and made up in either distilled or cathodic water on endogenous antioxidant activity (assessed as total antioxidant activity [TAA]), and shoot production

Experiments reported in this section were conducted on *T. emetica* only, due to lack of seed availability of the other species at that time. In Chapter 2, it was reported that excised axes of *T. emetica* required only excision under a DMSO solution and thereafter a post-excision soak in water for shoot production by a substantial number of the axes (Table 2.1). One should note that those results were recorded after 6 weeks in culture. After 20 weeks in culture, given adequate time for axes to reach maximum development in terms of shoot production, results showed that more of the excised axes of *T. emetica* (70%) produced shoots when treated with the additional post-excision soak in distilled water containing DMSO than in distilled water alone (35%; Fig. 3.6). This was consistent for axes from *T. dregeana* and *P. longifolia* (Table 2.1).

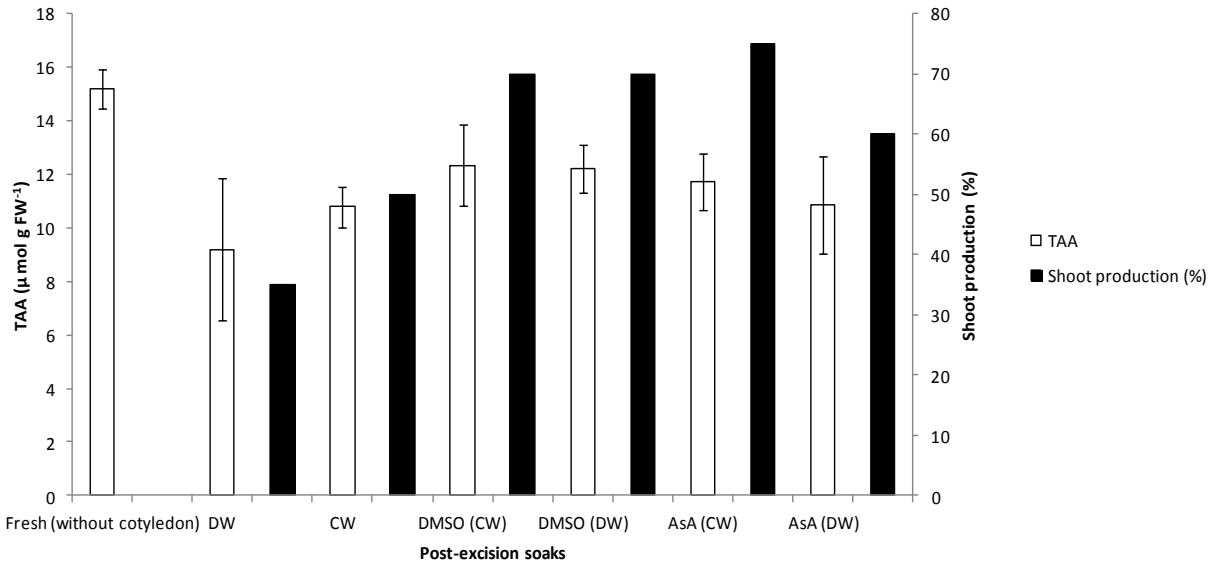


Fig. 3.6 : Total antioxidant activity and shoot production in excised axes of *T. emetica* after different post-excision soaking treatments (n=20). Error bars represent standard deviation.

X-axis annotations (Fig. 3.6):

DW: distilled water

CW: cathodic water

DMSO (CW): DMSO prepared in cathodic water

DMSO (DW): DMSO prepared in distilled water

AsA (CW): ascorbic acid prepared in cathodic water

AsA (DW): ascorbic acid prepared in distilled water

Figure 3.6 shows the effect of the spectrum of post-excision soaking treatments on shoot development and total antioxidant activity in excised axes of *T. emetica* compared with freshly excised, untreated embryonic axes. The soaking solution, whether made up in distilled or cathodic water, had no significant effect (ANOVA, $p < 0.05$) on axis TAA, and, in fact, the highest TAA was found in freshly excised axes not exposed to any post-excision soaking solution. Post-excision soaking in an antioxidant solution promoted shoot development (consistent with data presented in Chapter 2), with AsA made up in cathodic water resulting in the highest shoot production (Pearson Chi-square, $p < 0.05$). As there were no significant differences observed in TAA between various post-excision soaking treatments, it is unlikely that there is any relationship between TAA and shoot development. Testing with TTZ showed 100% viability of axes after all treatments tested.

3.3.4 Discussion: the effect of post-excision soaking solutions on TAA, viability and shoot development and the effect of cathodic water on the activity of DMSO and AsA

Thus far, the results presented in this study have established a relationship between the exogenous applications of antioxidants during stages of excision and shoot development (Fig. 3.6). The current evidence has shown that antioxidants such as DMSO promote shoot development, presumably by action of scavenging superoxide, especially at the stage of excision when an oxidative burst is likely to be most damaging because of the close proximity of the wounding sites to the shoot meristems (Naidoo *et al*., 2011). The purpose of investigating the effects of other antioxidants on shoot development was to establish if a less toxic, but equally efficacious, alternative to DMSO could be used throughout the cryopreservation process. Results presented in Fig. 3.6 have a few noteworthy implications for further studies on oxidative stress. Firstly, axis survival could be precluded because of physical or oxidative damage, or both (Walters *et al*., 2002). At the stage of excision, it has been consistently shown that survival is more influenced by the regulation or scavenging of free radical species than by physical damage (Table 2.1, Fig. 3.4 & Fig. 3.6). This conclusion is supported by the fact that shoot development occurred in *T. emetica* embryonic axes treated with antioxidants, and shoot necrosis is likely to have occurred in those that were untreated (controls), as reported for axes of another meliaceous species, *Ekebergia capensis*, by Perán *et al.* (2006). On the basis of damage and death being a consequence of oxidative stress at this stage, the first line of investigation focused on the mechanism of protection by exogenous antioxidants; hence the effects on TAA were studied. Here, it was observed that post-excision soaking, regardless of the solutes or solvents used, had no significant effect on

TAA (Fig. 3.6), with the exception of a significant difference between the control axes (where TAA was recorded as being highest) and any of those that were excised and exposed to pre-culture and soaking. However, there were significant differences observed in shoot development between treatments, suggesting that the effect of the antioxidants in the soaking solutions promoted shoot development by axes by means other than influencing the inherent endogenous antioxidant status. The observation that TAA was higher in control axes than in those receiving antioxidant treatments could be explained by a regulatory effect of external antioxidants, causing TAA to decline as a consequence of the external provision of antioxidants. The result that showed no shoot development to occur in control axes, despite these having the highest TAA, once again supports the suggestion that the promotion of shoot development by external antioxidants is a consequence of their ROS scavenging properties rather than their effect (such as in increase in concentration or activity of) endogenous antioxidant mechanisms. However, this is in contradiction to the report by Berjak *et al*. (2011) that enhanced TAA of both fresh and flash-dried axes of *Boophone disticha* accompanied by exposure to cathodic water, relative to treatment with CaMg solution. Moreover, those authors argued that the positive effects of cathodic water on the explants in terms of higher germination and vigour were most likely to have been the consequence of enhancement of activity of endogenous antioxidants, as also proposed by Hanaoka (2001).

The second point brought to light from this study is the enhanced shoot development by axes soaked in cathodic water containing AsA (Fig. 3.6). The use of cathodic water on its own did not, at this stage, promote shoot development to the extent that it did when combined with AsA. As the specific mechanism of action, in terms of enhancement of antioxidant properties by cathodic water is unknown, one could conjecture that a principle mode of action could be to enhance the activity of other exogenously-provided (or endogenous) antioxidants by way of increasing their re-reduction: i.e. increasing the reducing power of AsA in this case, to promote its efficiency in quenching ROS as suggested by Hanaoka (2001). Although not significantly different, an effect can also be seen in TAA between AsA+distilled water treatment and AsA+cathodic water treatments of excised axes, where the latter showed higher TAA. The proportion of axes producing shoots after DMSO post-excision soaking was somewhat (but not significantly) lower than that following treatment with cathodic water containing AsA. However, in view of possible cumulative cytotoxic effects of DMSO (e.g. upon dehydration, which would result in its intracellular concentration being increased), post-excision soaking in cathodic water containing AsA was selected for all further investigations on excised axes of *T. emetica* and *T. dregeana*. While significantly enhancing the effect of

exogenously applied AsA, cathodic water seemed to have no effect on the activity of DMSO in terms of promoting shoot development. One may draw on the modes of action of AsA and DMSO to suggest that the ROS scavenging action of DMSO may primarily be its inhibitory effect on ROS-generating respiratory pathways, while the primary mode of action of AsA may be direct interaction with free radicals, specifically as a proton donor when acting on superoxide anion radicals. As such, the reducing power of cathodic water may have more of an effect in promoting the activity of AsA, as shown by Hanaoka (2001), than on the activity of DMSO. In addition to the regeneration of AsA within the cell by the ASC-GSH cycle described earlier, the action of cathodic water may very well promote the spontaneous regeneration of endogenous and exogenously applied AsA (Berjak *et al*., 2011).

Studies by Hanaoka (2001) showed that at the elevated pH of reduced – i.e cathodic – water, a combined treatment of AsA and the water increased the superoxide dismutation activity of AsA due to an increase in the dissociation activity of the water, while reduced water on its own did not have this effect. Nontheless, at this basic pH, cathodic water on its own was able to reduce the levels of hydrogen peroxide within an *in vitro* system, as well as scavenge singlet oxygen and the hydroxyl radical, suggestedly by means of the dissolved atomic hydrogen produced by electrolysis of the water (Shirahata *et al*., 1997). Cathodic water was also shown to protect and maintain genetic integrity by suppressing ROS-induced (specifically those ROS produced by H_2O_2/Cu (II) and HQ/Cu (II) systems) single stranded breakages of DNA (Shirahata *et al*., 1997; Hanaoka, 2001). Based on studies thus far and current findings, the present investigation considered the effect of the pH of soaking solutions on shoot development and TAA, using pH-adjusted distilled and cathodic water as solvents for different treatments.

3.3.5 Responses: the effect of (1) stages of excision and (2) pH of exogenously-applied solutions on endogenous antioxidant activity (TAA), and shoot development

Figure 3.7 illustrates a significant difference in shoot production (Pearson Chi-Square, $p < 0.05$) and TAA (One-way ANOVA, $p < 0.05$) across treatments. In these stages prior to post-excision soaking, there was once again no trend between TAA and shoot production. Final excision, even in the presence of an antioxidant to quench the initial oxidative burst in response to wounding, is suggested to have imposed damage on the tissue which resulted in lower shoot development.

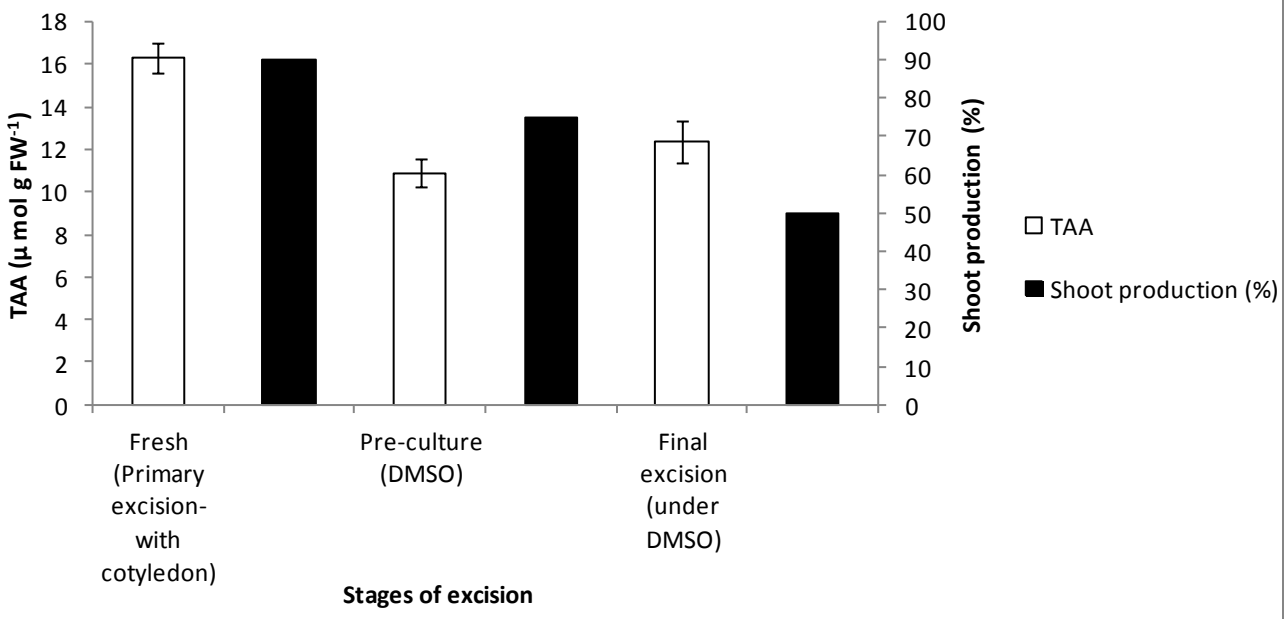


Fig. 3.7: Total antioxidant activity of, and shoot development (20 weeks in culture) by, excised axes of *T. emetica* after stages of excision (n=20). Error bars represent standard deviation.

Figure 3.8 illustrates the effect of pH of post-excision soaking treatments on shoot development and TAA, where the trend shows both soaking solutions to be more effective in promoting shoot production when at their original pH, i.e. distilled water at pH 8.2 and cathodic water at pH 11.2, and significantly different from treatments where the pH had been adjusted (Pearson Chi-Square, $p < 0.05$). There were significant differences in shoot production within cathodic water and distilled water treatments as well as between distilled water and cathodic water treatments (Pearson Chi-Square, $p < 0.05$) but no significant differences in TAA were observed within or across treatments (One-way ANOVA, $p > 0.05$). The pH of each post-excision treatment therefore had a significant effect on shoot production.

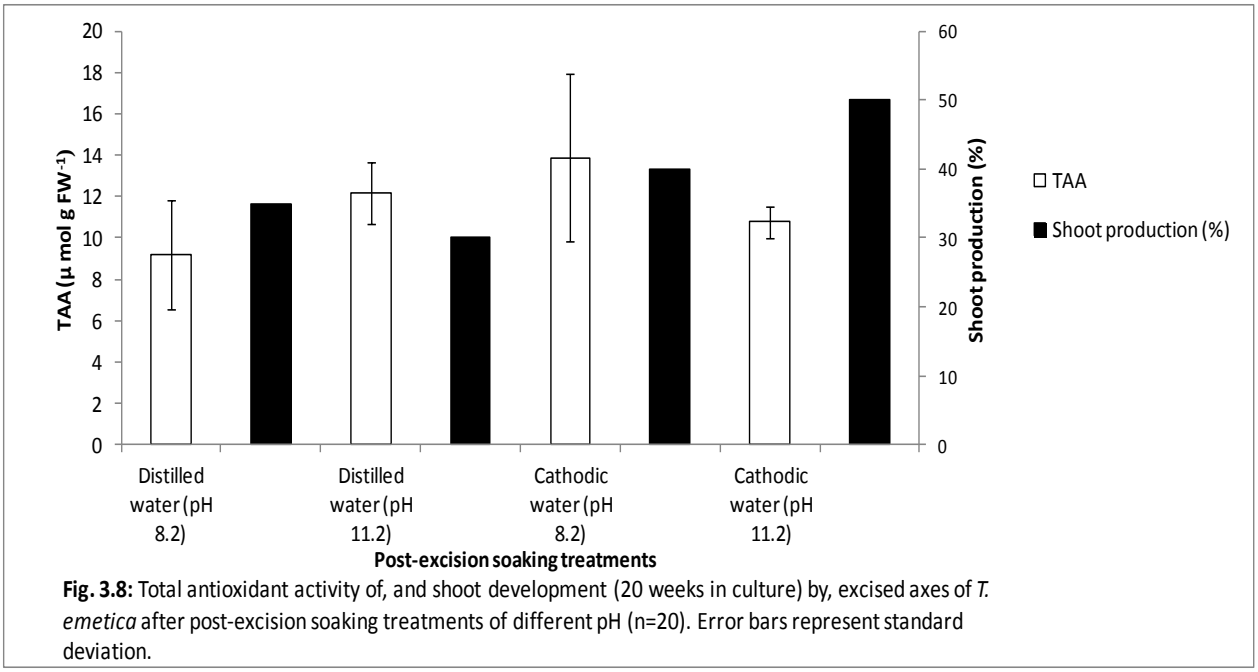


Fig. 3.8: Total antioxidant activity of, and shoot development (20 weeks in culture) by, excised axes of *T. emetica* after post-excision soaking treatments of different pH (n=20). Error bars represent standard deviation.

Figure 3.9 illustrates the effect of pH of DMSO solutions, made up in distilled water or cathodic water, on TAA and shoot development. DMSO made up in cathodic water and in distilled water were slightly more effective in terms of shoot development when left at their original pH i.e. DMSO (cathodic water) at pH 11.4 and DMSO (distilled water) at pH 7.6. There were no significant differences in shoot production within DMSO (cathodic water) and DMSO (distilled water) treatments i.e. pH did not have a significant effect on shoot development (Pearson Chi-Square, $p > 0.05$). There were no significant differences in TAA within DMSO (cathodic water) and DMSO (distilled water) treatments irrespective of pH,nor across DMSO (cathodic water) and DMSO (distilled water) treatments (One-way ANOVA, $p > 0.05$); illustrating once again that pH does not have a significant effect on endogenous TAA or on shoot production in this case. No relationship was observed between TAA and shoot development.

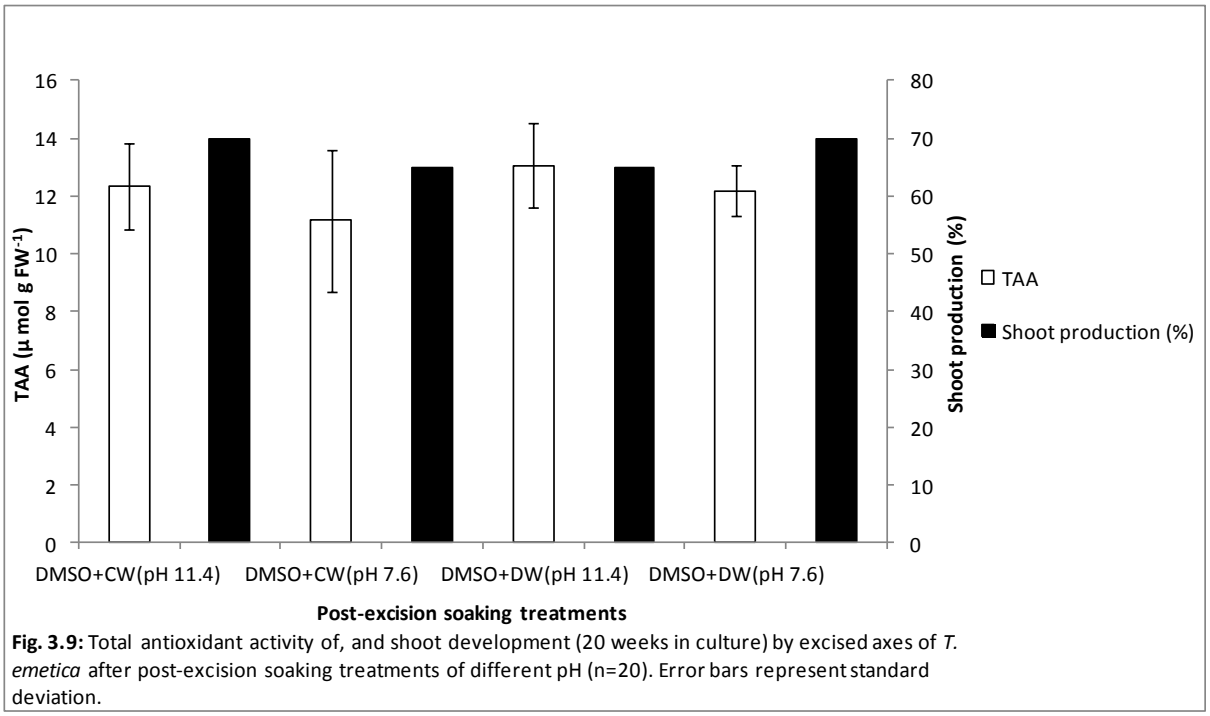


Fig. 3.9: Total antioxidant activity of, and shoot development (20 weeks in culture) by excised axes of *T. emetica* after post-excision soaking treatments of different pH (n=20). Error bars represent standard deviation.

X-axis annotations (Fig. 3.9):

DMSO+CW: DMSO prepared in cathodic water

DMSO+DW: DMSO prepared in distilled water

Figure 3.10 illustrates the effect of pH of AsA solutions prepared in cathodic and distilled water on shoot development and TAA by excised axes of *T. emetica.* Ascorbic acid solutions prepared in either distilled or cathodic water were more effective in promoting shoot development when left at their original pH i.e pH 2.8 and pH 3.0 respectively. There were significant differences in shoot production within AsA (distilled water; pH 2.8 and pH 3.0) and AsA (cathodic water; pH 3.0 and pH 2.8) treatments as well as between AsA (distilled water) and AsA (cathodic water) treatments (Pearson Chi-Square, $p < 0.05$). Despite the differences being very small, the pH of an AsA soaking solution, prepared in either distilled or cathodic water, had a significant effect on shoot development. No significant differences in TAA were observed within or between treatments (One-way ANOVA, $p > 0.05$). There was once more no observed relationship between endogenous TAA and shoot development in axes treated with soaking solutions at different pH.

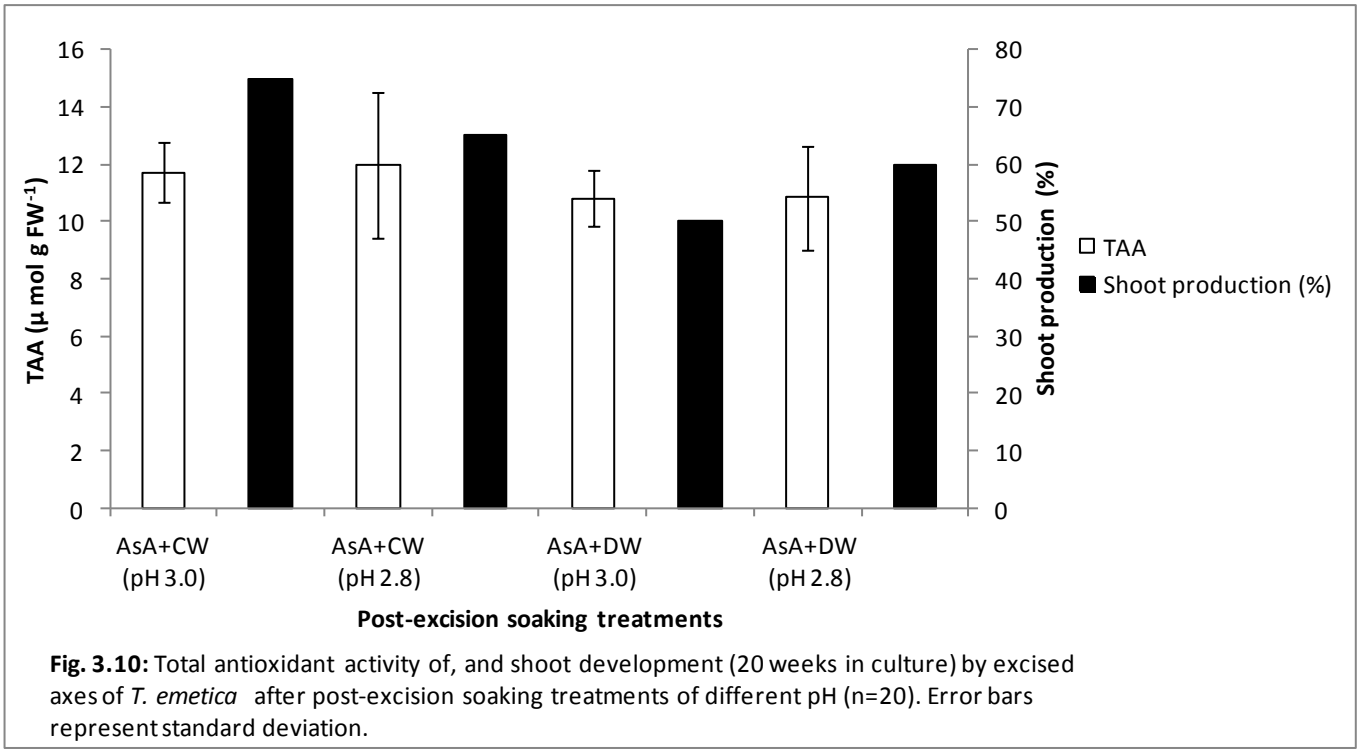


Fig. 3.10: Total antioxidant activity of, and shoot development (20 weeks in culture) by excised axes of *T. emetica* after post-excision soaking treatments of different pH (n=20). Error bars represent standard deviation.

X-axis annotations (Fig. 3.10):

AsA+CW: ascorbic acid prepared in cathodic water

AsA+DW: ascorbic acid prepared in distilled water

3.3.6 Discussion: the effect of stages of excision and pH of exogenously applied antioxidant solutions on TAA and shoot development

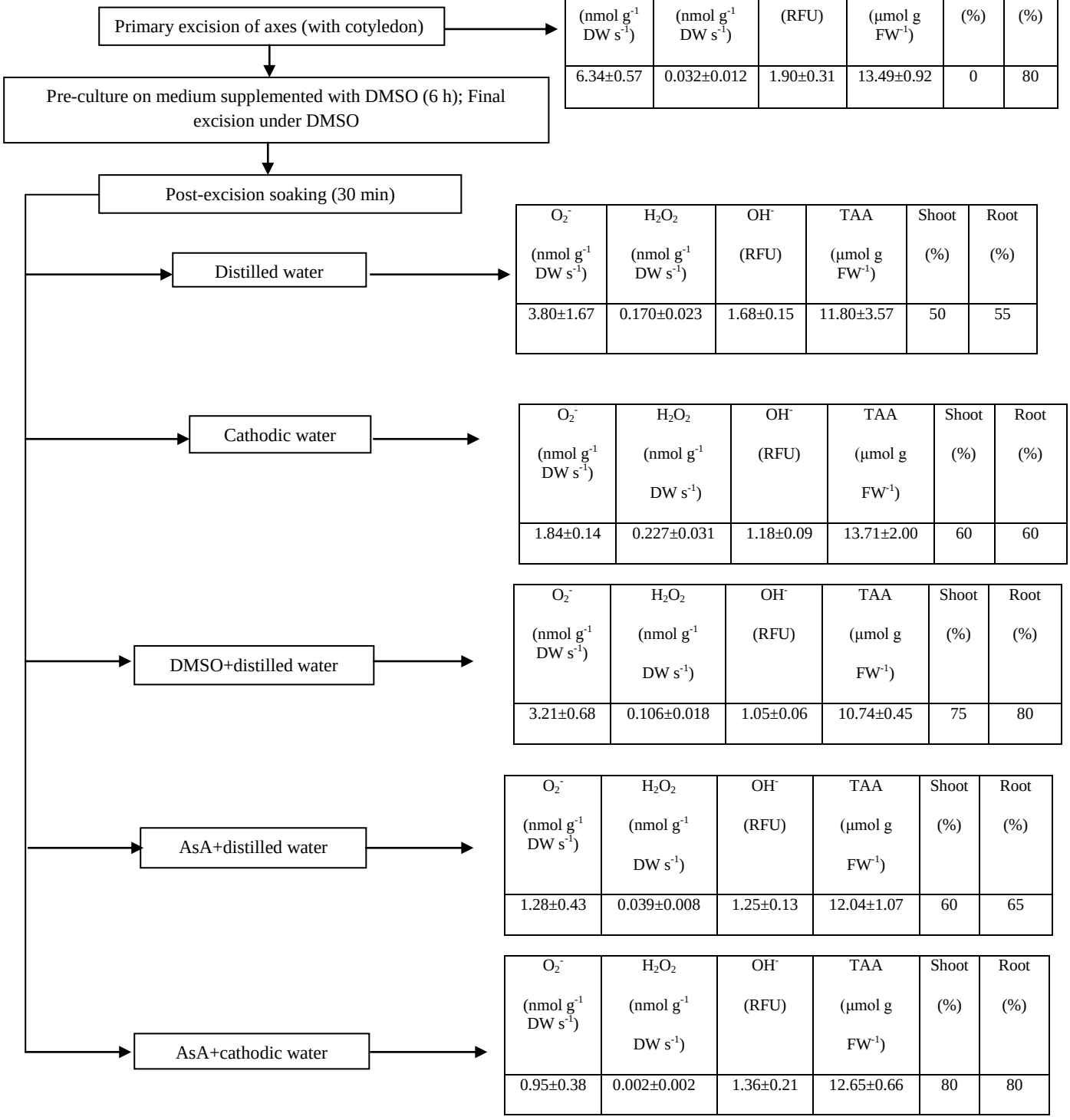
The first set of results presented in this section (Fig. 3.7) show that increased TAA did not always correspond to an increase in the percentage of axes developing shoots, i.e. while the treatment where axes showed the highest TAA also promoted the highest shoot development, the treatment where axes had the lowest TAA was not the treatment where shoot production was the lowest. However, one should take note that these treatments were applied to different explant types, with the effects best explained as follows: Where primary excision of the explant produced the highest shoot development because the cotyledonary blocks were still attached, after final excision the cotyledons were completely removed with the resultant lesions being on the axis surface and the oxidative burst upon excision consequently originating much closer to the shoot meristem. This is held to have compromised shoot development by a substantial proportion of the axes. Although there were differences seen in TAA between the treatments, it would be difficult to relate TAA to shoot development due to the difference in explant type. At this point, the results from Fig. 3.7 merely show that there was a difference in TAA between untreated axes and those exposed to DMSO, and that severing of the cotyledons has a significant negative effect on shoot development which corresponded to lower TAA in these axes than those that were freshly excised.

Results presented in Fig. 3.8 show significant differences in shoot development between cathodic water and distilled water treatments, as well as between the original and adjusted pH within these treatments, but no significant pH-related differences occurred in TAA between treatments. It was also observed that a higher percentage of axes developed shoots in those treatments where each soaking solution (distilled water, cathodic water, AsA + distilled water, AsA + cathodic water, DMSO + distilled water, DMSO + cathodic water) was left at its original pH. These trends were consistent across soaking treatments, as can be seen in Figures 3.9 and 3.10. Here again, throughout all treatments, AsA prepared in cathodic water promoted shoot production by the highest percentage of excised axes, and yet it was not the treatment that showed axes to have the highest TAA. It must be noted here that unlike the studies conducted by Hanaoka (2001), where the pH of AsA prepared in cathodic water was brought up to the pH (11.2) of cathodic water, in the present experiments, the pH was changed according to the pH of the solution when the selected solute was dissolved in distilled water (as shown in Table 3.1). As there was not a drastic change in pH between the original and adjusted soaking solution when AsA was the solute, it is feasible that the effect

of pH on endogenous TAA would be minimal. Seemingly improved capacity by axes for shoot development (Fig. 3.10) is presently difficult to explain, as the pH differences where AsA was included in the soaking solution, were extremely small.

It is important to note that high expression of antioxidant production does not necessarily translate to an enhancement of antioxidant defences, and therefore increased antioxidant capacity within the cell may not always result in a higher degree of protection (Blokhina *et al*., 2003). The competence of an antioxidant system is largely influenced by the unconstrained integration of many aspects such as ROS compartmentalisation, antioxidant localisation, transport of antioxidants and the ability to induce an antioxidant defence under stressful conditions (Blokhina *et al*., 2003; Francini *et al*., 2006; Halliwell & Gutteridge, 2007). The inferences from results presented in this part of the study called for further investigation into the effect of antioxidants throughout steps of the cryopreservation procedure on specific free radical species, the corresponding response by the endogenous antioxidant system and the repercussions of oxidative metabolism on shoot and root development by excised axes.

3.3.7 Responses: the effect of stages of cryopreservation on ROS production, TAA and viability in the presence and absence of exogenous antioxidants



O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
6.34±0.57	0.032±0.012	1.90±0.31	13.49±0.92	0	80

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
3.80±1.67	0.170±0.023	1.68±0.15	11.80±3.57	50	55

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
1.84±0.14	0.227±0.031	1.18±0.09	13.71±2.00	60	60

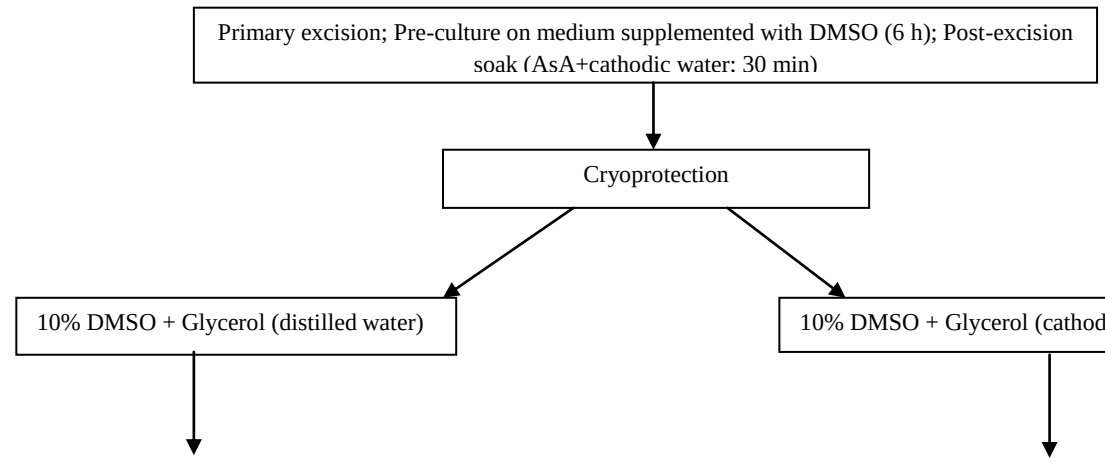
O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
3.21±0.68	0.106±0.018	1.05±0.06	10.74±0.45	75	80

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
1.28±0.43	0.039±0.008	1.25±0.13	12.04±1.07	60	65

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
0.95±0.38	0.002±0.002	1.36±0.21	12.65±0.66	80	80

Fig. 3.11: Illustrating the steps of excision and various post-excision soaking treatments tested, accompanied by quantitative measures of free radical species, total antioxidant activity and viability for excised axes of *T. dregeana* (n=20).

Assessment of free radicals and antioxidants during stages of cryopreservation was conducted on axes from *T. dregeana* only, in the context of restricted seed availability. Based on the fact that cathodic water did not show any effect on the activity of DMSO in terms of facilitating shoot development, a DMSO soaking solution prepared in cathodic water was not tested. Results presented in Fig. 3.11 show the cumulative effect of the excision process and each soaking treatment on selected parameters, as all explants were exposed to a DMSO pre-culture and were excised under a DMSO solution prior to exposure to various soaks. Significant differences within any one radical species occurred between the soaking solutions and the control (distilled water) (One-way ANOVA, $p < 0.05$). Additionally, there were significant differences in radical production between antioxidant soaks (One-way ANOVA, $p < 0.05$). No significant differences were observed in TAA between antioxidant soaking treatments and the control or between individual antioxidant treatments (One-way ANOVA, $p < 0.05$). Significant differences were noted in shoot and root production between treatments and the control and between individual treatments (Pearson Chi-Square, $p < 0.05$). The post-excision soaking treatment containing AsA prepared in cathodic water had the lowest production of superoxide (0.95 nmol/g DW/s) and hydrogen peroxide (0.002 nmol/g DW/s) as well as the best response – 80% – in terms of root and shoot production. Based on the bifurcating nature of the experimental design, this was the post-excision soaking treatment chosen for this step prior to further processing of the axes. Despite DMSO being significantly more efficacious in scavenging the hydroxyl radical (One-way ANOVA, $p < 0.05$) (which would be likely to be detrimental to the tissue if not regulated) shoot production after an AsA+cathodic water treatment was marginally better and production of superoxide and hydrogen peroxide was significantly lower (One-way ANOVA, $p < 0.05$).



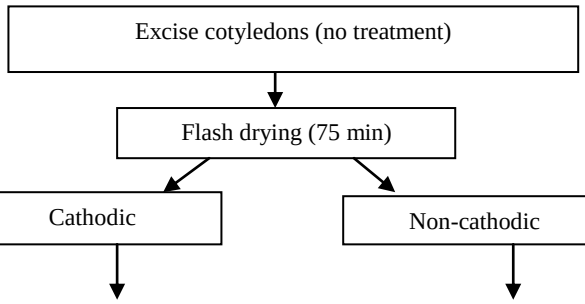
O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
0.92±0.23	***0.015±0.007**	1.67±0.23	14.14±0.72	**0**	**0**

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
0.78±0.11	***0.003±0.000**	1.49±0.31	14.77±1.84	**40**	**50**

*Significant differences between treatments denoted by bold font

Fig. 3.12: Illustrating the steps of excision and two cryoprotectant treatments tested, accompanied by the quantitative measures of free radical species, total antioxidant activity and viability, for excised axes of *T. dregeana* (n=20).

Figure 3.12 provides an overview of results showing significant differences in hydrogen peroxide production by axes between cryoprotection treatments (Independent Samples Test, $p < 0.05$). No significant differences were observed for the other two radical species or for TAA between treatments (Independent Samples Test, $p > 0.05$; Mann-Whitney-U, $p < 0.05$). There was a significant difference in viability data between treatments (Pearson Chi-Square, $p < 0.05$): exposure of excised axes to cryoprotectants prepared in cathodic water resulted in 40% and 50% shoot and root production, respectively, compared with no root or shoot production at all, after exposure to cryoprotectants made up in distilled water. The trend showed that cryoprotectants made up in cathodic water reduced production of superoxide and the hydroxyl radical to some extent but significantly lowered the occurrence of H_2O_2, as well as marginally increasing TAA. Additionally, as root and shoot development followed exposure only when the cryoprotectants were made up in cathodic water, this was the cryoprotection treatment selected for subsequent trials.



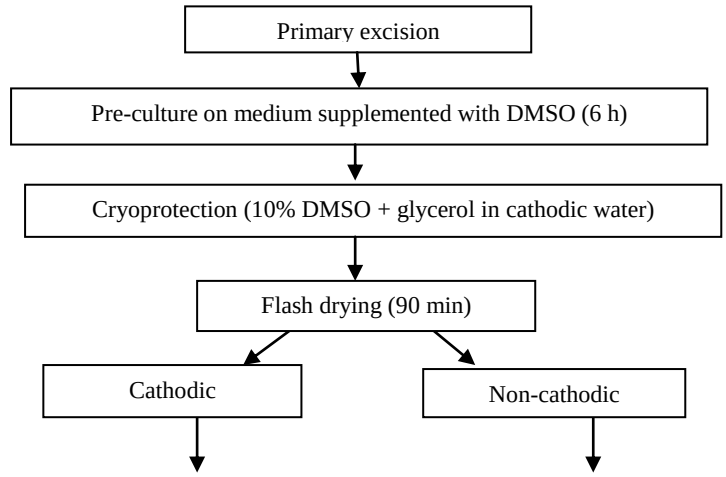
O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***3.75±0.35**	***0.011±0.002**	1.83±0.21	42.30±2.11	0	0

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***7.18±0.97**	***0.015±0.001**	2.08±0.28	40.94±4.81	0	0

*Significant differences between treatments denoted by bold font

Fig. 3.13: Illustrating the steps of excision and cathodic and non-cathodic flash drying for 75 min, accompanied by the quantitative measures of free radical species, total antioxidant activity and viability after rehydration in the CaMg solution excised axes of *T. dregeana* which had neither been pre-treated, nor cryoprotected (n=20).

Fig. 3.13 shows significant differences in production of superoxide and hydrogen peroxide (Independent Samples test, $p < 0.05$) between treatments. No significant difference was observed in hydroxyl radical production (Independent Sample test, $p > 0.05$) or TAA (Mann-Whitney-U test, $p > 0.05$) between axes flash-dried with or without dry cathodic treatments. However, the data show that dry cathodic protection significantly reduced superoxide production, as well as depressing production of hydrogen peroxide somewhat. Total antioxidant activity was also noted to be somewhat higher (although not statistically different) in axes that were cathodically protected during flash drying than in those that were not. Axes exposed to different flash-drying treatments and then cultured on regeneration medium were first rehydrated in CaMg as has been the conventional rehydration protocol in our laboratory. As the parameters were assessed specifically for the effect of desiccation and not rehydration, rehydration in cathodic water was not carried out at this stage. However, irrespective of whether or not cathodic protection was applied during flash drying, after rehydration with the CaMg solution, no axes produced either a root or a shoot. There were no differences observed in viability between treatments as no survival occurred in either.



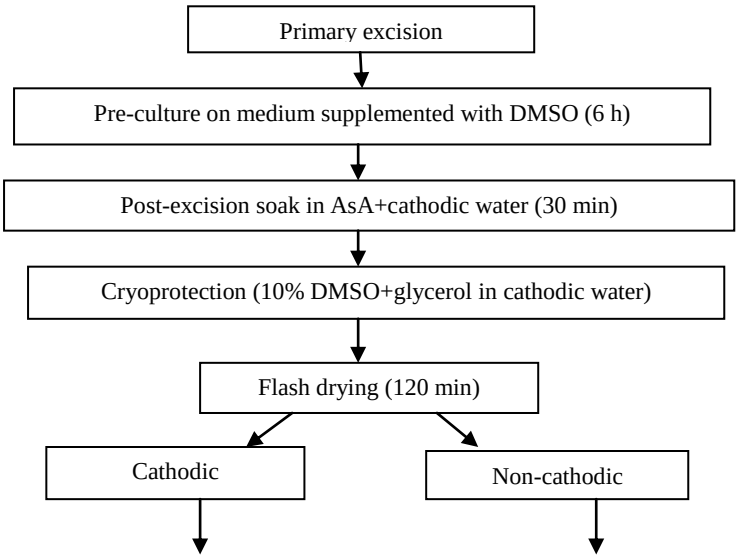
O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***0.49±0.25**	***0.012±0.002**	2.27±0.26	55.95±1.88	0	0

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***0.92±0.04**	***0.036±0.016**	2.34±0.58	51.60±6.18	0	0

*Significant differences between treatments denoted by bold font

Fig. 3.14: Illustrating the steps of excision, pre-culture, cryoprotection and cathodic and non-cathodic flash drying (90 min), accompanied by the quantitative measures of free radical species, total antioxidant activity and viability after rehydration in the CaMg solution, for excised axes of *T. dregeana*. Axes were pre-cultured and had been exposed to the selected cryoprotectant solution (n=20).

Fig. 3.14 shows that there was a significant difference in superoxide and hydrogen peroxide production (Independent Samples test, $p < 0.05$) but no significant differences were observed in hydroxyl radical production or TAA (Mann-Whitney-U test, $p > 0.05$) for pre-cultured, cryoprotected axes between cathodic and non cathodic drying regimes. Cathodic protection was associated with marginally increased TAA in axes desiccated with dry cathodic protection as opposed to those desiccated conventionally (non-cathodic drying). It must be noted that in this drying regime, even those axes exposed to non-cathodic drying had been subjected to pre-culture and cryoprotection treatments, both of which included AsA, DMSO or cathodic water. It is therefore not surprising that differences between treatments in terms of radical production and TAA were not significant. Despite the trends indicating reduction of superoxide and hydrogen peroxide following flash drying where cathodic protection was provided, no axis produced either a root or a shoot after rehydration in the CaMg solution. The same situation pertained after conventional flash drying.



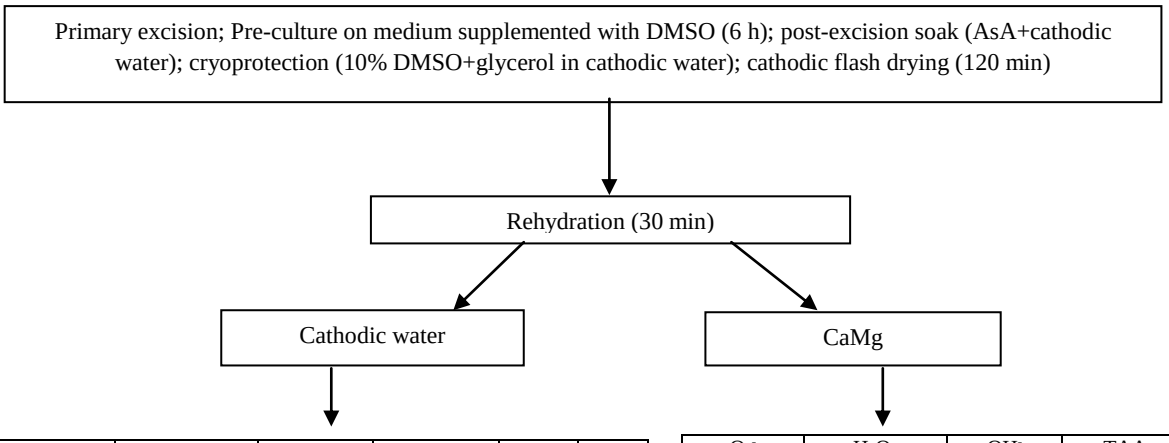
O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***0.75±0.04**	***0.003±0.001**	2.43±0.62	36.56±3.10	0	**10**

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***0.89±0.03**	***0.012±0.002**	2.60±0.92	35.43±2.90	0	**0**

*Significant differences between treatments denoted by bold font

Fig. 3.15: Illustrating the steps of excision, pre-culture, post-excision soaking, cryoprotection and cathodic and non-cathodic flash drying for 120 min, accompanied by the quantitative measures of free radical species, total antioxidant activity and viability after rehydration in the CaMg solution, for excised axes of *T. dregeana* (n=20).

Figure 3.15 details results for axes which had been pre-cultured, post-excision soaked and cryoprotected,which show that there were significant differences in superoxide and hydroxyl radical production (Independent Samples test, $p < 0.05$) between treatments: dry cathodic protection was significantly more efficacious in reducing these radicals compared with non-cathodic drying. No significant differences in hydroxyl radical production or TAA (Independent Samples test, $p > 0.05$) between these treatments were observed. Nevertheless, the trend shows dry cathodic protection to efficiently have reduced the occurrence of two of the three radical species and marginally to have increased TAA. Further to this, 10% of axes exposed to a pre-culture on DMSO, AsA+cathodic water post-excision soak, cryoprotection (cathodic water) and cathodic flash-drying produced roots in culture after rehydration in CaMg. Based on these data, this was the drying regime selected to use prior to cooling. However, before cooling trials were undertaken, it was necessary to assess the possible effects that rehydration in cathodic water (instead of the CaMg solution) would have.



O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***1.01±0.16**	***0.010±0.002**	3.81±1.21	18.69±3.83	**40**	**50**

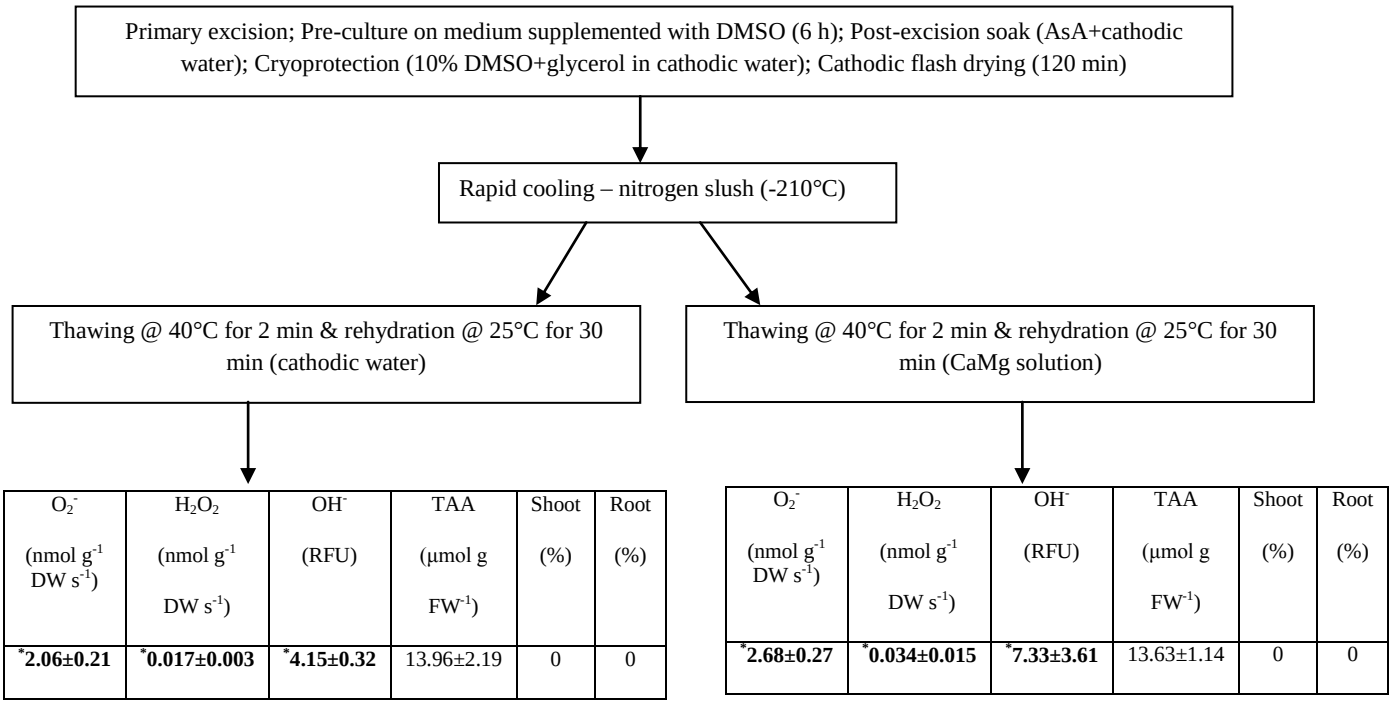
O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***1.33±0.19**	***0.018±0.005**	4.10±0.73	18.04±1.07	**0**	**10**

*Significant differences between treatments denoted by bold font

Fig. 3.16: Illustrating the steps of the selected flash drying regime and two rehydration treatments tested, accompanied by the quantitative measures of free radical species, total antioxidant activity and viability for excised axes of *T. dregeana* (n=20).

Figure 3.16 illustrates significant differences in superoxide and hydrogen peroxide production by axes between two rehydration regimes, where rehydration in cathodic water after the selected drying regime (Fig. 3.15) significantly reduced production of both these ROS (Independent Samples test, $p < 0.05$). No significant differences were observed in hydroxyl radical production (Independent Samples test, $p > 0.05$) or TAA (Mann-Whitney-U test, $p > 0.05$) between treatments. The trend shows that the rehydration regime employing cathodic water significantly reduced two of the three free radical species (also being associated with some reduction in occurrence of the hydroxyl radical) and marginally increased TAA in excised axes. Significant differences were observed in viability data between treatments in terms of shoot and root production (Pearson Chi-Square, $p < 0.05$). Forty and 50% of axes rehydrated with cathodic water produced shoots and roots, respectively, while 10% of those exposed to a CaMg rehydration showed the capacity for onwards development in terms of root production only. These results were highly significant, particularly since shoot production had never been achieved by excised axes of *T. dregeana*

after drying, prior to the use of cathodic protection during dehydration, and provision of cathodic water as the rehydration medium.



O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***2.06±0.21**	***0.017±0.003**	***4.15±0.32**	13.96±2.19	0	0

O_2^- (nmol g^{-1} DW s^{-1})	H_2O_2 (nmol g^{-1} DW s^{-1})	OH^- (RFU)	TAA (µmol g FW^{-1})	Shoot (%)	Root (%)
***2.68±0.27**	***0.034±0.015**	***7.33±3.61**	13.63±1.14	0	0

*Significant differences between treatments denoted by bold font

Fig. 3.17: Illustrating the steps of the selected cooling regime and two thawing and rehydration treatments tested, accompanied by their quantitative measures of free radical species, total antioxidant activity and viability assessed on excised axes of *T. dregeana* (n=20).

Figure 3.17 shows results after the selected excision, cryoprotection and drying regimes were applied to axes prior to cooling, the axes cooled in nitrogen slush and then thawed and rehydrated in either cathodic water or CaMg solution. Thawing and rehydration of axes with cathodic water significantly reduced all ROS (Independent Samples test, $p < 0.05$) and very marginally increased TAA relative to those thawed and rehydrated with CaMg solution. Hydroxyl radical production after cooling, thawing and rehydration in CaMg (7.33 RFU) was substantially higher than in all other procedural stages of cryopreservation. However, no root or shoot production by axes after retrieval from cryogenic conditions

occurred in either case despite the fact that rehydration in cathodic water significantly decreased the incidence of all radical species assessed.

3.3.8 Discussion: the effect of procedural steps associated with cryopreservation on ROS activity, TAA and capacity for root and/or shoot production in the presence or absence of selected antioxidants

The cellular state may be defined as the total active state that regulates cells such that adaptability to their immediate surrounding environment can occur (Shao *et al*., 2008). Therefore, strict regulation of the cellular state is of paramount importance when axes are being cryogenically manipulated, particularly because of the stress-inducing nature of composite stages (Berjak *et al*., 2011). In this phase of the work, the study aimed to dissect oxidative events in cellular metabolism by measuring ROS, as a marker of oxidative damage (Halliwell and Gutteridge, 2007) and the related antioxidant activity within the tissues during the procedural stages of cryopreservation, in order to determine if poor survival of recalcitrant germplasm could be attributed to an unbalanced cellular state (Kranner *et al*., 2006; Varghese *et al*., 2011). In parallel with this primary objective, the successful use of exogenously applied antioxidants in scavenging ROS and facilitating shoot development by excised axes evidenced thus far in the study (Chapter 2; Naidoo *et al*., 2011) prompted the further investigation of changes in oxidative metabolism and the corresponding capacity for onwards development of axes in the context of the provision of antioxidant treatments.

Results presented in Figures 3.11 to 3.17 showed certain significant trends in ROS production and antioxidant activity and their effect on root and/or shoot development associated with the different steps of cryopreservation. While death or survival at each of these stages could well be attributed to causes other than the influence of the cellular state, it is the effect of oxidative metabolism that is associated with each stage on survival that will be discussed here.

Data for excised axes of *T. emetica* treated with a pre-culture (in DMSO for 6 h) and various post-excision soaking treatments (Fig. 3.6) showed shoot development by axes treated with AsA+cathodic water to be significantly higher than after any other treatment during this stage. Similarly, Fig. 3.11 illustrates that this post-excision soak promoted shoot development by the greatest number of excised axes of *T. dregeana*. The assessment of oxidative parameters showed that the best capacity for root and shoot development associated with this treatment is most likely due to the overall lowest production of ROS, despite this treatment not promoting the highest TAA within axes after excision. Ascorbic acid used in

combination with cathodic water significantly lowered superoxide and hydrogen peroxide production compared with any other soaking treatment (Fig. 3.11). This supports the action of cathodic water as an enhancer of proton donors and suggests that superoxide and hydrogen peroxide are efficiently scavenged by this treatment because of the increased dissociation activity of AsA when dissolved in cathodic water (Hanaoka, 2001). This treatment was not, however, correlated with the lowest level of hydroxyl radical production, which was apparent in axes treated with a DMSO (distilled water) treatment. This result was not surprising, as DMSO has been documented to be a particularly potent hydroxyl radical scavenger (Rosenblum & El-Sabban, 1982; Yu & Quinn, 1994); nevertheless, DMSO did not lower hydroxyl radical ocurrence to values that were significantly different from production by axes treated with AsA (in cathodic water) or by axes exposed to any other soaking treatment. It is noted, however, that all axes were exposed to DMSO at the point of excision and it may have been at this point that hydroxyl radical production during the oxidative burst in response to wounding was lowered in all cases. The highest TAA did not correlate with the lowest ROS production or highest capacity for shoot development (Fig. 3.10). Responses of cells to deal with oxidative stress may not always involve increasing levels of antioxidants as, according to Halliwell (2006), external provision of protection can act in inhibiting ROS-producing systems, enhancing other mechanisms such as chaperones for transport of antioxidants, or even inducing changes in oxidative damage targets. The details of the mechanism by which AsA (in cathodic water) best promoted shoot development and reduced superoxide and hydrogen peroxide levels after excision, remain to be resolved. However, in view of the results presently obtained, and considering the synergistic function of AsA with an array of other antioxidants in ROS-scavenging, and its role in maintaining membrane and lipoprotein integrity and promoting cell growth and development (Halliwell, 1994; Potters *et al*., 2002; Shao *et al*., 2008) this combination was as the post-excision treatment of choice.

Curbing oxidative stress prior to desiccation and cooling may promote survival subsequent to these stages, but the addition of external antioxidants during the excision process alone may be adequate only to mitigate oxidative damage induced at this stage, and not be effective against stress-induced damage associated with subsequent processes. It was therefore considered probable that the provision of antioxidant protection would be necessary throughout the cryopreservation procedure.

Excised axes of *T. dregeana* have never before reported to produce shoots or to show substantial root development after cryoprotection, despite considerable effort (Kioko, 2003; Goveia, 2007) where callus production or swelling was reported as the most successful

assessment of survival. While Goveia (2007) showed that the use of cryoprotectants did not significantly improve survival after cooling of *T. dregeana* axes, the benefits of cryoprotection during the cooling stage have, nonetheless, been detailed in Chapter 2. Survival after cryoprotection reported in this study is definitely a step forward in axis cryopreservation of this species, especially since Kioko *et al*. (1998) showed groups of cells – suggested to be callus precursors – in cryoprotected axes of *T. dregeana* to retain an ultrastructural integrity, while cells from non-cryoprotected axes were observed to undergo extensive subcellular damage, after freezing. In the present study it was hypothesised that the preparation of selected cryoprotectants in cathodic water may have a positive effect on survival, and the results (see Fig. 3.12) proved this to be so. In terms of oxidative metabolism, there were significant differences seen only in the reduction of hydrogen peroxide occurrence. Using cathodic water as a solvent for DMSO and glycerol significantly promoted both shoot and root development, compared with the complete lack of onwards development by those axes cryoprotected by DMSO and glycerol prepared in distilled water. However, the trends shown during cryoprotection, and all other procedural stages which followed, demonstrated the lowered incidence of both superoxide and hydrogen peroxide and the increased activity of antioxidants (although this was not significant) in those treatments where wet and/or or dry cathodic protection was included.

Considering that both cryoprotective treatments exposed axes to the same concentration of cryoprotectants for the same duration, the inability of axes to survive conventional cryoprotection (when the solvent was distilled water) is not a consequence of cryoprotectant toxicity, nor is it a result of osmotically-induced damage by the 'shrink-swell' action suggested to be characteristic of penetrative cryoprotectants (Rall, 1993). In the present study, survival of cryoprotection is suggested to be a direct consequence of the significant scavenging of hydrogen peroxide by cathodic water as was demonstrated by Hanaoka (2001). It is possible that generation of hydrogen peroxide during conventional cryoprotection has markedly cytotoxic effects, and the scavenging of this ROS alone could facilitate survival. Based on the results of oxidative parameters, it is also reasonable to infer that survival after cryoprotection using cathodic water as the solvent can be attributed to a degree of regulation of oxidative metabolism afforded by either the action of cathodic water alone or by the interactive effect of cathodic water with DMSO and/or glycerol. By extension, it is feasible to suggest that, at the cryoprotection stage, and during the stages of drying and rehydration, the positive effects of cathodic protection stem from the modulation of oxidative metabolism such that ROS are kept at levels high enough for their effective role

in signalling but not so high as to induce a state of oxidative stress. Cathodic protection is suggested to facilitate such regulation by reducing and increasing ROS and antioxidants, respectively, to levels appropriate for normal cellular homeostasis, which translates to survival of axes if physical damage can be avoided or is not too severe.

With regard to the optimisation of a cryopreservation protocol, these results are significant in that it is extremely beneficial to have axes survive the step of cryoprotection where both DMSO and cathodic water are present: i.e., while cathodic water may provide the means to regulate oxidative metabolism, DMSO could reduce the Tn (ice-nucleation temperature) of the cell soluble phase (cytoplasm) and thereby avoid phase changes in membranes and by doing so protect cell membranes (Yu & Quinn, 1994) from irreversible damage induced by the freezing process which is known to be the primary cause of injury at low and ultra-low temperatures in plant germplasm (Levitt, 1980; Benson, 1990). Both proposed phenomena would serve to promote survival post-cooling.

In each of the three drying regimes, parameters were assessed within each regime between the treatment affording dry-cathodic protection and the one that did not. Dry-cathodic protection was observed consistently and significantly to reduce superoxide and hydrogen peroxide levels across regimes (Figs 3.13 – 3.15). However, no root or shoot production occurred unless the full spectrum of pre-culture, post-excision soaking, cryoprotection and dry cathodic protection during dehydration was applied, under which circumstances 10% of the axes each produced a root only (Fig. 3.15, cf. Figs 3.13 & 3.14). The indication from these results is that desiccation confers so severe a stress that exposure of axes to dry cathodic protection (Fig. 3.13), or to an AsA (cathodic water) soak followed by dry cathodic protection (Fig. 3.14), or an AsA (cathodic water) soak, cryoprotection in the presence of cathodic water and then dry cathodic protection (Fig 3.15) is not adequate to counteract oxidative stress which precludes shoot development. These results highlight that cathodic protection, while successful in regulating oxidative stress during stages prior to desiccation of axes of *T. dregeana*, was not adequate to mitigate ROS-induced damage, presumably particularly of the shoot meristematic region.

As discussed in Chapter 2, the rate of drying may very likely affect root and shoot tissue differentially, and while assays of oxidative status indicate ROS levels in the entire explant, they cannot identify where the concentrations may be higher. As indicated by Kioko (2003), the axis shoot tip dries more rapidly than does the root pole: hence the shoot apical meristem may become far more adversely affected as a result of the duration of the applied stress. During desiccation, water from the cytoplasm is removed and, as a consequence of

disruptive metabolism, ROS are generated (Smirnoff, 1993). It is known that free radicals are produced at different localisations in the cell and are also compartmentalised throughout the tissue, and similarly certain antioxidants are found in higher concentrations in specific compartments: for example, ascorbate and glutathione are present in particularly high concentrations in the chloroplast (Mittler, 2002). It has also been shown that at low water contents, metabolic balance is weakened (Pukacka & Ratajczak, 2006) and antioxidant defences are hindered under water deficit conditions. In this regard, there is a large decrease in the ascorbate pool as a result of even slight removal of water from the cell (Smirnoff, 1993). It is therefore probable that unbalanced metabolism is initiated by drying and the situation is exacerbated in the shoot pole by the duration needed to dehydrate the whole axis to a predetermined water content range which would facilitate minimally-injurious cooling upon cryogen exposure. Additionally, effect of possible mechanical damage accompanying drying, could induce secondary oxidative events such as lipid peroxidation while concomitantly reducing antioxidant capacity (Bailly, 2004; Varghese *et al*., 2011) thus severely affecting first the shoot meristematic region and subsequently, the root pole. All-in-all, based on root development after drying of *T. dregeana* axes as reported in other studies (Kioko, 2003; Goveia, 2007) and presently (after axes were exposed to post-excision soaking, cryoprotection and cathodic drying), it is likely that the shoot meristem is subjected to more severe stress, both oxidative and mechanical, than are other regions of the axis.

Superoxide levels were noted be particularly high after all drying treatments, albeit lower after wet and dry cathodic protection. Superoxide is known to inactivate numerous enzymes that are fundamental in energy production and amino acid metabolism (Halliwell, 2006), hence elevated levels could result in accelerated inactivation rates of enzymes which translate to inhibition of metabolic pathways crucial to growth and repair, ultimately resulting in death. It is possible that the levels of the major scavengers of superoxide and hydrogen peroxide, i.e. SOD and CAT, respectively (Halliwell, 2006) might have declined or behaved dysfunctionally under water stressed conditions in recalcitrant tissues (Chaitanya & Naithani, 1994). The results of the experiments discussed to this point tend to indicate that external provision of antioxidants is inadequate to overcome the oxidative stressed state evidenced by the measured levels of superoxide and hydrogen peroxide immediately after desiccation, which bring about localised necrosis in, or even death of, axes. Regardless of the minimal capacity for ongoing development (10% of axes producing roots) obtained using the procedure detailed in Figure 3.14, this was the procedure selected for assessment of rehydration and cooling on each parameter.

Following this drying procedure, but subsequently rehydrating axes in cathodic water for 30 min (Fig. 3.16) facilitated significantly enhanced onwards development of the excised axes, characterised by substantial shoot and root production. This result has never before been achieved after desiccating and rehydrating axes of *T. dregeana*. There was significant reduction of both superoxide and hydrogen peroxide in axes rehydrated in cathodic water, as well as slightly lower production of the hydroxyl radical and somewhat (but not significantly) higher TAA observed in these axes. Mayaba *et al*. (2002) showed there to be a burst of hydrogen peroxide during rehydration after desiccation in the desiccation-tolerant moss, *Atrichum androgynum*, and while the oxidative burst in that study was suggested to be a response in defence against pathogens, here the unregulated production of hydrogen peroxide could be damaging to desiccation-sensitive tissue. The rehydration step in itself could impose a stress disrupting the oxidative balance in desiccated recalcitrant axes (Smirnoff, 1993), such that survival is uncertain. Nevertheless, the use of cathodic water at this stage seems to curb excessive production of radical species adequately, and is suggested to alleviate oxidative stress and hence facilitate survival. However, it is not suggested that the use of cathodic water alone at the rehydration stage is the key promoter of survival, as one should note that axes have, up to this point in the procedure, been exposed to protection in the form of AsA, DMSO and wet and dry cathodic protection. Rather, it is suggested that it is the cumulative effect of protective mechanisms at each procedural stage that fine-tuned oxidative metabolism so as to allow survival of axes after rapid dehydration.

At the final stage of rapid cooling, thawing and rehydration, it was observed that once again, processing with cathodic water subsequent to cooling resulted in significant lowered levels of the three ROS measured as well as a marginal increase in TAA compared with the situation when CaMg was used for these purposes (Fig. 3.17). Despite the seemingly efficient reduction of oxidative indicators, neither root nor shoot production by axes occurred post-cooling. However, superoxide and hydroxyl radical production was considerably higher after cooling compared with levels in axes that had not been cooled (Fig. 3.17 cf. 3.16), even for those axes thawed and rehydrated in cathodic water. One could conjecture two reasons for the axes being presumed to be dead at this stage, the first one being that they succumbed to severe oxidative stress brought on by free radical production completely overwhelming the antioxidant capacity within the cells (Benson & Bremner, 2004) even when reducing power was provided by the cathodic water, and, the second being physical damage incurred by the axes as a result of intracellular ice formation (Benson, 1990; Pegg, 2001). It is most probable

that death resulted from a combination of both, but it will require detailed experimentation to test this.

The higher production of free radicals post-cooling (Sershen *et al*., 2012c) as compared to any other procedural stage can be accredited to cumulative stress at this stage, and while provision of antioxidant protection may have been adequate to counter-balance oxidative metabolism after each individual stage of cryopreservation, the accumulated stress after cooling could be too great to overcome.

The durations of dehydration - even under flash drying conditions – and after cryoprotection, were long enough to presume that metabolism-liked damage would have occurred. This damage might not be discerned in terms of ROS accumulation, as the damage would have already occurred, so it is necessary to undertake other studies to reveal the extent and location of damage (Chapter 4).

The freezing process itself is highly injurious to recalcitrant germplasm and it is highly probably that solution water was still present in the axes, even at the water content range optimised for the axes to be exposed to cooling. In this case, studies have to be conducted to assess freezable water that may still remain in the tissue (Chapter 4).

The exogenous application of protective mechanisms in the form of cathodic protection, AsA and DMSO at each stage of the cryopreservation procedure was ineffective to combat oxidative stress and facilitate survival after cooling. While survival of excised axes was obtained during procedural stages up to the point of cooling when employing the above mentioned means of protection, a procedure to overcome freeze-induced stress, both biochemical and physical, still requires investigation.

Results presented in this study have shown significant progress towards cryopreservation of the selected recalcitrant-seeded species, and has opened many avenues for further investigation into cryopreservation of recalcitrant plant germplasm, which will be detailed under future considerations and studies (Chapter 4).

CHAPTER 4: Conclusions and Recommendations for Future Studies

The efforts to successfully cryopreserve germplasm of the selected recalcitrant-seeded species in this study made incremental progress, but onwards development of evidenced by root and shoot production after cooling remained elusive. Although freezing damage is conjectured to have been the most likely cause of this, it is suggested that there still are gaps in our understanding of cryopreservation of recalcitrant germplasm. Nontheless, this study was significant in indicating that one of the possibly fundamental underlying causes for failure of excised axes to produce normal seedlings after procedural stages of cryopreservation could be oxidative stress and unbalanced oxidative metabolism. Significantly, the implementation of certain procedures which regulated oxidative stress (pre-culture in a medium supplemented with DMSO and pre-soak in various antioxidant solutions before excision, etc.), facilitated the normal growth of axes of *T. dregeana*, *T. emetica* and *P. longifolia* after the necessary stage of excision; and, in the case of *T. dregeana*, after the cryogenic stages of cryoprotection and desiccation as well.

The use of cathodic protection, especially cathodic water at procedural stages of cryopreservation has allowed for survival of excised axes of *T. dregeana* after excision, cryoprotection, desiccation and rehydration, assessed as the production of normal seedlings from the embryonic axes. Throughout these stages, it was inferred that oxidative metabolism had been regulated by a substantial decrease in ROS production, particularly the levels of superoxide and hydrogen peroxide. Whitaker *et al*. (2010) showed apoplastic peroxidases (EPOXC), that are generally associated with superoxide production, to be loosely bound to cell walls in *T. dregeana*. These peroxidases commonly have an optimum neutral pH (around 7.2), but it is unlikely that their potential inactivation could have been involved, as pH adjustments of the CaMg solution and of cathodic water did not have a major effect. Alternatively, as suggested by Shirahata *et al*. (1997) the action of cathodic water in regulating oxidative metabolism could be a result of its direct ROS scavenging activity, particularly of hydrogen peroxide, or the enhancement of endogenous antioxidant activity as shown by Hanaoka (2001) where cathodic water was shown to increase the efficacy of AsA as a proton donor (as was also shown in this study). Dry cathodic protection on its own, and employed in combination with post-excision soaking and cryoprotection, was not adequate to promote shoot development; however, rehydrating with cathodic water after this treatment achieved some success. Assessment of oxidative parameters in conjunction with viability, suggests that cathodic and other antioxidant protection at procedural stages of

cryopreservation promotes survival by regulation of oxidative metabolism via strict control over the ROS-antioxidant interaction interface, which is central to normal growth and development of tissue.

Protocols to achieve survival of recalcitrant germplasm at low temperatures require a multi-faceted, systems-based approach, where biochemical, biophysical, genetic, molecular and structural damage are acknowledged as possible causes of death. As such, all facets should be equally investigated to provide protection and promotion of growth at each of these levels.

The use of embryonic axes as explants for embryo cryopreservation has widely been acknowledged as a useful tool for the establishment of germplasm banks for recalcitrant- and intermediate-seeded species, and those which are rare or threatened (Chaudhury & Malik, 1998). For future studies to develop cryopreservation protocols of these recalcitrant species, bearing in mind results and progress made in this study, general suggestions are improvement of protocols in terms of efficiency and wider applicability across species. When investigating the physiological and biophysical/mechanical causes of death during the cryopreservation process itself, future studies will concentrate on increasing drying rates, so that explant viability is retained at, or very near, non-freezable water contents, as lethal damage by ice crystallisation is likely to have been the major underlying cause of the lack of onwards development by axes of *T. dregeana* after cryogenic cooling. In this vein, differential scanning calorimetric (DSC) studies have to be undertaken to establish if freezable water is present in the tissue and, if so, the quantity present. Means to speed up the rate of water loss by flash dying in order to (further) curtail metabolism-linked damage, are currently underway in our laboratory. Short-term culture of excised axes on media containing somewhat elevated sucrose concentrations (as used for other types of explants (Reed, 2008) could be a means to lower water content osmotically, prior to flash drying. Technical approaches to protect the shoot meristematic region during rapid drying should also be investigated.

Implementation of various cooling rates too, and their possible differential effect on root and shoot development should be undertaken. Differential scanning calorimetry (DSC) can also be used to investigate these parameters and these assessments will form an integral part of ongoing investigations.

As reflected above, survival of axes after all the cryogenic procedures culminating in cryogen exposure was not attained, suggesting that the cause of death is more than an oxidative problem. Alternate/additional cause of failure, particularly the possible involvement of mechanical/structural damage at both a cellular/tissue level (cell collapse on the removal

of water as well as ice crystal damage), and at the ultrastructural level (membrane appression on loss of cellular water) must be investigated. Some of these effects are desiccation-related, rather than a consequence of low temperature, but physical damage induced by all parts of the cryopreservation protocol must be addressed. Physical damage, especially during desiccation, can be assessed using quantitative indicators such as leakage of solutes and can be quantitatively assessed at the ultrastructural level using electron microscopy.

The oxidative metabolism studies in one tropical recalcitrant system, i.e. excised embryonic axes of *T. dregeana*, highlights the need to undertake similar investigations using axes of other tropical, and of temperate, recalcitrant-seeded species, in an attempt to further understanding of responses of desiccation sensitive germplasm by way of comparative studies. Tropical recalcitrant seeds have a much higher lethal minimum temperature than those of temperate recalcitrant species (Bonner, 2008) and therefore seeds of the latter variety are considered to be more amenable to cryopreservation procedures. Investigations of the responses of temperate recalcitrant germplasm to cryopreservation by way of studying oxidative events should be valuable in advancing the frontiers of knowledge in the field of plant germplasm cryobiology/cryopreservation.

The possibility of using different antioxidants and compounds such as desferrioxamine, which sequesters iron and thus disturbs Fenton chemistry that generates hydroxyl radicals (Benson *et al*., 2005); flavonoids, polyols, sugars and proline which are known ROS-scavengers during water deficit (Smirnoff, 1993) should be considered, either alone or in conjuction with cathodic protection. Here it is essential to note that external antioxidant protection requires the co-ordination of an arsenal of redox reactions, many of which are interlinked via the tight coupling of redox cycling mechanisms with the related co-factors and intermediates. Such protocols, which involve the addition of antioxidants, must consider the interdependent effects of each additive (Benson, 2000b). In addition to external provision of antioxidants, studies have shown the phenylpropanoid pathway to be responsible for a diverse array of phenolic metabolites that are often induced by stress and serve a role in plant protection. There has been evidence to suggest that the phenylpropanoid metabolite, chlorogenic acid (CGA; 5-*O*-caffeoylquinic acid), has a novel antioxidant function under abiotic stress conditions and phenylpropanoid biosynthesis may provide an alternate pathway for energy dissipation and the promotion of enhanced antioxidant capacity within the cell (Grace & Logan, 2000). This would also be an avenue for investigation.

The use of the GSSG/GSH couple as an indicator of redox potential in the tissue (Varghese *et al*., 2011) will advance the oxidative work conducted thus far and will be measured across selected species.

Intended investigations, regardless of whether focus is directed on a physiological, molecular or genetic level should be undertaken concomitantly with ultrastructural studies as far as possible. Histochemical and cytochemical studies would involve the *in situ* localisation of superoxide (Beckett *et al*., 2003) and hydrogen peroxide (Bailly & Kranner, 2011) by light microscopy; ultrastructural localisation of hydrogen peroxide (Bestwick *et al*., 1997) and tissue localisation of ROS via fluorescence/confocal microscopy (Bailly & Kranner, 2011). These studies will yield important information not only regarding the magnitude of ROS produced but also specific areas where uncontrolled ROS is generated.

Studies involving the identification of genes responsible for desiccation and chilling tolerance, as well as those that play a role in expression of antioxidants, should be undertaken in temperate and tropical recalcitrant material to advance remediation of tissue damage during cryopreservation from a genetic approach, if upregulation of genes to promote desiccation/chilling tolerance and antioxidant activity is in fact possible. Specific genes that have been shown to be expressed during the acquisition of desiccation tolerance, such as COR (cold-regulated proteins) and LEAs [Late-Embryogenesis Abundant proteins; specifically MtEm6 (group 1), DHN3 (dehydrins and MtPM25 (group 5)] (Boudet *et al*., 2006), can possibly confer resistance to dehydration and cold stress, and since desiccation sensitivity particularly is such a fundamental limiting factor to cryopreservation of recalcitrant germplasm, studies of this nature may be of considerable value.

All studies undertaken in the future should also include assessment of DNA integrity (by the COMET assay [Fairbairn *et al*., 1995; Olive & Banáth, 2006]) before and after cryogenic processes.

In conclusion, the current study made significant progress towards the cryopreservation of three recalcitrant seeded-species, particularly in the case of *T. dregeana*. The outcomes have substantially elucidated the possible role of unbalanced oxidative metabolism in cryopreservation-induced damage as well as made advances towards ameliorating the consequences of oxidative stress in recalcitrant axis cryopreservation. Importantly, this study highlights the benefits of understanding the underlying causes of failure in cryopreservation protocols and conclusively suggests the integrative exploration of the physical and biochemical basis of recalcitrance in future cryopreservation efforts.

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