The effect of developmental status and excision injury on the success of cryopreservation of germplasm from non-orthodox seeds

by

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Submitted in fulfillment of the academic requirements for the degree of

Master of Science

in the

School of Biological and Conservation Sciences

University of KwaZulu-Natal

Durban

2007

Preface

The experimental work described in this thesis was carried out in the Plant Germplasm Conservation Research Laboratory, School of Biological and Conservation Sciences, Howard College Campus, University of KwaZulu-Natal, Durban, under the supervision of Dr Joseph Kioko and Profs Patricia Berjak and Norman Pammenter.

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

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Meagan Goveia

Acknowledgements

I would like to thank God, because through Him, all things are possible.

I would also like to thank my supervisors, Dr Joseph Kioko and Profs Patricia Berjak and Norman Pammenter, for their support, encouragement, guidance and the occasional shove throughout the course of this study. I really am grateful.

To the past and present members of the Plant Germplasm Conservation Research Group for their understanding and flexibility and most importantly for just being there for me if I needed them, I am truly indebted.

To the beam team, James, Pirrie and Sharon, thanks for your extended patience with me. I would also like to thank Prof. Richard Beckett for advice with the ROS assay.

To my family, thank you for always listening to my troubles and supporting me through to the end.

To Verdine, I cannot thank you enough for your patience, support and the endless hours of seed cleaning!

I would like to acknowledge and express my gratitude to the National Research Foundation, and to the Darwin Initiative in collaboration with the Millennium Seed Bank, Kew, U.K., for financial support.

Abstract

The zygotic germplasm of plant species producing desiccation-sensitive seeds can be conserved in the long-term only by cryopreservation. Usually the embryonic axis is excised from the cotyledons and is used as the explant for cryopreservation as it is small and provides a large surface area:volume ratio. However the shoot of the axis of most species studied does not develop after excision, with the result that survival after cryopreservation is often recorded as callus production or simply explant enlargement and/or greening. Thus, besides explant size, factors such as *in vitro* regeneration techniques, physical injury induced upon excision and developmental status of the seed could compromise the success of cryopreservation.

This study investigated the effect of the factors mentioned above, with particular attention to the developmental status of the seeds on explant *in vitro* development (section 3.1), response to dehydration (section 3.2) and cryopreservation of the desiccation-sensitive embryonic axes (section 3.3) of two species: *Trichilia dregeana*, *T. emetica* and embryos of a third, *Strychnos gerrardii*. For all three species, investigations were conducted on the embryonic axes/embryos excised from mature seeds immediately after fruit harvesting and from mature seeds stored under hydrated conditions for different periods (in order to achieve different degrees of development). In addition, preliminary studies were carried out on axes of *T. dregeana* to assess whether generation of reactive oxygen species (ROS) occurs in response to wounding upon axis excision (section 3.4).

Excised embryonic axes of *T. dregeana* and *T. emetica* did not develop shoots *in vitro* unless the explants included attached cotyledonary segments. Following the development associated with short-term storage, however, the excised axes could develop shoots after complete cotyledon excision. The embryos from the (endospermous) seeds of *S. gerrardii* which included the paper-thin cotyledons, developed normally *in vitro*, with percentage germination increasing with seed storage time. For all three species, *in vitro* axis germination was promoted when activated

charcoal was included in the germination medium, regardless of the developmental stage of the seeds.

When dehydrated to approximately 0.3 g H_2O g^{-1} dry mass (g g^{-1}), embryonic axes from all three species failed to develop shoots even though a minimum of 50% produced roots in all cases. Hence, shoot production was shown to be more sensitive to desiccation than was root production. Furthermore, the sensitivity of the shoot apical meristem to desiccation was not ameliorated with seed storage for *T. dregeana* and *T. emetica*, but did decrease for *S. gerrardii* when seeds were stored for 6 – 8 weeks.

The application of certain cryoprotectants did facilitate production of shoots after dehydration by a few axes of both *Trichilia* spp. For *T. dregeana* explants, combination of glycerol and sucrose allowed for 10% of the axes to retain the ability for shoot production after dehydration while for *T. emetica* explants, the combination of DMSO and glycerol (10 - 20% shoot production after dehydration) was best. The efficacy of the cryoprotectants was not influenced by storage period. The provision of cryoprotectants still needs to be tested for *S. gerrardii*.

Survival of subsequent cryopreservation of *T. dregeana* and *S. gerrardii* explants was best achieved with rapid cooling in nitrogen slush, with the cooling procedure for *T. emetica* explants still to be optimized. The highest post-cryopreservation survival of *T. dregeana* axes was achieved when seeds had been stored for three months, while the seed storage period did not affect post-thaw survival of the axes of *T. emetica* or *S. gerrardii*. A small proportion of *S. gerrardii* explants only, produced shoots after cryopreservation, whereas the surviving embryonic axes of *T. dregeana* and *T. emetica* regenerated only as non-embryogenic callus. Although callus production is less desirable than successful seedling establishment, it has the potential for micropropagation if embryogenicity can be induced.

Ultrastructural examination of the shoot apical meristem of *T. dregeana* after a 3-d recovery period, following excision, revealed considerable cellular derangement, although damage of individual organelles could not be resolved microscopically.

Preliminary studies on *T. dregeana* involving a colorimetric assay using epinephrine, confirmed the generation of ROS in response to wounding associated with axis excision. Reactive oxygen species generated appeared to persist over prolonged periods rather than occurring only as a single oxidative burst. Hence, ROS production at the wound site could be the primary factor contributing to lack of shoot development. Axes immersed in the anti-oxidant, ascorbic acid (AsA) immediately after excision, showed lower ROS production and 10% shoot development when cultured *in vitro*, indicating that the oxidative burst coincident with, and after excision might be counteracted if immediate ROS production can be adequately quenched. Future investigations should aim to identify the specific ROS associated with wounding and optimize an anti-oxidant treatment(s) that will facilitate shoot development.

Thus, the successful cryopreservation of the germplasm of the species tested, and others producing recalcitrant seeds, depends on a spectrum of species-specific factors, some still to be elucidated.

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List of Abbreviations

°C	degrees celcius
%	percentage
µM	micromolar
µm	micrometer
g	gram
g g^{-1}	gram of water per gram dry mass
g l^{-1}	gram per litre
l	litre
m	metre
ml	milliliter
ml l^{-1}	millilitre per litre
mm	millimeter
M	molar
mM	millimolar
mg l^{-1}	milligram per litre
nm	nanometre
v/v	volume per volume
w/v	weight per volume
Ca^{2+}	calcium cation
Mg^{2+}	magnesium cation
h	hours
min	minutes
d	day
pH	hydrogen ion concentration
Tween 20 / 80	polyoxyethylene sorbitan monolaurate
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
NAA	α-Naphthalene acetic acid
dmb	dry mass basis
mol. wt	molecular weight
Conc.	concentration

$CaCl_2$	calcium chloride
CaOCl	calcium hypochlorite
$HgCl_2$	mercuric chloride
$MgCl_2$	magnesium chloride
NaOCl	sodium hypochlorite
H_2O	water
DMSO	dimethylsulfoxide
PVP	polyvinyl-pyrrolidone
PBS	phosphate buffered saline
LEA	late embryogenic abundant/accumulating
DNA	deoxyribonucleic acid
ANOVA	analysis of variance
AC	activated charcoal
AsA	ascorbic acid
CA	citric acid
RH	relative humidity
ROS	reactive oxygen species
MS	Murashige and Skoog medium (1965)
WPM	Woody Plant Medium (1981)

1. INTRODUCTION

1.1 Biodiversity and the need for conservation

Biodiversity, which can be defined as the variety of all life on earth or within a particular habitat, includes taxonomic, ecological and genetic diversity (Lovejoy, 1996). Today, human activities negatively impact on both plant and animal diversity. The increasing population, poverty, social and commercial activities are factors which contribute to the exploitation and degradation of natural environments. Species-rich areas are being destroyed by desertification, erosion, deforestation, highways, housing, farming, and recreation (Geldenhuys, 2000).

Plants, in particular, are endangered not only by the above factors, but also by environmental and climatic changes, invasive alien species and the medicinal plant trade. Plants afforded the first medicines used to treat illness before pharmaceuticals were developed, creating a ready and ongoing market for medicinal plants. The World Wildlife Fund (WWF) has identified China and India as being two of the largest markets for medicinal plants because of their large populations and ancient heritage (WWF, online). In South Africa, and KwaZulu-Natal (KZN) particularly, the Zulu culture is the primary driving force behind the trade. This culture requires the assistance of diviners and herbalists to identify and treat illness or misfortune encountered by an individual, using practices that require the use of indigenous medicinal plants. The KZN Wildlife Commission has estimated that approximately two thirds of the province's population relies on traditional medicine for primary health care, with the result that more than 1 020 plant species are used for traditional medicines and approximately 4 500 tonnes of plant parts are traded per year for this purpose (KZN Wildlife, online). Gathering and selling medicinal plants provides a means-to-an-end for many unemployed people. However, entire plant populations are sought after for their roots or bulbs and are often depleted instead of being sustainably harvested (KZN Wildlife, online). Alternatively, where the bark of trees is required for its medicinal properties, excessive bark-stripping often kills the trees, as in the case of *Warburgia salutaris* (Kioko *et al*., 2003b). As a result of over-exploitation of medicinal plants, many indigenous species have become threatened, demanding more effective conservation strategies.

Conservation involves not only species or populations, but also genetic diversity. This, is the variation within and between populations of species, and contributes to total biodiversity (Lovejoy, 1996). The loss of genetic diversity in plants can be partly attributed to agriculture. Although agricultural practices do not necessarily use indigenous plants, the aim is to produce crops with better and higher yields on larger scales (Ford-Lloyd and Jackson, 1991). Consequently, the lower yielding strains are replaced by their higher-yielding counterparts, hence narrowing the genetic base and encouraging genetic uniformity. There are many commercial and economical advantages in breeding plants with higher yields and resistance to pests and disease. However, these are jeopardized if a stress is encountered to which the plant populations cannot adapt because of the lack of diversity in their gene pool. Thus, the maintenance of genetic diversity is essential for plant conservation.

It is for these reasons that the Global Strategy for Plant Conservation was developed (Convention on Biological Diversity, online). This strategy is ultimately aimed at halting the loss of plant biodiversity by: Understanding and documenting plant biodiversity; conserving plant biodiversity; using plant biodiversity sustainably; promoting education and awareness about plant biodiversity and building the capacity for the conservation of plant biodiversity (Convention on Biological Diversity, online). To achieve these goals, 16 targets have been set to be accomplished by 2010, including: The protection of 50% of the world's most important areas of plant diversity, the conservation of 70% of the genetic diversity of crops and other socio-economically valuable plant species, the conservation of 60% of the world's threatened species *in situ* and 60% of threatened species in accessible *ex situ* collections with 10% included in recovery and restoration programmes.

1.2 Germplasm conservation

Plant genetic resources or germplasm describes the total genetic diversity of cultivated species and their wild relatives (Ford-Lloyd and Jackson, 1991), and conservation of germplasm is the most fundamental aspect of biological conservation. Plant germplasm can be derived from a variety of sources, which will in turn determine the method of its conservation.

1.3 Methods of germplasm conservation

Plant germplasm can be conserved either *in situ* or *ex situ* (Figure 1.1). *In situ* conservation involves the maintenance of plants in their natural environments, (e.g. in nature reserves or national parks), which allows natural evolution of the species in its biological environment (Ford-Lloyd and Jackson, 1991; Krogstrup *et al*., 1992; Shands, 1993; Meilleur and Hodgkin, 2004). There are, however, social and economic factors which also need to be considered such as the availability of a sufficient number of individuals to maintain viable populations, the availability of land, and maintenance costs of the natural reserve (Berjak *et al*., 1996).

Ex situ conservation involves the removal of plants from their natural habitats for cultivation in botanic gardens, fields, plantations or for seed storage in seed banks or genebanks (Ford-Lloyd and Jackson, 1991). These practices can be labour-and land-intensive, but provide the only alternative where natural habitats are destroyed, as is the case for cycads in China (Xiao and Gong, 2006). In order to obtain a genetically representative sample, it is critical to investigate the diversity within a species before germplasm is collected, and to determine the appropriate sample size necessary (Krogstrup *et al*., 1992; Berjak *et al*., 1996; McFerson, 1998).

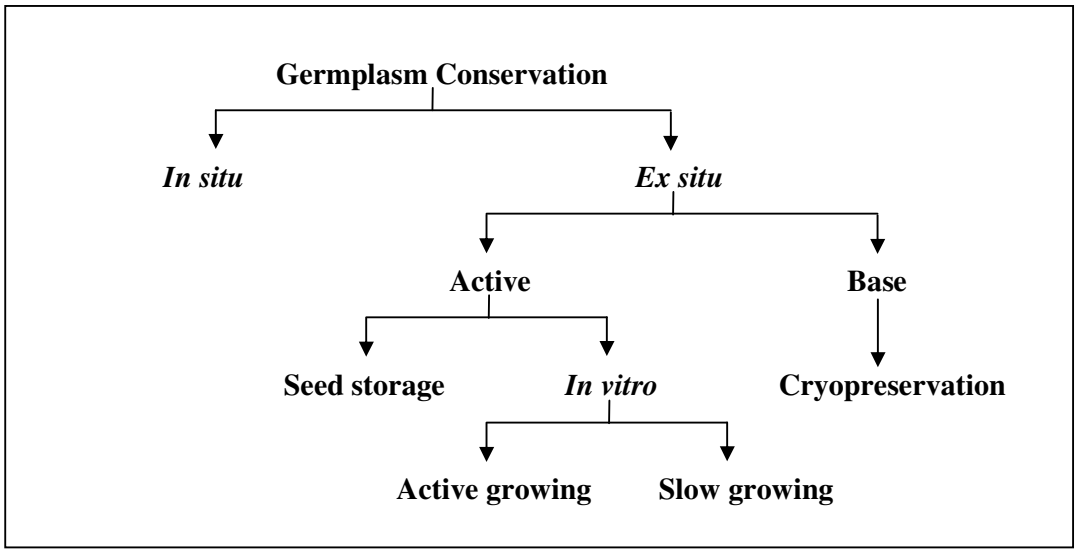


Figure 1.1: Mechanisms of germplasm conservation.

Germplasm for *ex situ* collections is obtained from either an active or a base collection (Figure 1.1). Active collections allow for short-to-medium term storage of germplasm while base collections are intended for germplasm conservation in the long-term and often serve as back-up for active collections (Krogstrup *et al*., 1992; Watt *et al*., 2000).

1.3.1 Active collection: seed storage

Seed storage is the most common method used to conserve plant germplasm because of the convenience and relatively low costs involved. The main factors determining the longevity of seeds in storage are temperature and water content, and good quality seeds can be stored for decades or more when kept at temperatures at or below -18°C and 5 - 7% relative humidity (RH) (IBPGR, 1976; Ford-Lloyd and Jackson, 1991; Krogstrup *et al*., 1992; Ellis and Hong, 2006).

This strategy, however, cannot be applied to all plant species as the post-harvest behaviour of their seeds, which determines the most suitable method of conservation, differs. As discussed in further in Section 1.4., seeds are termed either 'orthodox', 'recalcitrant' (Roberts, 1973), or intermediate (Ellis *et al*., 1990a; 1991) depending on their physiological response to desiccation and low temperatures. While orthodox seeds can be dried to low water contents and can tolerate freezing temperatures, recalcitrant seeds are sensitive to desiccation, freezing and possibly chilling (Ford-Lloyd and Jackson, 1991; Krogstrup *et al*., 1992; Berjak *et al*., 1996). Thus, for recalcitrant seeds, methods of germplasm conservation other than the conventional low-temperature/low RH approach are required.

1.3.2 Active collection: *in vitro* storage

In vitro storage requires the application of tissue culture practices, by which cells, tissues or organs are excised from the parent plant, surface sterilized and then transferred to an artificial growth medium *in vitro* (Krogstrup *et al*., 1992; Mandal *et al*., 2000). The medium can be manipulated to produce different cultures such as unorganized, undifferentiated callus or organized tissues and organs that will continue development. Although genetic conservation aims to preserve specific and unique genotypes, somaclonal variation (which is the variation generated during culture) can

occur (Krogstrup *et al*., 1992). To minimize this risk, it is preferable to culture organized systems such as embryos, meristems and shoot tips for *in vitro* storage, as these are more stable and reproducible genetic systems (Ford-Lloyd and Jackson, 1991; Krogstrup *et al*., 1992; Berjak *et al*., 1996; Engelmann, 1997; Mandal *et al*., 2000).

Cultures can be stored *in vitro* by three methods: Cryopreservation (which is discussed in more detail in section 1.3.3), actively growing cultures or slow growth cultures. Maintaining actively growing cultures requires the regular transfer of material onto new media, usually at monthly intervals (Krogstrup *et al*., 1992; Mandal *et al*., 2000, Mycock *et al*., 2004). This method does risk losing cultures due to contamination or physiological decay; however, the remaining material can potentially be rapidly micropropagated to bulk up reserves (Krogstrup *et al*., 1992; Razdan and Cocking, 1997; Mandal *et al*., 2000).

Minimal growth storage exposes cultures to factors which limit growth; these can be either chemical or physical and include use of growth retardants, reduced temperatures and reduced oxygen (Krogstrup *et al*., 1992; Reed and Chang, 1997; Staritsky, 1997). Before material kept under these conditions can be manipulated, a prolonged lag phase is needed to restore the normal physiology. This method of *in vitro* storage may impose selection pressures and environmental stresses yielding plants with genetic modifications (Krogstrup *et al*., 1992; Reed and Chang, 1997; Staritsky, 1997).

1.3.3 Base collection: cryopreservation

Cryopreservation describes the freezing of biological material and subsequent storage at ultra-low temperatures, normally at or near -196°C, the temperature of liquid nitrogen (Finkel and Ulrich, 1983; Withers, 1988; Kartha and Engelmann, 1994; Pritchard, 1995; Sakai, 1997; Berjak *et al*., 1999a). Cryopreservation can be used as a tool to preserve genetic stability as all metabolic activity ceases at ultra-low temperatures, thus minimizing, if not precluding, genetic changes (Krogstrup *et al*., 1992; Krishnapillay, 2000; Lynch, 2000; Kioko *et al*., 2003a; Engelmann, 2004; Harding, 2004).

Unless involving small, intact orthodox seeds, which can be directly planted out (Walters *et al*., 2005), cryopreservation requires *in vitro* methods because *in vitro* media and techniques are necessary to assess viability of the explants before, and following, cryopreservation, and to generate plants after cryostorage (Reed *et al*., 2001; Berjak and Pammenter, 2004a).

Plant material and protocol for cryopreservation

A broad spectrum of plant material can be used for cryopreservation, including buds, shoots, meristems, cell cultures, protoplast cultures, anthers, pollen, somatic and zygotic embryos, callus, and whole seeds (Dumet *et al*., 1997; Mandal, 1997; Anthony *et al*., 2000; Krishnapillay, 2000; Seitz, 2000). In this study, the cryopreservation of embryos or embryonic axes of species producing recalcitrant seeds (Figure 1.2) has been investigated, and is reviewed further below.

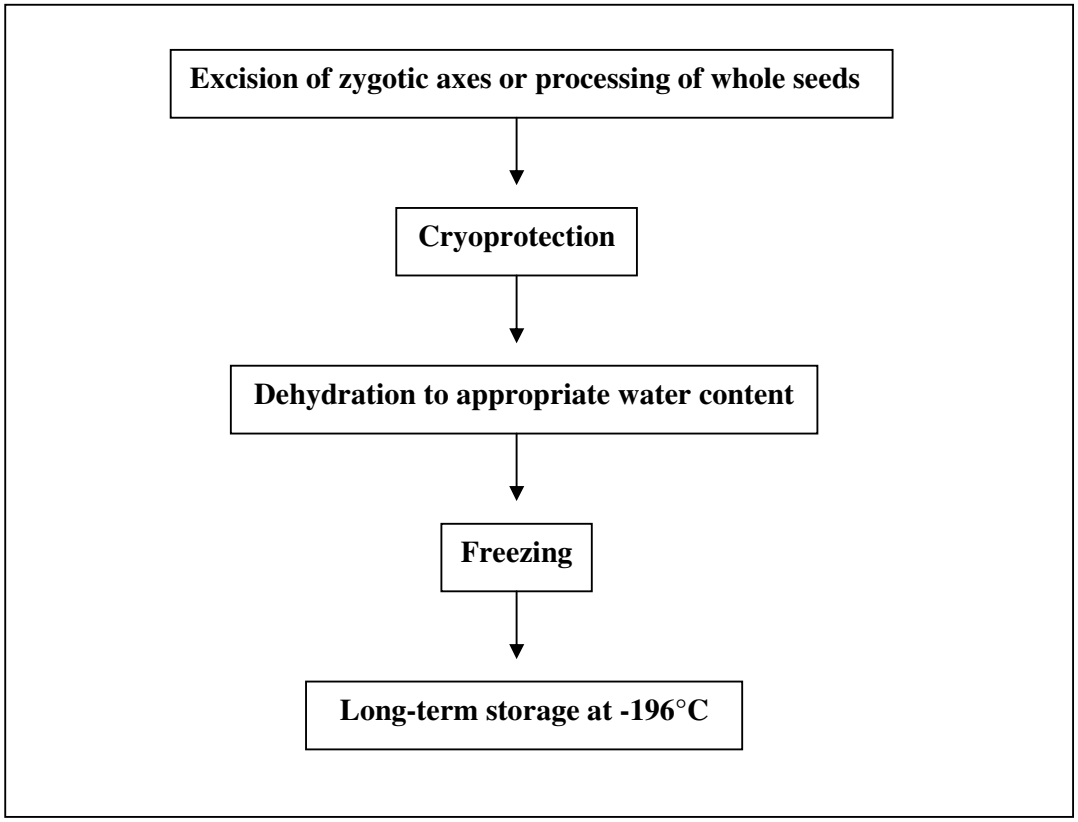


Figure 1.2: Procedure for cryopreservation of recalcitrant material. (Adapted from Ford-Lloyd and Jackson, 1991).

1.3.3.1 Cryopreservation of excised zygotic axes and whole embryos within seeds

Cryopreservation of recalcitrant seed germplasm requires the smallest mass:volume ratio to facilitate rapid dehydration and thus minimize desiccation damage (Berjak *et al*., 1996; Berjak and Pammenter, 2001; Wesley-Smith *et al*., 1999, 2001a). Most recalcitrant seeds are relatively large and have high water contents. However, in most cases the bulk of these seeds is constituted by cotyledons or endosperm, with the embryonic/zygotic axis comprising only a small fraction of the entire volume. It is therefore generally necessary to excise the small embryonic axis and use it as the explant for cryopreservation. Whole seeds may also be used for cryopreservation provided they are sufficiently small and relatively desiccation-tolerant as has been demonstrated for *Azadirachta indica* (Berjak and Dumet, 1996) and *Warburgia salutaris* (Kioko *et al*., 2003b).

1.3.3.2 Cryoprotection

Cryoprotectants reduce freezing injury in hydrated tissues and should provide protection during thawing and after cryostorage (Kartha and Engelmann, 1994; Lynch, 2000). Cryoprotectants can be categorized as either penetrating or non-penetrating (Meryman and Williams, 1985). Penetrating cryoprotectants counteract freezing damage by reducing the quantity of electrolytes in non-freezable water. Conversely, non-penetrating cryoprotectants withdraw water from cells by osmosis, hence reducing the amount of free water available for intracellular ice crystal formation (Meryman and Williams, 1985; Kartha and Engelmann, 1994; Fuller, 2004). In both cases, the net effect of the cryoprotectants is to lower tissue water concentration, i.e. to effect a measure of dehydration.

Cryoprotection is achieved either by soaking explants in the cryoprotectant solution for the appropriate period (e.g. Meryman and Williams, 1985; Mycock *et al*., 1995; Potts and Lumpkin, 1997; Mycock, 1999; Valladares *et al*., 2004), or by preculturing the explants on a growth medium enriched with cryoprotectants, usually sucrose (e.g. Dumet *et al*., 1994; Thierry *et al*., 1997; Hitmi *et al*., 1999; Thammasiri, 1999; Lynch, 2000; Blakesley and Kiernan, 2001). Cryoprotectants are not always favoured because they can become cytotoxic at higher concentrations (Finkle *et al*., 1985; Berjak *et al*.,

1999a) and optimal cryoprotective treatments are often species-specific (Wesley-Smith, 2002).

1.3.3.3 Dehydration to appropriate water contents

The hydrated nature of recalcitrant seeds makes them unsuitable for cryopreservation because the ice crystals formed as water freezes may cause lethal damage to the tissue (Berjak and Pammenter, 2001; Wesley-Smith *et al*., 2001b). Thus, to avoid freezing injury, it is necessary to dehydrate the tissue without compromising its viability. The slower water is removed, the greater the metabolism-linked damage, as metabolism becomes unbalanced over time (Pammenter *et al*., 1998; Berjak *et al*., 1999b; Berjak and Pammenter, 2001; Walters *et al*., 2001). The faster recalcitrant seeds can be dried, the lower the water content they can tolerate before viability is lost (e.g. Pammenter *et al*., 1998). Therefore, the use of excised embryonic axes confers the advantage of rapid dehydration because of their relatively small size.

Embryonic axes can be dried in a laminar air flow (e.g. Normah *et al*., 1986; Pritchard and Prendergast, 1986; Corredoira *et al*., 2004). However, faster drying can be achieved by flash-drying. This technique exposes axes to moving dry air, to facilitate rapid drying within minutes to hours (Berjak and Pammenter, 2001; Pammenter *et al*., 2002). Optimal water content for cryopreservation varies with species, but flash-drying achieves the optimal water content quickly, thereby reducing exposure time to potentially harmful intermediate water contents (Wesley-Smith *et al*., 1992; Vertucci and Farrant, 1995; Berjak and Pammenter, 2007).

1.3.3.4 Freezing methods

Cryopreservation techniques have been grouped into two broad categories, termed classical and new techniques (Engelmann, 1997).

Classical freezing is often achieved in two steps and hence has also been termed ‘two step freezing’ (Krogstrup *et al*., 1992). Following treatment with cryoprotectants, the first step involves slow controlled cooling to a defined pre-freezing temperature, usually around -40°C (Krogstrup *et al*., 1992; Engelmann, 1997), achieved by programmable

freezers. In this step, extra-cellular ice is formed and the cells become dehydrated as intracellular water is lost to the exterior. Thus, freezable water leaves the cells, preventing (or at least minimizing) intracellular ice crystal formation upon transfer into liquid nitrogen (step 2) (Kartha and Engelmann, 1994; Engelmann, 1997).

Rapid freezing provides an alternative to the method described above and involves the plunging of explants directly into liquid nitrogen or sub-cooled nitrogen. This allows the explants to pass rapidly through the temperature range (from -2°C to -80°C) at which ice crystal formation occurs, thus minimizing or ideally preventing the damage caused by ice formation (Kartha and Engelmann, 1994). This technique extends the water content window in which explants will survive freezing (Wesley-Smith *et* al., 1992; Berjak *et al*., 1996) and its simplicity makes it an attractive cooling method.

New freezing techniques are based on vitrification - the formation of an amorphous glass. These techniques avoid ice crystal formation by dehydration of explants, rather than preventing freezing injury within the tissues as in the classical techniques. By using high concentrations of cryoprotectants and vitrification solutions, explants can be dehydrated to low water contents preventing ice crystal formation when immersed into liquid nitrogen (Krogstrup *et al*., 1992; Engelmann, 1997). Seven vitrification procedures have been established and are elaborated by Engelmann (1997). These are: Encapsulation dehydration vitrification; encapsulation vitrification; desiccation; pregrowth; pregrowth desiccation and droplet freezing.

Once the explants have been frozen, they can be theoretically stored at the ultra-low temperature of liquid nitrogen (-196°C) indefinitely (Kartha and Engelmann, 1994; Pritchard, 1995; Sakai, 1997; Berjak *et al*., 1999a; Walters *et al*., 2004). At this temperature all metabolic activity will have ceased without damaging the tissue, therefore allowing long-term preservation. However, according to Benson and Bremner (2004), the probability of very slow free radical generation during cryopreservation, cannot be overlooked.

Cryopreservation may offer the only feasible means for the long-term preservation of the germplasm of plants producing non-orthodox seeds (Kartha and Engelmann, 1994; Pritchard, 1995; Dumet *et al*., 1997; Berjak *et al*., 1999a; Krishnapillay, 2000; Berjak and Pammenter, 2001, 2004a).

1.4 Post-harvest seed behaviour

As discussed above, seeds provide a source of plant germplasm which can be stored in active or base collections as a means of conservation and that the mode of conservation is dependent on the behaviour of the seeds once they have been shed from the parent plant. Initially two categories of seed behaviour were described (Roberts, 1973). Seeds defined by that author as orthodox are characterized by their ability to remain viable for predictably long periods at low temperatures after dehydration to low water contents. At the other extreme, seeds categorized as recalcitrant have relatively high water contents, are damaged by dehydration and are generally short-lived and unstable (Roberts, 1973). A third category of seed behaviour, termed intermediate, was described by Ellis *et al*. (1990a) in experiments using coffee seeds. Seeds in this category, such as those of oil palm and royal palm (Ellis *et al*., 1991), are relatively desiccation-tolerant, but do not remain viable for long periods at reduced water contents. Those of tropical origin appear also to be chilling-sensitive, at least when dry (Hong and Ellis, 1996) However, a continuum of seed behaviour has been suggested (Berjak and Pammenter, 1994, 2007; Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002) as many species cannot unequivocally be assigned to any one of these categories. This view is strongly supported by recent investigations of Daws *et al*. (2006b) who have shown that *Acer pseudoplatanus* seeds of northerly provenance in the U.K. show typically recalcitrant behaviour, while those from the Mediterranean exhibit intermediate traits.

Although most commercial crops show orthodox seed behaviour, crops constitute less than 0.1% of all higher plants (Berjak and Pammenter, 2001, 2004b). As seed biology studies are extended, non-orthodox behaviour is encountered in increasingly more species.

1.5 Seed recalcitrance

There are several factors which have been identified as distinguishing orthodox and non-orthodox behaviour, as described below:

1.5.1 Seed development

Orthodox seed development is clearly divided into three phases, *viz*., histodifferentiation, reserve deposition and maturation drying (Kermode and Finch-Savage, 2002). During histodifferentiation, undifferentiated cells divide and specialize, thus developing tissues with specific functions. These events take place in both monocots (Olsen *et al*., 1992) and dicots (Wang and Hedley, 1991). In the second phase, reserves in various forms are deposited in the endosperm or cotyledons providing nutrients that will sustain seedling development. Both stages thus far increase the dry and fresh mass of the seeds. Ultimately, maximum dry mass is reached and seeds are described as physiologically mature. A rapid loss of water, and thus fresh mass, follows in the phase of maturation drying, accompanied by preparation for germination, replacing events intrinsic to seed development. Once all three phases are complete, seeds are shed and remain quiescent until water becomes available for germination (Kermode and Finch-Savage, 2002). (Note that physiological dormancy occurs in seeds of many species.)

In recalcitrant seeds, the final phase of maturation drying is absent (Berjak *et al*., 1989, 1990; Farnsworth, 2000; Connor and Sowa, 2002; Kermode and Finch-Savage, 2002) resulting in seeds being shed at relatively to very high water contents (0.3 - 4.0 g g^{-1} dry mass basis [dmb] depending on the species [Berjak and Pammenter, 2001, 2004b]).

In addition to facilitating intracellular order and structural stability (Berjak, 2006) water is involved in virtually every dynamic process in living cells because it provides the solvent medium in which movement of solutes and biochemical reactions take place (Sun, 2002). Recalcitrant seeds remain metabolically active when shed, which is facilitated by appropriate water contents. Studies to assess various characteristics of the relationship between water status and seed development have been carried out on seeds of *Quercus robur* (Grange and Finch-Savage, 1992; Finch-Savage, 1992a; Finch-

Savage *et al*., 1992), *Avicennia marina* (Farrant *et al*., 1993) and *Podocarpus henkelii* (Dodd *et al*., 1989) showing the latter stages of seed development of non-orthodox seeds to differ from that in orthodox types. As shown by those authors, in recalcitrant seeds the final stage of maturation drying is absent, or very curtailed.

1.5.2 Desiccation

The response to desiccation is a major factor differentiating non-orthodox from orthodox seeds. Orthodox seeds have evolved mechanisms for desiccation tolerance which allow for survival during and after maturation drying. Recalcitrant seeds lack, or only partially, express these mechanisms (Berjak and Pammenter, 2001), thus limiting them from completing the final phase of development and ultimately defining the basis for their desiccation sensitivity.

1.5.2.1 Desiccation tolerance mechanisms

Desiccation tolerance is the ability to dry to equilibrium with RH of the air and resume normal function when rehydrated (Alpert and Oliver, 2002; Phillips *et al*., 2002). In the strict sense, desiccation-tolerant organisms (or life-cycle stages of an organism), will survive for protracted periods at tissue water contents in the range 0.05 – 0.15 g g^{-1} dmb (reviewed by Berjak, 2006; Berjak *et al*., 2007). Several mechanisms have been proposed that together confer the ability for desiccation tolerance.

1.5.2.1.1 Intracellular physical characteristics

Physical protection against desiccation in orthodox seeds is provided by a large reduction in fluid-filled vacuoles, caused either by their breakdown into smaller ones and/or filling them with insoluble reserve material (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2004b). The accumulation of starch or lipid bodies (Vertucci and Farrant, 1995) also assists in increasing the resilience of cells to the water stress accompanying extreme dehydration. Studies carried out on various species described as highly or moderately recalcitrant or orthodox showed that the degree of vacuolation in axis cells at maturity was directly related to the desiccation sensitivity of the seeds (Berjak *et al*., 1984; Farrant *et al*., 1989, 1997).

The plant cytoskeleton, comprised of microtubules and microfilaments, organizes the intracellular milieu spatially and also provides internal support (Wyatt and Carpita, 1993; Hoffman and Vaughn, 1995). In orthodox seeds, the cytoskeleton is thought to dissociate upon dehydration and reassemble upon imbibition (Pammenter and Berjak, 1999). Experiments on the embryonic axes of the recalcitrant seeds of *Quercus robur* (Mycock *et al*., 2000); *Inga vera* (Faria *et al*., 2004) and *Bridelia micrantha* (Merhar *et al*., 2004); revealed that dissociation of the cytoskeleton occurred upon injurious levels of dehydration, and that complete reassembly upon rehydration was not achieved. In contrast, Faria *et al*. (2005) showed that the cytoskeleton was rapidly reconstituted during imbibition of orthodox *Medicago truncutula* seeds. The desiccation sensitivity of the cytoskeleton therefore must have physiological and structural consequences in recalcitrant tissues.

It is also important to maintain nuclear integrity upon dehydration, during the dry state and upon rehydration (Pammenter and Berjak, 1999). Osborne and Boubriak (1994) have shown that DNA becomes highly degraded during dehydration as a result of non-repaired, double-strand breaks in desiccation-sensitive tissue, which must contribute significantly to viability loss. In contrast, those authors indicated that DNA in desiccation-tolerant tissue is stable and has the appropriate mechanisms to withstand desiccation.

1.5.2.1.2 Intracellular dedifferentiation and metabolic 'switch-off'

At the onset of maturation drying in orthodox seeds, intracellular organelles such as mitochondria and plastids de-differentiate, thus losing their internal structure. In addition, endomembranes such as rough endoplasmic reticulum become reduced and cisternae of Golgi bodies become dissociated (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2004b). Concomitantly, metabolic activity, including respiration, membrane synthesis and processing, and DNA processing, ceases. This controlled shutdown of activity and dismantling of structures renders orthodox seeds ametabolic during maturation drying. Recalcitrant seeds do not undergo maturation drying, and apparently do not have these mechanisms, hence remaining metabolically active

throughout development (Faria *et al*., 2004). The lack of these mechanisms contributes to the vulnerability of the seeds to dehydration.

1.5.2.1.3 Free radicals and anti-oxidant systems

Free radicals and reactive oxygen species (ROS) are strong oxidizing agents produced during normal metabolism (e.g. during the reduction of mitochondrial cytochromes [Leprince *et al*., 1994; Leprince *et al*., 2000a]). In hydrated cells, ROS levels are controlled by anti-oxidant systems thus preventing injurious consequences of escaped free radicals (Pammenter and Berjak, 1999; Bailly *et al*., 2001; Berjak and Pammenter, 2001, 2004b; Buitink *et al*., 2002; Haslekås *et al*., 2003; Mayaba and Beckett, 2003; Kranner and Birtic, 2005). The most commonly studied consequence of free radical overproduction is lipid peroxidation, which ultimately leads to leaky membranes during imbibition as displayed in neem (Varghese and Naithani, 2000) cucumber and pea (Leprince *et al*., 2000b) and water lettuce (Sinha *et al*., 2005). When metabolism is disturbed, as is the case when dehydration occurs, there is potential for unregulated free radical production (Leprince and Hoekstra, 1998; Farrant *et al*., 2004). To prevent cellular damage, operation of anti-oxidant systems must be effective (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2004b; Oliver *et al*., 2001; Kranner *et al*., 2005). Although metabolism is 'switched off' in orthodox seeds during maturation drying, components of their anti-oxidant systems still remain active, facilitating safe dehydration and rehydration (Bailly *et al*., 2001; 2004; Türkan *et al*., 2005; reviewed by Berjak, 2006; Berjak *et al*., 2007).

Recalcitrant seeds have also been suggested to have anti-oxidant and free radical scavenging mechanisms (Chaitanya *et al*., 2000). However, these may become impaired or otherwise unable to cope with the level of free radicals generated as a consequence of water stress (Hendry *et al*., 1992; Leprince *et al*., 1999) especially during slow dehydration (Pammenter *et al*., 1998). Uncontrolled free radical generation and consequent oxidative damage caused by desiccation has been demonstrated for neem (Varghese and Naithani, 2000) but not for sycamore seeds (Greggains *et al*., 2000), thus demonstrating the variation in the presence and activity of anti-oxidant systems among seeds of non-orthodox species.

1.5.2.1.4 The presence of protective molecules

Sucrose and certain oligosaccharides

Orthodox seeds are known to accumulate sucrose and oligosaccharides as they mature (Koster and Leopold, 1988). At low water concentrations, these constituents contribute towards a highly viscous, supersaturated solution known as a glass, which can curtail molecular diffusion, thus minimizing unregulated metabolism (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2004b; Hoekstra *et al*., 2001; Kermode and Finch-Savage, 2002; Alpert, 2006). The glassy (vitrified) state may contribute to the prolonged life span of orthodox seeds (Leopold *et al*., 1994) and the ultimate breakdown of the glass may underlie seed deterioration in seed storage. However, the contribution of these carbohydrates to the glassy state, must be considered in conjunction with other cytomatrical constituents (see LEAs below).

Sugars and oligosaccharides are present in some recalcitrant seeds (Koster, 1991; Pritchard *et al*., 1995a). However, the formation of glass in seeds of these species can only occur at sub-zero temperatures and at water contents that do not support viability (Koster, 1991; Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2004b).

Late Embryogenic Accumulating/Abundant proteins (LEAs)

These proteins are involved in the acquisition and maintenance of desiccation tolerance in orthodox seeds. It is possible that their amphipathic nature allows for interaction with a wide range of macromolecules, thus preventing their denaturation under dehydrating conditions (Blackman *et al*., 1995; Stupnikova *et al*., 2006). In recalcitrant seeds, LEAs have been shown to be absent in some species (e.g. *Avicennia marina* [Farrant *et al*., 1993]) and present in others (Kermode, 1997). Ultimately, the acquisition and maintenance of desiccation tolerance must be the result of the interaction between a variety of factors, sucrose and LEA accumulation being prominent.

Berjak (2006) reviewed recent views of the glassy state in desiccation-tolerant organisms. That author strongly emphasized that the intracellular glass matrix in seeds cannot be considered to contain only sucrose and certain oligosaccharides, but must also include an array of other cytomatrical constituents. Principally, LEAs are now

considered vital components of the glassy matrix, adding tensile strength and suggestedly, stability to the matrix (Berjak, 2006).

1.5.2.1.5 The ability for damage repair on rehydration

The ability of orthodox seeds to withstand desiccation does not depend only on their protective mechanisms before and during desiccation (thus avoiding and minimizing damage), but also on their ability to repair desiccation damage upon rehydration. This had been demonstrated in the DNA repair mechanisms during early imbibition by Boubriak *et al*. (1997).

There are very few studies showing repair in damaged recalcitrant seeds. However Boubriak *et al*. (2000) showed that DNA repair does not fully recover after 8% water loss and that damage is unrepairable after 22% water loss, indicating the presence of inadequate repair mechanisms. Thus, it appears that repair mechanisms, if present, are also sensitive to water loss. Connor and Sowa (2003) have shown that as viability of recalcitrant *Quercus alba* acorns declines, so does the ability to reverse the gel to liquid crystalline phase in membranes.

1.5.2.2 Desiccation damage

As water is removed from cells, physical and physiological properties of cells change. These properties may be reversible once water is added; therefore desiccation damage is indicated not by the differences between the hydrated and dry state, but by the resumption of normal activity upon rehydration (Walters *et al*., 2002a).

Damage caused by desiccation may be structural, mechanical, molecular, or metabolically derived. The first indication of mechanical and structural damage is the loss of turgor pressure of cells (Walters *et al*., 2002a; Corbineau *et al*., 2004). Subsequently cells become highly vacuolated (Berjak *et al*., 1984), organelles such as the mitochondria and chloroplasts become disorganized (Raja *et al*., 2005) and, as discussed above, the cytoskeleton is dismantled and will not assemble normally upon rehydration.

At the level of membrane structure in desiccation-sensitive cells, demixing of membrane components can occur in dehydrated cells. This involves the fusion of the cell membrane and endomembranes, resulting in the exclusion of integral proteins from within the membranes and micelle formation between the membranes. Upon rehydration, separate lipid bilayers may be reformed. However, these are a combination of more than one type of membrane (Cordova-Tellez and Burris, 2002; Walters *et al*., 2002a). Furthermore, the proteins excluded are likely to be denatured (e.g. Connor and Sowa, 2003) and, in any case, would not be re-incorporated. Consequently, cellular contents leak from the cell because the membranes are non-functional.

Metabolically-derived damage causes the loss of viability because metabolism continues during mild water stress, which inevitably leads to a greater demand for high water potentials (Berjak *et al*., 1989; Pammenter *et al*., 1994; Walters *et al*., 2002a). The consequence of slow dehydration on desiccation-sensitive tissues is a matter of unbalanced metabolism which is suggested to be associated with accumulation of potentially damaging chemical species, particularly free radicals (Pammenter *et al*., 1998; Walters *et al*., 2001), in conjunction with ineffective anti-oxidant mechanisms.

1.5.3 Dehydration and drying rate

During the maturation drying phase, orthodox seeds undergo controlled shutdown of metabolic processes accompanied by the loss of freezable or solution water (Vertucci and Farrant, 1995; Berjak, 2006; Berjak *et al*., 2007). Further dehydration to below water contents of approximately 0.3 g g^{-1} (dmb) results in the removal of non-freezable water i.e. the water bound to intracellular surfaces (Vertucci and Farrant, 1995). While this does not have deleterious consequences in mature orthodox seeds because cells have acquired the mechanisms to protect against extreme desiccation, recalcitrant seeds are adversely affected, as demonstrated by Pammenter *et al.* (1991) for the seeds of *Landolphia kirkii*, and a range of other species (Pammenter *et al*., 1993).

As recalcitrant seeds are generally large structures that remain metabolically active after shedding and do not lose water readily, they naturally dry slowly (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2004b). During the progressive reduction in

freezable water, metabolism continues but becomes progressively unbalanced with dehydration (Berjak *et al*., 1990; Pammenter *et al*., 1998; Pammenter and Berjak, 1999; Walters *et al*., 2001). Slow dehydration allows more time for unbalanced metabolism to occur and, without any or only inadequate protective mechanisms, the consequences are lethal. Slow dehydration of the seeds of C*amellia sinensis* (Berjak *et al*., 1993), *Quercus nigra* (Bonner, 1996), *Wasabia japonica* (Potts and Lumpkin, 1997), *Trichilia dregeana* (Kioko *et al*., 1998), *Ekebergia capensis* (Pammenter *et al*., 1998), *Artocarpus hetrophyllus* (Wesley-Smith *et al*., 2001b), *Warburgia salutaris* (Kioko *et al*., 2003b) and *Trichilia emetica* (Kioko *et al*., 2006) was shown to be markedly more deleterious than rapid dehydration.

Thus, to conserve viability despite water loss, recent experiments on recalcitrant seeds apply a rapid dehydration technique, usually the use of a laminar air-flow (e.g. Corredoira *et al*., 2004; Makeen *et al*., 2005) or a flash-drier (e.g. Walters *et al*., 2002a; Perán *et al*., 2006). As the relatively large size of recalcitrant seeds of most species makes it difficult or impossible to dry them sufficiently rapidly, the excised embryonic axes from the seeds are commonly used. If axes are rapidly dried, freezable water can be removed without unbalancing metabolic processes (Pammenter *et al*., 1991; Thammasiri, 1999; Kioko *et al*., 2006). However, the removal of non-freezable water results in loss of integrity of cellular and structural organization (e.g. McKersie and Tomes, 1980), termed desiccation damage *sensu stricto* (Pammenter and Berjak, 1999; Pammenter *et al*., 2000; Walters *et al*., 2001; Kim *et al*., 2005). In this regard, Liang and Sun (2002) have proposed a model to determine the optimal dehydration rate to minimize desiccation damage.

Desiccation damage (either *sensu stricto* or metabolism-linked) in recalcitrant seeds is a consequence of the absence or inadequate mechanisms necessary to tolerate the loss of freezable and non-freezable water. As discussed above, non-freezable water cannot be removed from recalcitrant seeds/axes (irrespective of drying rate) without lethal consequences, but sufficiently rapid removal of freezable water can be tolerated to relatively low water contents simply because insufficient time is available for metabolism-linked damage to reach significantly injurious proportions (Pammenter *et*

al., 1998; Pammenter and Berjak, 1999; Walters *et al*., 2001; Berjak and Pammenter, 2007).

1.5.4 Seed storage potential

Orthodox seeds can be stored for long periods at low water contents under conditions of low temperature and RH (Roberts, 1973; 1981; Kozlowski and Pallardy, 2002). This has been reported for orthodox seeds of numerous species including lettuce (Walters *et al*., 1998, 2004, 2005), rice, millet and peanut (Hu *et al*., 1998), sesame and soybean (Chai *et al*., 1998), cucumber (Zeng *et al*., 1998), wheat (Stefani *et al*., 2000), cotton (Abdelmagid and Osman, 1975) and rye (Specht and Börner, 1998). Other studies have shown that seed longevity can be further extended if the temperature is lowered (Sacandé *et al*., 1998; Shen and Qi, 1998; Walters *et al*., 2005). However, even under near-ideal conditions, seed longevity is finite (Walters *et al*., 2005) and the water content below which longevity cannot be improved is considered a critical factor (Ellis *et al*., 1986, 1988, 1990b, 1990c; Vertucci and Roos, 1990). Furthermore, below a critical water content, further removal of water will not be beneficial (e.g. Ellis *et al*., 1990b; 1990c) and has actually been shown to increase deterioration of stored seeds (Vertucci and Roos, 1990; Walters 1998; Walters and Engels, 1998; Buitink *et al*., 2000). Deterioration of ultra-dry stored seeds, manifested as a decline in vigour and viability, has been attributed to the fact that water should be viewed not only as the medium within cells, but also a structural component of proteins and other macromolecules (Bernal-Lugo and Leopold, 1992). Thus, upon extreme dehydration denaturation of these macromolecules can occur, thereby adversely affecting viability of seeds.

Even under optimal conditions, conventional storage is not suitable for recalcitrant seeds which maintain viability only when stored in a hydrated state (Berjak, 1989; Drew *et al*., 2000; Andrade, 2001; Eggers *et al*., 2007). However, hydrated storage is successful only in the short-to-medium term (Berjak, 1989). Storage responses across a range of recalcitrant seeds have been studied, including *Scadoxus membranaceus* and *Landolphia kirkii* (Farrant *et al*., 1989), A*raucaria hunsteinii* (Pritchard *et al*., 1995b), *Symphonia globuifera* (Corbineau and Côme, 1986; 1988) *Azadirachta indica* (Nayal *et*

al., 2000, Neya *et al*., 2004) and *Quercus* spp. (Connor and Sowa, 2002). Those authors collectively show storage periods ranging between 20 days to 12 months, depending on the post-harvest behaviour of the seeds. Generally, it is seeds of only the temperate species (e.g. *Quercus* spp.) that can be stored for several months as recalcitrant species of temperate regions are more desiccation-tolerant and have been shown to have a longer life span than those of tropical origin (Pammenter and Berjak, 2000; Daws *et al*., 2006a) As seeds stored in the hydrated state are metabolically active, they undergo germination-associated changes culminating in extensive vacuolation and initiation of cell division (Farrant *et al*., 1988; Berjak *et al*., 1989; Pammenter *et al*., 1994). As discussed by those authors, these changes require water additional to that present in the seed at the time of shedding, thus exposing seeds to an initially mild, but increasingly severe, water stress. Consequently the deleterious events associated with water stress (such as the breakdown of co-coordinated metabolism, leading to free radical damage) lead to the death of the tissue (Dussert *et al*., 2006; Ratajczak and Pukacka, 2006).

The longevity of seeds under hydrated conditions is also affected by microorganisms such as fungi and bacteria. Fungi, in particular, are harboured on, and in, the seeds (Colpas *et al*., 2003; Hong *et al*., 2005) and proliferate under the favourable conditions necessary for hydrated storage (Berjak, 1996; Calistru *et al*., 2000; Sutherland *et al*., 2002; Anguelova-Merhar *et al*., 2003). Although a fungal species may dominate at any one time, inoculum of a range of mycoflora is harboured (Sutherland *et al*., 2002; Fakhrunnisa *et al*., 2006). Fungi have a significant impact at the stage where uncontrolled metabolism increases (Berjak, 1996; Schafer and Kotanen, 2004; Pucko *et al*., 2005, Gundel *et al*., 2006), thus contributing to a loss of viability under hydrated conditions.

Considering the factors raised above, storage of recalcitrant seeds may be possible in the short-term, but is unfeasible in the long-term. Thus, the only currently applicable option for long-term storage of recalcitrant seeds is cryopreservation.

1.6 Factors influencing successful cryopreservation

Successful cryopreservation is defines as the ability of the explants to produce normal seedlings or plantlets upon regeneration (Berjak and Pammenter, 2001). This is affected by a series of factors, listed below:

1.6.1 Explant size, water content and cooling and thawing rates

The smallest possible explants are preferable for cryopreservation as these can be rapidly dehydrated, minimizing the time available for unbalanced metabolism to occur. Thus, much of the solution/freezable water, likely to form ice crystals upon freezing is removed without jeopardizing viability of the explant (Wesley-Smith *et al*., 2004a). As almost invariably, some freezable water will be present, small explant sizes also facilitate rapid cooling which prevents (or minimizes) ice crystal formation, as explants rapidly pass though the temperature range at which ice crystal formation can occur.

Generally, the higher the final water content after flash-drying, the more rapid the rate of cooling must be to restrict crystallization and damage (Wesley-Smith *et al*., 1992). Using the embryonic axes of *Poncirus trifoliata*, Wesley-Smith *et al*. (2004b) showed that at low axis water contents intracellular viscosity is high, which slows ice crystal formation, making survival largely independent of cooling rate. However, at high axis water contents, the intracellular viscosity is lower, which facilitates rapid ice crystal formation (Wesley-Smith *et al*., 2004b). Thus, explants must be rapidly cooled to prevent damage, and care must also be taken to ensure that water contents below non-freezable limits are not reached.

Explants sufficiently-small for cryopreservation, can be obtained from various sources. For example, shoot tips provide suitable explants for cryopreservation and have been used successfully with rapid cooling rates for *Chrysanthemum* sp. (Hitmi *et al*., 1999), cassava (Charoensub *et al*., 1999), pineapple (Gámez-Pastrana *et al*., 2004), apple (Zhao *et al*., 1999a, 1999b), *Gentiana* spp. (Tanaka *et al*., 2004), *Prunus* sp. (Helliot and de Boucaud, 1997), *Holostema annulare* (Decruse *et al*., 2004) and *Pinus roxburghii* (Malabadi and Nataraja, 2006).

Axillary buds (Blakesley and Kiernan, 2001) and somatic embryos (Stewart *et al*., 2001; Fang *et al*., 2004; Valladares *et al*., 2004) also provide suitably-sized explants for successful cryopreservation. However, use of somatic embryos in particular, does not offer the genetic diversity afforded by seed-derived zygotic axes, and may also be compounded by somaclonal variation during their production (Krogstrup *et al*., 1992).

Whole non-orthodox seeds may also be cryopreserved, provided they are sufficiently small. Rapid rates of cooling must usually be used for whole seed cryopreservation to prevent ice crystal formation, as sufficiently low water contents may be difficult to achieve rapidly. Examples of successful cryopreservation of orthodox whole seeds include those of various orchid species (Thammasiri, 2000; Nikishina *et al*., 2001; Popov *et al*., 2004), *Piper nigrum* (Chaudhury and Chandel, 1994), and *Dendrobium candidum* (Wang *et al*., 1998). Cryopreservation of non-orthodox whole seeds has also been achieved for *Wasabia japonica* (Potts and Lumpkin, 1997), *Warburgia salutaris* (Kioko *et al*., 2003b) and *Azardirachta indica* (Berjak and Dumet, 1996).

Zygotic embryonic axes are the most commonly used explants for cryopreservation of recalcitrant germplasm. Their size makes them suitable for cryopreservation as they can be rapidly dehydrated to sufficiently low water contents thus rendering them less susceptible to ice crystal formation during freezing. Cryopreservation of axes following rapid cooling rates has been reported for a range of species, including *Artocarpus heterophyllus* (Chandel *et al*., 1995; Thammasiri, 1999), *Prunus amygdalus* (Chaudhury and Chandel, 1995), hydrated *Pisum sativum* (Mycock *et al*., 1991), *Camellia sinensis*, (Wesley-Smith *et al*., 1992), *Azadirachta indica* (Berjak and Dumet, 1996), *Trichilia emetica* (Kioko *et al*., 2006), Q*uercus* spp. (Pence 1990; Berjak *et al*., 1999b), *Aesculus hippocastanum* (Wesley-Smith *et al*., 2001a), *Zizania texana* (Walters *et al*., 2002b), *Poncirus trifoliata* (Wesley-Smith *et al*., 2004b), *Castanea sativa* (Corredoira *et al*., 2004), and *Citrus suhuiensis* (Makeen *et al*., 2005). However, in several cases (Pence 1990; Vertucci *et al*., 1991; Kioko *et al*., 1998; Berjak *et al*., 1999b) survival was scored by callus formation, which is less than ideal, as direct seedling establishment is the outcome desired. Survival after cryopreservation following slow cooling has been demonstrated for coffee (Dussert *et al*., 1997), immature wheat embryos (Kendall *et al*.,

1993), butternut (Beardmore and Vong, 1998) and *Landolphia kirkii* (Vertucci *et al*., 1991).

1.6.2 Thawing

Before regeneration, frozen tissue must first be thawed without allowing ice crystal growth during warming. This can be achieved only by rapid thawing, usually by immersing explants in a thawing medium placed in a temperature-controlled water bath (Berjak *et al*., 1999c; Dumet *et al*., 2002).

Water uptake on rehydration (imbibition) may have deleterious effects on dehydrated cryopreserved material (Osborne *et al*., 2002; Dussert *et al*., 2003), and initial water content, temperature of the imbibitional medium and rate of water uptake determine the sensitivity of dry/partially dry specimens to imbibitional injury (Hoekstra *et al*., 1999; Osborne *et al*., 2002; Perán *et al*., 2004) as these factors effect the structure and organization of intracellular membranes and the conformation of DNA. The addition of Ca^{2+} and Mg^{2+} cations to the rehydration solution used for cryostored recalcitrant explants has marked positive effects on their regeneration (Berjak *et al*., 1999c; Mycock, 1999). At appropriate conditions, these cations facilitate the reconstitution of cytoskeletal elements upon rehydration (Berjak and Mycock, 2004). Furthermore, Perán *et al*. (2004) showed that for recalcitrant axes, direct explant immersion is preferable to initial equilibrium with saturated RH. According to those authors, slow rehydration by equilibration will facilitate an undesirable period of unbalanced metabolism.

1.6.3 *In vitro* regeneration

1.6.3.1 Culture conditions

Given the variety and specific nature of factors affecting recovery after cryopreservation, specific *in vitro* conditions need to be optimized before cryopreservation can be attempted. Successful *in vitro* germination demands aseptic conditions as microorganisms will compete for nutrients and in any case, are likely to degrade the explant tissues rapidly (Kioko, 2003; Reed *et al*., 2004). Thus, the sterilization of the culture medium, and decontamination of explants, and the work surface is imperative.

The optimum culture medium for *in vitro* regeneration must also be ascertained. The appropriate composition of nutrients, minerals and vitamins in the medium is dependent on the species. However, depending on the morphogenic result required, the medium can be manipulated by the addition (and balance) of plant growth regulators such as auxins and cytokinins to stimulate either root production, shoot production, or callus growth (Ryynänen, 1998; Nhut *et al*., 2001; Turner *et al*., 2001; Burch and Wilkinson, 2002; Renau-Morata *et al*., 2005; Zhao *et al*., 2005); or by the alteration of environmental conditions such as light and temperature (Paolini *et al*., 2001; Reed *et al*., 2004; Keller, 2005).

1.6.3.2 Excision of explants

In many cryopreservation studies where the explants are embryonic axes, shoot formation is not achieved. Instead, survival has been recorded either as greening (e.g. *Landolphia kirkii* [Vertucci *et al*., 1991]), callus production (e.g. *Quercus*, *Aesculus*, *Carya* and *Juglans* spp. [Pence, 1990]), or development at the root pole only (e.g. for *Trichilia dregeana* [Kioko *et al*., 1998] and numerous other species [Engelmann, 1999]). Lack of shoot development may be attributed to the factors described below, which relate to the excision process itself.

1.6.3.2.1 Wounding

The general mode of axis excision in dicotyledonous seeds is to cut through the cotyledonary attachments flush with the axis surface. In so doing, explant volume is minimized (Kioko *et al*., 1998; Berjak *et al*., 1999b). However, explants excised in this manner, even before dehydration and/or cryostorage, frequently fail to develop shoots with the shoot pole becoming necrotic or, at best, forming callus (Kioko *et al*., 1998; Perán *et al*., 2006). Formation of callus at the shoot pole may form in response to wounding as shown in by Grunweld *et al*. (2002), Stobbe *et al*. (2002) and Kusumoto and Suzuki (2003) in other species.

1.6.3.2.2 Free radicals and Reactive Oxygen Species (ROS)

The precise nature of the causative agent damaging shoot apices is not clear. Although inhibition of genes controlling the development of meristematic tissue has been shown

in response to wounding (Souer *et al*., 1996), it is more likely that oxidative damage as a result of free radical production is the cause (e.g. Minibayeva *et al*., 2001, 2003).

Free radicals are not always hazardous as they can act as signaling molecules (Kovtun *et al*., 2000; Lopez-Huertas *et al*., 2000; Ashley *et al*., 2006) and play a role against pathogen attack (Minibayeva and Beckett, 2001; Bolwell *et al*., 1999, 2002; Loseva *et al*., 2002; Mayaba *et al*., 2002; Beckett *et al*., 2003; 2005). Such chemical species may promote elongation and growth (Fry, 1998; Liszkay *et al*., 2004) and gene activation in response to wounding (Orozco-Cárdenas *et al*., 2001; Watanabe *et al*., 2001; Pellinen *et al*., 2002).

However, free radicals are also produced in an oxidative burst as a wounding response in some plant tissues/organs studied, e.g. wheat roots (Vglegzhanina *et al*., 2001; Minibayeva *et al*., 2001, 2003), *Arabidopsis thaliana* leaves (Chang *et al*., 2004; Flors *et al*., 2006), and potato tubers (Razem and Bernards, 2003). This response has also been reported in lichens (Beckett and Minibayeva, 2003) and in macroalgae (Ross *et al*., 2006). Orthodox seeds have well-developed anti-oxidant mechanisms (Abdallah *et al*., 1997; Chandru *et al*., 2003; Edreva, 2005) that scavenge free radicals while anti-oxidant mechanisms are suggested to be either inadequate, impaired or not present in recalcitrant seeds (reviewed by Berjak and Pammenter, 2007). If free radicals are not scavenged efficiently, their build-up will have deleterious consequences (Hendry, 1993; Bailly, 2004; Bailly *et al*., 2004).

1.6.3.2.3 Developmental status of the seed/embryo

It has been previously suggested that axis development continues after shedding in recalcitrant seeds that can be stored for weeks to months under hydrated conditions before signs of germination become macroscopically visible (Berjak *et al*., 1989). For example, seeds of *T. dregeana* could be kept in hydrated storage conditions for five months before showing visible signs of germination (Kioko, 2003). It has also been shown by Finch-Savage and Blake (1994) and Pina-Rodrigues and Figliolia (2005) that seeds are shed at different developmental stages and development continues after shedding.

It is likely that the developmental status of the axes when excised will influence ongoing development whether negatively or positively, and that this critical factor needs to be resolved before any further manipulations for cryopreservation can be attempted.

Despite the many biochemical and procedural complications underlying successful cryostorage, there presently is no alternative for *ex situ* long-term conservation of the genetic resources of species producing recalcitrant seeds.

1.7 The present study

This study was intended to provide a broad understanding of the reasons underlying successful/unsuccessful axis dehydration and cryopreservation, and to contribute to development of procedures for successful preservation of the germplasm species investigated.

Thus, this study aimed to optimize parameters for successful cryopreservation of the embryonic axes of *T. dregeana*, *T. emetica* and *S. gerrardii*.

This involved determination of appropriate water content and freezing parameters for explants with no or different-sized cotyledon segments attached at different storage intervals to assess the effect of developmental status. These aspects were investigated for the three species to compare any trends between the species of the same family and between the two unrelated families.

The study also sought to optimize the *in vitro* culture protocols, as these are species-specific and will affect the recovery after cryopreservation.

In addition, ROS production was quantified to relate it to damage to the shoot region upon excision.

1.8 Species studied

1.8.1 *Trichilia dregeana* Sond.

Trichilia dregeana (Meliaceae), is commonly known as the forest mahogany or *umKhuhlu* (Zulu) and is indigenous to southern and eastern Africa. The name *Trichilia* is of Greek origin and refers to the three-lobed fruits which enclose the seeds (Pooley, 1993). Trees are known to reach heights of between 10 - 35 m and, although not common in the coastal forests of KwaZulu-Natal, South Africa, is also cultivated as a street tree in the province (Pooley, 1993; Kioko, 2003). The leaflets are shiny, have an almost hairless undersurface and are pointed at the tips. In southern Africa the plant flowers between October and December and prominent fruits are produced between March and May (and sometimes later). Each fruit capsule yields six black seeds, each almost completely enclosed by a scarlet aril (Pooley, 1993; Figure 1.3). The fruit is eaten by not only birds, but also by people as a milky soup with the addition of spinach (Pooley, 1993).

Products from this tree are used commercially (wood for furniture and oil for soap and cosmetic use), and also have medicinal properties. Preparations of the bark are used in the treatment of backache, stomach problems, kidney ailments and its usage as a fish poison has also been reported (Pooley, 1993; Hutchings *et al*., 1996; van Wyk and Gericke, 2000). Five limonoids have been chemically isolated from these seeds (Mulholland and Taylor, 1980) including trichilin A and dregeanin, which could contribute to the medicinal properties of this species and/or could have anti-fungal activity as reported for *Khaya ivorensis*, another species in this family (Abdelgaleil *et al*., 2005).

The seeds, which contain up to 65% lipid by weight, were reported to be recalcitrant and chilling sensitive (Choinski, 1990). Since then, other in-depth studies have been carried out on seeds of this species to determine strategies for field collection (e.g. Berjak *et al*., 2004), the potential for sub-imbibed storage (Drew *et al*., 2000; Eggers *et al*., 2007), the effects of dehydration on the nucleoskeleton (Merhar *et al*., 2002) and to assess if the absence of dehydrin-related molecules contributes to their desiccation sensitivity (Han *et al*., 1997). Work carried out by Kioko *et al*. (1998) and Kioko (2003)

showing the lack of shoot development after cryopreservation is of particular interest in the current study, which is aimed at ameliorating this deficiency and perhaps provide information pertaining to the failure of shoot development.

1.8.2 *Trichilia emetica* Vahl.

This is the second of the two *Trichilia* spp. found in Africa and is commonly known as the Natal mahogany and shares the same Zulu name with *T. dregeana* (*umKhuhlu*). In South Africa, the species occupies a similar habitat as *T. dregeana*, but does not extend as far south in KwaZulu-Natal. There is a striking superficial similarity in appearance between the two trees in this genus. However, while *T. dregeana* range between heights of 10 - 35 m, *T. emetica* trees reach heights of between 5 - 10 m (Pooley, 1993). The leaflets and fruit are also superficially morphologically similar; however, the leaflets of *T. emetica* are not as shiny as those of *T. dregeana*, have hairs on the undersurface and have rounded tips. There are also differences between the flowering and fruiting seasons, which are September to November and January to April, respectively, for *T. emetica*. The fruits have a distinct neck (absent in *T. dregeana*) and are slightly smaller than those of *T. dregeana*. The seeds, however, although slightly smaller in *T. emetica*, are very similar (compare Figures 1.3 and 1.4). Fruits are eaten by baboons, monkeys, antelope, and are also used to make a milky soup similar to that of *T. dregeana* (Pooley, 1993).

Preparations of bark, roots, leaves, as well as seed oils, are used for medicinal purposes against stomach, intestinal and kidney ailments, indigestion, fever, parasites and eczema (Hutchings *et al*., 1996; van Wyk *et al*., 1997). Resin and tannin is found in the bark (Hutchings *et al*., 1996) and limonoids such as trichilin A have also been isolated (van Wyk *et al*., 1997) and may contribute to the medicinal properties of this species.

Few studies have been published on the behaviour of the seeds of this species, but they have been classified as recalcitrant (Maghembe and Msanga, 1988; Kioko *et al*., 2006).

1.8.3 *Strychnos gerrardii* N.E. Br.

This species belongs to the family Loganiaceae and is commonly known as the black monkey orange or *umGuluguhla* (Zulu). The family is widespread throughout the world, with seven species represented in South Africa. *Strychnos gerrardii* is often confused with *S. madagascariensis* and also has the same English common name. Trees of *S. gerrardii* are found along the coastal and dune forests of KwaZulu-Natal and reach heights of 10 - 25 m. The leaves are shiny, hairless, fairly thin and taper at the tip and the base. These trees flower between November and January and fruit from April to November (Pooley, 1993). Fruits contain multiple seeds and have a substantial woody pericarp/exocarp that changes from green to pale yellow upon ripening (Pooley, 1993; Figure 1.5).

Although seeds of this species may have mildly toxic properties, the fruit pulp is eaten by people and monkeys. The species with toxic seeds, known for producing strychnine (*S. nux-vomica*) and curare poison (*S. toxifera*), are found in India and South America, respectively (Pooley, 1993; Philippe *et al*., 2004).

A few studies have been carried out on the branches and leaves (Itoh *et al*., 2005 and Hoet *et al*., 2006, respectively) of another closely related species, *S. spinosa* to determine its chemical properties. However, very little is known about the post-harvest seed behaviour of any *Strychnos* spp. indigenous to South Africa. Preliminary studies carried out by Khuzwayo (2002), however, indicated that the seeds of *S. gerrardii* show recalcitrant behaviour.

Figure 1.3: Three-lobed fruit and seeds of *T. dregeana*. Bar = 10 mm.

Figure 1.4: Fruit and seeds of *T. emetica*. Bar = 10 mm.

Figure 1.5: Woody pericarp/exocarp of ripe of *S. gerrardii* and seeds. Bar = 10 mm.

2. MATERIALS AND METHODS

2.1 Seed collection and cleaning

Open fruits were directly harvested from *T. dregeana* trees in the Glenwood area, Durban (between March - June) and from *T. emetica* trees from coastal LaLucia, north of Durban (between January - February) and also in Queensburgh which is slightly inland (between February - March). The only seeds collected from the ground were those newly-shed, characterized by their intact, smooth, bright aril as opposed to those where the aril showed any signs of deterioration and damage. Once in the laboratory, the seeds were removed from the fruit and those seeds damaged by insects (Figure 2.1) were discarded. The aril and seed coat were removed on the same day of collection (Figure 2.2), and the seeds processed further as described below.

Fruits of *S. gerrardii* were collected from the ground at the Burman Bush Nature Reserve, Durban. The hard, woody fruit covering, although often scarred by monkeys (Figure 2.3), provided ample protection for the seeds. The fruits were temporarily stored at 16°C over the period of seeds extraction and cleaning, as the removal of pulp surrounding the seeds was very time consuming (Figure 2.4).

Figure 2.1: Seeds of *T. dregeana* damaged by insects. Bar = 5 mm.

Figure 2.2: Cleaned seeds of *T. dregeana*. Bar = 10 mm.

Figure 2.3: Scratches on the fruit shell of *S. gerrardii*. Bar = 10 mm.

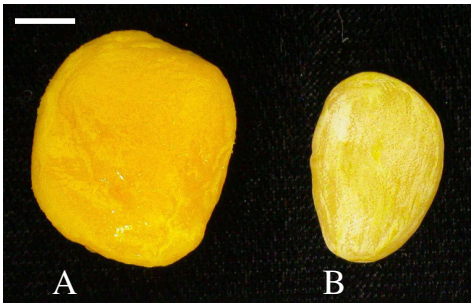


Figure 2.4: *S. gerrardii* seed before (A) and after (B) cleaning. Bar = 5 mm.

2.2 Seed processing

2.2.1 Surface sterilization

Cleaned seeds were surface sterilized using a 1% NaOCl solution containing a few drops of Tween 20/80®, a wetting agent, for 20 min (*T. dregeana* and *S. gerrardii*) or 10 min (*T. emetica*). Seeds were subsequently soaked in an anti-fungal 'cocktail' comprising 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*), 120 min (*S. gerrardii*) and 240 min (*T. dregeana*). These fungicides have been shown to curtail fungal contamination in storage (Calistru *et al*., 2000; Berjak *et al*., 2004). Seeds were then placed on paper towel on a laboratory bench, and dried back to the original batch fresh weight, and either used immediately or prepared for storage.

2.2.2 Seed storage

Seeds of all three species were dusted with Benomyl 500 WP (active ingredient, benzimidizole; Villa Protection, S. Africa) and stored at 16°C as a monolayer on a sterilized plastic mesh suspended 200 mm over water saturated paper-towel in a sealed 5-l bucket. Buckets and the plastic mesh used had been soaked for 30 min in a 1% NaOCl solution beforehand and allowed to dry.

2.3 Germination

2.3.1 Whole seeds

To ascertain their germination potential, whole seeds of *S. gerrardii* at three different maturity stages, indicated by fruit colour (green, yellow/green and yellow as seen in Figure 2.5), were planted out in Perlite®:vermiculte (1:1) in seedling trays. These were then kept in a germination room at 25°C and 12 h light/dark photoperiod.

2.3.1.1 Gravimetric determination of water content

Explants were weighed, and then placed in an oven at 80°C for 48 h before being re-weighed to determine water content of the embryonic axis and cotyledons/endosperm, separately. Water content was expressed on a dry mass basis, i.e. g H_2O g^{-1} dry mass.

Figure 2.5: Different seed maturity stages of *S. gerrardii* indicated by fruit colour. From left to right: Green (immature), yellow/green (intermediate) and yellow (mature). Bar = 10 mm.

2.3.2 Embryonic axes

The seeds of all three species are too large to be cryostored successfully, and therefore preliminary studies were carried out on *in vitro* culture of the embryonic axes. *Trichilia dregeana* and *T. emetica* produce exendospermous, dicotyledonous seeds, with the embryonic axis representing only a small proportion of the volume in relation to the cotyledons (Figure 2.6). *Strychnos gerrardii* produces endospermous dicotyledonous seeds, with the embryo comprising the axis, and the two relatively small, paper-thin cotyledons (Figure 2.7).

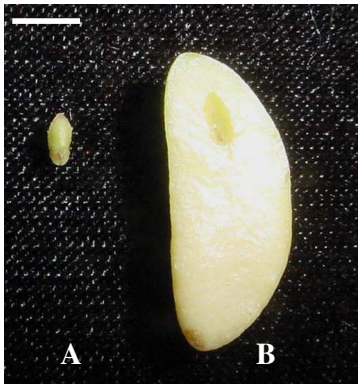


Figure 2.6: Difference between axis (A) and cotyledon (B) size in *T. dregeana*. (Axes and cotyledons of *T. emetica* are similar in appearance and relative size.)
Bar = 5 mm.

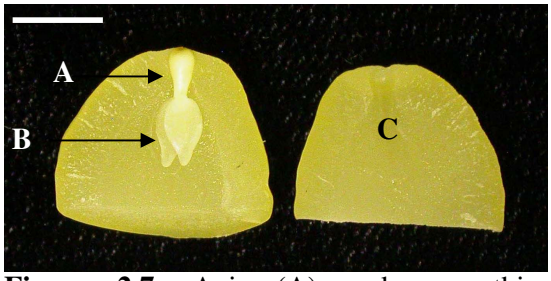


Figure 2.7: Axis (A) and paper-thin cotyledons (B) surrounded by endosperm (C) in *S. gerrardii*. Bar = 5 mm.

2.3.2.1 Explant type

Embryonic axes of *T. dregeana* and *T. emetica* were excised as follows: Axes without any cotyledon attached, axes with the basal cotyledonary segments only left intact, and axes with attached cotyledonary blocks each 2-mm wide (Figure 2.8). These cotyledon segments are referred to as none, basal segment and 2-mm segment, hereafter.

The cotyledons of *S. gerrardii* contribute only slightly to the mass of the embryo, thus explants were excised either with or without the cotyledons from seeds from mature (yellow) fruit.

Figure 2.8: *T. dregeana* explants with differently-sized cotyledon segments used for *in vitro* culture. None (A); basal segment of cotyledon (B); 2-mm cotyledon segment (C). Explants of *T. emetica* were similar. Bar = 1 mm.

2.3.2.2 Surface sterilization

The surface sterilization/decontamination procedures used for the embryonic axes were those determined by Kioko (2003) for *T. dregeana* and *T. emetica*, and by Khuzwayo (2002) for *S. gerrardii*. *Trichilia dregeana* explants were decontaminated in a laminar air-flow by soaking serially in 2% Hibitane® for 2 min, 70% ethanol for 2 min and 1% NaOCl for 5 min. Axes were then rinsed three times with sterile distilled water and sterilized for a further 5 min with 1% NaOCl before being rinsed a further three times with sterile distilled water.

Trichilia emetica and *S. gerrardii* explants were decontaminated in a laminar air-flow, by immersion in 0.02% (w/v) $HgCl_2$ for 2 min and 2% (w/v) CaOCl for 5 min, respectively, before three rinses with sterile distilled water.

2.3.2.3 *In vitro* germination medium

The basic nutrient media established as successful for *in vitro* culture of *T. dregeana* and *T. emetica* axes by Kioko (2003) were WPM (Lloyd and McCown, 1981) and MS (Murashige and Skoog, 1962), respectively. Murashige and Skoog medium was also optimal for *S. gerrardii* axes (Khuzwayo, 2002). Thus the basic *in vitro* culture media were comprised of either WPM or MS with sucrose (30 g l^{-1}) and agar (10 g l^{-1}), at pH 5.6 – 5.8.

Explants cultured on the above-mentioned media, however, had been found not to produce shoots if no vestiges of cotyledons remained attached to the axes (Khuzwayo, 2002; Kioko, 2003). The ultimate objective of this study was successful explant cryopreservation: Hence, since cotyledons add to the thermal mass of explants and could adversely affect their survival of cryopreservation, media were manipulated for each species in attempt to stimulate shoot development in the absence of attached cotyledonary tissue. For *T. dregeana*, explants were cultured on 'plain' WPM and WPM supplemented with either 0.1 mM ascorbic acid (AsA), 10 g l^{-1} citric acid (CA), or 4 g l^{-1} activated charcoal (AC). Ascorbic acid and CA are anti-oxidants, and thus could potentially scavenge any reactive oxygen species (ROS) potentially produced from wound sites. Activated charcoal is known to adsorb various organic compounds

including excess hormones, vitamins, abscisic acid, phenolic metabolites and ethylene (van Winkle *et al*., 2003).

For *T. emetica*, MS was used alone and in combination with 0.1mM AsA, or CA (10 g l^{-1}), or AC (4 g l^{-1}). In addition, 6-Benzylaminopurine (BAP; 1 mg l^{-1}) was added in combination with AC to MS medium to stimulate shoot development. The concentration of BAP used was based on a recent study carried out on the similar explants of *Ekebergia capensis* (Perán *et al*., 2006), another member of the Meliaceae producing recalcitrant seeds.

The MS medium for *S. gerrardii* was manipulated by using various concentrations of MS basal salts in the presence or absence of AC. Thus, four media were tested: half-strength MS, half-strength MS with AC, full-strength MS and full-strength MS with AC. The effect of three different concentrations of BAP (0.5 mg l^{-1}, 1 mg l^{-1}, 2 mg l^{-1}) on shoot development was also tested.

Explants for all treatments were cultured in 65 mm Petri dishes (five explants per Petri dish) and maintained under 16 h/8 h light/dark photoperiod at 23 – 25°C. The medium showing the best germination and seedling growth for each species was used as the regeneration medium for cryopreservation trials. The initial embryonic axis water content of all explant sizes for each of the three species was determined (see section 2.3.1.1 for gravimetric determination of water content).

2.4 Dehydration

2.4.1 Whole seeds

Seeds of *S. gerrardii* at different maturity stages (extracted from green, yellow/green and yellow fruits) were dehydrated in sealed bags containing activated silica gel for the following periods: 6, 12, 18, 24, 36, 48, 72, and 96 h. Seeds were sampled for viability after each drying period as described for whole seed germination (section 2.3.1.) and for axis and endosperm water content (section 2.3.1.1).

2.4.2 Embryonic axes

2.4.2.1 Laminar air-flow drying

The different types of explants (see above) of *T. emetica* were dried on sterile filter paper in a laminar air-flow for 2.5, 3 and 3.5 h. Following dehydration, the water content was determined for a sub-sample, while the remaining explants were rehydrated using a 1:1 solution of 1 μM $CaCl_2.2H_2O$ and 1 mM $MgCl_2.6H_2O$ for 30 min, according to the method of Berjak *et al*. (1999c), and cultured on the optimal *in vitro* germination medium established.

2.4.2.2 Flash-drying

Flash-drying entailed circulating silica-gel-dried air over the explants placed on a mesh, over by a fan, within a closed container (Pammenter *et al*., 2002). The explants with different-sized cotyledon segments were flash-dried and sampled for water content and germination assessment at 30 min intervals for 180 min for *T. dregeana*, and for 120 min for *T. emetica*. Explants of *S. gerrardii* from mature (yellow) fruits were flash-dried being sampled for water content and germination after the following periods: 10, 15, 20, 25, 30, 35, 40, 45, 50 min, with a final sample taken after 60 min. In addition, the two explant types of *S. gerrardii* at different maturities were flash-dried for 15, 30 and 50 min to compare responses with slow dehydration of the whole seed.

All explants assessed for *in vitro* germination after dehydration were first rehydrated for 30 min in the calcium/magnesium solution described above, and cultured on the germination medium established for each species.

2.5 Cryoprotection and cryopreservation

2.5.1 Cryoprotection

Explants of *T. dregeana* and *T. emetica* with no cotyledon, basal segments or 2-mm blocks were treated with the following cryoprotectants alone and in combination (Table 2.1): DMSO and glycerol (5 and 10% [v/v] each); PVP and dextran (5 and 10% [w/v] each); and sucrose (0.5 M and 1 M, Mycock *et al*., 1997). No cryoprotectants were tested on *S. gerrardii* explants as the aim was to establish a cryopreservation method without the use of such protectants. Cryoprotection was achieved by soaking the

explants in the lower concentration cryoprotectant solution for 60 min followed by transfer into the higher concentration solutions for 60 min, before dehydration. Effects of each cryoprotectant solution on water content and germination were assessed.

Table 2.1: Cryoprotectants tested in this study.

			Species Tested	
Cryoprotectant	**Type**	**mol. wt**	***T. dregeana***	***T. emetica***
None			+	+
DMSO	Penetrating	78.1	+	+
Glycerol (Gly)	Penetrating	92.1	+	+
Sucrose (Suc)	Non-penetrating	342.3	+	+
DMSO + Glycerol (D+G)	Penetrating		+	+
DMSO + Sucrose (D+S)			+	+
Glycerol + Sucrose (G+S)			+	+
PVP	Non-penetrating	15 000	+	-
Dextran (Dex)	Non-penetrating	40 000	+	-

2.5.2 Cryopreservation

Slower and rapid cooling were tested for explant cryopreservation. For slower cooling, explants were placed into cryotubes after cryoprotection and/or dehydration, and the cryotubes slotted into cryocanes, rapidly immersed into liquid nitrogen and left for at least 1 h. Rapid cooling entailed sub-cooling liquid nitrogen under vacuum to form nitrogen slush at -210°C. Explants were directly mixed with the sub-cooled nitrogen in a polystyrene cup immediately after cryoprotection and/or dehydration and left for approximately 10 min after the sub-cooled nitrogen slush returned to its liquid form.

After cryostorage, all material, irrespective of the cooling rate, was thawed by immersing in a 1:1 solution of 1µM $CaCl_2.2H_2O$ and 1mM $MgCl_2.6H_2O$ at 40°C for 2 min, followed by transfer to the same solution at room temperature for 30 min in the dark (Berjak *et al*., 1999c) to effect rehydration.

2.5.2.1 Explant culture post-cryopreservation

The rehydrated explants were appropriately surface-sterilized (see section 2.3.2.2 above), and cultured on the optimal *in vitro* medium (section 2.3.2.3). These cultures were kept in the dark at 23 - 25°C until growth was observed.

Callus proliferation was stimulated in explants surviving cryopreservation by sub-culture onto the basic nutrient medium supplemented with either 2,4-D (1 mg l^{-1}) or NAA (1 mg l^{-1}). In an attempt to stimulate embryogenicity, varying concentrations of BAP (0.5 mg l^{-1} and 0.25 mg l^{-1}) were included as variants of both media.

2.6 Assessment of the effect of developmental status on successful cryopreservation

Once the various factors were optimized (*in vitro* germination medium, cryoprotection, dehydration, and cooling rate) using embryos/embryonic axes from fresh seeds, they were applied to seeds of all three species stored for different periods. For *T. dregeana*, seeds were sampled after three and five months in hydrated storage as previous observations in our laboratory had shown that these seeds could be stored for at least six months without losing viability. Because of fungal proliferation, the seeds of *T. emetica* were storable (under hydrated conditions) for only two weeks and were sampled at 7 d intervals. The stored seeds of *S. gerrardii* were sampled every second week for eight weeks, until none were left.

2.7 Quantification of ROS production

These experiments were carried out on the explants of *T. dregeana* because it was the only species with seeds available at the time when this phase of the study was conducted. Axes excised from hydrated pea seeds were assessed for comparative purposes. Extra-cellular ROS production was measured for the different-sized explants of *T. dregeana* using the colorimetric superoxide assay described by Beckett *et al.* (2004), which estimates ROS production by the oxidation of epinephrine (Sigma, Germany) to adrenochrome, the quantification of which is done spectrophotometrically. Explants (n = 10) of different sizes were placed into separate 20 ml vials and 3.5 ml of 1 mM of epinephrine (pH 7.0) was added to each vial and shaken in the dark for 15 min.

The absorbance of each was then measured against the standard (unreacted epinephrine) at 490 nm using a Biochrom Ultrospec 1000 spectrophotometer.

Changes in ROS production by excised axes with time were also measured over 3 h. For this purpose, the incubation solution was decanted at 15 min sampling intervals and replaced with fresh epinephrine. The absorbance of the individual aliquots of decanted solution was then measured. The changes in ROS production by explants with no cotyledon, basal segments, and with 2-mm cotyledon blocks were measured for *T. dregeana* and compared with similar explants excised from hydrated pea axes which had been imbibed for 24 h. Whole seeds were used as (unwounded) controls, while the explants with different-sized cotyledon segments represented wounded (no cotyledon) and unwounded (basal segments and 2-mm cotyledon blocks) embryonic axes.

In addition, directly after excision, explants with no cotyledon were pretreated with a 0.05 mM, 0.1 mM or 0.2 mM solution of ascorbic acid (AsA), for 20, 40 and 60 min, to determine if the anti-oxidant would quench free radicals produced. From these results, the treatment offering the highest reduction in ROS was used to treat explants before *in vitro* culture, to give an indication of the impact of reduced ROS on shoot development.

2.8 Microscopy

All microscopical studies were carried out using the explants of *T. dregeana*. Seeds from this species being produced over the longest period.

2.8.1 Light microscopy

For assessment of physical changes in the shoot apex of *T. dregeana* axes during seed storage, whole axis specimens were prepared using a standard wax embedding protocol. Explants were initially fixed in a 2:1:10 v/v formalin:acetic acid:ethyl alcohol (FAA) solution for 24 h at room temperature. They were then dehydrated using a series of butanol:ethanol:water solutions (v/v) where butanol was increased from 10% to 15%, 25%, 40%, 55%, 70%, 85% and 100% for periods of 30, 30, 30, 30, 45, 60, 90 min, and overnight, respectively. Following this, the specimens were infiltrated through a series of wax:butanol mixtures (1:3 for 2 h, 1:1 for 3 h and 3:1 for 3 h) before being embedded

in 100% paraffin wax overnight. Sections between 30 and 40 μm thickness were prepared using an American Optical 820 microtome, and placed on slides coated with Haupts adhesive. Sections were de-waxed in xylene, and rehydrated in a graded ethanol series before being stained with 0.1% (w/v) toluidine blue in phosphate buffered saline (PBS) and mounted. All light microscopy images were viewed and images captured using a Nikon Biphot® Photomicroscope.

2.8.2 Transmission electron microscopy

Shoot apices of *T. dregeana* excised with and without cotyledon, were ultrastructually assessed immediately after excision and after three days in culture on plain WPM. Specimens were fixed in 2.5% phosphate-buffered glutaraldhyde (0.1 M, pH 7.2) for 24 h at 4°C. Following several rinses with phosphate buffer, specimens were post-fixed in a 0.5% aqueous osmium tetroxide for 1 h at room temperature and rinsed three times with phosphate buffer. Specimens were then dehydrated in a graded acetone series (30%, 50%, 75%, each for 5 min and 100% for 10 min) followed by infiltration and embedding in low-viscosity resin (Spurr, 1969) and polymerization for 8 h at 70°C. Ultra-thin sections in the range 60-100 nm were obtained with a Reichert Ultracut E Microtome, collected on copper grids and post-stained sequentially with saturated (2.5%) uranyl acetate and lead citrate (Reynolds, 1963) for 10 min each. Sections were viewed using a Jeol 1010 transmission electron microscope.

2.8.3 Scanning electron microscopy

Axes with no cotyledon attachments from newly-harvested seeds of *T. dregeana* were directly sputter-coated with gold in a Polaron E5100 sputter coater following critical point drying, and the surface structure viewed using a Leo 1450 scanning electron microscope.

2.9 Statistical analysis and photography

All data were analyzed using the statistical program SPSS 11.5 for Windows. Data were initially tested for normal distribution using a Kolmogorov-Smirnov test. As all data were normally distributed, a Chi-squared test was used to analyze germination data and a one sample t-test or a Univariate ANOVA used to analyze differences between seedling lengths. Differences in water content were determined using a One-way ANOVA, and a Univariate ANOVA combined with a *post hoc* Scheffé test was used to distinguish differences in ROS data (Zar, 1999). Sample sizes for each treatment ranged from 20 to 25 explants except for water content (five explants), biochemical assay (10 explants per treatments and three replicates of each) and microscopy (5-10 specimens).

All digital images were captured using a Nikon Coolpix® digital camera.

3. RESULTS AND DISCUSSION

All results are presented initially for fresh, non-stored material (investigations carried out to optimize various parameters) and then for stored material, in order to illustrate any effect of post-shedding developmental status on the characteristics observed.

3.1 Germination responses

3.1.1 Whole seeds

The germination responses of whole seeds of *Trichilia dregeana* and *Trichilia emetica* have been previously studied by Kioko (2003) who showed that germination performance was best when the aril and seed coat were removed. As those studies were exclusive and conclusive, germination trials of the seeds of the *Trichilia* spp. were not repeated in the present investigation. For *S. gerrardii*, whole seeds from fruits at different maturity stages were set out to germinate in seedling trays. Although all seeds germinated and developed into complete seedlings (i.e. roots and shoots produced) irrespective of the maturity stage, those from yellow fruits were significantly shorter (t – test, $p = 0.029$) as is shown in Table 3.1.

Table 3.1: Percentage germination and mean length of seedlings of *S. gerrardii* (roots and shoots) developed from whole seeds from fruits at different maturity stages. All data were recorded two months after planting. n = 20, standard deviation represented by ± values.

Fruit colour	% germination	Mean length
Green	100	81 ± 25.1
Yellow/green	100	70 ± 16.5
Yellow	100	43 ± 16.3

3.1.1.1 Whole seed germination: concluding remarks

The high germination percentages of seeds from fruit at different developmental stages indicate that all were physiologically competent for ongoing development and seedling establishment.

It is also noteworthy that seeds were not pre-treated before the germination trials, and in all cases germinated readily after extraction from the newly-shed fruits, although at

different rates (see below). Studies on other species such as *Psychotria stachyoides*, *Sorbus commixta* and *Olea europaea* have shown that the fruit pulp can inhibit germination (Leal and Oliveira *et al*., 1998; Yagihashi *et al*., 1998; Aerts *et al*., 2006, respectively) thus, meticulous seed cleaning is crucial for unambiguous results of germination trials.

Seeds from green fruits began germinating within the first week after sowing while those from the more ripened fruits initiated germination only three weeks after sowing. This could indicate that seeds from green fruits enter directly into the germination phase while seeds from yellow/green and yellow fruits may have entrained a relatively quiescent stage before germination (Kermode and Finch-Savage, 2002). It is possible, however, that the imposition of a slight degree of dormancy accompanies fruit ripening, as has been reported for non-orthodox *Phoenix reclinata* seeds (von Fintel *et al*., 2004). Alternatively, as a decline in seedling length also accompanied progressive fruit ripening, it is possible that seed quality/vigour had become compromised. Such observations have been reported for recalcitrant seeds of other species (reviewed by Berjak and Pammenter, 2004b). No reports on post-harvest seed behaviour of species within the same genus have been published to the present author's knowledge. However, investigations on three other *Strychnos* species are presently ongoing within our research group.

3.1.2 Embryonic axes

The germination response of embryonic axes from the three species was monitored to optimize *in vitro* conditions for seedling regeneration after cryopreservation. Various *in vitro* conditions were tested initially for embryonic axes from newly-harvested seeds and later on axes from seeds stored in the hydrated state in the short-term, once a suitable medium was established.

3.1.2.1 Newly-harvested seeds

Trichilia dregeana

Trichilia dregeana explants were excised with different-sized cotyledon attachments (also referred to as explants of different size) from seeds of newly-harvested fruits. In this regard, axes were excised with no vestiges of cotyledonary tissue; with the basal segments only; and with a 2-mm segment of each cotyledon (Figure 2.8). The explants were germinated on four different media *viz.* 'plain' WPM, WPM + AC (activated charcoal), WPM + AsA (ascorbic acid) and WPM + CA (citric acid). The addition of CA as an anti-oxidant made the medium harder than the other three media, thus making it difficult to plate out the explants. Also, explants plated on this medium became contaminated, possibly due to manipulation and protracted handling during the efforts to embed the axes partially into the medium. The hardening of the medium could be attributed to the quantity of citric acid used (10 g l^{-1}) when compared with values (between 25 – 200 mg l^{-1}) reported from a few other *in vitro* regeneration procedures (Komalavalli and Rao, 2000; Arya *et al*., 2003; Deb and Temjensangba, 2005).

Thus, results reported below are for explants plated on the remaining three media only. All explants, irrespective of size, developed roots on all media used. However, shoot production occurred only in explants with the basal segment or a 2-mm portion of cotyledon (Table 3.2). In these cases, there was no significant difference between shoot production from explants with different-sized segments of cotyledon as determined by Chi-squared tests ($p > 0.05$) on any of the media tested, except when comparing shoot production between explants with basal and 2-mm segments germinated on WPM only (Table 3.2). The percentage of explants developing shoots was significantly greater

(Chi-squared, $p < 0.05$), when plated on a medium supplemented with activated charcoal (Table 3.2).

Table 3.2: Percentage of explants with different-sized cotyledonary segments, from non-stored seeds of *T. dregeana*, developing roots and shoots when plated on three germination media and maintained for 12 weeks. n = 20.

	WPM Only		**WPM + AC**		**WPM + AsA**	
Cotyledon segment	% roots	% shoots	% roots	% shoots	% roots	% shoots
None	100	0	100	0	100	0
Basal	100	50	100	85	100	70
2-mm	100	85	100	75	100	60

Differences between root and shoot length on the different media were also monitored, and showed that root length was similar for all explant sizes across all three media (Figure 3.1) with no significant difference (Univariate ANOVA, $p > 0.05$). However, shoot length appeared to be affected by the size of the cotyledonary segments and by supplementation of the medium (Figure 3.2). Statistical analysis confirmed a significant difference between the explants on different media, and between explant sizes (Univariate ANOVA, $p < 0.05$), where the longest shoots grew in the presence of activated charcoal, indicating that phyto-toxic substances may be present in the medium, and activated charcoal may serve to adsorb these substances.

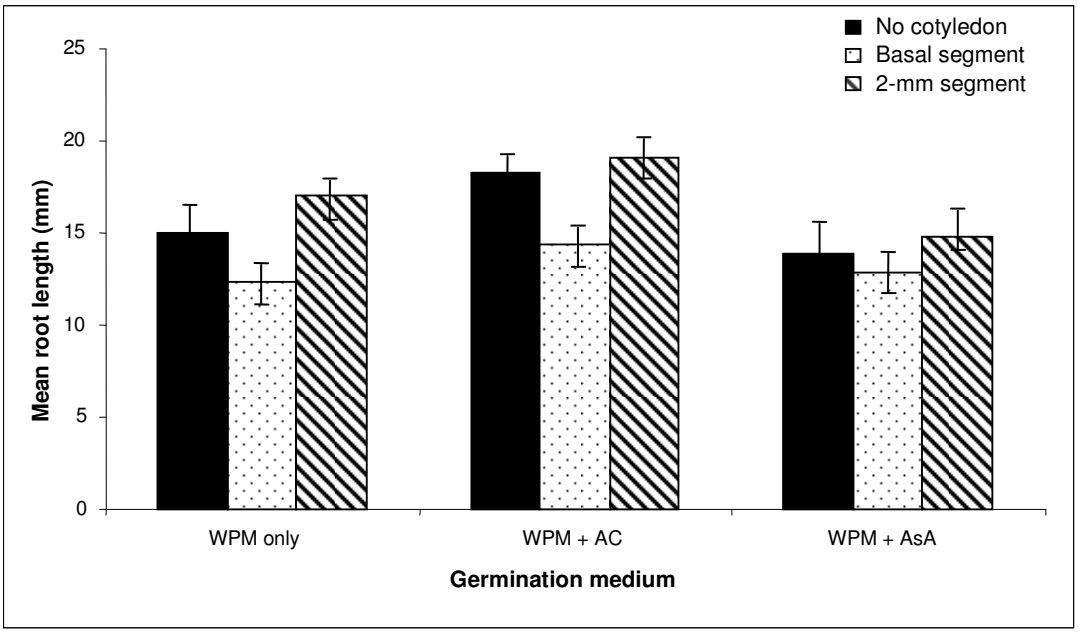


Figure 3.1: Root lengths of *T. dregeana* explants, with different-sized cotyledonary segments, excised from freshly-harvested seeds and plated on different germination media for 12 weeks. Bars indicate standard deviation. n = 20.

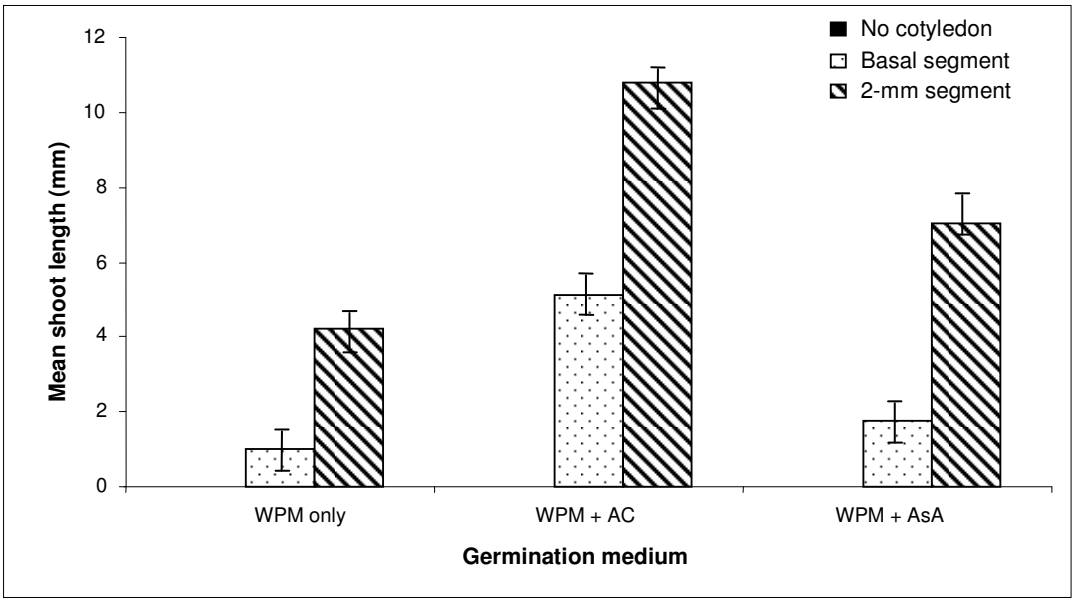
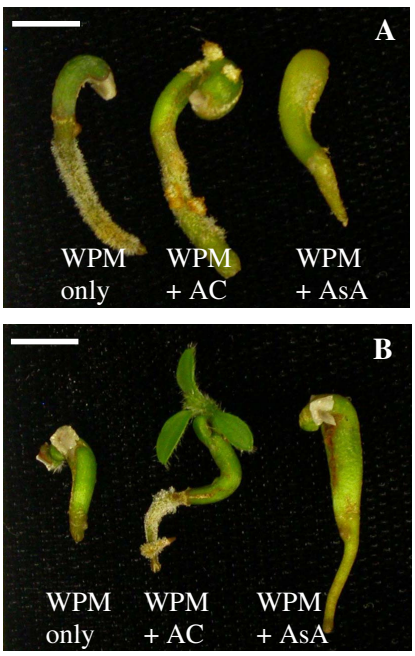


Figure 3.2: Shoot lengths of *T. dregeana* explants, with different-sized cotyledonary segments, excised from freshly-harvested seeds and plated on different germination media for 12 weeks. Bars indicate standard deviation. n = 20.

Differences in seedling development are clearly illustrated in Figure 3.3 showing shoot production only in the presence of cotyledon segments and that the increase in their size facilitates better shoot production.



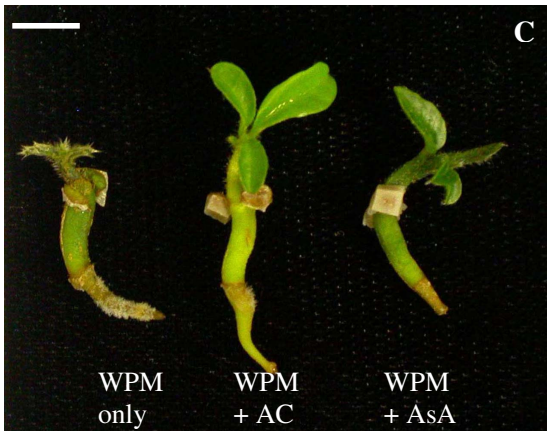


Figure 3.3: Seedling development, on different media, from explants of *T. dregeana* with no cotyledon (A); basal segment (B); and 2-mm segment of cotyledon (C) after 12 weeks in culture. Bar = 5 mm.

As WPM supplemented with either AC or AsA, was more beneficial to explants than the plain medium, the supplemented media were used in subsequent trials with seeds of *T. dregeana*.

Trichilia emetica

Explants from freshly-harvested seeds were cultured *in vitro* on the four media, *viz.*, MS, MS + AC, MS + AsA and MS + CA. As was the case for *T. dregeana*, all explants plated on the medium supplemented with citric acid became contaminated, thus the results for the remaining three media only, are reported below.

Table 3.3: Percentage of explants with different-sized cotyledonary segments, from non-stored seeds of *T. emetica*, developing roots and shoots when plated on three germination media and maintained for eight weeks. n = 20.

	MS Only		**MS + AC**		**MS + AsA**	
Cotyledon segment	% roots	% shoots	% roots	% shoots	% roots	% shoots
None	100	5	100	0	100	0
Basal	100	60	100	80	100	60
2-mm	100	90	100	100	100	95

The development of shoots noted in 1/20 of the explants apparently with no vestiges of cotyledon attached cultured on plain MS medium, may be accounted for by the possible incomplete excision of the cotyledon attachments. This one case had a negligible effect on the mean shoot length of the sample (Figure 3.5). Shoot production from explants with different-sized cotyledon segments was significantly higher when AC was added to the medium (Chi-squared, $p < 0.05$). The percentage shoot development was only significantly higher (Chi-squared, $p < 0.05$) in explants with basal cotyledonary segments, when plated on a medium supplemented with activated charcoal (Table 3.3).

Roots grew significantly longer (Univariate ANOVA, $p < 0.05$) when explants were plated on the medium supplemented with AC, than those grown on plain MS or on MS supplemented with AsA, in that order (Figure 3.4). Similarly, the longest shoots were observed on the medium supplemented with AC, and shoot length increased with an increase in the size of the cotyledon segment (Figure 3.5, Univariate ANOVA, $p < 0.05$).

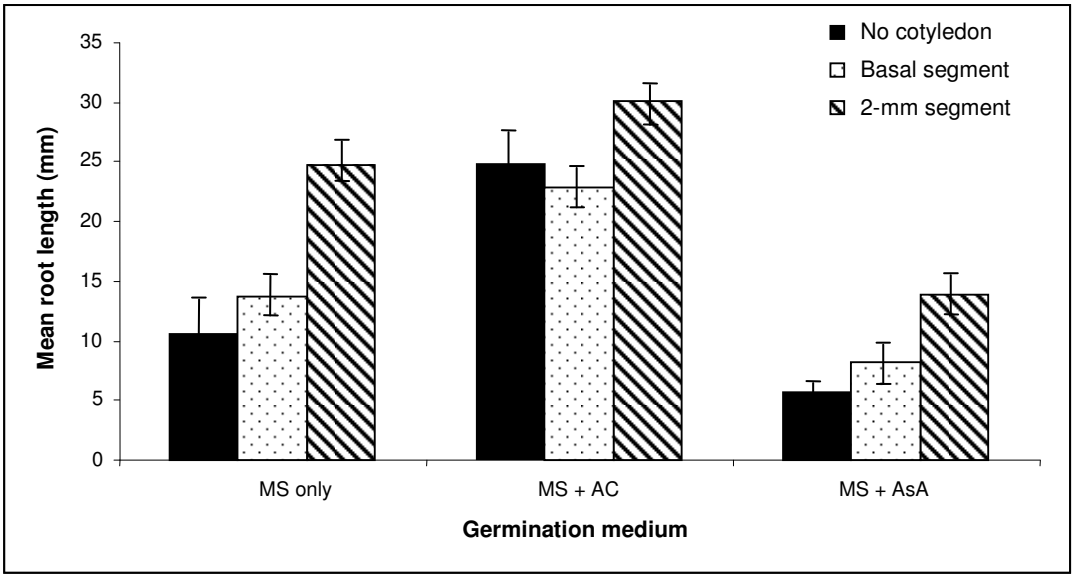


Figure 3.4: Root lengths of *T. emetica* explants, with different-sized cotyledonary segments, excised from freshly-harvested seeds and plated on different germination media for eight weeks. Bars indicate standard deviation. n = 20.

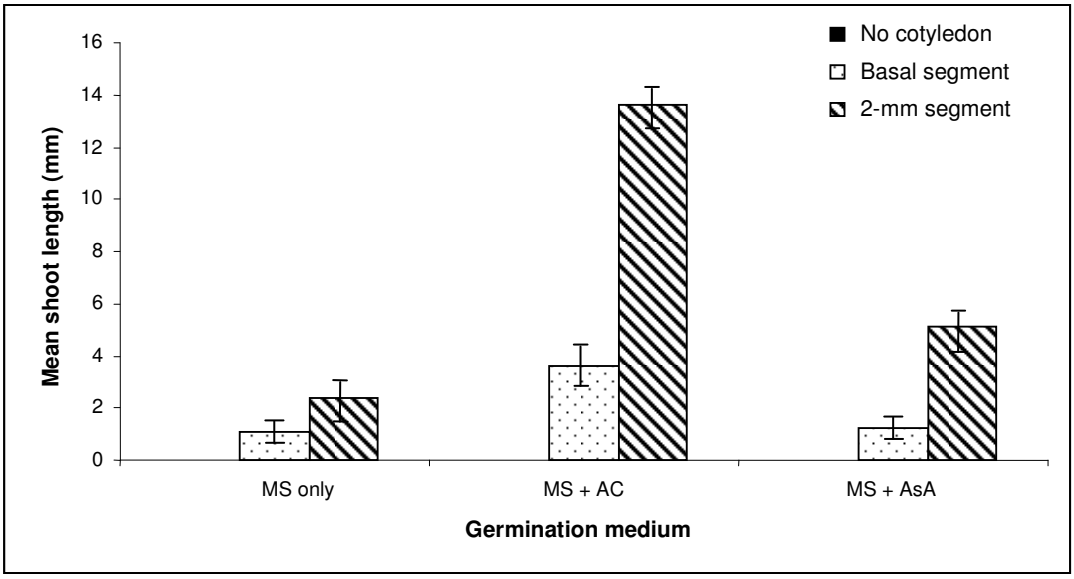
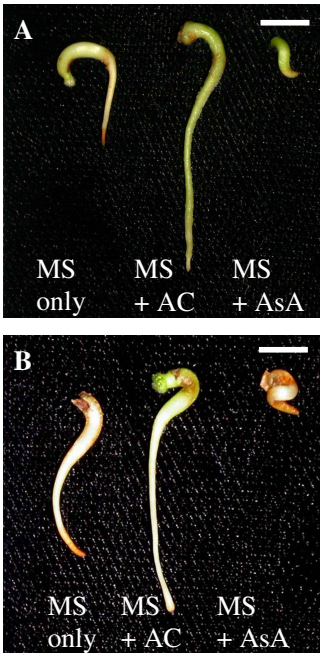


Figure 3.5: Shoot lengths of *T. emetica* explants, with different-sized cotyledonary segments, excised from freshly-harvested seeds and plated on different germination media for eight weeks. Bars indicate standard deviation. n = 20.

The appearance of seedlings differed, depending on size of the attached cotyledon segments and germination media. Explants with a 2-mm cotyledon segment, cultured on a medium supplemented with AC developed into the longest seedlings (Figure 3.6C).



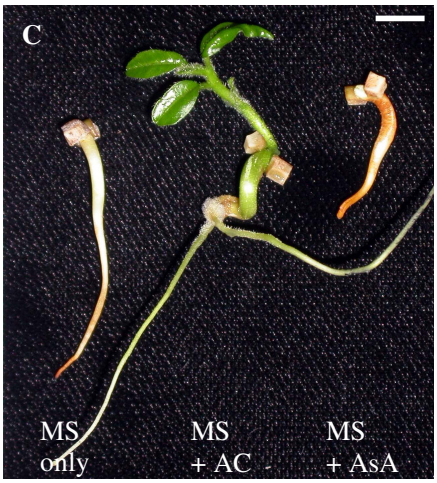


Figure 3.6: Seedling development on different media from explants of *T. emetica* with no cotyledon (A); basal segment (B); and 2-mm segment of cotyledon (C) after eight weeks in culture. Bar = 5 mm.

Strychnos gerrardii

The seeds of *S. gerrardii* are endospermous, the nutrient supply being provided by the endosperm surrounding the mature embryo, rather than directly from the cotyledons. As a result, bulky cotyledons (as seen in the *Trichilia* species), are replaced by those that are small and paper-thin. Explants of *S. gerrardii* were excised with or without cotyledons and were germinated on MS media of different strengths supplemented with AC. Roots were produced by all explants, irrespective of the germination medium (Table 3.4) while percentage shoot production was significantly higher (Chi-squared, $p < 0.05$) on media containing AC (Table 3.4). Percentage shoot production from explants with cotyledons was significantly higher than explants without cotyledons only when plated on unsupplemented ½ MS medium (Chi-squared, $p < 0.05$).

Table 3.4: Percentage of explants, with or without cotyledons, from non-stored seeds of *S. gerrardii*, developing roots and shoots when plated on four germination media and maintained for 10 weeks. n = 20.

	½ MS	**Only**	**½ MS**	**+ AC**	**MS**	**Only**	**MS**	**+ AC**
Explant size	roots	shoots	Roots	shoots	roots	shoots	roots	shoots
No cotyledon	100	0	100	60	100	0	100	60
Cotyledon	100	40	100	40	100	0	100	40

The different germination media did not significantly affect root length (Univariate ANOVA, $p > 0.05$). However, explants with cotyledons attached had significantly longer roots than those explants without cotyledons (Univariate ANOVA, $p < 0.05$) with the exception of those explants plated on ½ MS (Figure 3.7). When cultured on ½ MS, no shoots developed from axes from which cotyledons had been excised, nor did shoots develop from complete axes (no cotyledon excision) on full-strength MS. When cultured on ½ MS, however, the shoots which were produced by complete axes were significantly shorter (Univariate ANOVA, $p < 0.05$) than those produced by complete axes on either of the AC-supplemented media. Explants with cotyledons attached had significantly longer shoots (Univariate ANOVA, $p < 0.05$) than those explants without cotyledons (Figure 3.8) on both the AC-supplemented media. Thus, the deleterious effect of cotyledon removal is present even if the cotyledons comprise very little of the embryo mass. Although the extent of axis injury *per se* after cotyledon excision was not assessed for either *Trichilia* spp. or *S. gerrardii*, it is likely to have been considerably greater in the former cases (cf. Figures 2.6 and 2.8 with 2.7). It is consequently suggested that the deleterious effects of cotyledon excision on the apical shoot meristem would be correspondingly greater for *Trichilia* spp. axes, which was apparent in the preclusion of all shoot formation.

From these preliminary results, MS and ½ MS media supplemented with AC yielded similar percentages of roots and shoots when *S. gerrardii* axes were excised with cotyledons, hence ½ MS with AC was the selected for subsequent experiments.

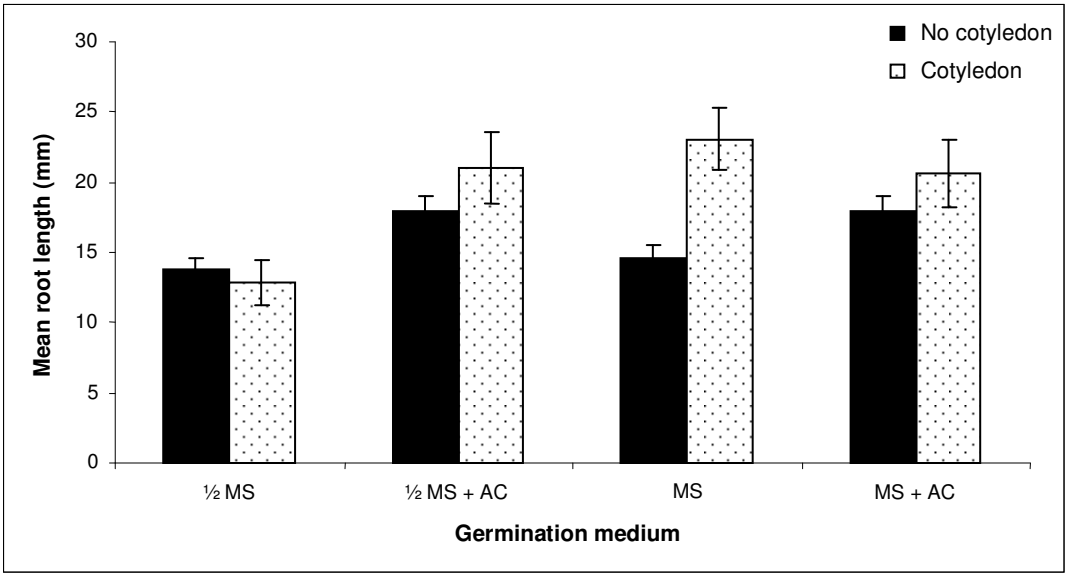


Figure 3.7: Root lengths of *S. gerrardii* explants, with and without cotyledons, excised from freshly-harvested seeds and plated on different germination media for 10 weeks. Bars indicate standard deviation. n = 20.

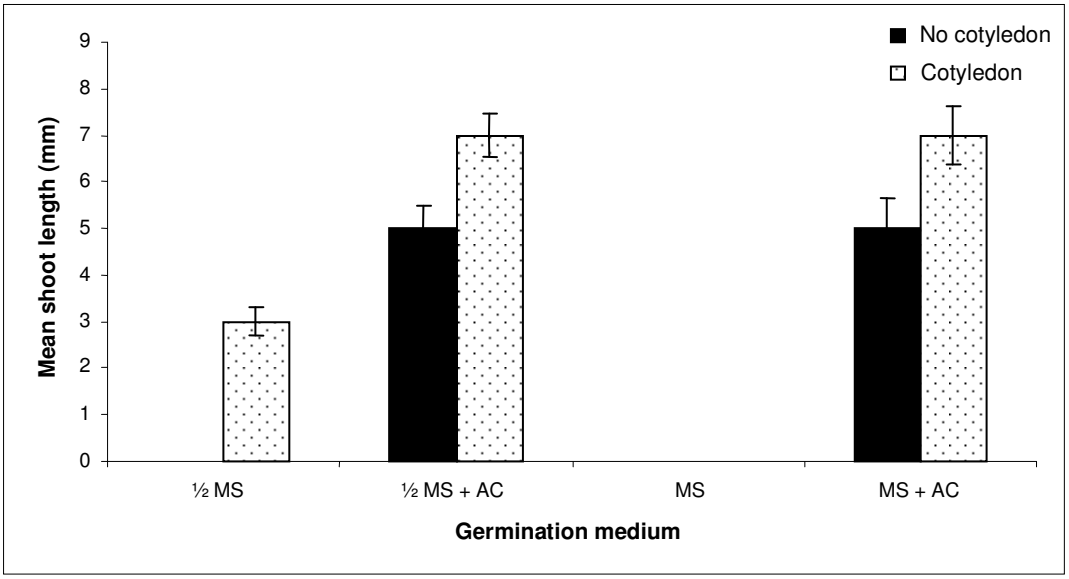


Figure 3.8: Shoot lengths of *S. gerrardii* explants, with and without cotyledons excised from freshly-harvested seeds and plated on different germination media for 10 weeks. Bars indicate standard deviation. n = 20.

3.1.2.1.1 Newly-harvested seeds – germination of excised embryonic axes: concluding remarks

Studies by Kioko (2003) were aimed at optimizing the germination medium for shoot production for axes of *T. dregeana* and *T. emetica* from which the cotyledons had been removed flush with the [axis] surface. The experiments reported by that author involved the supplementation of the medium with a variety of compounds, including: different ratios of auxin:cytokinin, nitrogen in either the oxidized or reduced form, adenine sulphate or ground cotyledons in either a particulate form or liquid extract. All these attempts were unsuccessful in stimulation of shoot production and it was later established that a segment of cotyledon was necessary to facilitate shoot development (Kioko, 2003).

The negative effects observed on addition of ground cotyledons or cotyledonary extract to the germination media led to the suggestion that homogenization of the cotyledons releases compounds toxic to the axes. This was not investigated further here or by Kioko (2003), thus the supplements to the basic nutrient medium in this study were selected on their properties to combat a range of toxic compounds and/or free radicals.

The addition of activated charcoal (AC) to a medium is known to adsorb various organic compounds, including excess hormones, vitamins, abscisic acid, phenolic metabolites and ethylene (van Winkle *et al*., 2003). Thus, the addition of AC to the medium was expected to facilitate seedling regeneration from wounded explants.

Although no shoot production was observed in explants excised without any vestiges of the cotyledons attached from newly-harvested seeds of *T. dregeana* or *T. emetica*, the addition of AC to the germination medium did increase shoot production in explants with basal segments of cotyledons (Tables 3.2 and 3.3). It is noteworthy that in all trials, cut surfaces of cotyledons were in contact with the medium, which had previously been shown to have inhibitory effects (Goveia, 2003). Hence, substances postulated to have leached from the cut surfaces of the cotyledons are suggested to have been adsorbed by the AC in the medium. The addition of AC to the medium has a positive effect not only on shoot production, but also on seedling length (Figures 3.2 and 3.5). Thus it can be

suggested that a toxic compound/s leaches from the wound sites into the germination media and has a direct effect on seedling growth and shoot production. The trend observed in *S. gerrardii* (Table 3.4) confirmed that the addition of AC to the germination media is beneficial to shoot production and seedling length (Figure 3.8).

In response to wounding, free radicals are produced in an oxidative burst as has been shown in wheat roots (Vglegzhanina *et al*., 2001; Minibayeva *et al*., 2001, 2003). If free radicals are not scavenged efficiently, their build-up is reported to have deleterious consequences (Hendry, 1993; Bailly, 2004) such as lipid peroxidation, which ultimately leads to leaky membranes as displayed in neem seeds (Varghese and Naithani, 2000).

Ascorbic acid (AsA), also known as vitamin C, plays an important role in plants because when ionized into ascorbate it acts as an anti-oxidant which protects plants against oxidative damage resulting from aerobic metabolism (Smirnoff, 1996). Citric acid (CA) also has anti-oxidant properties (Ström *et al*., 1994). However, in the present work, CA supplementation of the media was abandoned because of technical problems of medium density. Citric acid at a concentration of 10 g l^{-1} was previously used in studies carried out by Cheruyiot (pers. comm.[1]) for excised axes of *Syzigium cordatum*. However, this may not have been suitable for either *Trichilia* spp. and will require optimization in future studies.

From these results, *in vitro* germination of explants from all three species studied was best on a medium supplemented with AC as this allowed for better root and shoot development.

[1] Chepkorir Cheruyiot, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban

3.1.2.2 Effect of short-term hydrated seed storage on *in vitro* embryo germination

Some of the work embodied in this section has been published. See Goveia *et al*. (2004). *Seed Science Research* **14**: 241-248.

Trichilia dregeana

Hydrated storage for up to five months, had no effects on the ability of axes to produce roots, this being 100% from both newly-harvested and stored seeds. Shoot production occurred on media supplemented with either AC or AsA, regardless of whether the axes were excised from newly-harvested or stored seeds, provided that there was a segment of cotyledon attached (Table 3.5A and Table 3.5B). On the other hand, seed storage prior to axis excision had a positive effect, as explants excised with no vestiges of cotyledon attached, developed shoots after seeds had been stored for five months. These trends were observed on both germination media (WPM supplemented with either AC or AsA) tested (Table 3.5A and Table 3.5B), although more explants devoid of any cotyledon vestiges produced shoots when the WPM was supplement with AC than with AsA. Shoot production for all explant types increased significantly as seed storage period increased (Chi-squared, $p < 0.05$).

Table 3.5A: Percentage of *T. dregeana* explants with different-sized cotyledonary segments developing shoots when cultured for 12 weeks on WPM containing AC. Explants were excised from seeds stored for different periods. n = 20.

	Storage period		
Cotyledon segment	Not stored	3 month	5 month
None	0	0	25
Basal	85	85	90
2-mm	75	85	95

Table 3.5B: Percentage of *T. dregeana* explants with different-sized cotyledonary segments developing shoots when cultured for 12 weeks on WPM containing AsA. Explants were excised from seeds stored for different periods. n = 20.

	Storage period		
Cotyledon segment	Not stored	3 month	5 month
None	0	0	10
Basal	70	70	80
2-mm	60	80	90

Explants excised from seeds stored for three (Figure 3.9A) and five months (Figure 3.9B) showed not only a higher percentage germination, but also developed significantly longer seedlings when cultured on a medium supplemented with AC than on the medium supplemented with AsA (Univariate ANOVA, $p < 0.05$).

Seedling length refers to the combined length of both the root and shoot. However, explants excised with no vestiges of cotyledon attached developed shoots only after storage for five months (Table 3.5B) thus, the seedling length of this explant type after three month storage (Figure 3.9A) is based on root length only.

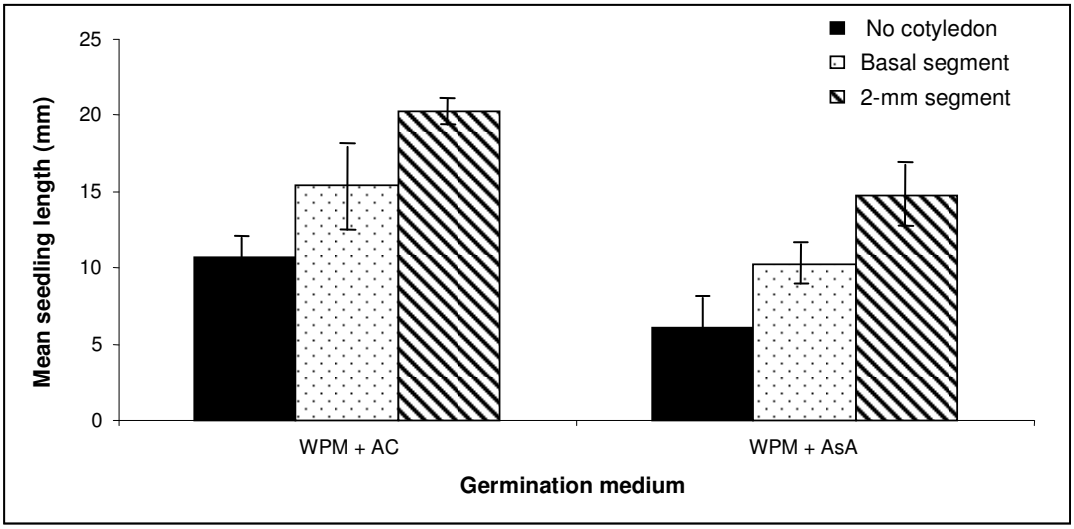


Figure 3.9A: Seedling lengths of explants with different-sized cotyledonary segments from *T. dregeana* seeds stored for three months and cultured for 12 weeks on WPM containing either activated charcoal (WPM + AC) or ascorbic acid (WPM + AsA). Bars indicate standard deviation. n = 20.

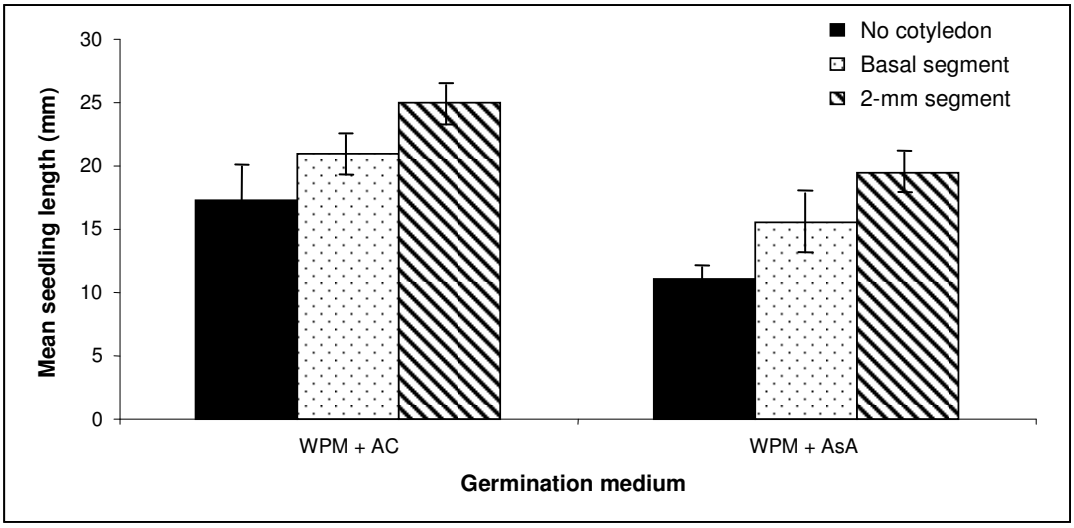


Figure 3.9B: Seedling lengths of explants with different-sized cotyledonary segments from *T. dregeana* seeds stored for five months and cultured for 12 weeks on WPM containing either activated charcoal (WPM + AC) or ascorbic acid (WPM + AsA). Bars indicate standard deviation. n = 20.

Although the seedling length increased as size of attached cotyledon increased in both cases, seedlings were generally longer when cultured on WPM containing activated charcoal (WPM + AC) than when cultured on the medium supplemented with AsA and when explants from seeds stored for five months were used (Univariate ANOVA, $p < 0.05$).

Trichilia emetica

Plain MS medium and MS containing either activated charcoal (MS + AC) or ascorbic acid (MS + AsA) were tested in experiments with stored seeds as these media had been established as optimal for axis germination (see section 3.1.2.1).

Root production remained at 100% irrespective of storage period (data not shown). Although the size of cotyledon segment significantly affected shoot production (Chi-squared, $p < 0.05$), storage period had no significant effect on shoot production (Chi-squared, $p > 0.05$). When excised from stored seeds, shoot production in explants with no cotyledon vestiges remaining, was significantly higher (Chi-squared, $p < 0.05$) than for similar explants from newly-harvested seeds (cf. Tables 3.6 and 3.3). Shoot production by these explants was also significantly higher (Chi-squared, $p < 0.05$) on

MS medium supplemented with AC (Table 3.6) than on medium supplemented with AsA, as was seen for *T. dregeana*.

Table 3.6: Percentage of *T. emetica* explants with different-sized cotyledonary segments developing shoots when cultured on three germination media and maintained for eight weeks. Explants were excised from seeds stored for different periods. n = 20.

	MS Only		**MS + AC**		**MS + AsA**	
			Storage period			
Cotyledon segment	1 week	2 weeks	1 week	2 weeks	1 week	2 weeks
None	20	20	70	50	15	20
Basal	60	60	80	90	60	55
2-mm	85	90	100	100	90	90

In addition, shoots were significantly longer (Univariate ANOVA, $p < 0.05$) when cultured on a medium containing AC (Figure 3.10) while lengths were similar when explants were cultured on MS only and MS containing ascorbic acid (MS + AsA). Storage period did not lead to significant differences in shoot lengths within treatments (explants with similar-sized cotyledonary segments), possibly because all the seeds had progressed substantially towards germination during storage (Berjak *et al*., 1989; Kioko *et al*., 2006). However, shoots of explants with a 2-mm cotyledon segment grew significantly longer than the other explants for all media tested (Univariate ANOVA, $p < 0.05$) and explants excised without any vestiges of cotyledon attached grew the best when excised from seeds stored for one week and cultured on MS + AC (Figure 3.10).

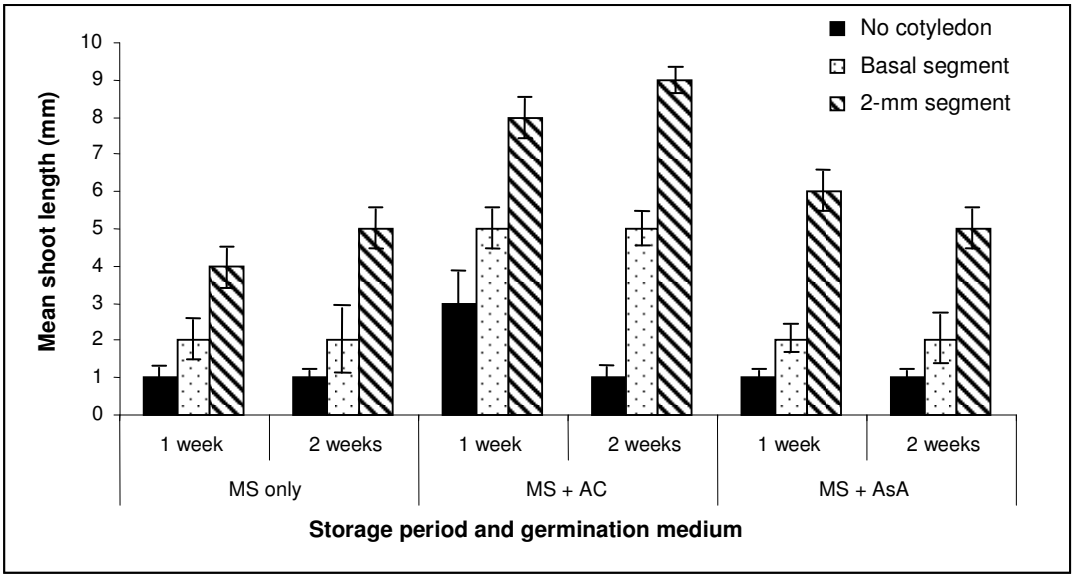


Figure 3.10: Shoot lengths of explants with different-sized cotyledonary segments excised from *T. emetica* seeds stored for different periods and cultured on different germination media for eight weeks. Bars indicate standard deviation. n = 20.

Experiments to determine the effect of a plant growth regulator (BAP) on shoot production were also carried out for explants excised with no vestiges of cotyledon attached, for both newly-harvested and stored seeds. However, the addition of BAP to the medium (Table 3.7) did not significantly affect the number of explants forming shoots (Chi-squared, $p > 0.05$). Although the concentration of BAP used was based on a previous study carried out on explants of *Ekebergia capensis* (Perán *et al*., 2006), another member of the Meliaceae producing recalcitrant seeds, it seems that BAP does not promote shoot development in *T. emetca*, at the concentration tested.

Table 3.7: Percentage of *T. emetica* axes excised with no cotyledonary attachment developing shoots when cultured with and without the addition of BAP to germination media and maintained for six weeks. Explants were excised from seeds stored for different periods. n = 20.

Storage period	MS only	MS + BAP	MS + AC	MS + AC + BAP
Not stored	0	0	0	0
1 week	20	25	70	65
2 weeks	20	25	50	50

Strychnos gerrardii

Sampling was carried out every second week for eight weeks. Only one explant type, *viz.* axes with cotyledons attached, was tested, because the latter contribute very little to embryo mass, and also because germination of embryos *in vitro* was higher when the cotyledons were attached (see section 3.1.2.1). Percentage root formation and root length were not affected by storage period (Table 3.8), while percentage shoot production and length decreased as storage time increased (Table 3.8). However, the differences were not significant (Univariate ANOVA, $p > 0.05$).

Table 3.8: Development of roots and shoots by *S. gerrardii* explants with cotyledons when cultured on ½ MS + AC for 12 weeks. Explants were excised from seeds removed from yellow fruits and stored for different periods. n = 20, standard deviations represented by ± values.

	Roots		**Shoots**	
Storage period	% germination	Mean length (mm)	% germination	Mean length (mm)
Not stored	100	18 ± 6.5	40	9 ± 10.4
2 weeks	95	16 ± 3.9	35	7 ± 3.4
4 weeks	95	17 ± 4.4	30	5 ± 3.7
6 weeks	100	16 ± 1.6	30	5 ± 3.7
8 weeks	100	16 ± 1.3	30	5 ± 3.8

Seedling length differed greatly when comparing whole seed germination (Table 3.1) with embryonic axis germination (Table 3.9). This may be because the *in vitro* germination medium could not provide all the nutrients and/or other growth factors available in the endosperm. An additional factor may be excision injury, although the embryos have no intimate contact with the endosperm macroscopically. However, the presence or not, of haustorial outgrowths from the cotyledon surfaces in contact with the endosperm, (as illustrated for *Avena fatua* [Bewley and Black, 1994]) needs to be confirmed. If present then such structures could be considerably damaged upon embryo excision. Furthermore, seedling development was incomplete for *in vitro* explants, with only a small percentage developing both roots and shoots (Chi-squared, $p < 0.05$). These were from the yellow/green and yellow fruits (Table 3.9). Interestingly, no significant difference (t-test, $p > 0.05$) in seedling lengths was observed among seedlings from seeds at different maturity stages, as was observed for those developing

from whole seeds. This argues for an effect of the seed structures themselves, which is obviated when axes are excised.

Table 3.9: Development of roots and shoots by *S. gerrardii* explants with cotyledons when cultured on ½ MS + AC for 12 weeks. Explants were excised from seeds from different-coloured fruits. n = 20, standard deviation represented by ± values.

	Roots		**Shoots**	
Fruit colour	% germination	Mean length (mm)	% germination	Mean length (mm)
Green	100	16 ± 2.1	0	0 ± 0.0
Yellow/green	100	15 ± 1.7	5	3 ± 1.1
Yellow	100	11 ± 1.2	20	5 ± 2.3

In addition, experiments to determine the effect of BAP on shoot development were conducted on explants from seeds stored for six weeks. This storage period was selected because no newly-harvested material was available at the time of the experiment, hence seeds stored under hydrated conditions after extraction from yellow fruits were used. These explants were cultured on a plain ½ MS medium supplemented with different concentrations of BAP to determine whether this cytokinin would facilitate shoot development. Although there was some effect, irrespective of the concentration of BAP added to medium, neither shoot development nor length was significantly affected (Chi-squared and t-test respectively, $p > 0.05$), as shown in Table 3.10 and Figure 3.11. Hence, further research is needed to find an appropriate hormone and concentration to stimulate shoot production.

Table 3.10: Development of roots and shoots by *S. gerrardii* explants with cotyledons when cultured on ½ MS with different concentrations of BAP for six weeks. Explants were excised from seeds removed from yellow fruits and stored hydrated for six weeks. n = 20, standard deviation represented by ± values.

	Roots		**Shoots**	
BAP conc.	% germination	Mean length (mm)	% germination	Mean length (mm)
0.5 mg l^{-1}	100	11 ± 1.8	25	1 ± 1.7
1 mg l^{-1}	100	9 ± 2.2	30	4 ± 2.5
2 mg l^{-1}	100	10 ± 1.8	25	6 ± 1.8

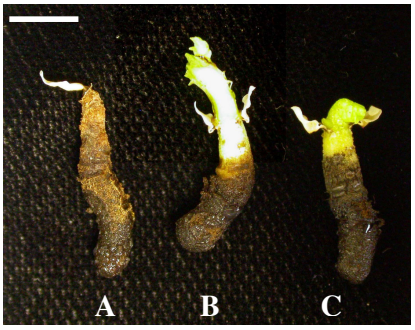


Figure 3.11: Seedling development from *S. gerrardii* explants with cotyledons, cultured on media with 0.5 mg l^{-1} BAP (A); 1 mg l^{-1} BAP (B); and 2 mg l^{-1} BAP (C), excised from seeds removed from yellow fruits and stored for six weeks. Bar = 5 mm.

3.1.2.2.1 Short-term hydrated seed storage – germination of excised embryonic axes: concluding remarks

The storage periods utilized in this study for the seeds of *T. dregeana* and *T. emetica* differed from those of some previous studies. Drew *et al*. (2000) reported a loss in *T. dregeana* seed viability after 16-21 days while Kioko (2003) stored seeds for up to five months, as was possible in this study (Tables 3.5A and 3.5B). In contrast, the seeds of *T. emetica* were stored for two months by Kioko (2003), while in this study, it was possible to store seeds for only two weeks before they were overcome by fungi. This variability in storage longevity of recalcitrant seeds is known to occur among species, between different harvests within species and within seeds from the same harvest (Berjak *et al*., 1989; Finch-Savage and Blake, 1994). Variability can be attributed to time elapsed between seed shedding and collection, the maturity at harvest and also the proliferation of micro-organisms, especially fungi, in storage.

When excised embryonic axes were cultured, the trend observed from the stored seeds of *T. dregeana* and *T. emetica* revealed an increase in the proportion of explants producing shoots with increasing storage period irrespective of the germination medium (Tables 3.5A, 3.5B, 3.6). This supports the study by Finch-Savage (1992a) and Finch-Savage and Blake (1994), showing that recalcitrant seeds are shed at varying developmental stages and continue to develop after shedding. The improved ability of explants with no vestiges of cotyledon attached to develop shoots after a period of seed storage indicated the under-developed nature of the axis when shed, and that subsequent development in storage had occurred. The addition of AC to the medium also facilitated shoot formation in explants from stored seeds, indicating that toxic substances were still

leaching from cut cotyledons from the more mature explants, and that these explants did not have an inherent mechanism to counteract the effect.

In contrast to the observations on *Trichilia* species (Meliaceae), up to 40% of the explants from newly-harvested seeds of *S. gerrardii* (Loganiaceae) were able to produce shoots when cultured *in vitro*, and this ability for shoot production was essentially unchanged over the storage period (Table 3.8). This indicates that developmental maturity of the seeds/axes had been reached when *S. gerrardii* fruits were shed. Futhermore, an increased ability for seedling production by excised axes was observed as fruits ripened from green to yellow (Table 3.9), which is consistent with advancing stages of seed development. This study has established a preliminary baseline for the storage potential of the seeds of *S. gerrardii*, for which no published data are available.

The seeds of *T. dregeana* and *T. emetica* are exendospermous, the mature seed being composed entirely of an embryonic axis and two bulky, nutrient-storing cotyledons. On the other hand, *S. gerrardii* seeds are endospermous; the seed storage reserves are not found in the paper-thin cotyledons, but rather remain accumulated in the tissue surrounding the embryo. The separation of the embryo from the endosperm leaves no nutritive tissue for seedling development during germination, and whole seeds have the ability for more rapid and complete germination as opposed to those embryos plated on a nutrient medium (compare Tables 3.1 and 3.9). Although the nutrient medium provides the carbon source and the essential amino acids and minerals necessary for growth, it may lack growth compounds and/or other factors found in the endosperm. It is also possible that microscopic damage to the abaxial cotyledon surfaces may have occurred upon embryo separation from the endosperm, as suggested above.

Whole seeds extracted from green *S. gerrardii* fruits germinated more rapidly than those from riper fruits. As the endosperm of *S. gerrardii* seeds becomes increasingly hard with maturation, these observations suggest that while all the nutrients and other factors for germination are available at both stages the seeds may develop a measure of dormancy. Alternatively this slower germination could be associated with slower nutrient accessibility from the hardened endosperm. Another possibility might be a

transient coat-imposed dormancy, possibly by consolidation of the testa (not presently investigated) that temporarily restricts gaseous exchange and/or water uptake. von Fintel *et al*. (2004) reported similar observations for seeds of the palm *Phoenix reclinata*, where seeds extracted from less mature fruits germinated more readily than those at later fruit maturity stages. Although *P. reclinata* is a monocot, a similar situation of endosperm/seed hardening accompanies maturation (von Fintel *et al*., 2004).

Although the *S. gerrardii* seeds were fully germinable at all stages tested, shoot development of many axes was compromised after excision, although no physical damage could be discerned macroscopically. Plant growth hormones are often used to establish a desired morphogenic result when culturing explants *in vitro*, with auxins and cytokinins being the main categories of hormones used in plant tissue culture. The cytokinin BAP is commonly used to facilitate shoot formation across species, e.g. *Ficus* sp. (Jaiswal and Narayan, 1985), jackfruit (Rahman and Blake, 1988), ginger (Balachandran *et al*., 1990), legumes (Angeloni *et al*., 1992; Nandwani and Ramawat, 1992), *Sterculia* sp. (Purohit and Dave, 1995), cotton (Agrawal *et al*., 1997) and sunflower (Charriere *et al*., 1999). In a recent study by Perán *et al*. (2006), BAP was used on *Ekebergia capensis* axes from which the cotyledons had been completely removed, to induce shoot formation. In that study, numerous adventitious buds developed at the wound sites. This species, also a member of the Meliaceae (like the *Trichilia* spp.), has axes that fail to develop shoots unless excised with a portion of each cotyledon attached. Thus, based on these guidelines, the same BAP concentration was used in the medium for *T. emetica* explants from which the cotyledons were excised flush with the axis surface (Table 3.7). However, as BAP inclusion did not result in adventitious bud or shoot formation, it is possible that this was not the optimum concentration or perhaps, the most suitable cytokinin, for *T. emetica*. Nevertheless, it is imperative that the possibility of microscopically-visible damage to the abaxial cotyledon surfaces, which could be ascertained by scanning electron microscopy, be examined.

Experiments were aimed at determining the optimal BAP concentration that might stimulate shoot formation by explants from the seeds of *S. gerrardii*. However, the various concentrations of BAP tested did not make any significant difference to shoot production (Table 3.10). Thus, more rigorous tests must be conducted to ascertain the potential of BAP in future studies.

In the case of *S. gerrardii* the developmental stage of the seeds at the time of the experiment could be critical as the endosperm may contribute to the level of cytokinins found in the embryo. Studies on the seeds of *Theobroma cacao* showed that cytokinins were present throughout development (Dangou *et al*., 2002), initially in high concentrations corresponding to high mitotic activity of both embryo and endosperm, followed by decreased cytokinin concentrations as degradation of the endosperm was amplified (Dangou *et al*., 2002). Furthermore, studies on the seeds of *Pisum sativum* showed that the mother plant controls the flow of nutrients and other factors (including hormones) to the developing embryo via the endosperm (Wang and Hedley, 1991). It may be generalized that the cytokinin concentration within the embryo will vary depending on the stage of development of the seeds. Hence, the addition of cytokinins to the *in vitro* medium may not trigger shoot production unless the optimum concentration within the embryo is achieved. For excised axes of *S. gerrardii*, therefore, which have presently been shown to have the potential for shoot production *in vitro* (Table 3.8), the effects of a spectrum of cytokinins at various concentrations in the medium need to be ascertained.

3.2 Dehydration responses

3.2.1 Whole seeds

Dehydration responses were established for the seeds of *S. gerrardii* only as this had not been previously documented. In the present study, although the whole seed of *S. gerrardii* was used during dehydration in silica gel, the water content of the embryo was determined independently from the endosperm. The response of whole seeds of *T. dregeana* and *T. emetica* to dehydration has been previously studied by Kioko (2003) who found that axes dried in whole seeds took 4 and 8 days respectively, to reach water contents attained by those which were flash-dried. However, axes from seeds of *T. emetica* dried faster than those from *T. dregeana* which was attributed to their being smaller. Futhermore, a decline in germination was observed as axis water content declined, for both *Trichilia* spp. (Kioko, 2003).

Initial mean water contents of embryos of *S. gerrardii* seeds of all maturity stages were similar (1.0 – 1.1 g g^{-1}), as were the dehydration rates (Figure 3.12). After seed dehydration for 96 h, embryo water contents had decreased to 0.16 – 0.19 g g^{-1} for seeds of all maturity stages. Embryo water contents at different maturity stages before or after dehydration were not significantly different (One-way ANOVA, $p > 0.05$). For all maturity stages, the initial water content of the endosperm was significantly lower (One-way ANOVA, $p < 0.05$) when compared with that of the embryo (Figure 3.12). However, after dehydration for 12 h there was no significant difference between axis and endosperm water contents (One-way ANOVA, $p > 0.05$).

As embryo water content decreased below approximately 0.7 g g^{-1}, germinability (Figure 3.13) and average length (Figure 3.14) of seedlings also decreased, for seeds of all maturity stages. Percentage germination was significantly different between seeds of different maturity stages after dehydration to an embryo water content of approximately 0.3 g g^{-1} (Chi-squared, $p < 0.05$). After dehydration to a water content of 0.4 g g^{-1}, the maturity stage had a significant effect on seedling length (t-test, $p < 0.05$), with the longest seedlings developing from seeds from green fruits, followed by yellow/green and yellow fruits.

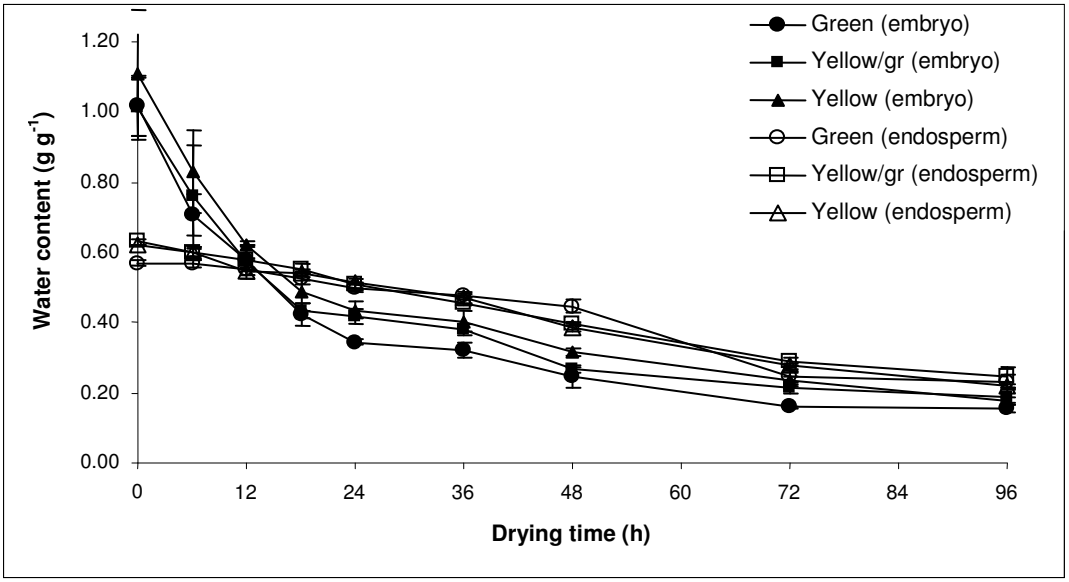


Figure 3.12: Changes in mean embryo and endosperm water content as dehydration period in silica gel increased for *S. gerrardii* seeds at different stages of maturity (as indicated by fruit colour). Bars indicate standard deviation. n = 5.

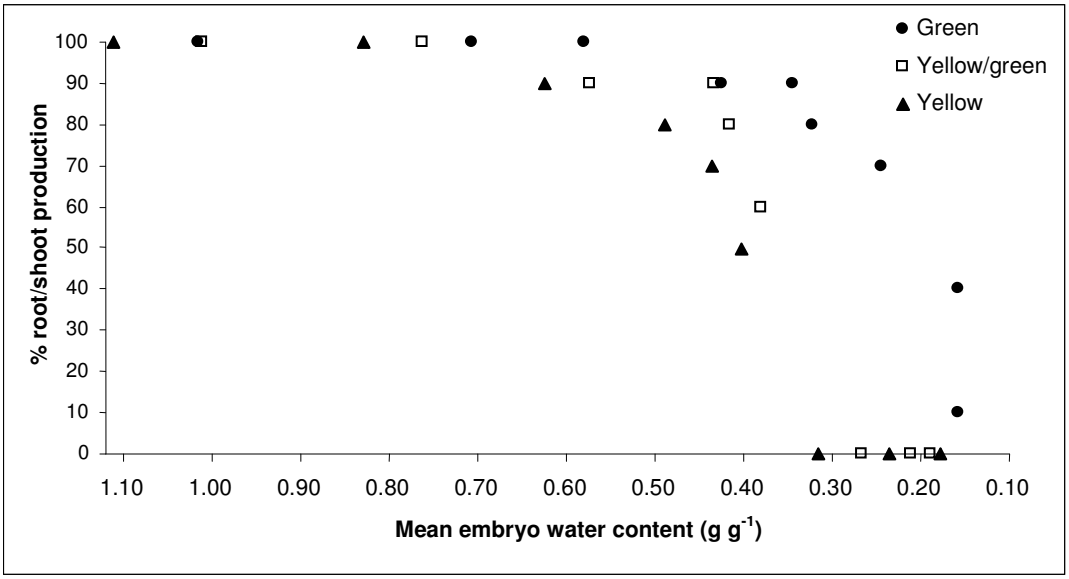


Figure 3.13: Changes in root and shoot (with root) production with reduction in the water content of *S. gerrardii* seeds at different stages of maturity (as indicated by fruit colour). Mean embryo water content (n = 5) interpolated from the same drying time-course as the seeds set out to germinate (n = 20).

Seeds from green fruits retained better germination capacity when dried to lower water contents than did those from fruits at other maturity stages: while there was no germination at embryo water contents of approximately 0.3 g g^{-1} in yellow/green and yellow fruits, 75% germination was still observed around this water content for seeds from green fruits (Figure 3.13). The length of resultant seedlings decreased with the extent of seed dehydration (Figure 3.14). It was also noteworthy that at any water content, the seedlings from the seeds of green fruits were the longest, with seedlings from yellow fruits being the shortest. For example, at 0.4 g g^{-1} seedling lengths were on average 60, 45 and 22 mm long for seeds from green, yellow/green and yellow fruits respectively, while at 0.3 g g^{-1}, only seeds from green fruits germinated, with seedling lengths of approximately 44 mm. Thus, it appears that as fruits ripen from green to yellow, the maturing seeds become more sensitive to desiccation.

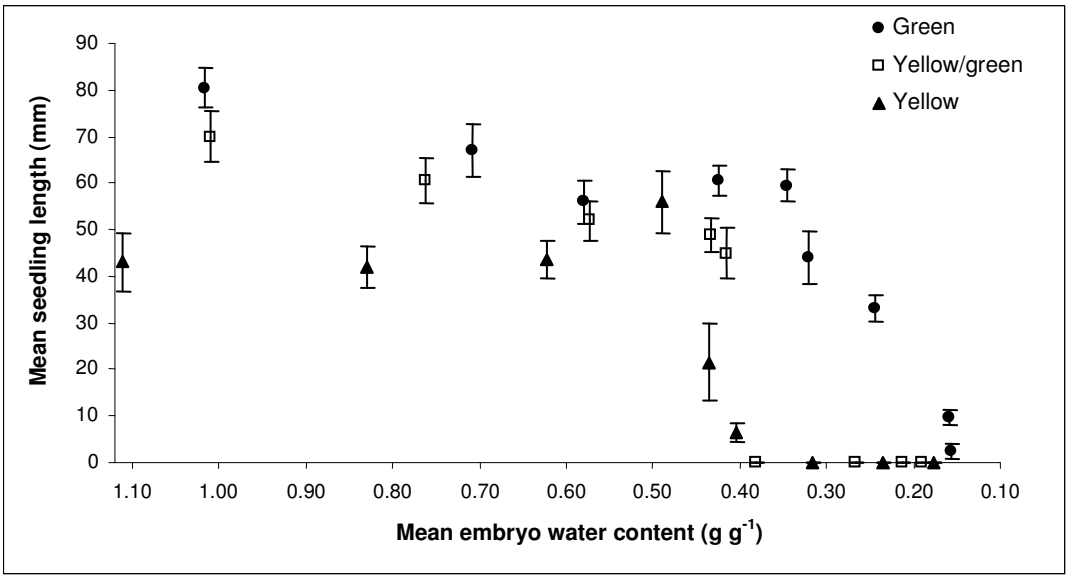


Figure 3.14: Changes in seedling length with reduction in the water content of *S. gerrardii* seeds at different stages of maturity (as indicated by fruit colour). Bars indicate standard deviation. Mean embryo water content (n = 5) interpolated from the same drying time-course as the seeds set out to germinate (n = 20).

3.2.1.1 Whole seed dehydration: concluding remarks

During the development of recalcitrant seeds, the final phase of maturation drying is absent. Thus, at maturity, these seeds often have had almost no net loss of water, they continue to accumulate dry mass, retain active metabolism, and remain desiccation-sensitive (Kermode and Finch-Savage, 2002).

In most recalcitrant seeds, tolerance to desiccation appears to increase somewhat as reserves are accumulated during development: concomitantly water concentration (content) decreases, not necessarily by water loss, but in relation to dry mass (Berjak and Pammenter, 1997; Kermode and Finch-Savage, 2002). A study conducted on the seeds of *Quercus robur* showed that shedding occurred at different times from the same tree and late-shed seeds had lower water contents than those shed earlier (Finch-Savage and Blake, 1994). Those authors showed a negative correlation between water content and desiccation tolerance. Thus, a parallel with orthodox seed development was suggested; however, full desiccation tolerance would not be acquired in these seeds because they were naturally shed before the final maturity stage. Hence, it has been suggested from those studies on *Q. robur*, that desiccation sensitivity may result from the premature termination of development (Finch-Savage, 1992a) with the variation in desiccation tolerance linked to the amount of matrix-bound water (Finch-Savage, 1992b; Grange and Finch-Savage 1992). Recent studies by Daws *et al.* (2004, 2006b) on seeds of *Aesculus hippocastanum* and *Acer pseudoplatanus*, respectively, have shown degree of development to be negatively correlated with relative desiccation-sensitivity along a North – South gradient. Seeds of both species from the most northerly provenance (in the Northern hemisphere) were least well-developed and most desiccation-sensitive at shedding.

In addition to those studies, Berjak *et al.* (1993) showed that the immature axes of *Camellia sinensis* in the histodifferentiation stage were also more sensitive to desiccation (damage at 0.7 g g^{-1}) than the mature axes in the reserve accumulation stage (damage at 0.4 g g^{-1}). Work on *Zizania palustris* and *Z. texana* embryos showed that the properties of water in these recalcitrant embryos change with development to resemble more those of desiccation-tolerant seeds (Vertucci *et al.*, 1994). For embryos of *Z.*

palustris and *Z. texana* it has also been demonstrated that the critical water content was highest in least mature embryos - i.e. the least mature embryos studied tolerated the least water loss (Vertucci *et al*., 1995). Seeds from species such as *Acer pseudoplatanus* (harvested in southern England) were shown to reach maximum desiccation tolerance before shedding but become dormant at maturity (Hong and Ellis, 1990) while the extreme desiccation sensitivity of seeds of *Avicennia marina*, is unaffected by the developmental stage at which they are harvested (Farrant *et al*., 1993). Hence, the relationship between desiccation sensitivity and developmental maturity of seeds when shed, varies with species.

Although the present study shows for *S. gerrardii* that embryo water contents at different maturity stages are not significantly different (Figure 3.12), seeds from green fruits were more desiccation-tolerant than seeds from riper fruit, maintaining germinability after dehydration below 0.3 g g^{-1} (Figure 3.13). These results are contrary to most of those described above as desiccation tolerance appears to decrease with maturity. However, a study on the recalcitrant seeds of *Clausena lansium* and *Litchi chinensis* by Fu *et al*. (1994) also showed that less mature seeds of both species were more desiccation-tolerant than fully mature or over-ripened seeds. Similar results were reported for the seeds of *Landolphia kirkii* by Berjak *et al*. (1992). As no maturation drying phase *sensu stricto* is present in recalcitrant seeds, they are shed at high water contents and are metabolically active when shed. Berjak *et al*. (1992) have shown that axes which have begun germinative processes are damaged at higher water contents than previous to this developmental stage. Thus it might be argued from the present results for *S. gerrardii*, that as the fruit colour changed from green to yellow, seeds progressed from initially being germinable, into germination metabolism *sensu stricto*, accompanied by increasing desiccation sensitivity. Concomitantly, metabolism-linked damage could be intensified as heightened metabolic rates accompanying germination. However this interpretation is complicated by desiccation responses on embryos excised from the fruits of different stages of ripening (see later).

Seedling vigour assessed as length x days after seeds were set to germinate, was also affected by the difference in fruit maturity stage, with vigour decreasing as fruits

yellowed (Figure 3.14). Similarly, wampee seeds also showed a decrease in vigour as seeds matured (Fu *et al*., 1994). A study by McKersie and Tomes (1980) attributed the decline in vigour of imbibed wild oats and birdsfoot trefoil seeds to a loss of membrane integrity caused by dehydration of these now desiccation-sensitive seeds. Based on that evidence, from the present observations it is suggested that the seeds of *S. gerrardii* lose vigour when held in yellowing/yellow fruits, as desiccation sensitivity increases. Thus, when dehydrated these seeds (from yellow fruits) may be more likely to lose membrane integrity, and hence to lose vigour more readily and at higher water contents, than seeds from green-coloured fruits when dehydrated.

Whole *S. gerrardii* seeds had to be dehydrated for 96 h before an embryo water content of approximately 0.35 g g^{-1} was reached. Such relatively slow, prolonged dehydration of seeds in silica gel maintains embryos at declining, but permissive water contents allowing (a) germinative metabolism to proceed accompanied by increasing desiccation sensitivity (e.g. Berjak *et al*., 1989); (b) concomitant metabolism-linked damage to occur (Walters *et al*., 2001). Relatively slow dehydration is thus suggested to increase the damage caused by desiccation, particularly in seeds from mature, yellow fruits. However, the results obtained with axes excised from seeds of fruits across the green to yellow range, are contradictory to this interpretation (see section 3.2.2, *S. gerrardii* below).

Whole seeds of *S. gerrardii* were more tolerant to desiccation when compared with those of *T. dregeana* and *T. emetica* previously determined by Kioko (2003). For example, at a water content of 0.4 g g^{-1}, between 90 – 50% of *S. gerrardii* seeds germinated (depending on fruit maturity), while seeds from both *T. dregeana* and *T. emetica* failed to germinate. Furthermore, only 40% of seeds of *T. dregeana* and *T. emetica* germinated at water contents of 0.8 g g^{-1} and 0.5 g g^{-1}, respectively (Kioko, 2003), while seeds from green-coloured fruits of *S. gerrardii* continued to germinate below a water content of 0.3 g g^{-1}. However, the seeds of *S. gerrardii* are still far too large to cryopreserve. Thus, further studies aimed at germplasm cryopreservation were carried out on the embryos.

3.2.2 Embryonic axes

Some of the work embodied in this section has been published. See Goveia *et al.* (2004). *Seed Science Research* **14**: 241-248.

The responses of axes from newly-harvested, and those from stored, seeds, were ascertained to compare the effects of different dehydration techniques.

Trichilia dregeana

When determining water content of explants with different-sized cotyledon segments from newly-harvested seeds, all cotyledonary tissue attached to the axis was removed after rapid dehydration, thus giving a true reflection of axis water content. The decline in axis water content of the explants with different-sized cotyledon segments was not significantly different during the course of dehydration (One-way ANOVA, $p > 0.05$). Initial mean water contents of 2.04 g g^{-1} decreased to 0.30 g g^{-1} and 0.25 g g^{-1} after dehydration for 150 min and 180 min, respectively (Figure 3.15).

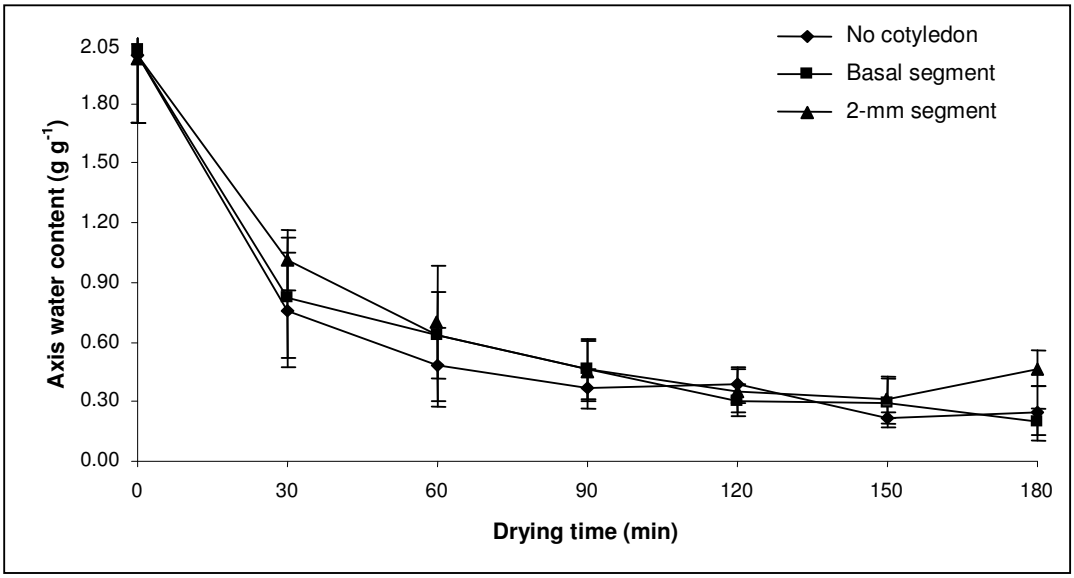


Figure 3.15: Changes in mean axis water content of explants without, and with, different-sized cotyledonary segments from newly-harvested seeds of *T. dregeana* during rapid dehydration by flash-drying. Bars indicate standard deviation. n = 5.

To germinate dehydrated explants, the two pre-selected media from the *in vitro* germination trials (WPM + AC and WPM + AsA) were used. However, the different media did not significantly affect percentage root or shoot production (Chi-squared,

p > 0.05; Figure 3.16A and Figure 3.16B respectively). Thus, WPM + AC was selected for further experiments as germination was marginally better on this medium.

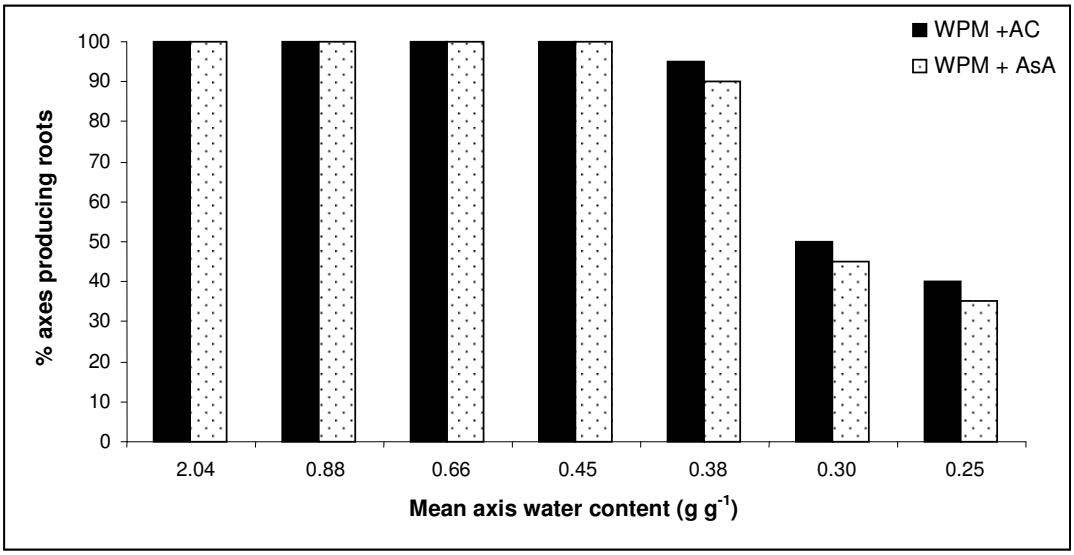


Figure 3.16A: Root production by axes of *T. dregeana* with attached basal cotyledonary segments, flash-dried to different water contents and germinated on either WPM + AC or WPM + AsA. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

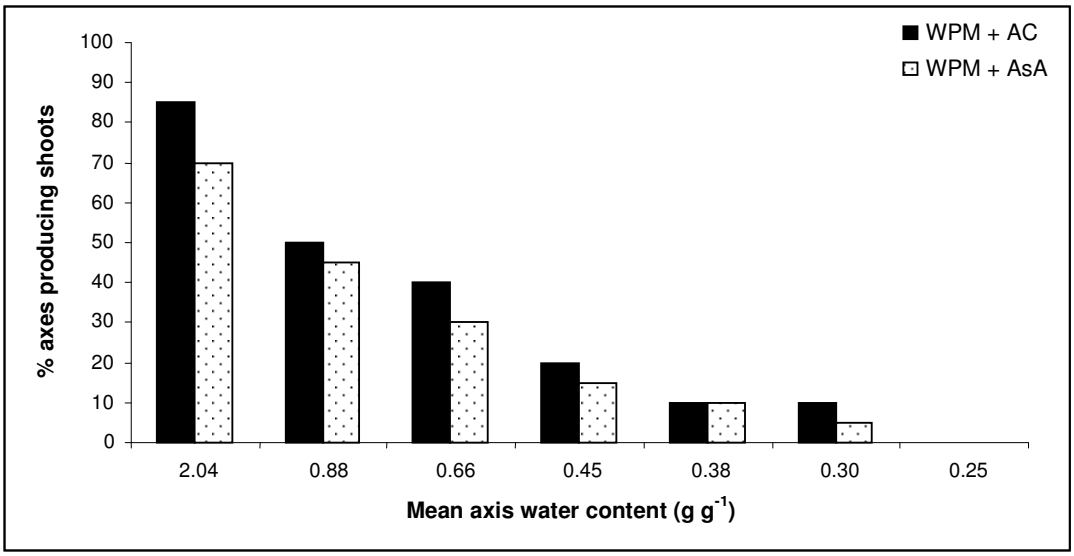


Figure 3.16B: Shoot (with root) production by axes of *T. dregeana* with attached basal cotyledonary segments, flash-dried to different water contents and germinated on either WPM + AC or WPM + AsA. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

The decline in percentage root production as water content decreased was not significantly affected by the amount of cotyledonary tissue remaining attached to the explants (Chi-squared, $p > 0.05$), as a steep drop in all occurred as water content reached 0.30 g g^{-1} (Figure 3.17A).

Shoot production was more sensitive to dehydration than root production, with a decline to less than 20% in the total number of explants producing shoots at a water content of 0.40 g g^{-1} (Figure 3.17B). Shoot production was not significantly affected by explants having different-sized cotyledon segments (Chi-squared, $p > 0.05$); however, axes with no vestiges of cotyledonary tissue attached did not develop shoots.

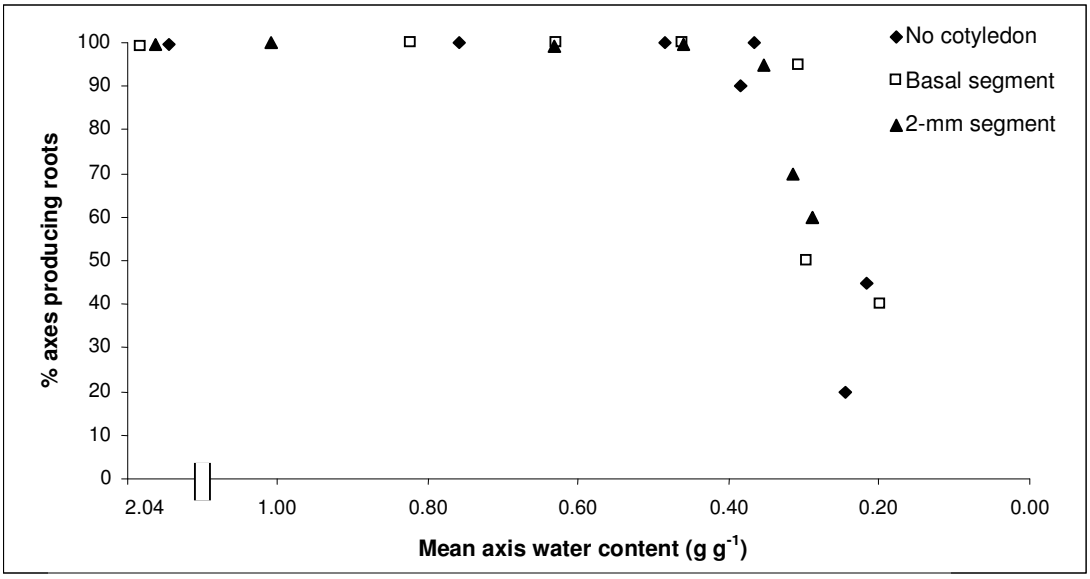


Figure 3.17A: Root production by explants without, and with, different-sized cotyledonary segments from newly-harvested seeds of *T. dregeana* flash-dried to different water contents and germinated on WPM + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

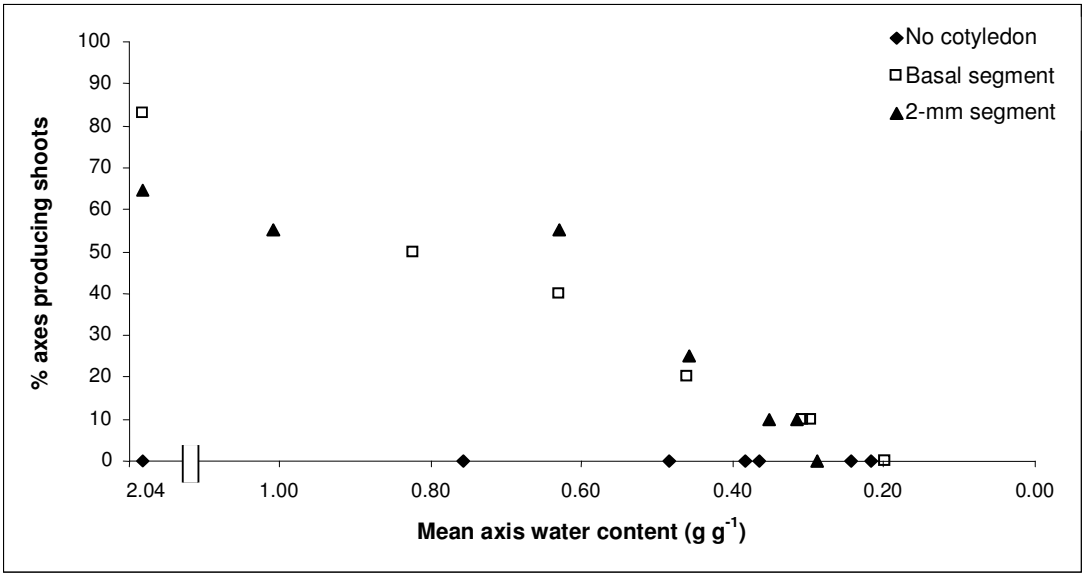


Figure 3.17B: Shoot (with root) production by explants without, and with, different-sized cotyledonary segments from newly-harvested seeds of *T. dregeana* flash-dried to different water contents and germinated on WPM + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

Explants from newly-harvested and three-/five-month stored seeds for the cryostorage trials were selected after rapid dehydration for 150 and 180 min as this allowed for water contents around 0.30 and 0.25 g g^{-1}, respectively. Although root and shoot production had been shown to decline, these water contents have been reported to be suitable for cryopreservation of explants of other species, as demonstrated for axes of *Landolphia kirkii* (Pammenter *et al*., 1991; Vertucci *et al*., 1991) *Camellia sinensis* (Berjak *et al*., 1993), *Coffea arabica* (Dussert *et al*., 1997) *Trichilia dregeana*, (Kioko *et al*., 1998) and *Quercus robur* (Berjak *et al*., 1999b). Reduction to lower water contents probably results in the removal of non-freezable water, i.e. the water bound to intracellular surfaces (Vertucci and Farrant, 1995), thus inducing a loss of integrity of cellular and structural organization (McKersie and Tomes, 1980; Vertucci *et al*., 1994; Pammenter and Berjak, 1999; Pammenter *et al*., 2000; Walters *et al*., 2001).

Axis water contents of explants from stored seeds were not significantly different from those from newly-harvested seeds before or after dehydration (One-way ANOVA, $p > 0.05$). Initial axis water contents ranged between 2.06 – 2.09 g g^{-1} for stored seeds

while water content after dehydration by flash-drying for 150 and 180 min was around 0.32 and 0.27 g g^{-1} respectively (Figure 3.18).

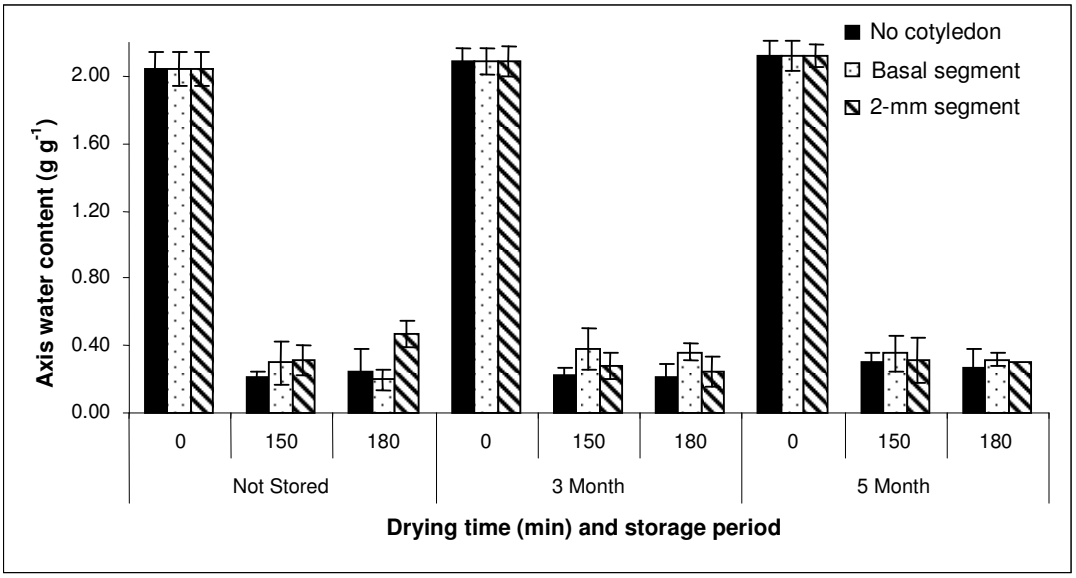


Figure 3.18: Changes in mean axis water content of explants without, and with, different-sized cotyledonary segments from newly-harvested and stored seeds of *T. dregeana* during rapid dehydration by flash-drying to 0.32 and 0.27 g g^{-1} (150 and 180 min, respectively). Bars indicate standard deviation. n = 5.

Dehydrated explants from all stored seeds were germinated on WPM + AC only based on the results obtained from experiments using newly-harvested seeds.

Percentage root production by explants from seeds stored for different periods was not significantly different after dehydration to water contents in the range 0.32 - 0.25 g g^{-1} (Chi-squared, $p > 0.05$). In addition, a Chi-squared test revealed no significant difference in root production between explants with different-sized cotyledon segments at any given water content (Figure 3.19A), although the percentage of explants producing roots after dehydration was consistently higher when a 2-mm segment of cotyledon was left attached.

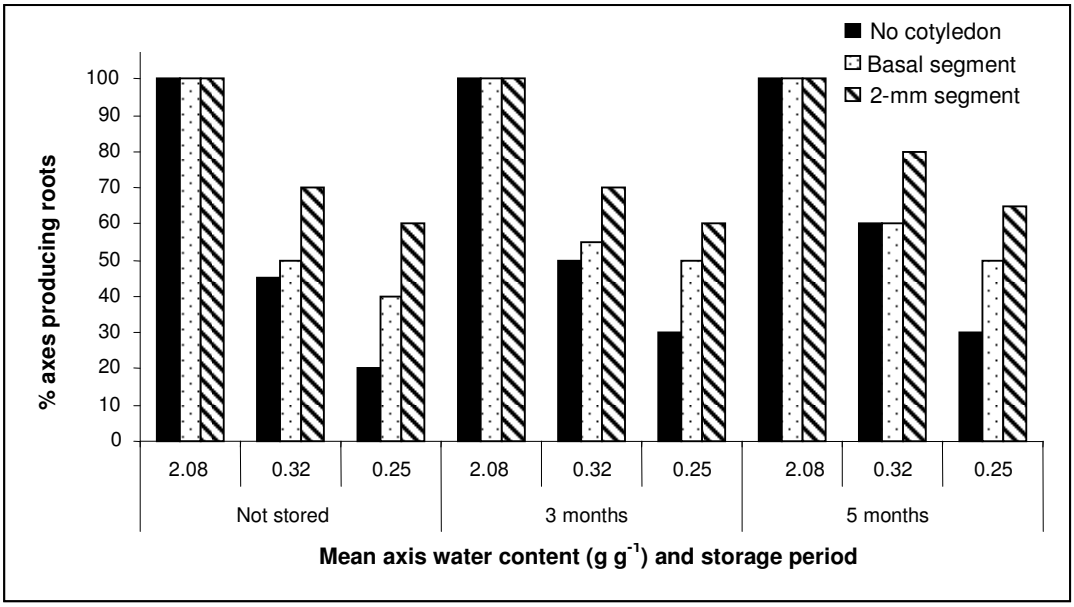


Figure 3.19A: Root production by explants without, and with, different-sized cotyledonary segments from newly-harvested and stored seeds of *T. dregeana* flash-dried to different water contents and germinated on WPM + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

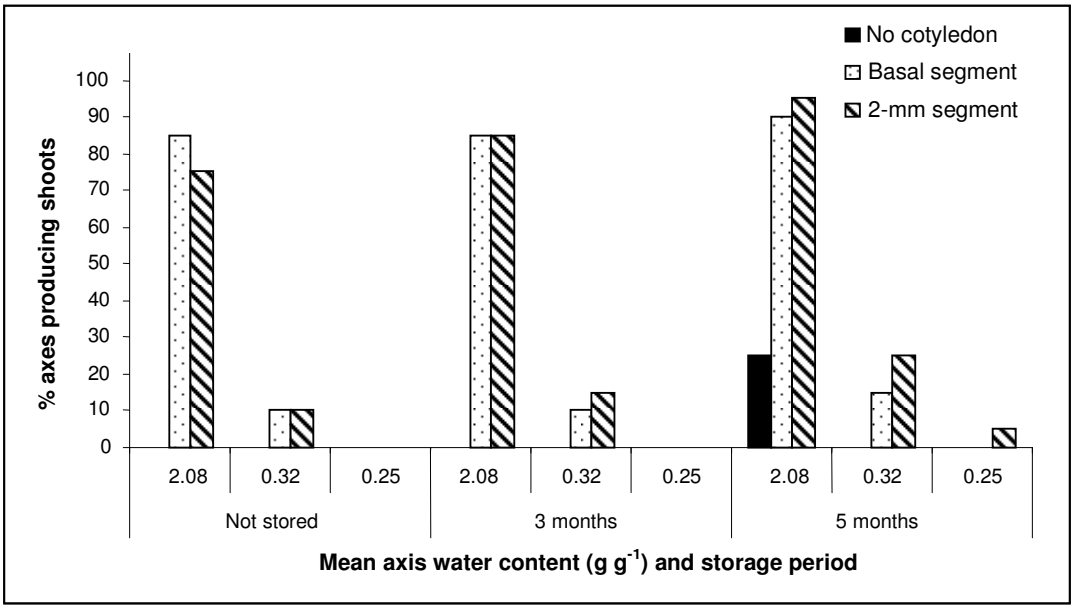


Figure 3.19B: Shoot (with root) production by explants without, and with, different-sized cotyledonary segments from newly-harvested and stored seeds of *T. dregeana* flash-dried to different water contents and germinated on WPM + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

Shoot production appeared more sensitive to dehydration than root production, with only 10 – 25% of explants producing shoots after flash-drying for 150 min (Figure 3.19B) to around 0.32 g g^{-1} (Figure 3.18) for explants with a segment of cotyledon attached. Although percentage shoot production by these explants increased with seed storage period, this was not significant (Chi-squared, $p > 0.05$). In addition, no shoot production was observed by any explants with no, or different-sized, cotyledonary segments after flash-drying to water contents of approximately 0.25 g g^{-1} (180 min), except after five months seed storage where 5% shoot production was observed in explants with a 2-mm segment of cotyledon (Figure 3.19B). This was, however, statistically not significant (Chi-squared, $p > 0.05$). It is also noteworthy that significant shoot production from explants with no vestiges of cotyledon attached (Chi-squared, $p < 0.05$) was observed only after seeds had been stored for five months, and axes not dehydrated (Figure 3.19B). In contrast, no equivalent explants from newly-harvested seeds produced shoots (Figure 3.17B and 3.19B)

Thus, storage period significantly increased the potential for shoot development by non-dried excised axes of *T. dregeana*.

Trichilia emetica

Explants from newly-harvested seeds of *T. emetica* were dehydrated using two methods. The first, slower method involved dehydrating explants on filter paper under sterile conditions in a laminar air-flow, while rapid flash-drying constituted the second method.

3.2.2.1 Laminar air-flow drying

Dehydration for 3 h was selected based on previous work carried out by Kioko (2003) and was adjusted by 30 min above and below this time to compare differences in dehydration responses as recalcitrant seeds are known to exhibit inter-seasonal variability (Berjak and Pammenter 2004a).

Seed availability was curtailed by the short fruiting season. Thus, axis water content following laminar air-flow dehydration was determined only for explants with no vestiges of cotyledon and those with just the basal cotyledonary segments attached. In addition, the effect of slow or laminar air-flow dehydration on germination was compared between newly-harvested seeds and those stored for two weeks. It was not possible to store seeds for longer than this because of a problem of fungal proliferation.

Attachment of the basal cotyledonary segment did not significantly affect axis water content either before or after seed storage or explant drying (One-way ANOVA, $p > 0.05$) as reflected in Figure 3.20. Water content was reduced to approximately 0.20 g g^{-1} after dehydration for 3 h and remained within the 0.22 - 0.19 g g^{-1} range after different drying times (Figure 3.20). Initial axis mean water content (1.75 g g^{-1}) of explants from two-week stored seeds was not significantly different from the initial water content of those from newly-harvested seeds, and this remained so after dehydration.

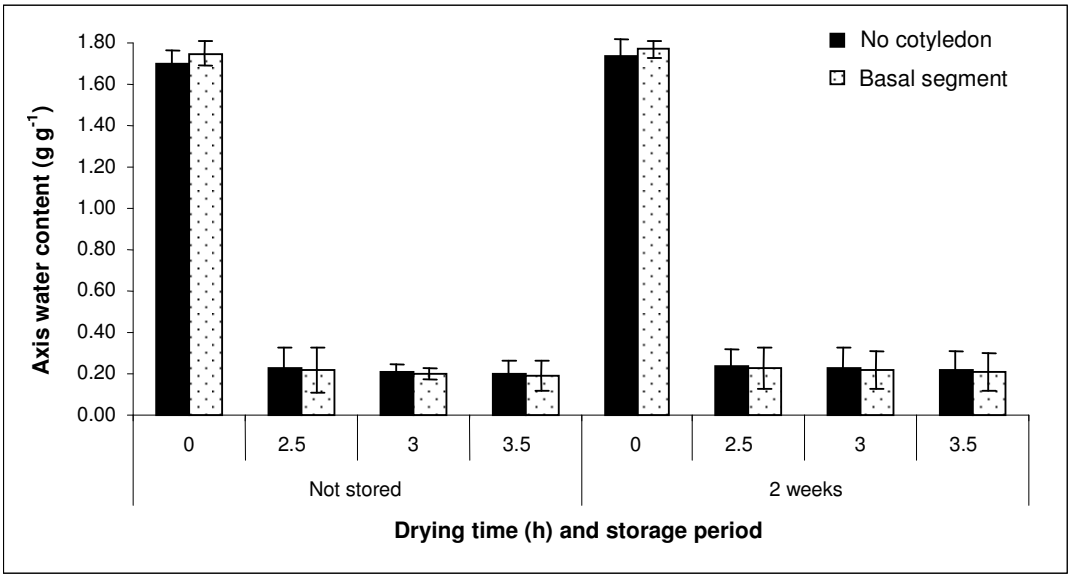


Figure 3.20: Changes in mean axis water content of explants without, and with, the basal cotyledonary segment from newly-harvested and two-week stored seeds of *T. emetica* before and after dehydration in a laminar air-flow. Bars indicate standard deviation. n = 5.

After each drying period, explants were plated on MS medium with, and without, activated charcoal (AC). However, the different media did not significantly affect (Chi-squared, $p > 0.05$) percentage root or shoot production (Figure 3.21A and Figure 3.21B respectively). Thus, the simpler medium, MS only, was selected for the remainder of this aspect of the study.

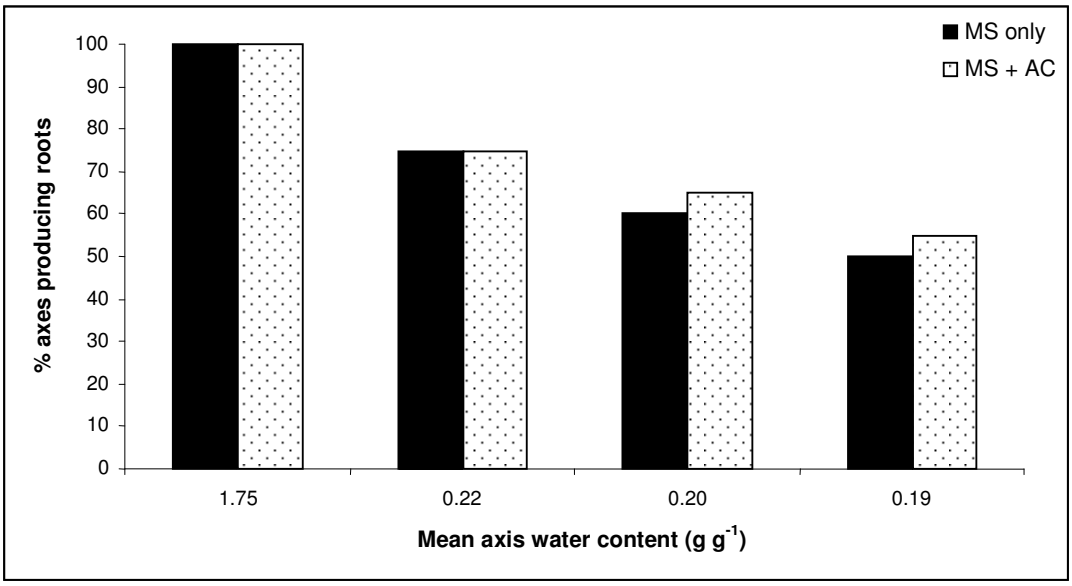


Figure 3.21A: Root production by axes of *T. emetica* with the basal cotyledonary segments attached, from newly-harvested seeds, dried in a laminar air-flow to different water contents and germinated on either MS or MS + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

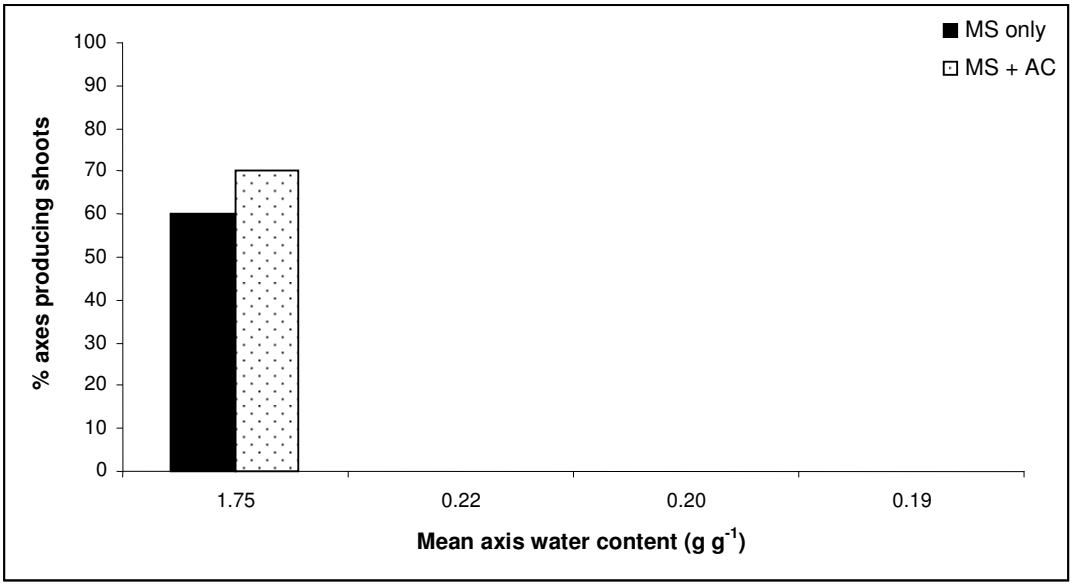


Figure 3.21B: Shoot (with root) production by axes of *T. emetica* with the basal cotyledonary segments attached, from newly-harvested seeds, dried in a laminar air-flow to different water contents and germinated on either MS or MS + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

Percentage root production prior to dehydration was not significantly different between explants with or without the basal cotyledonary segment. However, percentage shoot production of non-dehydrated explants with the basal cotyledonary segment or no remaining cotyledonary tissue, was significantly different (Chi-squared, $p < 0.05$). Of all non-dehydrated explants germinated with the segment of cotyledon attached, 55% developed shoots whereas only 5% of explants with no vestiges of cotyledon attached developed shoots (Figure 3.22). Shoots from explants of newly-harvested seeds were more sensitive to dehydration than were roots, as shoot development was negligible after dehydration irrespective of whether any cotyledonary tissue was left attached or not (Figure 3.22). Dehydration was also significantly correlated with decreased root production (Chi-squared, $p < 0.05$) for explants with or without basal cotyledonary segments (Figure 3.22). While percentage root formation was higher in explants with attached cotyledonary basal segments after dehydration in a laminar air-flow, this was not statistically significant (Chi-squared, $p > 0.05$).

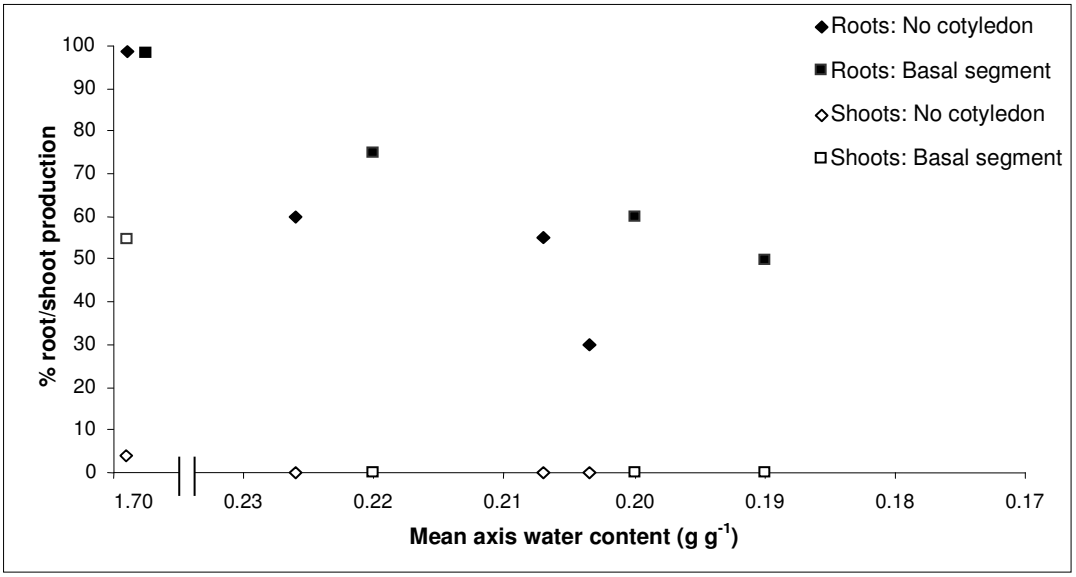


Figure 3.22: Root and shoot (with root) production by explants without, and with, attached cotyledonary basal segments from newly-harvested seeds of *T. emetica* dried in a laminar air-flow and germinated on MS. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

Although axis water content increased during storage (Figure 3.20), presumably by import from the cotyledons, it was not significantly different from that of newly-harvested seeds (One-way ANOVA, $p > 0.05$). The percentage of explants developing roots before dehydration remained at 100%. Percentage shoot production was significantly different between axes with and without the basal cotyledonary segments after excision from stored seeds prior to dehydration (Chi-squared, $p < 0.05$). However, after the two-week storage period, at the initial water content (approximately 1.74 g g^{-1}), explants without remaining cotyledonary vestiges showed significantly increased shoot production compared with similarly-excised axes from newly-shed seeds (Chi-squared, $p < 0.05$; compare Figures 3.22 and 3.23). Once dehydrated to water contents between 0.24 – 0.21 g g^{-1}, however, only 5% of explants with the basal cotyledonary segment developed shoots after dehydration (Figure 3.23; Chi-squared, $p > 0.05$). Root production at water contents between 0.24 – 0.21 g g^{-1} was higher in explants with the attached basal segments than those without (Figure 3.23). However, this was not significantly different (Chi-squared, $p > 0.05$).

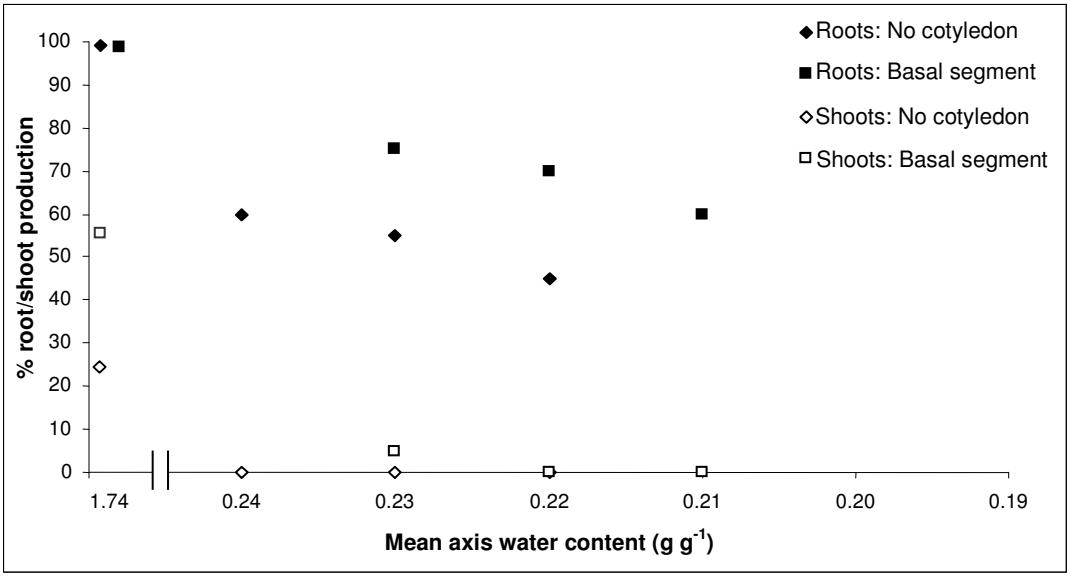


Figure 3.23: Root and shoot (with root) production by explants without, and with, attached cotyledonary basal segments from two-week stored seeds of *T. emetica* dried in a laminar air-flow and germinated on MS. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

While the two-week storage period significantly increased the potential for shoot development in non-dehydrated excised axes of *T. emetica*, the embryonic shoot apices remained essentially as sensitive to dehydration as those of axes from newly-harvested seeds.

In addition, the effect of BAP on shoot development was assessed for axes from which all cotyledonary tissue had been excised. This was done for both newly-harvested seeds, and those stored for two weeks using axes at the original water content and samples after 2.5 and 3.5 h of laminar air-flow drying. However, in all the cases the availability of BAP in the medium had no significant effect on shoot production (Table 3.11).

Table 3.11: Percentage of *T. emetica* axes with no cotyledonary attachment developing shoots (with roots) when dried in a laminar air-flow and cultured with, and without, the addition of BAP to the germination medium. Explants were excised from newly-harvested and stored seeds. n = 20.

		Not stored		2 Weeks	
Drying time (h)	**Water content (g g^{-1})**	MS	MS + BAP	MS	MS + BAP
0	1.75	0	0	20	25
2.5	0.22	0	0	0	0
3	0.20	0	0	0	0
3.5	0.19	0	0	0	0

Although no shoot development occurred after dehydration, 60% of explants treated with BAP showed callus production (Figure 3.24).

Figure 3.24: Response of embryonic axes of *T. emetica* excised without cotyledonary attachments from two-week stored seeds, cultured on a medium containing BAP. A, dried to 0.21 g g^{-1}; B, non-dried. Bar = 5 mm.

3.2.2.2 Flash-drying

Axis water contents were determined at 30 min intervals over 120 min of flash-drying for explants excised without, and with different-sized cotyledon segments from newly-harvested seeds. The presence or size of the attached cotyledonary segment did not significantly affect water content at any dehydration interval (One-way ANOVA, $p > 0.05$). The mean initial axis water content was 1.75 g g^{-1}, declining to around 0.48 g g^{-1} for all explant types after rapid dehydration for 120 min (Figure 3.25).

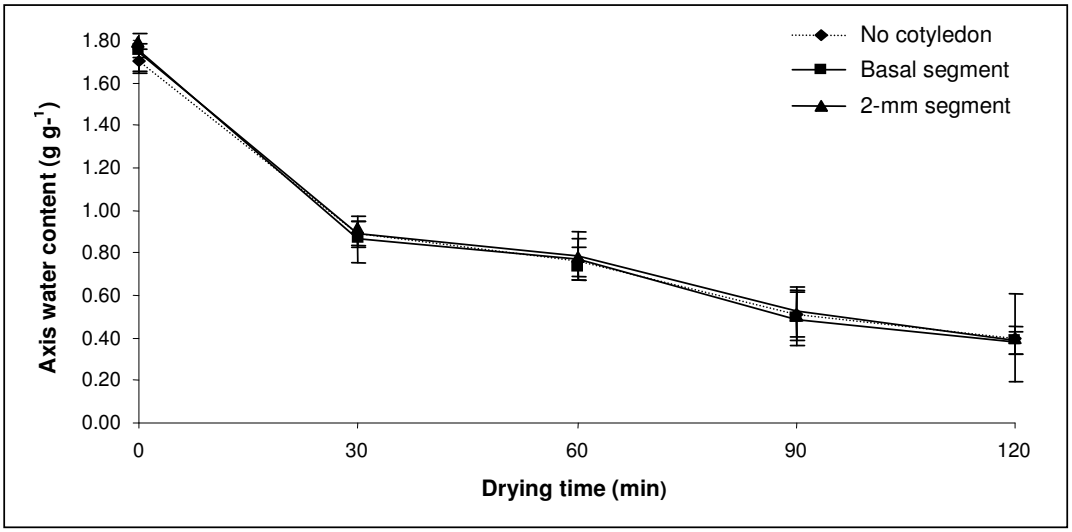


Figure 3.25: Changes in mean axis water content of explants without, and with, different-sized cotyledonary segments from newly-harvested seeds of *T. emetica* during rapid dehydration by flash-drying. Bars indicate standard deviation. n = 5.

As the size of attached cotyledonary segment did not significantly affect axis water content for newly-harvested (Figure 3.25) or stored seeds (data not shown), water contents of explants with the basal cotyledonary segments from newly-harvested seeds were compared with those from one-and two-week stored seeds (Figure 3.26). Axes from stored seeds were sampled at 30 min intervals for 90 min because of a shortage of seeds.

The water contents were not significantly different for axes when excised from newly-harvested or stored seeds, either before or after drying (One-way ANOVA, $p > 0.05$). The initial axis water contents ranged between 1.75 – 1.79 g g^{-1} before dehydration and these were reduced to approximately 0.51 – 0.56 g g^{-1} after dehydration for 90 min (Figure 3.26).

After each dehydration period explants were germinated on MS medium.

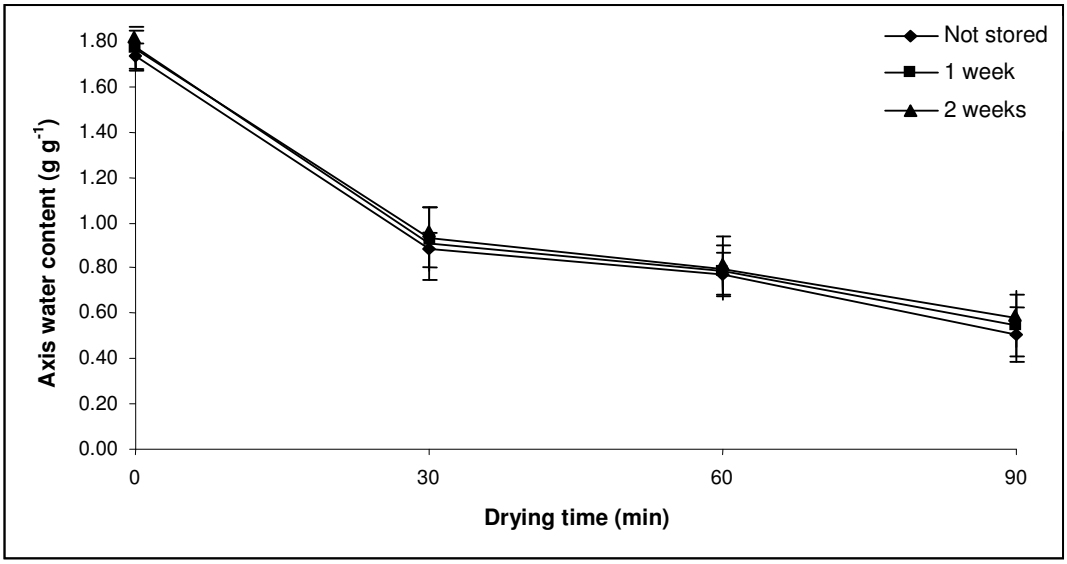


Figure 3.26: Changes in mean axis water content of explants with basal cotyledonary segments from newly-harvested and stored seeds of *T. emetica* during rapid dehydration by flash-drying. Bars indicate standard deviation. n = 5.

As had come to be expected (see section 3.1.2.1), root production was higher than shoot development at any water content (Figures 3.27A, B and C). The percentage of non-dried and dried explants producing roots did not differ significantly between explants with different-sized cotyledon segments irrespective of storage period (Chi-squared, $p > 0.05$). However, it is noteworthy that the lowest percentage of explants producing roots was when no vestiges of cotyledon were left attached, for both newly-harvested and stored seeds. Furthermore, the percentage of explants producing roots decreased significantly after dehydration (Chi-squared, $p < 0.05$) as shown in Figure 3.27A, B, and C.

For non-dried explants from newly-harvested seeds the absence, or presence and size, of attached cotyledonary segment significantly affected the percentage of shoots produced (Chi-squared, $p < 0.05$), shoots failing to develop from explants with no vestiges of cotyledon attached (Figure 3.27A). However, after storage for one and two weeks, these parameters were not significant for shoot production by non-dried explants (Figure 3.27B and Figure 3.27C respectively). Shoot production of explants with no vestiges of cotyledon attached was 40% after both seed storage periods.

After dehydration, even by the first sampling interval when mean water content was 0.90 g g^{-1} shoot production decreased significantly (Chi-squared, $p < 0.05$) in newly-harvested (Figure 3.27A), one-week stored (Figure 3.27B) and two-week stored seeds (Figure 3.27C), irrespective of the absence, or size of attached cotyledonary segment.

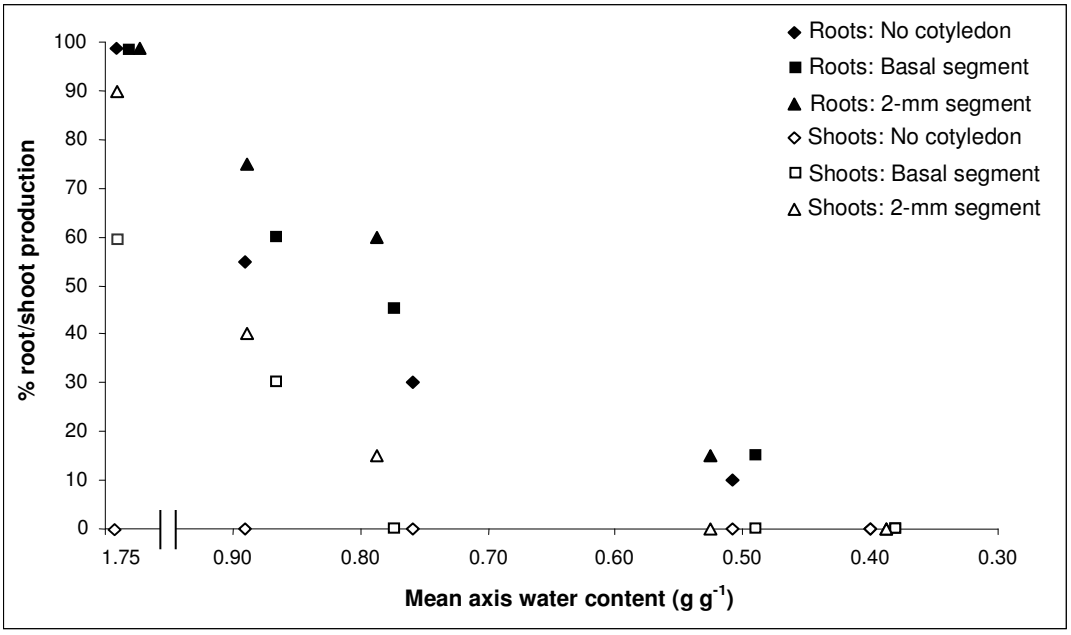


Figure 3.27A: Root and shoot (with root) production by explants without, and with, different-sized cotyledonary segments from newly-harvested seeds of *T. emetica* flash-dried and germinated on MS. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

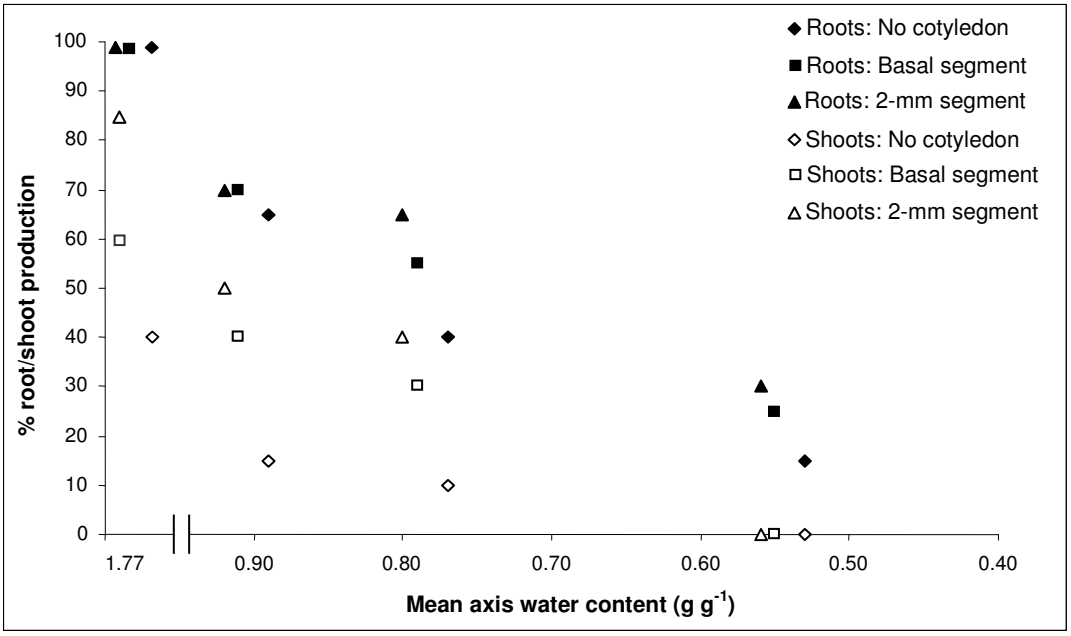


Figure 3.27B: Root and shoot (with root) production by explants without, and with, different-sized cotyledonary segments from one-week stored seeds of *T. emetica* flash-dried and germinated on MS. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

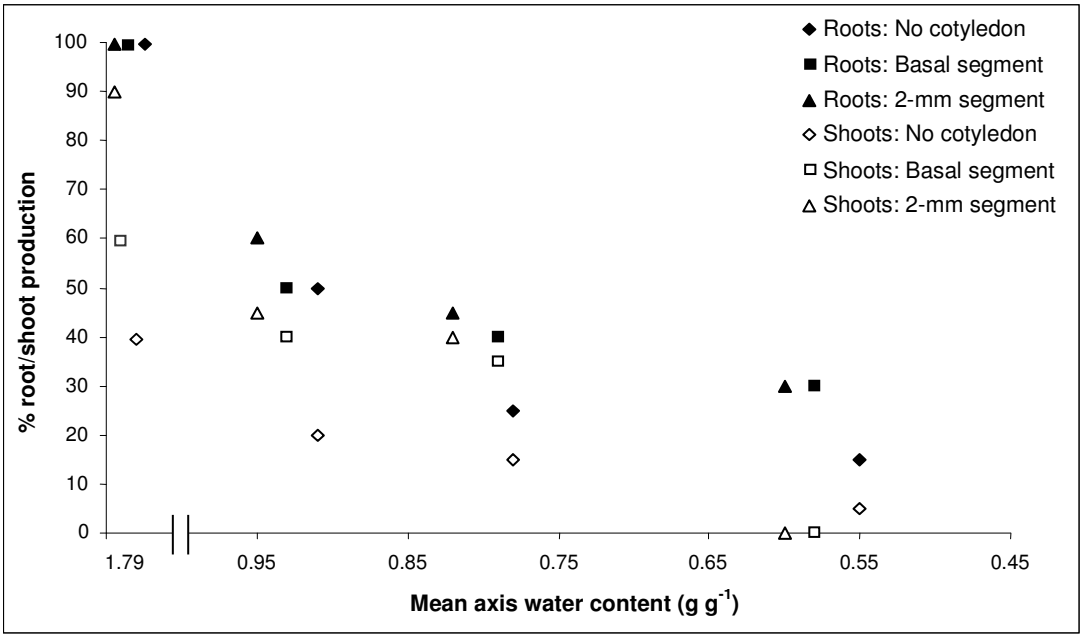


Figure 3.27C: Root and shoot (with root) production by explants without, and with, different-sized cotyledonary segments from two-week stored seeds of *T. emetica* flash-dried and germinated on MS. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

Overall, flash-drying below 0.8 g g^{-1} significantly decreased root and shoot production (Chi-squared, $p < 0.05$). However, root production either before or after rapid dehydration was not affected by the seed storage period (Chi-squared, $p > 0.05$). Shoot production significantly increased with storage period in non-dehydrated explants from which all vestiges of cotyledon had been removed (Chi-squared, $p < 0.05$). However, after dehydration there was a significant decrease in shoot production, irrespective of the seed storage period (Chi-squared, $p < 0.05$), indicating sensitivity of the shoot apex, irrespective of the degree of increased development that had been facilitated within two weeks.

The effect of BAP on shoot development was also monitored for non-dehydrated and flash-dried explants following cotyledon excision flush with the axis surface. In these experiments BAP had no significant effect (Chi-squared, $p > 0.05$) on dehydrated axes (Table 3.12).

Table 3.12: Percentage of *T. emetica* axes with no cotyledonary attachment developing shoots (with roots) when flash-dried and cultured with, and without, the addition of BAP to the germination medium. Explants were excised from newly-harvested and stored seeds. n = 20.

Drying time	**Water content**	**Not**	**Stored**	**1**	**week**	**2**	**weeks**
(min)	**(g g^{-1})**	MS	MS + BAP	MS	MS + BAP	MS	MS + BAP
0	1.75	0	0	40	45	40	45
30	0.90	0	0	15	15	20	25
60	0.77	0	0	10	15	15	20
90	0.56	0	0	0	0	5	5

Strychnos gerrardii

The changes in embryo water content as dehydration period increased were established for explants from seeds of newly-harvested yellow fruits of *S. gerrardii* (Figure 3.28). The axis in this endospermous seed has paper-thin cotyledons which were not removed for any of the investigations, as it was considered that they would not affect the thermal mass of the embryo during cooling for cryopreservation. After extraction from yellow fruits, initial mean embryo water content was 1.28 g g^{-1} and declined to around 0.12 g g^{-1} after rapid dehydration by flash-drying for 60 min (Figure 3.28).

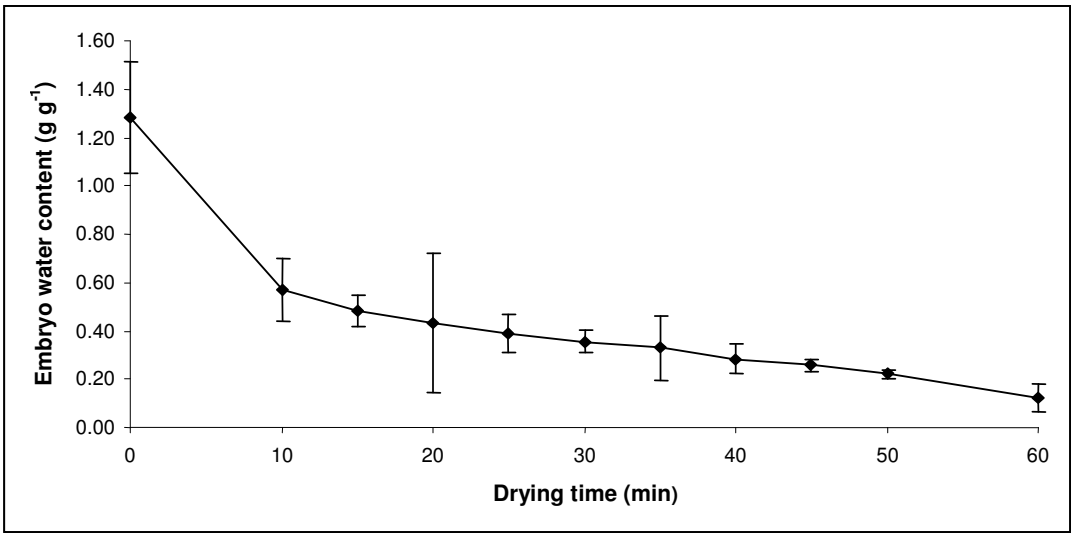


Figure 3.28: Changes in mean embryo water content during rapid dehydration by flash-drying of explants from newly-harvested seeds of *S. gerrardii* extracted from yellow fruits. Bars indicate standard deviation. n = 5.

The germination medium used [half-strength MS medium supplemented with activated charcoal (½ MS + AC)] was selected from studies on embryo germination (see section 3.1.2.2). As was observed for the other two species in this study, shoot apices were more sensitive to dehydration than root meristems. The percentage of embryos forming roots remained at 100% after dehydration and began to decline only once water contents of approximately 0.28 g g^{-1} were reached (Figure 3.29) with the decrease in root production after dehydration not being significant (Chi-squared, $p > 0.05$). However, only 40% of the total number of non-dehydrated embryos produced shoots (Figure 3.29), which declined significantly after dehydration (Chi-squared, $p < 0.05$) to 20% at a

mean water content of 0.57 g g^{-1}, and ceased at water contents lower than 0.39 g g^{-1} (Figure 3.29).

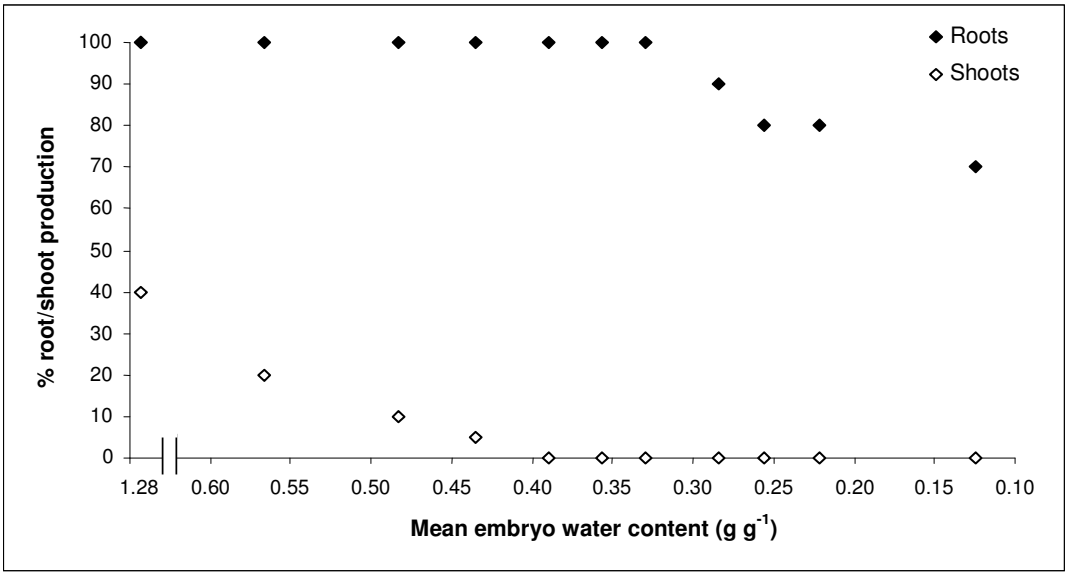


Figure 3.29: Root and shoot (with root) production by embryos from newly-harvested seeds of *S. gerrardii* extracted from yellow fruits flash-dried and germinated on ½ MS + AC. Mean embryo water content (n = 5) interpolated from the same drying time-course as the embryos set out to germinate (n = 20).

After extraction from yellow fruits, seeds were wet-stored (see section 2.2.2) for periods up to eight weeks. Initial water contents of embryos varied slightly with storage period, but were not significantly different (One-way ANOVA, $p > 0.05$). When subjected to flash-drying, water contents for explants became essentially similar, irrespective of the seed storage period (Figure 3.30). Drying times of 15, 30 and 50 min were selected for dehydration trials of embryonic axes from stored seeds as these times allowed for a water content range between 0.50 – 0.20 g g^{-1}.

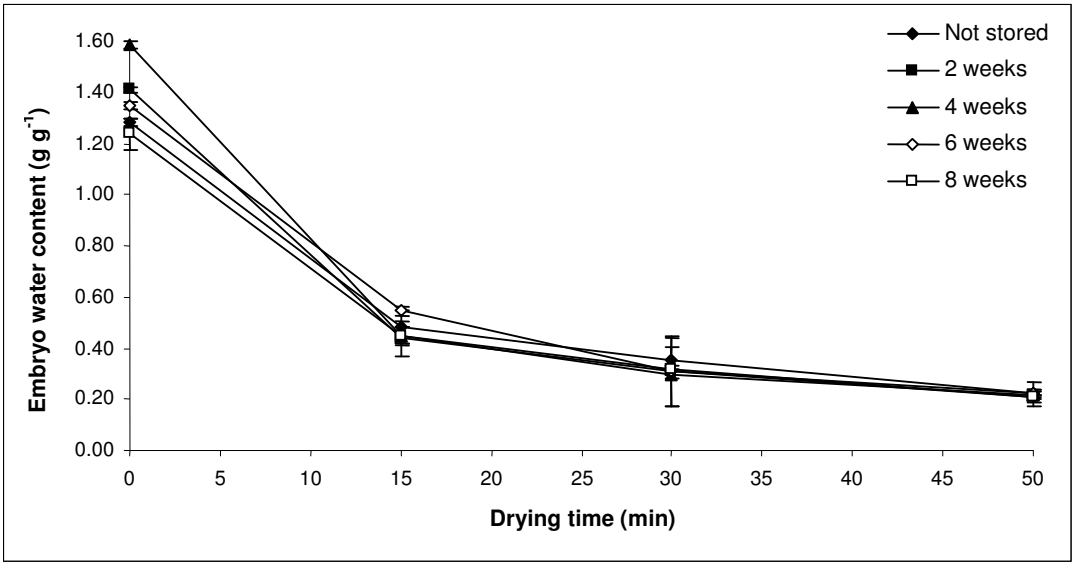


Figure 3.30: Changes in mean embryo water content of *S. gerrardii* explants of seeds from newly-harvested yellow fruits and seeds stored for two to eight weeks, during rapid dehydration by flash-drying. Bars indicate standard deviation. n = 5.

The percentage of non-dehydrated embryos forming roots was not significantly different for seeds stored for different periods (Chi-squared, $p > 0.05$). Although root production did begin to taper at water contents of approximately 0.30 g g^{-1} (Figure 3.31A), the decline was not significant (Chi-squared, $p > 0.05$), irrespective of the storage period.

Even though the percentage of non-dehydrated embryos forming shoots was highest in explants from newly-extracted (non-stored) seeds and decreased with storage period, the change was not significant (Chi-squared, $p > 0.05$). After dehydration, however, shoot production in explants from non-stored seeds had declined considerably when the mean water content dropped to 0.47 g g^{-1}, and no shoots were produced by embryos dried to 0.35 g g^{-1}. In contrast, embryos from stored seeds showed significantly higher shoot production at the lower water contents (Chi-squared, $p < 0.05$), with 15 – 20% of the explants from seeds stored for six and eight weeks producing shoots at a mean water content of 0.32 g g^{-1} (Figure 3.31B).

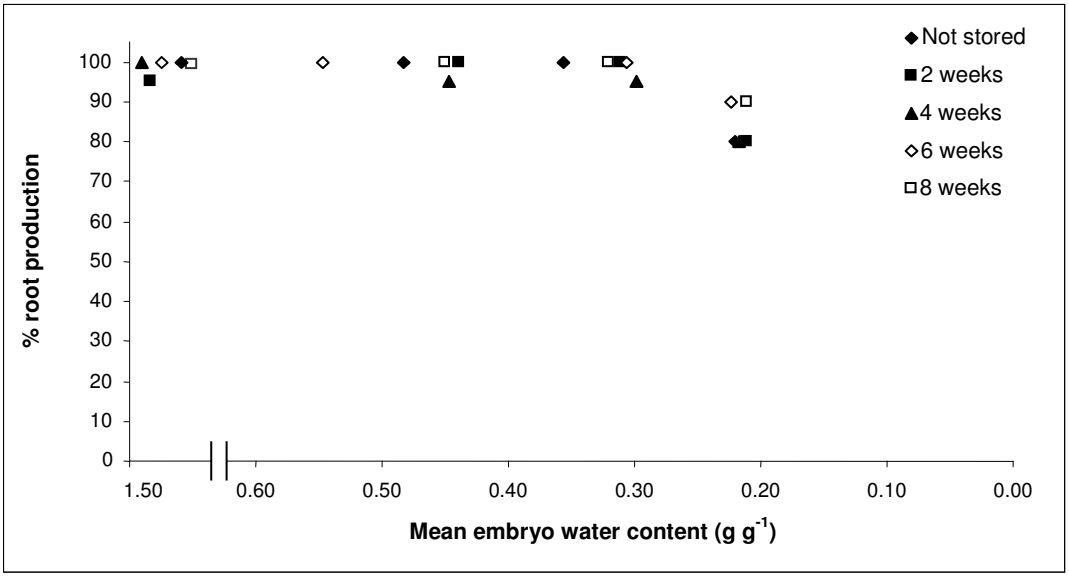


Figure 3.31A: Root production by embryos from newly-harvested and stored seeds of *S. gerrardii*, extracted from yellow fruit, flash-dried and germinated on ½ MS + AC. Mean embryo water content (n = 5) interpolated from the same drying time-course as the embryos set out to germinate (n = 20).

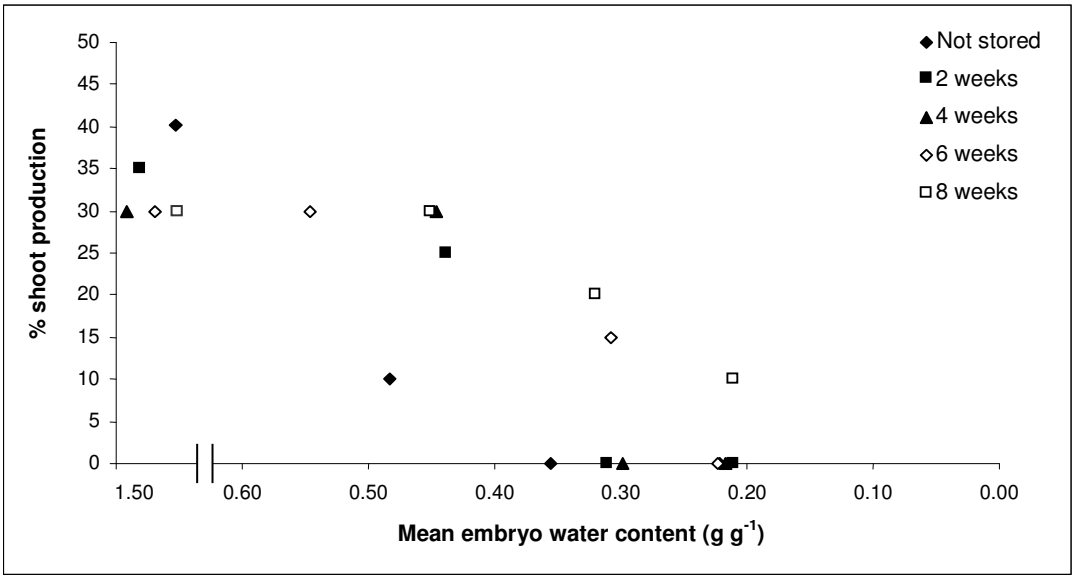


Figure 3.31B: Shoot (with root) production by embryos from newly-harvested and stored seeds of *S. gerrardii*, extracted from yellow fruits, flash-dried and germinated on ½ MS + AC. Mean embryo water content (n = 5) interpolated from the same drying time-course as the embryos set out to germinate (n = 20).

Overall, root production before or after rapid dehydration was not significantly affected by seed storage period (Chi-squared, $p > 0.05$), while shoot production after embryo dehydration was significantly better after seed storage (Chi-squared, $p < 0.05$).

3.2.2.3 Seed responses in relation to change in fruit colour

Embryos from seeds of fruits at three different maturity stages were also subjected to dehydration and the water contents and germinability determined. Initial embryo water contents increased as fruit colour changed from green to yellow, although this was not significant (One-way ANOVA, $p > 0.05$). On dehydration, the water content of embryos from less mature seeds of green-coloured fruits was consistently slightly lower than that of those from seeds of yellow/green and yellow fruits, independent of dehydration period (Figure 3.32). This however, was not significant (One-way ANOVA, $p > 0.05$).

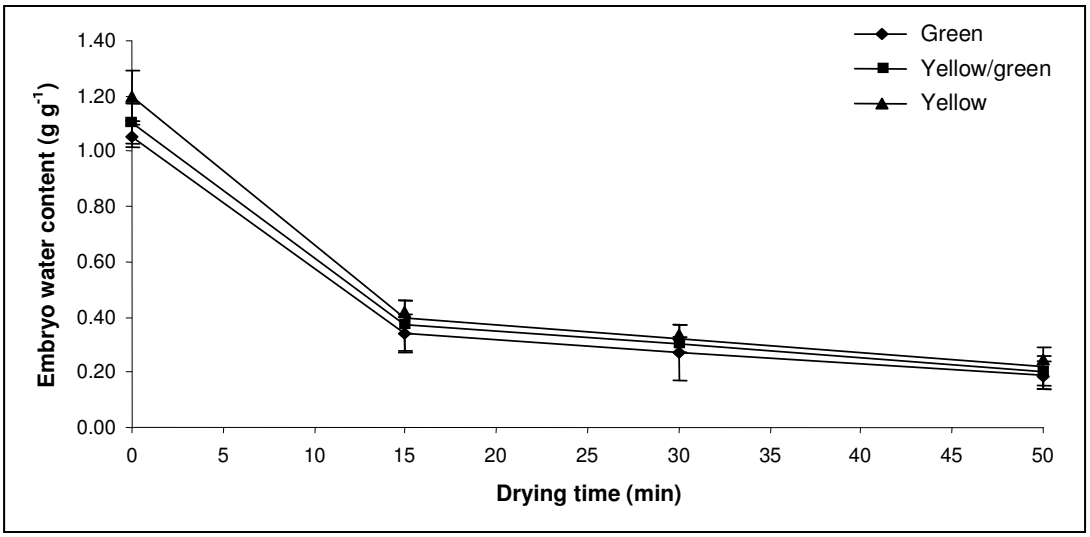


Figure 3.32: Changes in mean embryo water content of explants from *S. gerrardii* seeds at different stages of maturity (as indicated by fruit colour). Bars indicate standard deviation. n = 5.

The stage of seed maturity had no effect on root production in non-dehydrated embryos. Although on dehydration to lower water contents, embryos from more mature seeds had higher percentage root production than those from less mature seeds (Figure 3.33), these differences were not significant (Chi-squared, $p > 0.05$). The percentage of non-dehydrated embryos producing shoots was significantly affected by maturity, (Chi-squared, $p < 0.05$) with embryos from seeds from green-coloured fruits not producing shoots. Irrespective of the presumed state of maturity (coincident with fruit colour), the percentage of embryos forming shoots was zero after dehydration to a mean water content of 0.37 g g^{-1}, but the initial shoot production by non-dehydrated embryos was low for all maturity stages, being best at 20% for embryos of seeds from yellow fruits

(Figure 3.33). Therefore, dehydration *per se* may not be the reason for the lack of shoots.

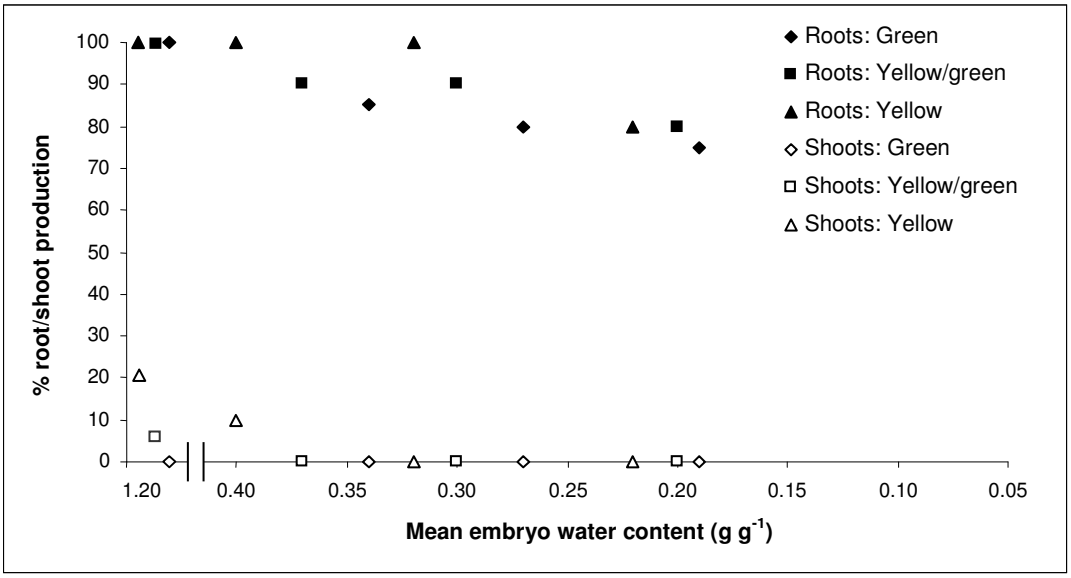


Figure 3.33: Root and shoot (with root) production by embryos excised from *S. gerrardii* seeds from fruits at different maturity stages (green - yellow). Embryos were flash-dried and germinated on ½ MS + AC. Mean embryo water content (n = 5) interpolated from the same drying time-course as the embryos set out to germinate (n = 20).

The effect of different concentrations of BAP on shoot production was assessed for embryos from seeds stored for six weeks after extraction from yellow fruits. In these experiments, embryos were flash-dried for 0 – 50 min and cultured onto a ½ MS medium supplemented with various concentrations of BAP. Irrespective of the concentration of BAP, no significant effects (Chi-squared, $p > 0.05$) on shoot production were recorded for either dehydrated or non-dehydrated embryos (Table 3.13).

Table 3.13: Percentage of *S. gerrardii* embryos producing shoots (in addition to roots). Embryos were excised from seeds removed from yellow fruits and stored hydrated for six weeks, flash-dried and cultured on a medium with or without BAP. n = 20.

Drying time (min)	Water content ($g\ g^{-1}$)	0 mg l^{-1} BAP	0.5 mg l^{-1} BAP	1 mg l^{-1} BAP	2 mg l^{-1} BAP
0	1.32	30	25	30	25
15	0.55	30	25	25	25
30	0.31	15	10	15	15
50	0.21	0	0	0	0

3.2.2.4 Dehydration of excised embryonic axes: concluding remarks

On dehydration, explants from newly-harvested seeds of all three species showed a decline in viability. This trend is typically displayed by recalcitrant seeds (reviewed by Berjak and Pammenter, 2004a). In addition, in this study, shoot production was found to be more adversely affected by desiccation than root production, as has previously been observed for the meliaceous species, *T. dregeana* and *T. emetica* (Kioko, 2003) and *Ekebergia capensis* (Perán *et al.*, 2006). Within these general trends, the following observations were made in this study: For *T. dregeana*, explants could be dehydrated from an initial mean water content of 2.04 g g^{-1} to water contents of approximately 0.30 g g^{-1} (Figure 3.15) with 40% root production and 10% shoot production (Figure 3.16A and Figure 3.16B respectively). Explants of *T. emetica* had a lower initial water content of 1.75 g g^{-1}, and could be dehydrated only to a water content of around 0.50 g g^{-1} before loss of the ability for shoot production (Figure 3.27A). The explants of *S. gerrardii* had the lowest initial mean water content of 1.28 g g^{-1} (Figure 3.28) and could be dehydrated to water contents as low as 0.20 g g^{-1} with 80% maintaining the ability for root production although shoots were not produced at that water content (Figure 3.29). These results were obtained for *T. dregeana* and *T. emetica* explants with cotyledonary attachments, as explants from newly-harvested seeds with no vestiges of cotyledon attached failed to develop shoots before, or after, dehydration. For *S. gerrardii*, cotyledons were neither wholly nor partially removed, yet the shoot apices were shown to be more sensitive to both excision and dehydration than was the root pole of the embryo. It is possible that although the cotyledons of *S. gerrardii* embryos were left intact, microscopically visible surface damage may have occurred on embryo excision. Although the embryonic axes of all three species were adversely affected by dehydration, in terms of injurious water contents, it appears that seeds of *T. emetica* are

the most desiccation-sensitive, followed by those of *T. dregeana*, while *S. gerrardii* seeds are the least desiccation-sensitive of those tested.

Many recalcitrant seeds continue development after shedding (Dodd *et al*., 1989; Berjak *et al*., 1992, 1993; Fu *et al*., 1994) and the extent of desiccation sensitivity varies among seeds of different species along the continuum of post-harvest behaviour (Sun and Liang, 2001). The difference in desiccation sensitivity among seeds of the three species in this study could be attributed to one or more of several factors. In the context of this study, the most obvious difference between the *Trichilia* spp. and *Strychnos gerrardii*, is the difference between fruit and seed structure. Coming from ultimately dehiscent fruits, seeds of *T. dregeana* and *T. emetica* have large, fleshy cotyledons. In contrast, mature fruits of *S. gerrardii* are pulpy and indehiscent, and the seeds are endospermous. Although there are no reports indicating that endospermous seeds, or their embryos, are less sensitive to desiccation, the cotyledons of *S. gerrardii* do not need to be severed from the axis to attain suitable- sized explants for cryopreservation. Hence, the embryonic axis is not compromised by direct injury prior to dehydration.

Although all fruits and seeds for the present study were harvested in the urban environment, the different natural habitats of the three species could also contribute to inherent differences in desiccation sensitivity. *Trichilia emetica* trees are naturally found in the coastal and riverine areas, *T. dregeana* trees occur in coastal forests more inland, and the natural provenance of *S. gerrardii* is in sandy habitats including dune forests (Pooley, 1993). In addition, the different seasonal periods of fruit development and harvesting times [January (*T. emetica*), May (*T. dregeana*) and November (*S. gerrardii*)] for fruits among the three species corresponded to seasonal differences in rainfall (104 mm, 29 mm and 96 mm for *T. dregeana*, *T. emetica* and *S. gerrardii* respectively[2]). In a study on 40 species in the family, Meliaceae, Hong and Ellis (1998) showed that species with desiccation-sensitive seeds typically occur in moist areas. Furthermore, Tweddle *et al*. (2003), Pritchard *et al*. (2004), and Daws *et al*. (2004, 2006b) have shown that a variation in seed desiccation sensitivity is related to geographical distribution of the trees, even within individual species. In general,

[2] SurGeo software package, developed for our laboratory by S3 Technologies: Postnet Suite 226, Private Bag X9118, Pietermaritzburg, 3200, South Africa.

recalcitrant-seeded species of temperate regions appear to be more desiccation-tolerant and have longer post-harvest life spans than those of tropical origin (Pammenter and Berjak, 2000, Daws *et al*., 2006a). In the present cases, however, all species studied have a sub-tropical to tropical natural distribution. Nevertheless, *S. gerrardii* grows in less protected habitats that are more prone to soil water evaporation than the forest habitats where the *Trichilia* spp. naturally occur. Thus, the increase in sensitivity to desiccation from *S. gerrardii* to *T. dregeana* and *T. emetica* could be at least partially explained in terms of their natural habitats.

Little is known about the evolutionary status of desiccation sensitivity shown by recalcitrant seeds. It has been postulated that desiccation-tolerance is the ancestral condition and that structural and morphological modifications, together with increased size and growth, could be related to the loss of tolerance (Dickie and Pritchard, 2002). However, Pammenter *et al.* (2000) suggested that the first seeds may well have been desiccation-sensitive with tolerance evolving early, and a number of times independently. Furthermore, although the trait of seed recalcitrance is somewhat more common within certain families, there is no widespread taxonomic link. Instead, as the several authors cited above have suggested, natural provenance may appears to be a major factor in the degree of seed desiccation sensitivity manifested.

Storing recalcitrant seeds under hydrated conditions facilitates the onset and continuation of germination processes, accompanied by increasing desiccation sensitivity, as first demonstrated for *Avicennia marina* (Farrant *et al*., 1986; 1993). However, *A. marina* seeds are poised for germination upon shedding, whereas recalcitrant seeds of other species are shed at a stage where a degree of development (which may take a prolonged time) is required before the onset of germination (Berjak *et al*., 1989). According to those authors, however, ongoing post-harvest development depends on the seeds not losing water, but also not requiring additional water from an extraneous source. In all three species presently studied, axis water content did not change significantly with storage period. Also, on dehydration, the pattern of water loss in fresh and stored seeds remained similar. As post-harvest development did not affect desiccation sensitivity in the seeds of the three species studied, it can be deduced that

none entrained germinative metabolism during storage unlike the situation of *A. marina* seeds (Farrant *et al*., 1986; 1993). Another study on three unrelated species producing recalcitrant seeds, showed that the rate at which subcellular germination events proceeded to the point at which additional water was required varied and appeared to determine the longevity of seeds maintained in storage (Farrant *et al*., 1989). Similarly, the seeds in the present study, although recalcitrant, could be stored for different periods before germination was observed, indicating that variation in degree of recalcitrant behaviour could well be related to their developmental status at shedding.

In seeds removed from storage, shoot development among non-dehydrated explants without cotyledon attachments increased to 20% (Figure 3.19B) and 40% (Figure 3.27B) for *T. dregeana* and *T. emetica* after five months, and one week of storage, respectively. This indicates that ongoing axis development during storage occurred, rendering the shoot apices somewhat less sensitive to the consequences of physical injury accompanying cotyledon excision. Axis continuity with the cotyledon occurs over two broad regions at the shoot pole in the embryos of *T. dregeana*. Thus, severing the cotyledons close to the axis leaves two lesions, one on either side of the shoot apex (see Figure 3.64). The physical damage upon cotyledon removal is in close proximity to the shoot apex, which may affect shoot development. By allowing the seeds to develop further under hydrated conditions, the shoot apex has been shown to elongate somewhat (Goveia *et al*., 2004); hence, the meristematic region becomes positioned further away from the wound sites caused by excision, and ultimately shoot development in some axes could occur. Thus, it is suggested that wounding of the axis during excision is the primary reason contributing to the lack of shoot development in explants from newly-harvested seeds.

The shoot region of explants from stored seeds also were relatively more desiccation-tolerant than in the newly-shed material, loss in shoot viability for *T. emetica* explants from which all cotyledonary tissue was removed occurring at approximately 0.70 g g^{-1} (Figure 3.27B and Figure 3.27C) rather than at 0.90 g g^{-1} as was the case for axes from non-stored seeds. Although even the lower water content is probably too high for cryopreservation, it indicates that if seeds were allowed to develop further, retention of

shoot apex integrity might be achieved at lower water contents, thus facilitating the use of zygotic explants for cryopreservation. Similarly, in *S. gerrardii*, desiccation tolerance at the shoot pole increased with storage period as some explants from seeds stored for six and eight weeks were able to develop shoots after dehydration to water contents of approximately 0.30 g g^{-1} (Figure 3.31B), whereas this was achievable only at 0.50 g g^{-1} by explants from non-stored seeds. Thus, it is proposed that seed/embryo/axis developmental status is a critical factor for successful cryopreservation as presently indicated for the three experimental species.

However, there appears to be a limit to the post-shedding (storage) development that can facilitate cryopreservation, as studies on amaryllids indicate that if seeds are allowed to develop too far in storage, the axes become less amenable to dehydration and cryopreservation (Naidoo, 2006; von Fintel, 2006). This is in accord with increasing desiccation sensitivity once germinative metabolism, *sensu stricto*, proceeds to the stage of cell division and increased vacuolation (Farrant *et al*., 1986; 1993; Berjak *et al*., 1989).

Besides developmental status, one of the other key factors determining the successful cryopreservation of recalcitrant explants is optimization of dehydration. Studies carried out by Berjak *et al*. (1993); Bonner (1996); Pammenter *et al*. (1998); Farrant *et al*. (1999); Walters *et al*. (2001) and Wesley-Smith *et al*. (1999, 2001b) showed that the more rapidly the desiccation-sensitive tissues are dried, the lower the water content they will tolerate before viability is lost, as the time allowed for unbalanced metabolism to occur is reduced. In this study two rapid dehydration techniques were tested using the embryonic axes of *T. emetica*. Laminar air-flow drying (which is somewhat slower) was the primary technique used based on previous work by Kioko (2003) and flash-drying was also applied to maintain uniformity with the other species.

The dehydration periods under the laminar air-flow were pre-selected based on previous experiments carried out by Kioko (2003). Thus, explants from freshly-harvested and stored seeds were dried to water contents between 0.20 and 0.25 g g^{-1} (Figure 3.20), which was similar to the water content of 0.26 g g^{-1} attained by Kioko (2003). On

dehydration, axes from newly-harvested seeds failed to produce shoots and 30 - 70% of explants produced roots, irrespective of whether cotyledons were completely excised, or not. The sensitivity of shoots and roots did not change after storage of *T. emetica* seeds for two weeks (Figure 3.23). When flash-dried, the explants reached water contents of 0.48 - 0.51 g g^{-1} after 90 min (Figure 3.26) but failed to produce shoots whether derived from stored or non-stored seeds. After dehydration, 20 - 60% of non-stored explants produced roots, irrespective of whether cotyledons were completely excised, or not; however, after two weeks of seed storage this range declined to 20 - 40% (Figure 3.27C).

From these results it appears that explants which were dried under the laminar-air flow yielded better results than flash-drying which is contrary to those observed by the authors cited above, as laminar air-flow drying is less rapid than flash-drying (Pammenter *et al*., 2002). However, seeds for the laminar-air-flow trials were collected from La Lucia, north of Durban whereas those for the flash-drying trials were collected from Queensburgh, slightly further south and a little inland. Seeds were therefore not collected from the same populations, hence the genetic variation might partly account for the unexpected response to rate of dehydration. Variability is also known to occur in different seasons among species, between different harvests within species and within seeds from the same harvest (Berjak *et al*., 1989; Finch-Savage and Blake, 1994; Drew *et al*., 2000) and could account for the responses. Furthermore, seeds collected from Queensburgh towards the end of the fruiting season were smaller and of a poorer quality visibly, and are therefore considered not to have given a true reflection of the potential for flash-drying.

The differences in the response to provision of BAP after dehydration of explants from newly-harvested and stored seeds of *T. emetica* were negligible, irrespective of the dehydration technique used (Table 3.11 and Table 3.12). Similarly, dehydrated explants of *S. gerrardii* showed no differences in shoot production when subsequently cultured on media with varying concentrations of BAP (Table 3.13). Although BAP is known to induce shoot production (see section 3.1.2.2), this pre-supposed that a sufficient proportion of apical meristem cells is not impaired either by excision or dehydration. A

widened range of BAP concentrations needs to be tested, but the outcome may well be the production of adventitious buds, as demonstrated by Perán *et al*. (2006) for explants of *Ekebergia capensis*.

Strychnos gerrardii explants from seeds from fruits at three different maturity stages could be flash-dried to water contents between 0.40 and 0.20 g g^{-1}, attained after 15 - 50 min (Figure 3.32)., Similar embryo water contents could be reached only after 72 - 96 h upon whole seed dehydration in silica gel. Thus, rapid dehydration is better achieved using excised embryonic axes than whole seeds, as reported for various species by Pammenter *et al*. (1998); Farrant *et al*. (1999); Walters *et al*. (2001) and Wesley-Smith *et al*. (2001b).

Embryonic axes are the most suitable explants for cryopreservation, but the ability for seedling generation is essential. When considering seeds from *S. gerrardii* fruits of different maturity stages, the percentage of dehydrated and non-dehydrated excised embryos producing roots was similar with 75 - 80% of explants dehydrated to 0.20 g g^{-1} (Figure 3.33) developing roots. Thus, the root pole of the explants is equivalently desiccation-tolerant irrespective of seed maturity. However, shoots failed to develop in embryos excised from seeds of green fruit before and after dehydration, and only those from seeds of yellow fruit produced shoots before (20%) and after (10%) dehydration to a water content of 0.40 g g^{-1} (Figure 3.33). Thus, desiccation sensitivity of the embryonic shoot apex appears to decrease in embryos extracted from fruits as they mature from green to yellow.

These results are directly contradictory to those obtained when whole seeds were excised from green, yellow/green and yellow fruits and subjected to relatively slower desiccation over 96 h. In those studies, seeds of green-coloured fruits which still had soft endosperm tissue, had embryos that were most tolerant to desiccation, when the seeds were dried over 96 h at the same rate as the hard endosperm seeds from yellow/green and yellow fruits. Furthermore, those seeds germinated more readily compared with seeds from yellow fruits, and grew more rapidly upon germination within the trial period.

The most feasible explanation of these apparently paradoxical responses to dehydration, may reside not in the embryos themselves, but in the nature and properties of the endosperm in seeds from green and yellow fruits in relation to the composition of the *in vitro* germination medium. The still soft endosperm of seeds from green-coloured fruits may have readily afforded nutrients for absorbtion by the embryo initiating germination even after dehydration. In contrast, it is possible that the naturally-consolidated state of the endosperm may have been rendered unfavourably hard upon experimental dehydration of seeds from yellow fruits, so imposing a state of sparingly-available nutrients for the embryos, thus compromising germination.

In the case of embryos excised from seeds of newly-harvested green and yellow fruits, it is suggested that while the *in vitro* medium favoured germination of embryos of the latter, it was less suitable for embryos from the seeds removed from green-coloured fruits. It is possible that these embryos lacked the mechanism(s) for adequate uptake of nutrients from the medium (as opposed to the endosperm), to support seedling establishment, as has been suggested for *Vicia sativa* and *Fagopyrum esculentum* by Müntz *et al*. (2001). Thus the apparently greater desiccation sensitivity of isolated *S. gerrardii* embryos from seeds of green-coloured fruits might rather be a manifestation of the unsuitability of a germination medium which favoured embryos from seeds of yellow, more mature fruits.

Obviously these conjectures must be tested by a series of carefully designed experiments assessing parameters individually.

3.3 Cryopreservation of embryonic explants

Cryopreservation of the whole seeds of species investigated was not attempted as the seeds were considered too large (Figures 2.2 and 2.4). The embryonic axes (*Trichilia* spp.)/embryos (*S. gerrardii*) provided potentially suitable explants for cryopreservation because of their size (Figures 2.6 and 2.7) and the appropriate water contents to which a proportion (although not a large sub-sample) could be dehydrated without compromising viability (Figures 3.19B, 3.23 and 3.31B)

Trichilia dregeana

3.3.1 Effect of cryoprotectants on water content and shoot formation

Before cryopreservation was attempted, a series of cryoprotectants was tested on explants with different-sized cotyledon segments, excised from newly-harvested seeds.

As described in Section 2.5, explants were soaked in various cryoprotectants and then dehydrated. The size of cotyledonary segment attached to explants had no significant effect (One-way ANOVA, $p > 0.05$) on water content after cryoprotection only, or after cryoprotection and subsequent dehydration. Thus, axis water contents for explants with basal cotyledonary segments attached are presented to compare differences between the effects of the cryoprotectant solutions. Axis water content of dried and non-dried explants was not significantly affected (One-way ANOVA, $p > 0.05$) by treatment with different cryoprotectants (Figure 3.34), although all cryoprotectants tested, with the exception of dextran (Dex), appeared to increase axis water content relative to that of non-cryoprotected axes. This effect was seen for non-dehydrated and dehydrated explants. Initial water contents ranged between 2.04 – 2.20 g g^{-1} and decreased to 0.40 – 0.21 g g^{-1} after dehydration (both with and without cryoprotection).

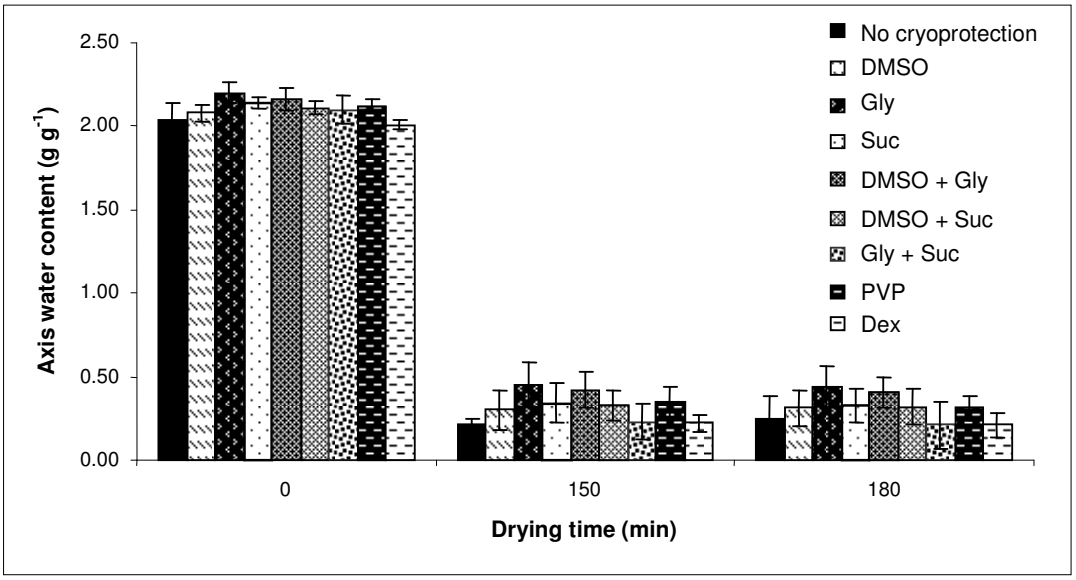


Figure 3.34: Changes in mean axis water content of explants with basal cotyledonary segments, from newly-harvested seeds of *T. dregeana*, treated or not treated with cryoprotectants and then rapidly dehydrated. Bars indicate standard deviation. n = 5.

Explants treated with cryoprotectants, but not dehydrated, were germinated on WPM containing AC. The cryoprotectants tested did not promote shoot development in explants with no vestiges of cotyledon remaining after excision. Noting that root production occurred in all cases, and that shoot production in explants with only the basal or 2-mm cotyledonary segments was similar (Chi-squared, $p > 0.05$, data not shown), shoot production of explants with basal cotyledonary segments only, has been shown to compare the responses to the various cryoprotectants.

The percentage of non-dried explants producing shoots when treated with cryoprotectants was not significantly different (Chi-squared, $p > 0.05$) from that obtained in the absence of cryoprotectants (Figure 3.35). After dehydration to a water content of approximately 0.25 g g^{-1} or higher, shoot production was achieved only by those explants not treated with cryoprotectant, or treated with sucrose, dextran, a combination of DMSO and sucrose (DMSO + Suc), and a combination of glycerol and sucrose (Gly + Suc). Of these, the highest number of axes producing shoots (Chi-squared, $p < 0.05$) occurred after the use of either dextran or the combination of glycerol and sucrose (Figure 3.35). Therefore, these two cryoprotectant treatments were selected for the subsequent cryopreservation trials.

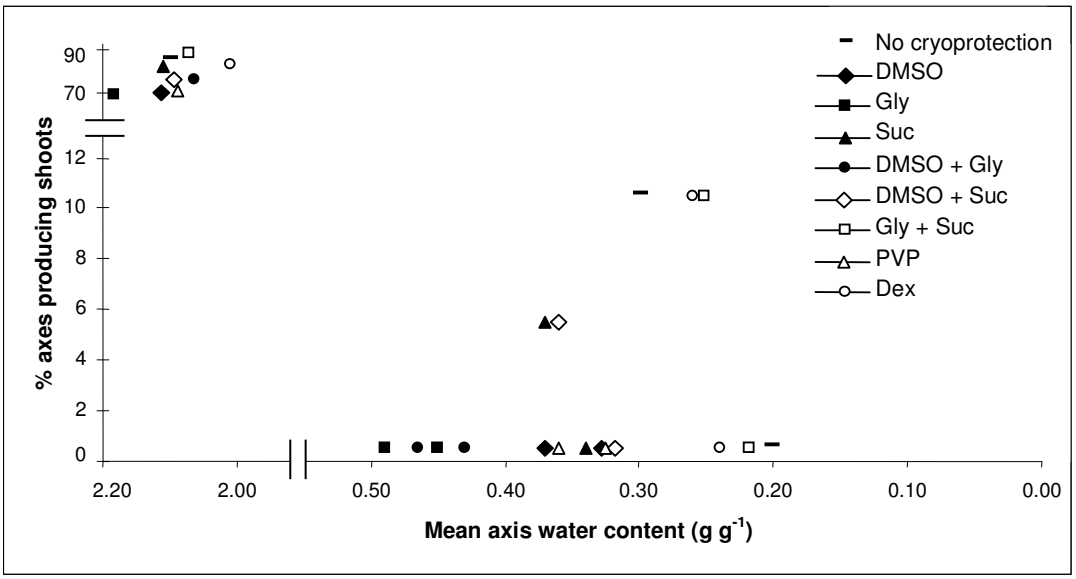


Figure 3.35: Shoot (with root) production by *T. dregeana* explants with basal cotyledonary segments, from newly-harvested seeds, treated or not treated with cryoprotectant solutions, flash-dried to different water contents and germinated on WPM + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

The size of cotyledonary segment attached to explants had no significant effect (One-way ANOVA, $p > 0.05$, data not shown) on water content of axes excised from seeds that had been stored. Thus, the axis water content for explants with basal cotyledonary segments was selected to compare differences between the effects of cryoprotectant solutions. Axis water content of dried and non-dried explants was not significantly affected (One-way ANOVA, $p > 0.05$) by storage period or different cryoprotectants (Figure 3.36).

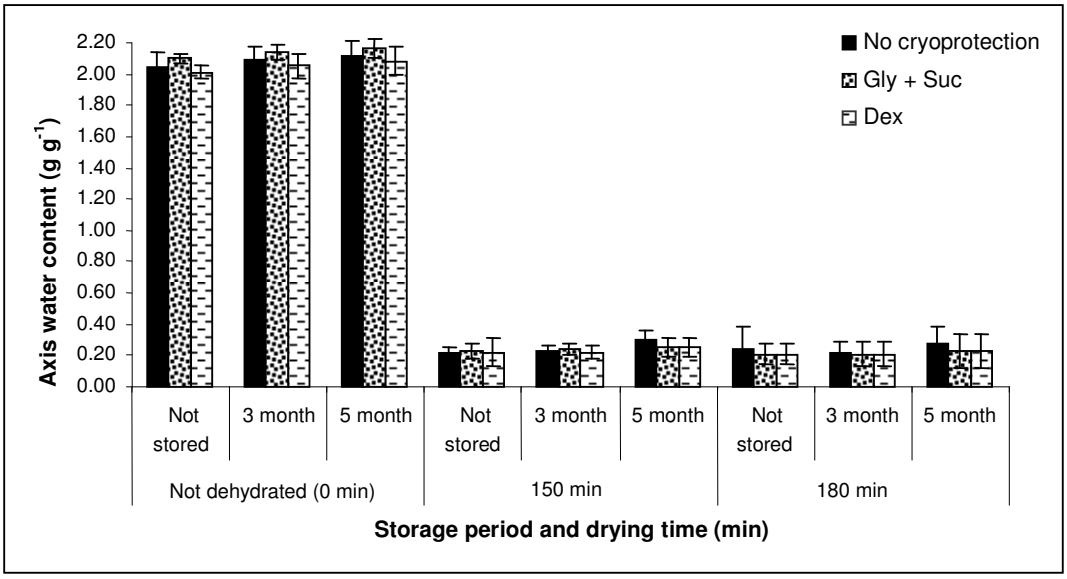


Figure 3.36: Changes in mean axis water content of explants with basal cotyledonary segments, from newly-harvested and stored seeds of *T. dregeana*, treated or not treated with selected cryoprotectants and rapidly dehydrated. Bars indicate standard deviation. n = 5.

The purpose of using cryoprotectant solutions is to improve recovery of cells after cryopreservation (Thammasiri, 1999; Fuller, 2004). These experiments assessed the effect of cryoprotectants prior to, and after, dehydration, with particular emphasis on shoot development. When axes were excised without cotyledonary vestiges from stored seeds, shoot production was not significantly affected (Chi-squared, $p > 0.05$) by the application of cryoprotectants, whether the explants were dehydrated or not (Table 3.14).

Table 3.14: Percentage of *Trichilia dregeana* axes producing shoots (with roots) after complete excision of the cotyledons. Seeds had been stored for five months, and axes were either treated or not treated with cryoprotectants, followed by flash-drying for up to 180 min. n = 20.

Drying time (min)	Water content (g g^{-1})	No cryoprotectant	Gly + Suc	Dex
0	2.10	25	15	10
150	0.30	0	0	0
180	0.21	0	0	0

Similarly, cryoprotection had no significant effect on shoot formation in explants with basal cotyledonary segments (Figure 3.37A) or 2-mm segments of cotyledons (Figure 3.37B), irrespective of the storage period or the extent of drying (Chi-squared, $p > 0.05$).

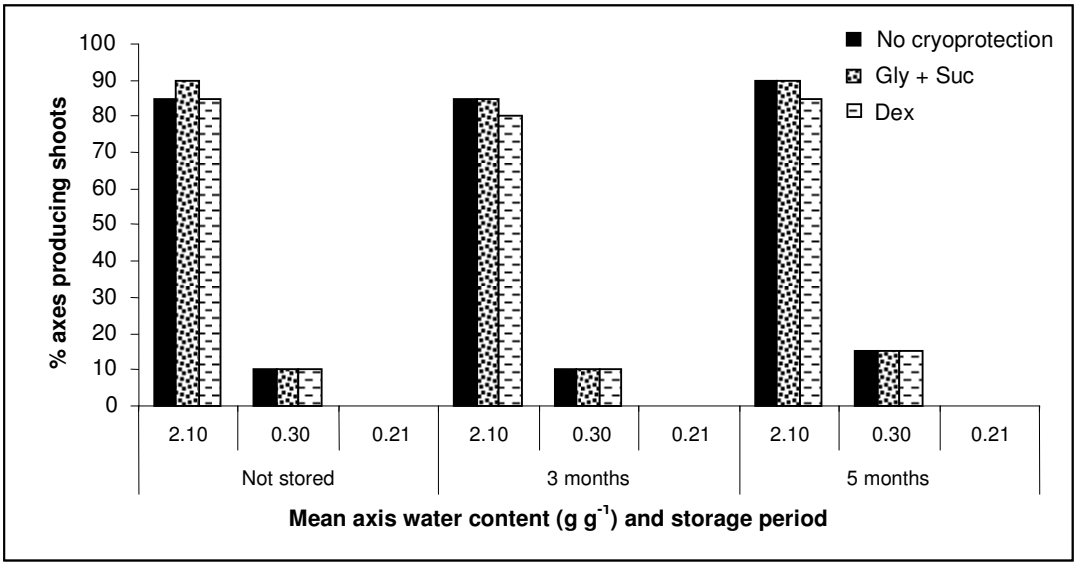


Figure 3.37A: Shoot (with root) production by *T. dregeana* explants with basal cotyledonary segments, from newly-harvested and stored seeds, treated or not treated with cryoprotectants, flash-dried to different water contents and germinated on WPM + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

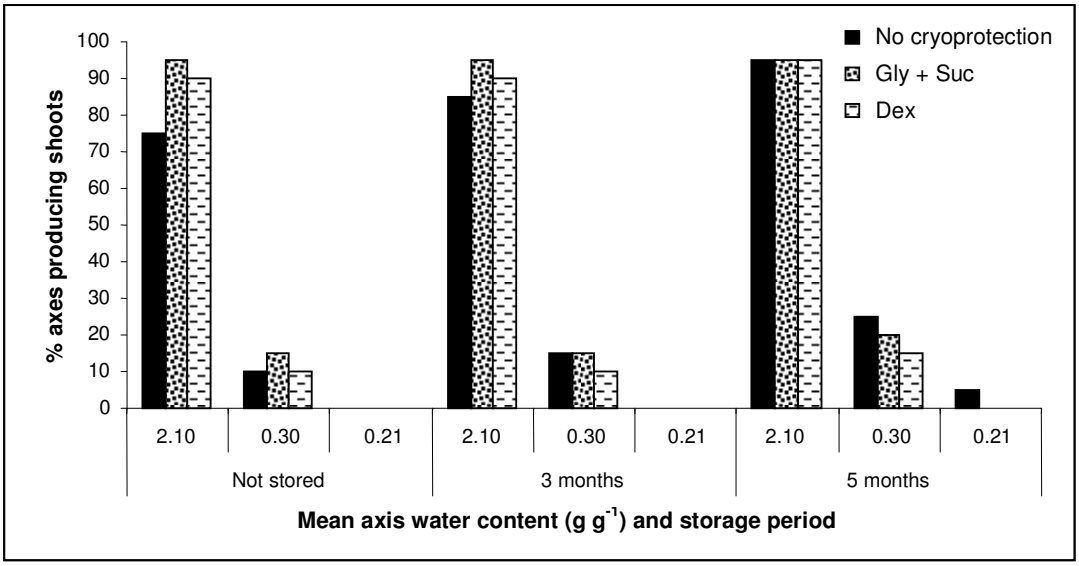


Figure 3.37B: Shoot (with root) production by *T. dregeana* explants with 2-mm cotyledonary segments, from newly-harvested and stored seeds, treated or not treated with cryoprotectants, flash-dried to different water contents and germinated on WPM + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

3.3.2 Cryopreservation: effects of explant size, dehydration, seed storage period and cryoprotection

Explants excised with different-sized segments of the cotyledons from newly-harvested and stored seeds were cryopreserved using two methods that achieved different cooling rates. As described in Section 2.5, slower cooling was done by placing explants into cryotubes which were then slotted into cryocanes and rapidly immersed into liquid nitrogen, while rapid cooling was achieved by mixing explants directly with sub-cooled nitrogen in a polystyrene cup immediately after cryoprotection and/or dehydration.

No explants from newly-harvested seeds survived cryopreservation when frozen in cryotubes, irrespective of water content after dehydration or the use of cryoprotectants. However, when explants were rapidly cooled, 15 - 20% survival (in terms of the potential to produce callus [see below]) was observed, but only for explants with basal and 2-mm cotyledonary segments, after dehydration to a water content range between 0.30 – 0.25 g g^{-1} (Table 3.15). No survival after cryopreservation was recorded after further exposure to dehydrating conditions.

Table 3.15: Percentage survival (potential for callus production) after cryopreservation of *T. dregeana* explants with different-sized cotyledonary segments from newly-harvested seeds either treated or not treated with cryoprotectants, flash-dried to 0.30 – 0.25 g g^{-1} and rapidly cooled. n = 20.

Cotyledon segment	No cryoprotectant	Gly + Suc	Dex
None	0	0	0
Basal	20	20	20
2-mm	20	15	15

The explant size had no significant effect on survival after cryopreservation, for explants of stored seeds (Chi-squared, $p > 0.05$, data not shown). Explants from stored seeds, dried to 0.30 – 0.25 g g^{-1} (Figure 3.38A) for 150 min had significantly higher survival rates after cryopreservation when the rapid cooling technique was used, rather than after slower freezing in cryotubes (Chi-squared, $p < 0.05$). Also, higher (but not significantly so) survival was achieved in explants from three month-stored seeds than from those that were newly-harvested, when rapidly cooled, irrespective of the cryoprotectant (Chi-squared, $p > 0.05$), except when treated with the combination of glycerol and sucrose (Figure 3.38A).

Cryoprotection did not increase survival after freezing – in fact, survival was significantly higher in the absence of cryoprotectants (Chi-squared, $p < 0.05$) as clearly indicated for explants from seeds stored for five months (Figure 3.38A). Ultimately, cryopreservation of explants from three-month stored seeds was the most successful as only those survived (retained the ability to produce callus) after both slower and rapid cooling, irrespective of the cryoprotectant.

Explants from stored seeds, dehydrated over 180 min to a mean water content of 0.22 - 0.21 g g^{-1} survived cryopreservation only when rapidly cooled (Figure 3.38B). At this water content, post-cryopreservation survival was significantly higher in explants from three-month than five-month stored seeds and also better in the absence of cryoprotectants (Chi-squared, $p < 0.05$). Although explants sustained the additional duration of drying, the extended exposure to the stress associated with desiccation is likely to have compromised the ability for survival after cryopreservation.

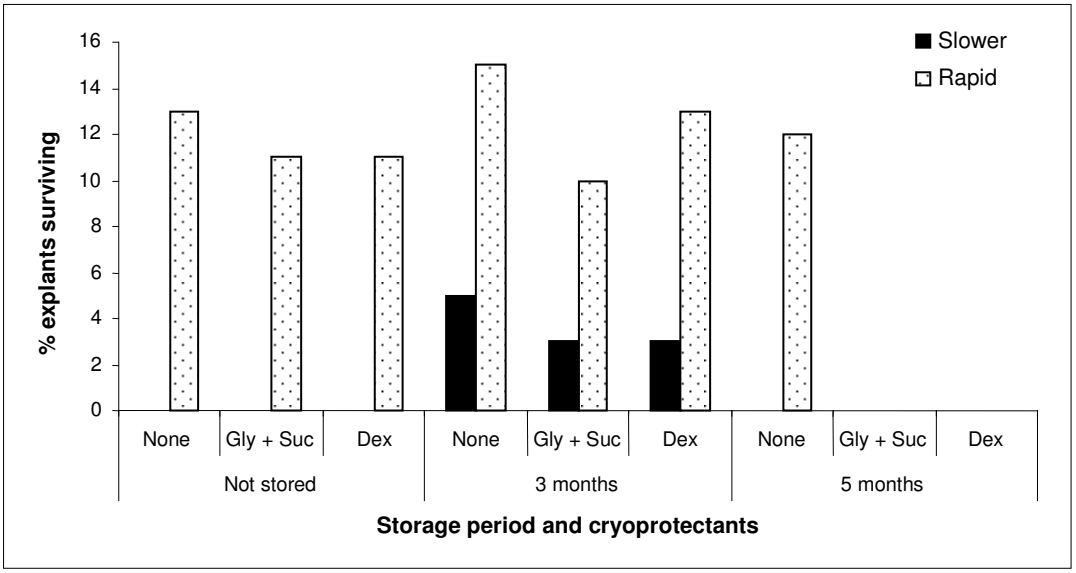


Figure 3.38A: Survival assessed as the ability to produce callus after cryopreservation of *T. dregeana* explants with basal cotyledonary segments from newly-harvested and stored seeds, either treated or not treated with cryoprotectants, flash-dried for 150 min to 0.30 – 0.25 g g^{-1} and cooled by the slower and rapid methods. n = 20.

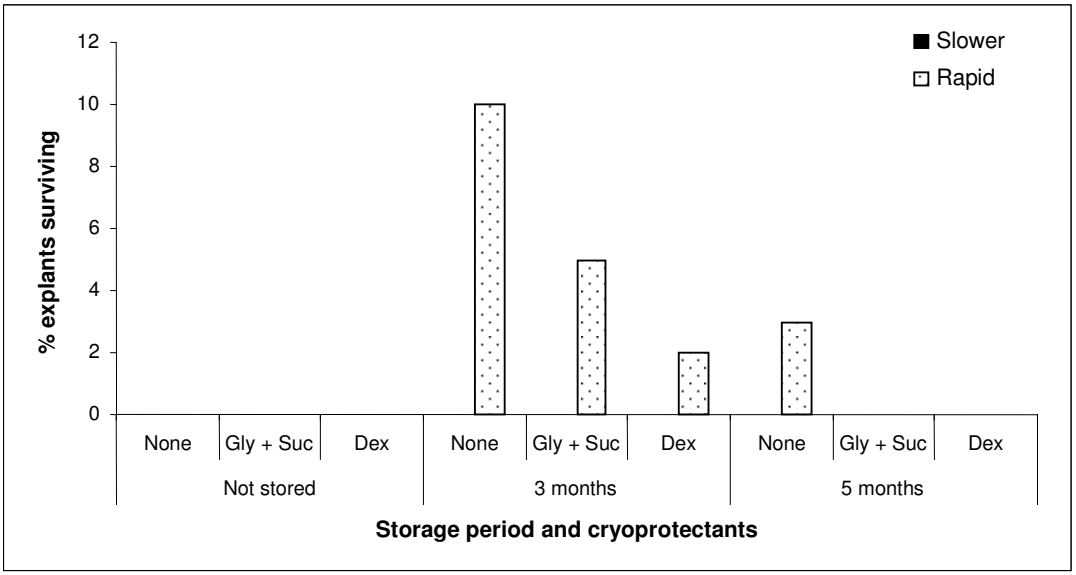


Figure 3.38B: Survival assessed as the ability to produce callus after cryopreservation of *T. dregeana* explants with basal cotyledonary segments from newly-harvested and stored seeds, either treated or not treated with cryoprotectants, flash-dried for 180 min to 0.22 - 0.21 g g^{-1} and cooled by the slower and rapid methods. Note that there was no survival after slower cooling. n = 20.

All survival after cryopreservation occurred in the form of callus produced at the root pole of the axis (Figure 3.39). In order to stimulate further callus production and, possibly embryogenicity of the callus, cryopreserved explants from stored seeds were sub-cultured onto WPM containing either 2,4-D (1 mg l^{-1}) or NAA (1 mg l^{-1}).

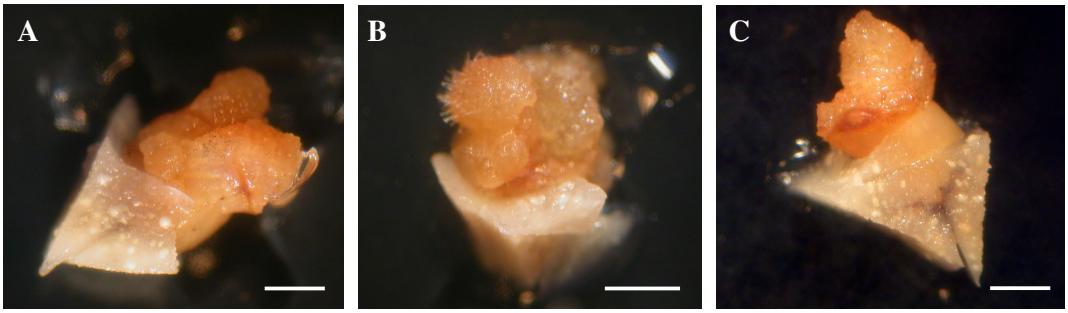


Figure 3.39: Survival after cryopreservation of *T. dregeana* axes with basal cotyledonary segments from newly-harvested (A); three month-stored (B) and five month-stored seeds (C), not treated with cryoprotectants, dried to a mean water content of 0.30 g g^{-1} and rapidly cooled. Bar = 0.5 mm.

Explants dried for 150 min to 0.30 - 0.25 g g^{-1} and frozen in cryotubes showed no further proliferation than was originally achieved (Figure 3.40) and the type of auxin had no significant effect on callus growth (Chi-squared, $p > 0.05$). Explants frozen similarly after dehydration for 180 min to 0.22 – 0.21 g g^{-1} and sub-cultured onto medium with the various auxins still did not initiate callus formation after sub-culture (data not shown).

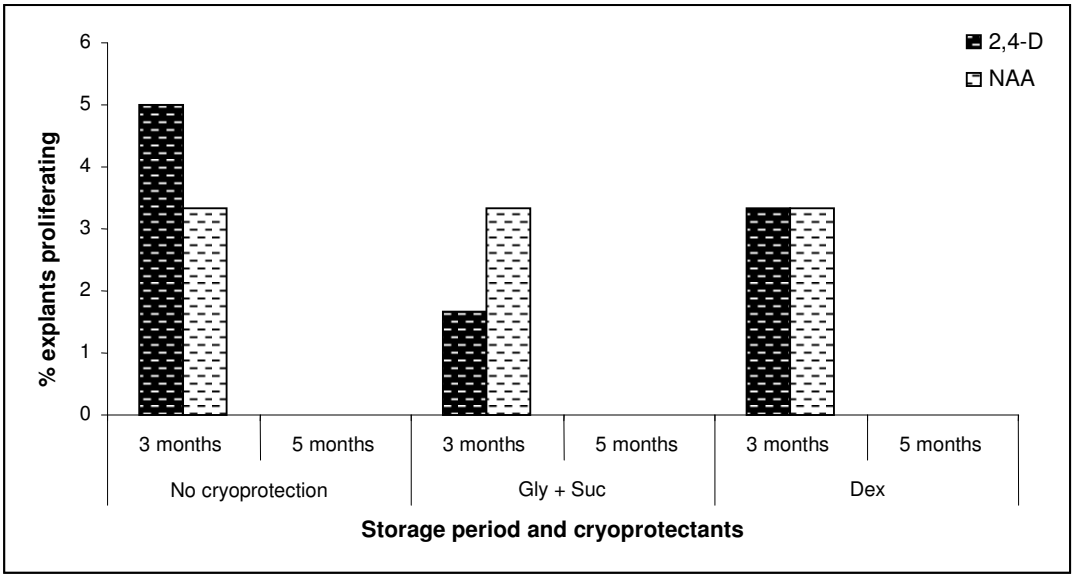


Figure 3.40: Percentage of explants producing callus from stored seeds of *T. dregeana*, treated or not treated with cryoprotectants, dried for 150 min to 0.30 - 0.25 g g^{-1}, frozen in cryotubes and sub-cultured on media with different auxins.

Explants which were rapidly cooled after dehydration for 150 min to 0.30 - 0.25 g g^{-1} showed significant increases in callus production on auxin-containing culture media, compared with those slowly frozen. In particular, explants from seeds stored for three months, and not treated with any cryoprotectants showed significantly higher callus production (Chi-squared, $p < 0.05$) upon the addition of either hormone to the regeneration medium (Figure 3.41A). Although callus proliferation occurred on both media tested, for these explants 2,4-D was significantly better (Chi-squared, $p < 0.05$) than NAA. Explants from seeds stored for five months responded equally on both media provided no cryoprotectants had been added (Figure 3.41A). However, their potential for callus production was significantly lower than explants from seeds that had been stored for three months (Chi-squared, $p < 0.05$).

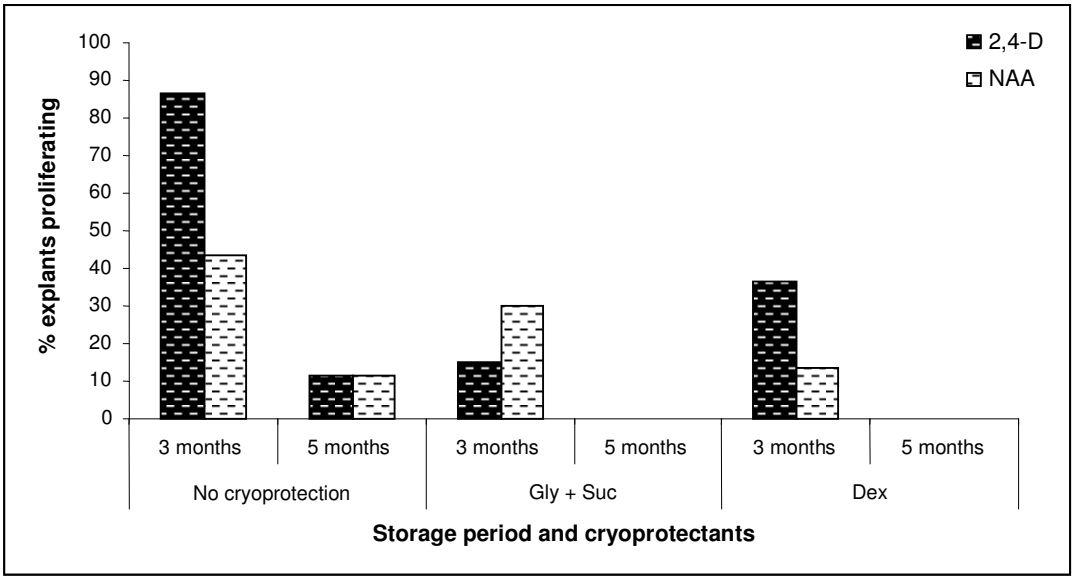


Figure 3.41A: Percentage of explants producing callus from stored seeds of *T. dregeana*, treated or not treated with cryoprotectants, dried for 150 min to 0.30 - 0.25 g g^{-1}, rapidly cooled and sub-cultured on media with different auxins.

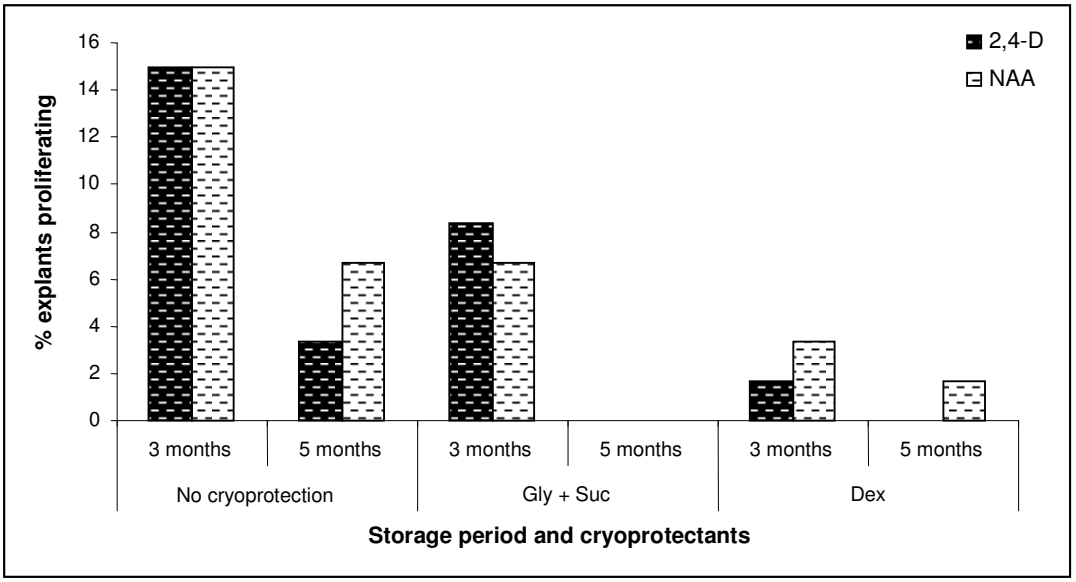


Figure 3.41B: Percentage of explants producing callus from stored seeds of *T. dregeana*, treated or not treated with cryoprotectants, dried for 180 min to 0.22 - 0.21 g g^{-1}, rapidly cooled and sub-cultured on media with different auxins.

Explants dehydrated to 0.22 – 0.21 g g^{-1} and cryopreserved by rapid cooling, also showed an increase in callus proliferation after sub-culture on an auxin-containing medium (Figure 3.41B), although this increase was not significant compared with

callus proliferation prior to sub-culture on an auxin-containing medium (Figure 3.38B). Explants that were not treated with cryoprotectants showed significantly higher callus proliferation than those with prior cryoprotectant treatment (Chi-squared, $p < 0.05$), on both auxin-containing (Chi-squared, $p > 0.05$).

All cryopreserved explants were rehydrated in aqueous 1μM $CaCl_2.2H_2O$ and 1mM $MgCl_2.6H_2O$ solution. While damage to the cryoprotected explants upon rehydration is possible due to the inrush of water from the aqueous solution, Perán *et al*. (2004) showed that slow rehydration has more deleterious effects than rapid rehydration.

The embryogenicity of the proliferated callus was then assessed in an attempt to develop a protocol to facilitate morphogenesis. However, the callus was assessed as non-embryogenic, based on the large size and elongated shape of the cells (Figure 3.42). Hence two different concentrations of BAP (0.25 mg l^{-1} and 0.5 mg l^{-1}) were then added to the media containing either 2,4-D or NAA to test for the induction of embryogenicity.

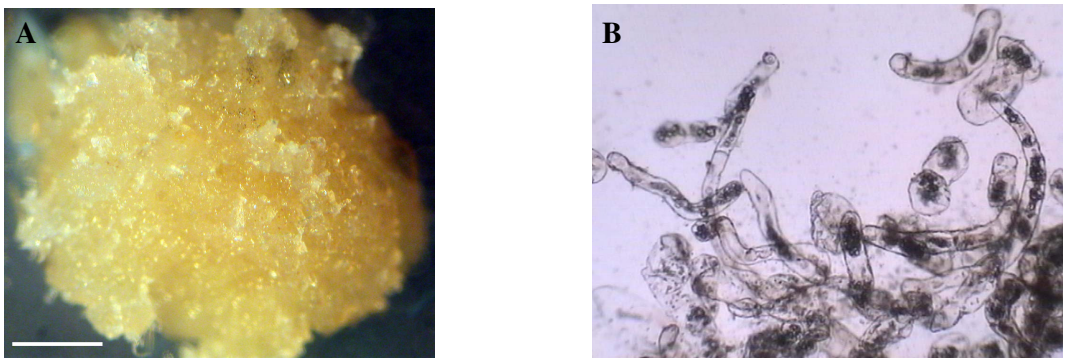


Figure 3.42: Callus produced after sub-culture onto a medium with 2,4-D (A, Bar = 0.3 mm) showing elongated non-embryogenic cells (B, Mag = 40X).

However, none of the media tested (supplemented with BAP, and either 2,4-D or NAA) induced embryogenicity, the callus remaining non-embryogenic. Thus, future experiments should be focus on inducing embryogenicity potential in callus produced from *T. dregeana* explants surviving cryopreservation.

Trichilia emetica

3.3.3 Effect of cryoprotectants on water content and shoot formation

Cryoprotectants were tested on explants with different-sized cotyledon segments, which were then dehydrated using the air stream of a laminar flow cabinet, as this had previously been established as the dehydration method of choice for embryonic axes of this species (Kioko *et al*., 2006).

The size of cotyledonary segment attached to explants had no significant effect (One-way ANOVA, $p > 0.05$, data not shown) on water content after cryoprotection only, or after cryoprotection and subsequent dehydration. Thus, axis water contents for explants with basal cotyledonary segments only, have been shown to compare differences after treatment with various cryoprotectant solutions. Axis water content of dried and non-dried explants was not significantly affected (One-way ANOVA, $p > 0.05$) by treatment with the various cryoprotectants (Figure 3.43). Initial water contents ranged between 1.73 – 1.78 g g^{-1} and decreased to 0.27 – 0.20 g g^{-1} after dehydration (both with and without cryoprotection), in all cases.

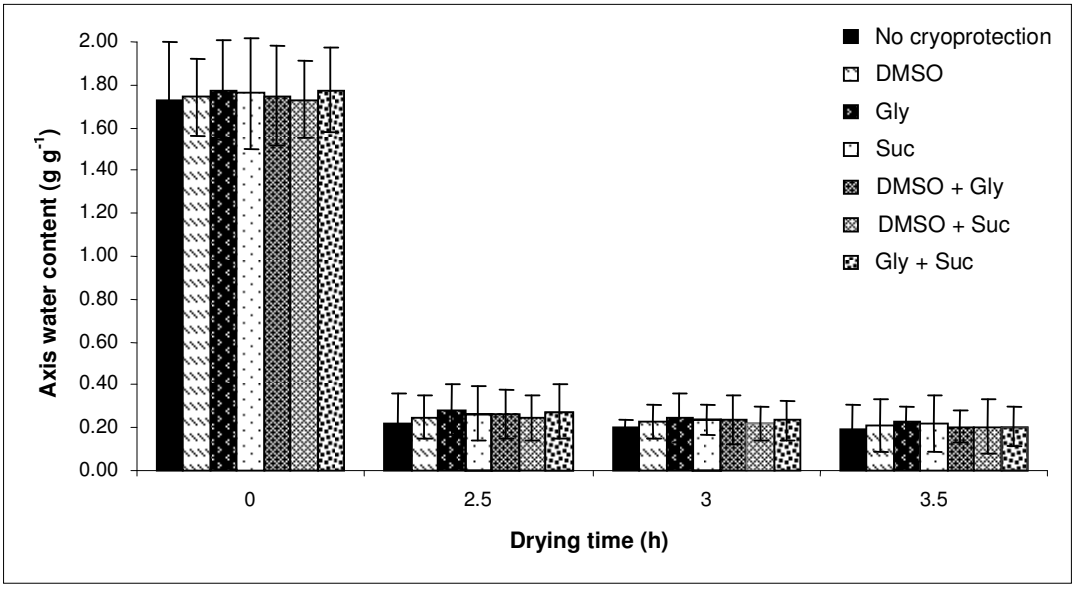


Figure 3.43: Changes in mean axis water content of explants with basal cotyledonary segments, from newly-harvested seeds of *T. emetica*, treated or not treated with cryoprotectants and dehydrated under a laminar air-flow. Bars indicate standard deviation. n = 5.

Explants treated with cryoprotectants were germinated on MS medium containing AC. However, explants for which all vestiges of cotyledonary tissue had been excised did not develop shoots, regardless of the cryoprotectant used. As shoot production in explants with only the basal cotyledonary segment or a 2-mm segment of cotyledon was similar (Chi-squared, $p > 0.05$, data not shown), shoot production of explants with basal cotyledonary segments only, has been shown to compare the response to the various cryoprotectants (Figure 3.44).

The percentage of non-dried explants with basal cotyledonary segments producing shoots (all of which also produced roots) after treatment with cryoprotectants was not significantly different (Chi-squared, $p > 0.05$) from that obtained in the absence of cryoprotectants (range between 70 – 85%, Figure 3.44). After dehydration, shoots developed only in a small proportion of those explants treated with sucrose (5% shoot production) or the combination of DMSO and glycerol (10 – 20% shoot production, Figure 3.44). Hence, these two cryoprotectant treatments were selected for the subsequent trials using axes from newly-harvested and stored seeds, and for cryopreservation.

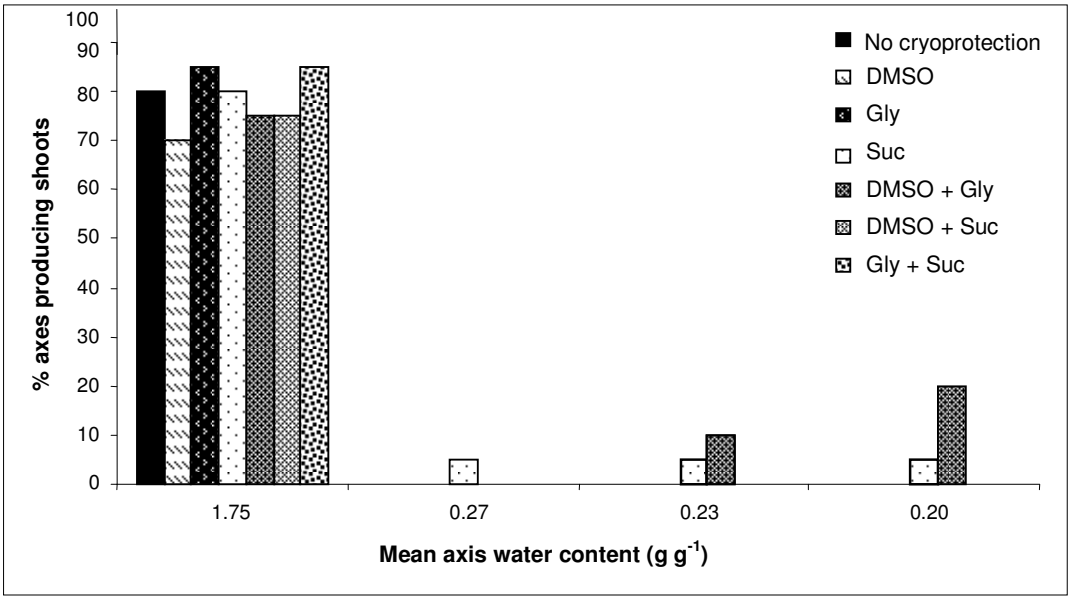


Figure 3.44: Shoot (with root) production by *T. emetica* explants with basal cotyledonary segments, from newly-harvested seeds, treated or not treated with cryoprotectants, dried under a laminar air-flow to different water contents and germinated on MS + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

The size of cotyledonary segment attached to explants had no significant effect (One-way ANOVA, $p > 0.05$, data not shown) on axis water content irrespective of seed storage period. Thus, the axis water content for explants with basal cotyledonary segments was selected to compare differences between the effects of cryoprotectant solutions. Also, axis water content of dried and non-dried explants was not significantly affected (One-way ANOVA, $p > 0.05$) by the two-week storage period or different cryoprotectant solutions applied (Figure 3.45).

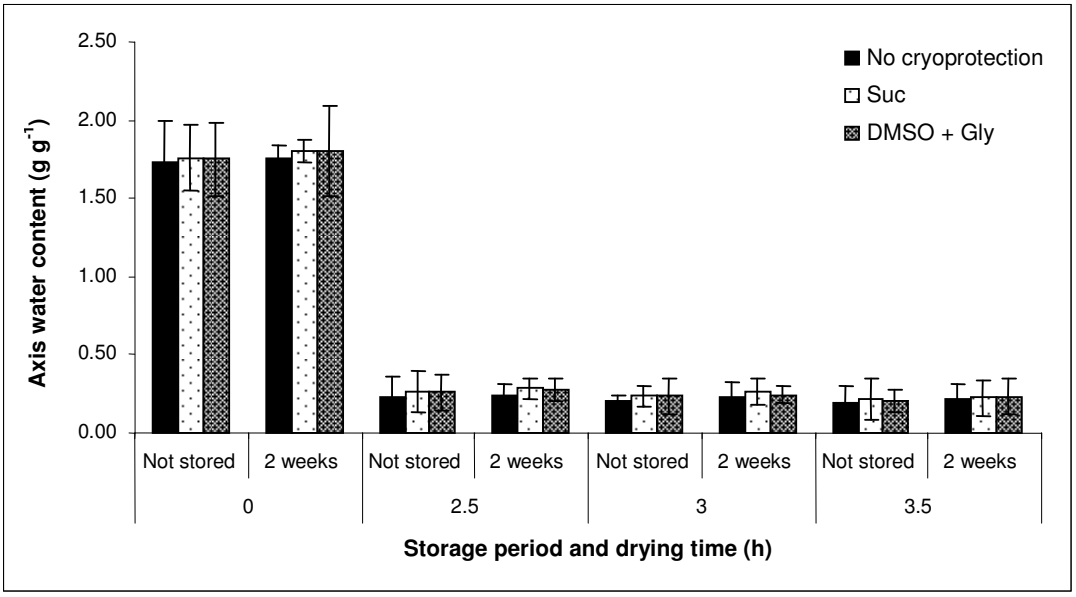


Figure 3.45: Changes in mean axis water content of explants with basal cotyledonary segments, from newly-harvested and stored seeds of *T. emetica*, treated or not treated with selected cryoprotectants and dehydrated under a laminar air-flow. Bars indicate standard deviation. n = 5.

Note that the seed storage periods (three and five months) *vs.* two weeks for *T. dregeana* and *T. emetica* respectively, are very different. This reflects the marked differences in post-harvest development rate, as established by Kioko (2003), as well as problems posed by fungal proliferation upon longer storage of *T. emetica* seeds. As was observed for *T. dregeana* explants, the provision of cryoprotectants did not promote shoot development in explants from *T. emetica* seeds after storage (Chi-squared, $p > 0.05$, Figure 3.46). In addition, the treatment with cryoprotectants did not ameliorate the lack of ability for shoot production by non-dehydrated axes with no attached cotyledonary vestiges (Table 3.16). After dehydration, only an insignificant proportion

of axes (Chi-squared, $p > 0.05$) treated with a combination of DMSO and glycerol produced shoots (Table 3.16).

Table 3.16: Percentage shoot (with root) production by *T. emetica* axes excised with no cotyledonary vestiges from seeds stored for two weeks, either treated or not treated with cryoprotectants, dried under a laminar air-flow for up to 3.5 h (mean axis water content indicated in brackets) and cultured on MS + AC. n = 20.

Cryoprotectant	**0 h (1.78 g g^{-1})**	**2.5 h (0.29 g g^{-1})**	**3 h (0.25 g g^{-1})**	**3.5 h (0.21 g g^{-1})**
No cryoprotection	50	0	0	0
DMSO	20	0	0	0
Gly	40	0	0	0
Suc	40	0	0	0
DMSO + Gly	30	0	0	5
DMSO + Suc	30	0	0	0
Gly + Suc	55	0	0	0

Cryoprotection had no significant effect on shoot production by non-dehydrated explants excised with basal cotyledonary segments from stored seeds (Figure 3.46, Chi-squared, $p > 0.05$). However, it is noteworthy that when compared with non-dried explants from newly-harvested seeds, a significant increase (Chi-squared, $p < 0.05$) in shoot development was observed when explants from seeds stored for two weeks were treated with either sucrose, a combination of DMSO and glycerol, a combination of DMSO and sucrose or a combination of glycerol and sucrose (cf. Figures 3.46 and 3.44).

After dehydration for 2.5 h to 0.29 – 0.27 g g^{-1}, shoot development was observed only in a small proportion of those explants treated with sucrose (5% shoot production) and those without any cryoprotection (10% shoot production). However, after further dehydration to lower water contents (0.25 -0.20 g g^{-1}) the ability for shoot production was maintained only by a small proportion of explants treated with either sucrose or a combination of DMSO and glycerol (Figure 3.46). Cryoprotection had no significant effect on shoot production by dehydrated explants with basal cotyledonary segments (Chi-squared, $p > 0.05$), despite the seed storage period.

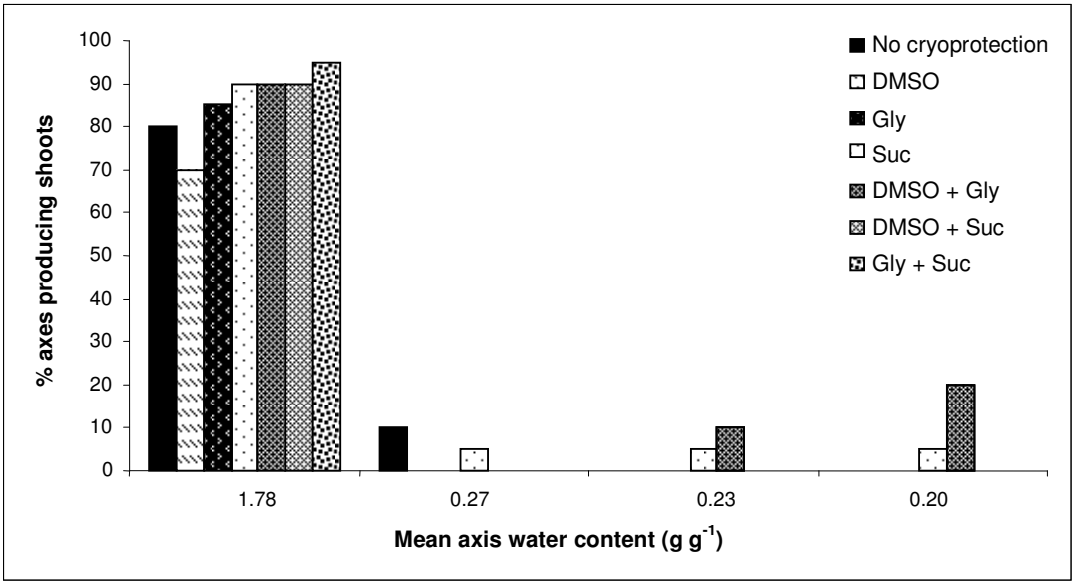


Figure 3.46: Shoot (with root) production by *T. emetica* explants with basal cotyledonary segments, from seeds stored for two weeks, treated or not treated with cryoprotectants, dried under a laminar air-flow to different water contents and germinated on MS + AC. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

Axes with all vestiges of cotyledons completely excised, from newly-harvested and two-week stored seeds were also treated with cryoprotectants, and then plated on MS medium supplemented with BAP, but without AC. For non-dried axes, BAP had no effect on shoot production, no explants from newly-harvested seeds producing shoots (Table 3.17). Explants from stored seeds produced shoots, but the percentage shoot production was not significantly higher (Chi-squared, $p > 0.05$) when BAP was added to the medium (Table 3.17). The differences in germination (i.e. root and shoot production) of non-dried explants (Table 3.17), compared with the figures shown in Column 2, Table 3.16 can be attributed to the differences in the medium on which explants were cultured, the presence of activated charcoal promoting both root and shoot production.

After dehydration under a laminar air-flow, axes with no cotyledonary vestiges failed to produce shoots (data not shown), even when cultured on a BAP-containing medium.

Table 3.17: Percentage shoot (with root) production of non-dried *T. emetica* axes excised with no cotyledonary vestiges from newly-harvested and stored seeds, either treated or not treated with cryoprotectants and cultured on MS medium with or without BAP. n = 20.

	Not stored		2 weeks	
Cryoprotectant	MS	MS + BAP	MS	MS + BAP
No cryoprotection	0	0	20	25
DMSO	0	0	10	10
Gly	0	0	20	25
Suc	0	0	20	20
DMSO + Gly	0	0	15	20
DMSO + Suc	0	0	15	15
Gly + Suc	0	0	25	25

3.3.4 Cryopreservation: effects of explant size, dehydration, storage period and cryoprotection

3.3.4.1 Explants dried in a laminar air-flow

Explants excised from newly-harvested and stored seeds with no vestiges of cotyledon and those with basal cotyledonary segments were dried in a laminar air-flow for up to 3.5 h to a mean water content range between 0.29 - 0.20 g g^{-1} (previously determined as the optimal water content range by Kioko, 2003) and were cryopreserved using the cryotube cooling technique.

Irrespective of the presence of the basal cotyledonary segments (Figure 3.48) or not, explants treated with all tested cryoprotectants were cryopreserved, but only those treated with sucrose or the combination of DMSO and glycerol survived (Figure 3.48). Survival after cryopreservation was in the form of callus only, either from the whole axis or from the root pole of the explant (Figure 3.47) and was not significantly affected by the presence or absence of cotyledonary segments attached to the axes (Chi-squared, $p > 0.05$).

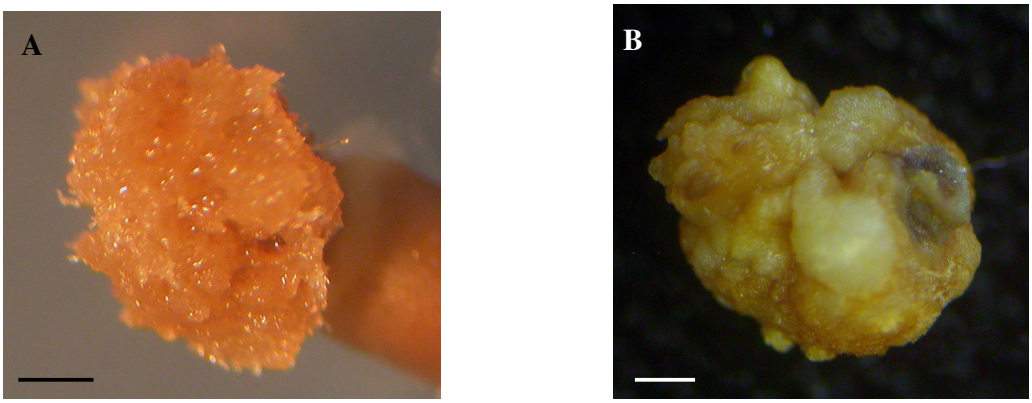


Figure 3.47: Survival of *T. emetica* explants (excised without cotyledonary vestiges) in the form of callus at the root pole (A); and from the whole axis (B), after cryopreservation by cooling in cryotubes. Bar = 0.5 mm.

Explants excised from newly-harvested seeds attained 5% survival when pre-treated with sucrose, and between 5 – 15% survival when pre-treated with a combination of DMSO and glycerol (Figure 3.48). At a water content between 0.21 and 0.20 g g^{-1} (dehydration for 3.5 h) the difference in explant survival after application of the two cryoprotectant treatments became statistically significant (Chi-squared, $p < 0.05$). Those explants excised from two-week-stored seeds survived cryopreservation only if dried for 3.5 h (to 0.21 – 0.20 g g^{-1}) after pretreatment with a combination of DMSO and glycerol (Figure 3.48).

From these experiments, cryopreservation appeared most successful using explants with, or without, cotyledonary segments from newly-harvested seeds, when the axes were pretreated with a cryoprotectant solution consisting of a combination of DMSO and glycerol, followed by dehydration in a laminar air-flow to a mean water content of 0.20 g g^{-1}.

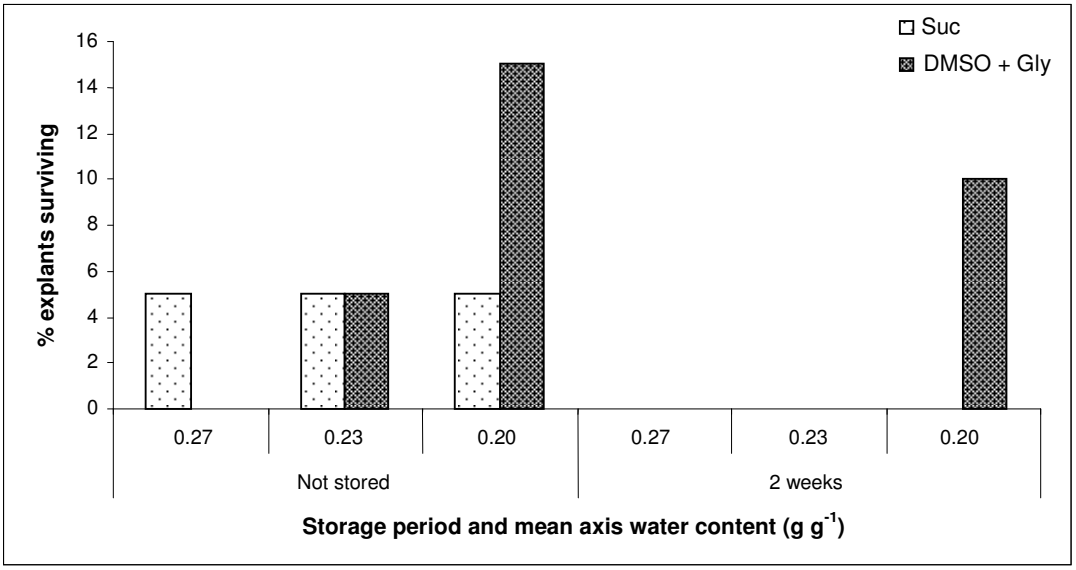


Figure 3.48: Survival assessed as the ability to produce callus after cryopreservation of *T. emetica* explants with basal cotyledonary segments from newly-harvested and two-week-stored seeds, treated with two cryoprotectants, dried under a laminar air-flow to different water contents and cooled in cryotubes. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

When MS medium supplemented with BAP was used to regenerate cryopreserved explants (excised with/without cotyledonary vestiges), there was an increase in the range of cryoprotected treatments which facilitated survival (cf. Figures 3.49 and 3.48). Survival of explants from newly-harvested seeds remained at 5 – 10% after cryopreservation when cryoprotected with either sucrose or a combination of DMSO and glycerol. In addition, 5 – 10% survival was also achieved for explants cryoprotected with a combination of DMSO and sucrose and a combination of glycerol and sucrose (Figure 3.49). Although the inclusion of BAP did facilitate post-thaw survival across a number of cryoprotectant treatments, there was no significant difference in the percentage survival attained from the treatments (Chi-squared, $p > 0.05$).

In explants excised from stored seeds, the addition of BAP to the regeneration medium did not affect survival after cryopreservation, with only 5% of explants (excised with/without cotyledonary vestiges) treated with a cryoprotectant solution containing DMSO and sucrose surviving (Figure 3.49). Also, survival was still in the form of callus, predominantly at the root pole (Figure 3.50).

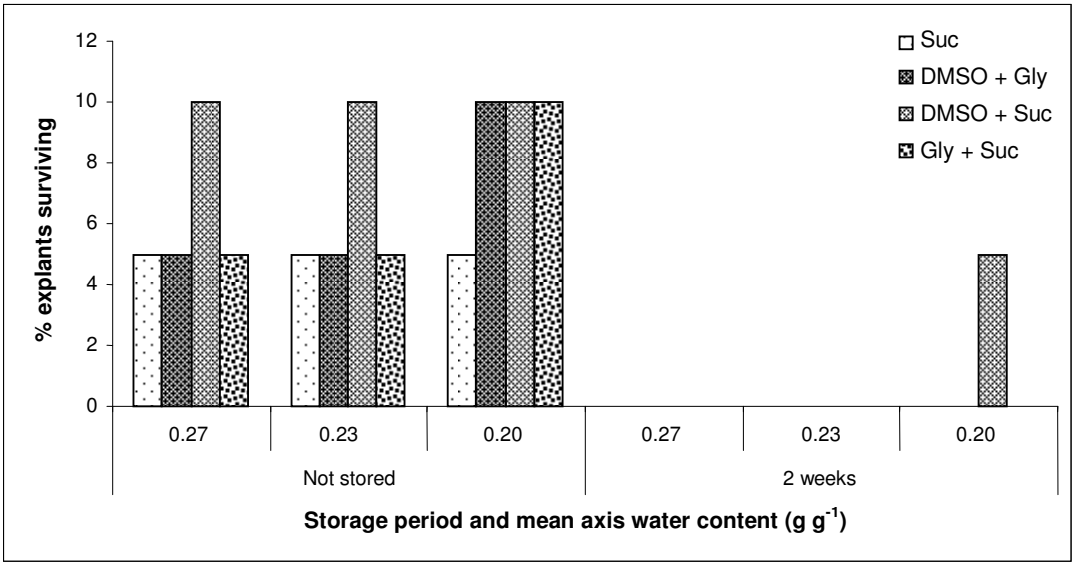


Figure 3.49: Survival assessed as the ability to produce callus after cryopreservation of *T. emetica* explants with basal cotyledonary segments from newly-harvested and two-week-stored seeds, treated with various cryoprotectants, dried under a laminar air-flow to different water contents, cooled in cryotubes and germinated on MS + BAP. Mean axis water content (n = 5) interpolated from the same drying time-course as the axes set out to germinate (n = 20).

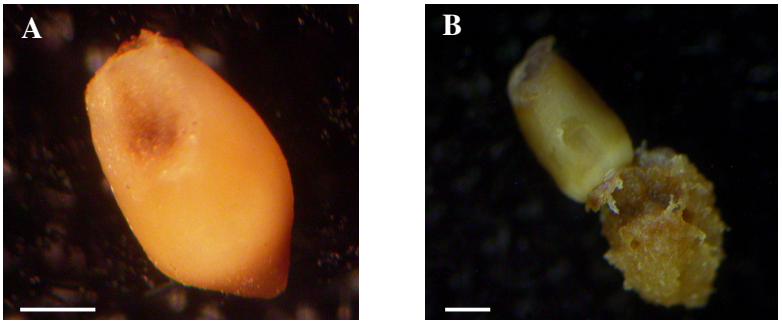


Figure 3.50: Response of embryonic axes of *T. emetica* excised with no cotyledonary vestiges from newly-harvested seeds, cryoprotected with DMSO + sucrose, and cooled in cryotubes. A, cultured without BAP; B, cultured with BAP. Bar = 0.5 mm.

Irrespective of the application of cryoprotectants or the addition of BAP to the medium, the calli produced from surviving explants (excised with/without cotyledonary vestiges) were non-embryogenic. Such cells are characterized by their large size and elongated shape, and also by lack of density of the cytoplasm (Figure 3.51).

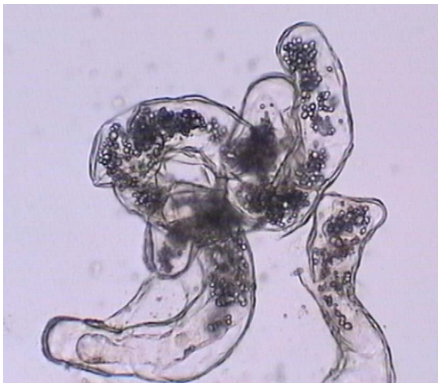


Figure 3.51: Non-embryogenic cells typical of the callus produced after cryopreservation of *T. emetica* explants. Mag = 100X.

3.3.4.2 Flash-dried explants

Explants excised with no cotyledonary vestiges, basal or 2-mm cotyledonary segments were flash-dried for 30, 60 and 90 min to water contents between 0.90 – 0.50 g g^{-1}, before cooling by the rapid technique in nitrogen slush. These explants were not treated with cryoprotectants beforehand due to the limited availability of seeds, and the same experiment was repeated for axes from newly-harvested seeds, and those stored for one and two weeks. The decline in seed availability also limited the use of both freezing techniques, hence the more rapid freezing technique was selected.

Explants were monitored for survival for 12 weeks after they were cultured on the regeneration media. These explants were assessed for callus production, greening or elongation to determine survival. The explants were considered to be dead if none of the aforementioned criteria were met or if the browning at the root and shoot pole occurred.

None of the explants survived cryopreservation using this cooling technique, irrespective of the explant type, drying time or the seed storage period (Figure 3.52). BAP was also added to the regeneneration medium for all treatments, after different storage intervals. However, this also did not have any effect on survival after cryopreservation. It is suggested that, axis water contents were probably too high, and that future studies should assess survival at lower water contents before cryopreservation. Furthermore, the use of cryoprotectants in combination with flash-drying needs to be investigated.

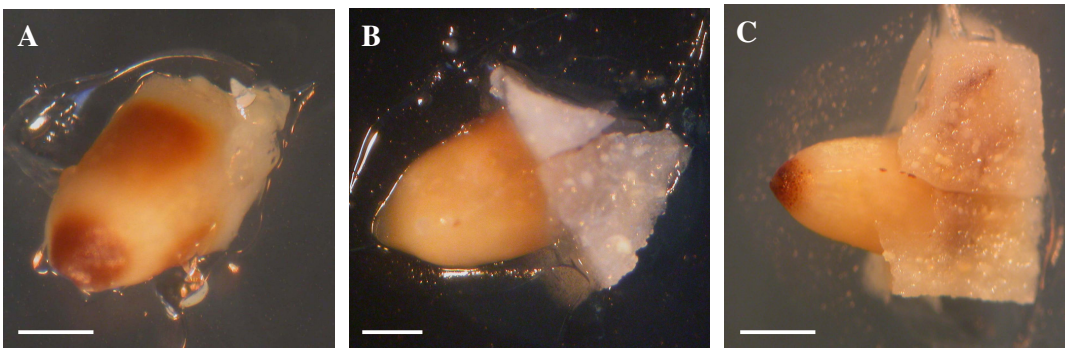


Figure 3.52: Response of *T. emetica* explants excised from one-week-stored seeds, flash-dried to 0.50 g g^{-1} and cryopreserved by rapid cooling. A, no cotyledonary vestiges; B, basal segments and C, 2-mm segments after 12 weeks in culture. Bar = 1 mm.

Strychnos gerrardii

3.3.5 Cryopreservation: effects of dehydration and storage period

All explants used for these experiments comprised the whole embryo i.e. the embryonic axis with both intact paper-thin cotyledons (Figure 2.7). *Strychnos gerrardii* embryos were cryopreserved using the cryotube and rapid cooling techniques, but were not cryoprotected prior to dehydration as the aim was to establish a cryopreservation method without the use of such protectants. The regeneration medium after cryopreservation comprised ½ MS containing AC (as previously established [section 3.1.2]).

Mean initial embryo water content was 1.41 g g^{-1} and decreased to 0.55 – 0.20 g g^{-1} after dehydration for various periods (see section 3.2). In contrast to the two other species tested in this study, survival after cryopreservation was not in the form of callus but rather root/hypocotyl elongation of the embryo. Survival after cryopreservation was not affected (Chi-squared, $p > 0.05$) by the cooling technique used (Figure 3.53). Although a slight decrease in survival was observed after dehydration for 50 min to a mean water content of 0.20 g g^{-1} in explants from stored and non-stored seeds (Figure 3.53), this was not significant (Chi-squared, $p > 0.05$). With the exception of embryos from seeds stored for four weeks (Chi-squared, $p < 0.05$), 80 – 100% survival after cryopreservation in the form of root/hypocotyl elongation was achieved for embryos from stored and non-stored seeds (Figure 3.53).

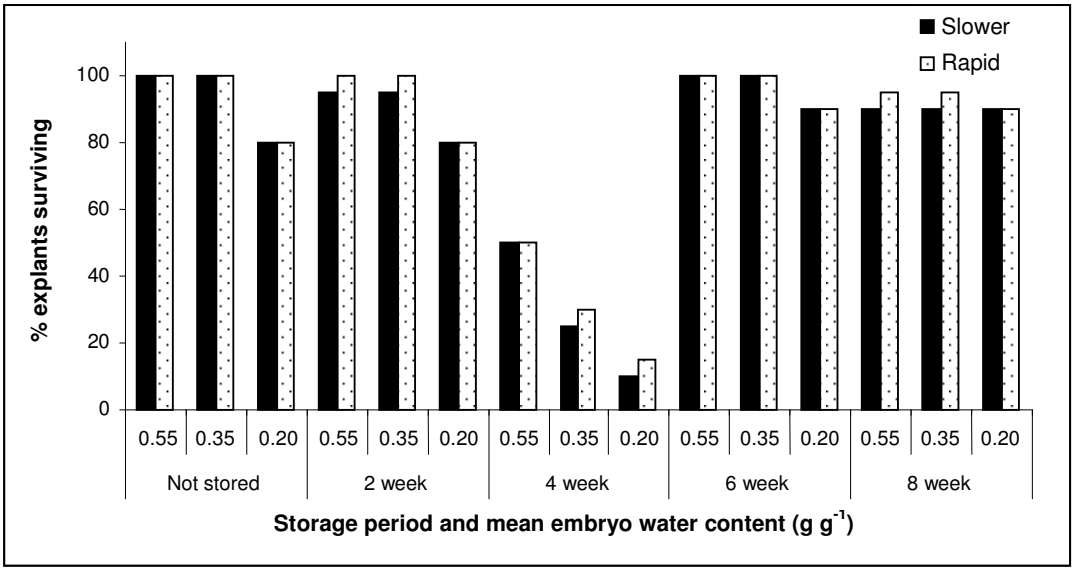


Figure 3.53: Survival assessed as the ability for root/hypocotyl elongation after cryopreservation of *S. gerrardii* embryos from newly-harvested and stored seeds from yellow fruits, flash-dried for 15, 30 and 50 min and cooled by the slower (cryotubes) and rapid (nitrogen slush) methods. Mean embryo water content (n = 5) interpolated from the same drying time-course as the embryos set out to germinate (n = 20).

The mode of post-cryopreservation growth was not affected by the cooling technique (Figure 3.54) or the drying period (Figure 3.55) with explant elongation occurring at the root pole (Chi-squared, $p > 0.05$). However, shoot development was adversely affected, being observed only for 10% of explants from non-stored seeds which were rapidly cooled after dehydration for 15 min to a mean water content of 0.55 g g^{-1} (Figure 3.56). The decrease in survival of explants from seeds stored for four weeks (Figures 3.53 and 3.56) is inconsistent with the dehydration responses of these embryos (see section 3.2) and considered to be as the result of a poor quality seed sample.

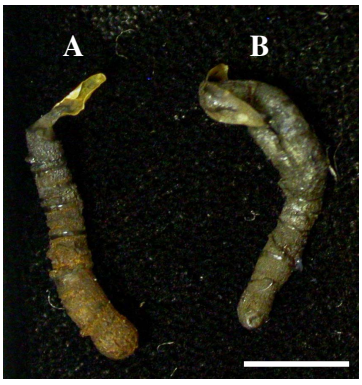


Figure 3.54: Seedlings of *S. gerrardii* from embryos which had been more slowly (A) and rapidly cooled (B). Embryos were excised from non-stored seeds and subjected to 30 min flash-drying. Bar = 5 mm.

Figure 3.55: Surviving *S. gerrardii* explants from two-week-stored seeds. Embryos flash-dried for 15 min (A); 30 min (B) and 50 min (C) before cryopreservation, are illustrated. Bar = 5 mm.

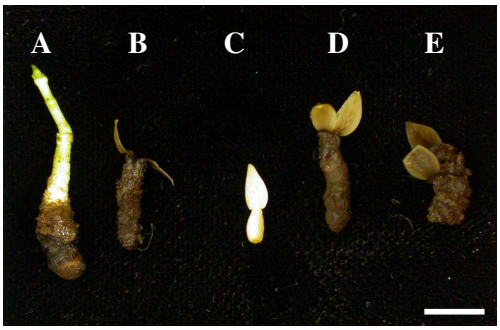


Figure 3.56: Surviving *S. gerrardii* explants from newly-harvested (A); two-week-stored (B); four-week-stored (C); six-week-stored (D) and eight-week-stored (E) seeds, dehydrated for 15 min and rapidly cooled. Bar = 5 mm.

3.3.5.1 Seed responses in relation to change in fruit colour

Explants from seeds extracted from fruits of different colours were also cryopreserved using both cooling techniques after the selected dehydration times (section 3.2.2.3).

Explant survival after cryopreservation was not affected by the cooling technique (Chi-squared, $p > 0.05$) and increased as fruits matured from green to yellow (Figure 3.57). Explants from seeds from green and yellow/green fruits survived best when dehydrated to a mean water content of 0.31 g g^{-1} (30 min) before cooling, indicating that the mean water content of 0.50 g g^{-1} after drying for 15 min was still unfavourably high, while after 50 min (0.21 g g^{-1}) explants were less likely to recover from desiccation damage *sensu stricto* (Figure 3.57). While extended dehydration (0.21 g g^{-1}) adversely affected survival of embryos from seeds of mature (yellow) fruits, these explants survived both slower and rapid cooling equally well if flash-dried for 15 or 30 min to mean water contents of 0.50 and 0.31 g g^{-1}, respectively. A significantly lower survival was observed only for embryos from seeds of green fruits, dried for 15 min (Chi-squared, $p < 0.05$).

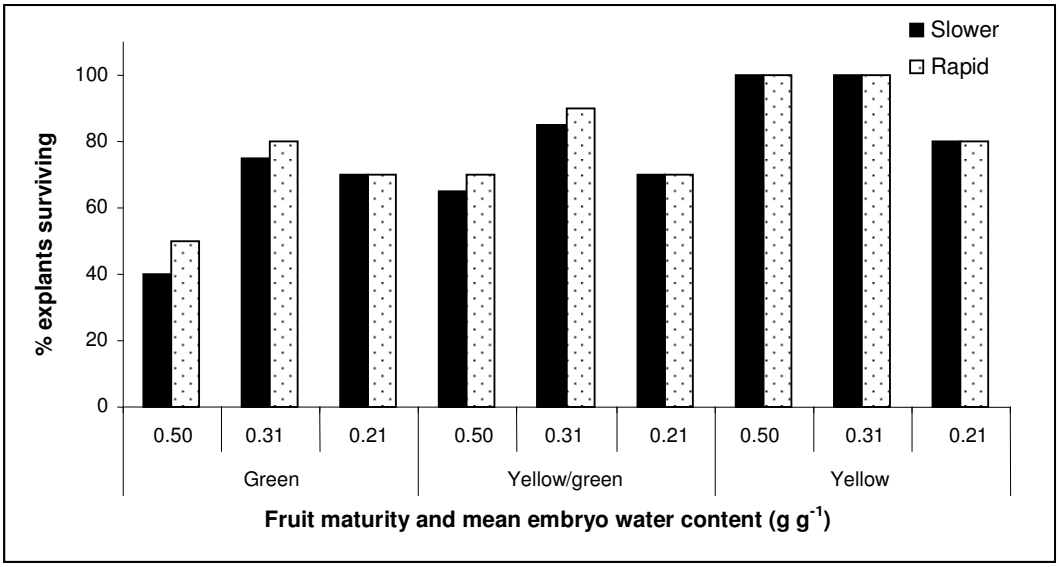


Figure 3.57: Survival assessed as the ability for root/hypocotyl elongation after cryopreservation of *S. gerrardii* embryos from seeds of fruits at different maturity stages, flash-dried for 15, 30 and 50 min and cooled by the slower (cryotubes) and rapid (nitrogen slush) methods. Mean embryo water content (n = 5) interpolated from the same drying time-course as the embryos set out to germinate (n = 20).

Although percentage embryo survival after cryopreservation was different in relation to fruit maturity stage, there was no difference in the mode of growth after cryopreservation, with elongation of the root/hypocotyl occurring (Figure 3.58). Shoot development failed to occur in explants from seeds of green or yellow/green fruits, only those embryos from seeds of yellow fruits, dehydrated to a mean water content of 0.50 g g^{-1} (15 min) and rapidly cooled, producing shoots (data not shown). However, even under these conditions, shoot production occurred in only 10% of the embryos.

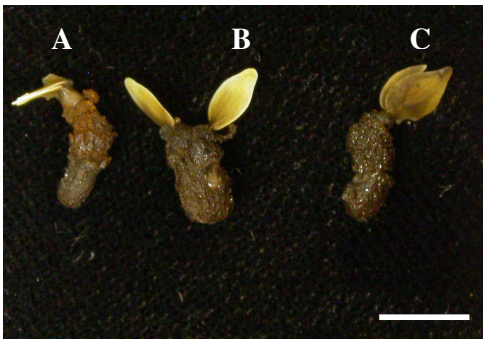


Figure 3.58: Surviving *S. gerrardii* explants from yellow (A); yellow/green (B) and green (C) fruits, dehydrated to 0.50 g g^{-1} and rapidly cooled. Bar = 5 mm.

In a further experiment, explants from seeds stored for six weeks were cryopreserved using the rapid cooling technique followed by regeneration on media containing various concentrations of BAP, to determine if shoot production would be stimulated.

The addition of BAP to the regeneration medium failed to facilitate shoot development, but affected the growth form of the resulting seedlings (see below). Survival after cryopreservation was highest in the absence of BAP for embryos dehydrated for 15 min to a mean water content of 0.50 g g^{-1}, while 2 mg l^{-1} BAP had a significantly adverse effect (Chi-squared, $p < 0.05$, Figure 3.59). While the highest concentration of BAP in the medium appeared to be less deleterious for embryos at a mean water content of 0.31 g g^{-1} (30 min flash-dried) survival was unaffected by BAP concentrations of 0.5 and 1.0 mg l^{-1} (Figure 3.59).

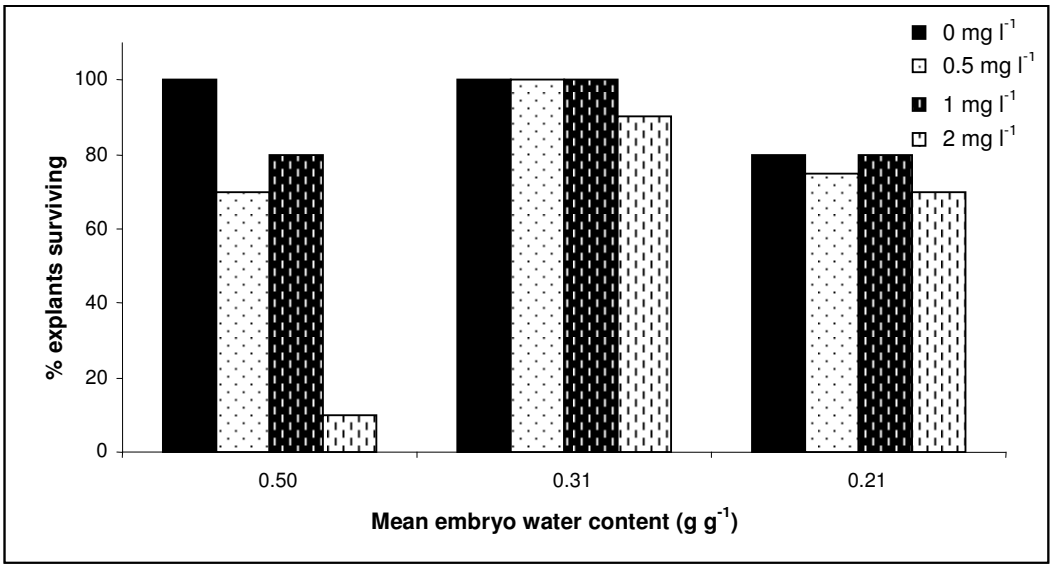


Figure 3.59: Survival assessed as the ability for root/hypocotyl elongation after cryopreservation of *S. gerrardii* explants from seeds extracted from yellow fruits and stored for six weeks, dried to different water contents, rapidly cooled and regenerated on ½ MS medium with various concentrations of BAP. Mean embryo water content (n = 5) interpolated from the same drying time-course as the embryos set out to germinate (n = 20).

The addition of different concentrations of BAP to the regeneration medium did not stimulate shoot development, and explants appeared more bulky showing surface tissue changes suggesting the potential for callus formation (cf. Figures 3.60 and 3.54). From

the outwards appearances of the explants, application of 0.5 mg l^{-1} BAP produced the least marked of these effects (Figure 3.60).

Future work on this species should be aimed at ascertaining the nature of the stress-/damage-response that negatively affects shoot development as well as manipulating regeneration media to stimulate and optimize shoot development. Such studies should also explore the potential benefits of cryoprotection.

Figure 3.60: Survival after cryopreservation of *S. gerrardii* explants, dried to 0.31 g g^{-1}, rapidly cooled and regenerated on ½MS medium with 0.5 mg l^{-1} BAP (A); 1 mg l^{-1} BAP (B) and 2 mg l^{-1} BAP (C). Bar = 5 mm.

3.3.6 Cryopreservation of excised embryonic axes: concluding remarks

Successful cryopreservation is dependent on a spectrum of often species-specific factors. In addition to optimizing the dehydration procedure, achieving optimum survival is often a combination of determining the best cryoprotectant, cooling and warming technique and regeneration medium (Kioko *et al*., 1998; Berjak *et al*., 1999a, 1999c; Krishnapillay, 2000).

The purpose of cryoprotectants is to reduce cooling injury in hydrated tissues and provide protection during thawing and recovery after cryostorage (Kartha and Engelmann, 1994; Lynch, 2000). Cryoprotection trials in both *Trichilia* spp. involved the use of penetrating (DMSO and glycerol used alone and in combination) and non-penetrating (sucrose, PVP and dextran) cryoprotectants. No significant difference in axis water content of *T. dregeana* and *T. emetica* explants was observed upon the addition of cryoprotectants (Figure 3.34 and Figure 3.43, respectively). Although explant viability was high when only cryoprotected, shoot viability decreased drastically when explants were subsequently dehydrated, although the provision of certain

cryoprotectants did facilitate production of shoots by a few axes of both *Trichilia* spp. (Figure 3.35 and Figure 3.44).

The effect of a cryoprotectant or a combination of cryoprotectants depends on its chemical composition, reaction time, toxicity level and the tissue with which it interacts (Finkle *et al*., 1985; Fuller, 2004). Thus, cryoprotectants facilitating the best shoot production were selected for use prior to explant cryopreservation. For *T. dregeana* explants, these were dextran and a combination of glycerol and sucrose, where 10% of the axes retained the ability for shoot production after dehydration (Figure 3.35). For *T. emetica* explants, sucrose (5% shoot production after dehydration) and the combination of DMSO and glycerol (10 - 20% shoot production after dehydration) were selected for cryopreservation experiments (Figure 3.44). Kioko (2003) observed similar trends for both species with 60% germination (root and shoot) in *T. dregeana* explants treated with glycerol and sucrose and 62.5% germination (root and shoot) in *T. emetica* explants treated with DMSO and glycerol. The poorer results obtained from this study could be a consequence of lower quality seeds being available, than those used in the study by Kioko (2003). Both inter-and intra-seasonal variability in recalcitrant seed quality has been documented for a variety of species (Berjak and Pammenter, 2004b)

The effects of cryoprotectants tested on explants from stored seeds were not significantly different after dehydration when compared with responses of fresh seeds, irrespective of the explant type for both *T. dregeana* (Table 3.14, Figure 3.37A and Figure 3.37B) and *T. emetica* (Table 3.16 and Figure 3.46). Thus, the efficacy of the cryoprotectants was not influenced by storage period.

Cryopreservation can be achieved by various cooling techniques, two of which were compared using the explants of *T. dregeana*. Slower cooling involved the plunging of cryotubes containing the explants into liquid nitrogen (-196°C) while, for rapid cooling, 'naked' explants were mixed with sub-cooled nitrogen (-210°C). Both methods, but especially the latter, allow the explants to pass rapidly through the temperature zone at which ice crystal formation occurs, thus offering the potential of reducing the damage caused by ice crystallization (Wesley-Smith *et al*., 1992; Kartha and Engelmann, 1994;

Berjak *et al*., 1996). The difference between the two techniques is the rate at which cooling takes place. The most commonly used method of cryopreservation is slower (cryotube) cooling and has been successful for explants of a range of species including embryonic axes of *Juglans regia* (de Boucaud *et al*., 1991); embryonic axes of *Azadirachta indica* (Berjak and Dumet, 1996); somatic embryos of *Daucus carota* (Thierry *et al*., 1997); shoot tips of *Manihot esculentum* (Charoensab *et al*., 1999); shoot tips of *Chrysanthemum cinerariaefolium* (Hitmi *et al*., 1999); somatic embryos of *Phoenix dactylifera* (Mycock, 1999) and seeds of *Miltonia flavescens* (Popov *et al*., 2004). Rapid cooling using sub-cooled nitrogen or 'slush' has also been successfully applied for cryopreservation of embryonic axes of *Camellia sinensis* (Wesley-Smith *et al*., 1992); *Aesculus hippocastanum* (Wesley-Smith *et al*., 2001a); *Acer saccharium* (Wesley-Smith, 2002) and *Poncirus trifoliate* (Wesley-Smith *et al*., 2004b). In this study, when explants from newly-harvested seeds of *T. dregeana* were cooled using cryotubes, none survived cryopreservation, irrespective of explant type or the cryoprotectant used. However, on rapid cooling, 15 - 20% survival manifested as the ability for callus production was observed in explants with cotyledonary segments attached, with or without cryoprotection (Table 3.15). Thus, cooling rate is suggested to be a major factor contributing to survival. However, the underlying reason for this is not entirely clear as ice crystal damage is unlikely at water contents below 0.30 g g^{-1} and the exact proportion of non-freezable water is unknown. Kioko *et al*. (1998) obtained considerably better survival - 49% of axes producing callus - for *T. dregeana* treated with a combination of DMSO and glycerol and rapidly cooled. While the quantitative difference in results might be attributed to the genetic variation within seeds, it is considered more likely to have resulted from the variability which is known to occur between different harvests within species (Berjak *et al*., 1989; Finch-Savage and Blake, 1994; Drew *et al*., 2000). Seeds collected for this experiment could have been of a somewhat a different genotype or, what is more probable considering that both the present collections and those of Kioko *et al*. (1998) were made from urban street trees in Durban, of an inferior quality. In this regard, the poor quality of *T. dregeana* seeds used by Drew *et al*. (2000) was considered to be the basis of their curtailed longevity under experimental conditions.

Explants from newly-harvested seeds of *T. emetica* were cooled in cryotubes after cryoprotection and laminar air-flow dehydration. Survival was highest (15%) when explants were dried to a mean water content of 0.20 g g^{-1} over 3.5 h after cryoprotection with a combination of DMSO and glycerol (Figure 3.48). Although not as good, these results concur with the 47% survival reported by Kioko (2003), confirming the best outcome when explants of *T. emetica* were treated with a combination of DMSO and glycerol. Seed storage period prior to experimentation did not affect survival after cryopreservation, possibly because after storage for only two weeks, seeds/embryos were still under-developed (see section 3.1.2.2). When explants from newly-harvested seeds of *T. emetica* were rapidly cooled after flash-drying, they failed to survive cryopreservation. There are several possible reasons for this, including their developmental status; water content after selected drying periods still being too high; and/or because they were not cryoprotected beforehand. In the present study, a comparison between slower and rapid cooling techniques was not undertaken because of the limitation in seed availability. Thus, the more effective cooling technique of the two could not presently be determined.

S. gerrardii explants from newly-harvested seeds were not treated with any cryoprotectants based on preliminary work carried out by Khuzwayo (2002) indicating that embryos could be cryopreserved without the use of cryoprotectants. These explants were flash-dried to water contents ranging between 0.55 – 0.20 g g^{-1} and cooled using both the slower and rapid techniques. No difference was observed between the effects of cooling rate, with both yielding 80 – 100% survival, assessed by root/hypocotyl elongation after cryopreservation (Figure 3.53). This corresponds directly with the results obtained by Khuzwayo (2002). Studies on *S. spinosa* explants have also shown 100% survival assessed as root/hypocotyl elongation after cryopreservation by slower and rapid cooling in the absence of cryoprotectants (Naidoo, pers. comm.[3]). The degree of dehydration tolerated, coupled with the high survival rates independent of cooling technique, suggest that the post-harvest behaviour of seeds of these species within the *Strychnos* genus could be described as minimally recalcitrant, or even intermediate. Species producing seeds in the intermediate category have been reported to survive

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cryopreservation without any cryoprotection treatment as seen for seeds and axes of *Azadirachta indica* (Berjak and Dumet, 1996), and *Warburgia salutaris* seeds (Kioko *et al*., 2003b).

With regard to the role of developmental status of axes in relation to cryopreservation, *T. dregeana* explants from three-month-stored seeds showed 3 – 5% survival when cooled using cryotubes and 10 – 15% survival when rapidly cooled, independent of explant type and cryoprotectant treatment (Figure 3.38A). Explants from five-month-stored seeds showed no survival when cooled using cryotubes and 12% survival independent of explant type when rapidly cooled, provided no cryoprotectant was applied (Figure 3.38A). While the percentage survival is similar for explants from stored and non-stored *T. dregeana* seeds when rapidly cooled, only those explants from stored seeds had the ability to survive without any cotyledonary attachment. Furthermore, explants from three-month-stored seeds only, survived slower cooling. Thus, it can be concluded that explants from three-month-stored seeds had developed sufficiently, rendering them less susceptible to dehydration and cooling damage, while five-month-stored seeds may have begun germination-associated events rendering them more sensitive to desiccation (e.g. Berjak *et al*., 1989) and cooling. Although Goveia *et al*. (2004) showed that axes excised without any cotyledonary vestiges from seeds stored for six months germinated and produced shoots, those axes were not dehydrated or cryopreserved. Hence, in those experiments the axes were not exposed to additional stresses which compromise survival.

Some explants of *T. emetica* from two-week-stored seeds, dried under a laminar air-flow and cooled using cryotubes survived (10%) only when treated with DMSO and glycerol (Figure 3.48), suggesting that cryoprotectants are probably essential for successful cryopreservation of that species. However, the optimum storage period of *T. emetica* seeds that would facilitate axes being in the most suitable developmental state, has not been ascertained. Rapidly cooled explants of *T. emetica* failed to survive cryopreservation when excised from seeds stored for one and two weeks (Figure 3.52). This is attributed to the combination of the relatively high water content of the explants upon cooling, and the lack of cryoprotection in addition to the developmental status of

the explant. Naidoo (2006) also observed a decline in survival for axes of numerous amaryllid species in relation to any one of these parameters.

Explants from all stored seeds of *S. gerrardii* (with the inexplicable exception of those stored for four weeks) showed 80 – 100% survival (assessed by root/hypocotyl elongation) after cryopreservation, independent of cooling rate or cryoprotection (Figure 3.53). Thus, in this species, developmental status does not appear to affect the potential for cryopreservation. It is possible that embryos from *S. gerrardii* seeds had developed sufficiently when the seeds were removed from the newly-harvested, yellow (most mature) fruits, to survive cryopreservation. However, as storage for up to eight weeks did not compromise survival after cryopreservation, it is suggested that the seeds had not reached the stage of incipient germination during storage as shown by Sershen *et al.* (2007) and von Fintel (2006) for various amaryllid species.

Explants from seeds of *S. gerrardii* fruits at different stages of ripening, corresponding to different seed maturities, were also cryopreserved using both slower and rapid cooling, but without cryoprotection. No cryoprotectants were tested on *S. gerrardii* explants as the aim was to ascertain whether a cryopreservation method could be developed without their use. As presently demonstrated, cooling rate had no effect on the survival of the explants after cryopreservation (Figure 3.57). However, survival of explants from seeds of green, less mature, fruits was significantly lower (40 – 80%) than from yellow/green (65 – 85%) and yellow, most mature (80 – 100%) fruits (Figure 3.57). These results are similar to others attained for seeds of tea, cocoa and jackfruit (Chandel *et al.*, 1995) showing that immature axes of the species concerned were less amenable to cryopreservation. It is probable that explants from seeds from green-coloured fruits had not yet reached a developmental stage to withstand and repair damage induced by excision, desiccation and cooling.

Although survival after cryopreservation was recorded for *T. dregeana*, explants did not develop into seedlings, instead showing callus proliferation at the root pole (Figure 3.39). Callus growth was further stimulated by sub-culture on media with either of the auxins 2,4-D or NAA. In terms of axes developing callus, the best results were obtained

from non-cryoprotected explants from three-month-stored seeds which were rapidly cooled and cultured on a medium containing 1 mg l^{-1} 2,4-D (Figure 3.41A). Callus production is commonly recorded to follow cryopreservation, and has been reported, for example in *Quercus* spp., *Aesculus hippocastanum* and *Carya* sp. (Pence, 1990) and was also noted in *Triticum aestivum* (Kendall *et al*., 1993). Kioko (2003) also observed callus production by *T. dregeana* explants which were cryopreserved using cryotubes. Callus production was also presently obtained from surviving explants of *T. emetica*, either at the root pole or over the entire surface of the axis (Figure 3.47A and Figure 3.47B respectively). In *S. gerrardii*, survival was in the form of elongation of the root/hypocotyl of the explant rather than callus formation (Figure 3.54), although, when 0.5 mg l^{-1} BAP was included in the recovery medium, initiation of what might be interpreted as callusing, appeared also to occur (Figure 3.60). Nevertheless, 10% of explants from seeds of newly-harvested, yellow fruits, produced shoots after cryopreservation (Figure 3.56). Shoot production was also observed for approximately 50% of non-dried cryopreserved explants of *S. spinosa* (Naidoo, pers. comm.[4]), indicating the potential for cryopreservation of the germplasm of *Strychnos* spp. Future studies on these species must focus on substantially improving the success rate, initially by cryoprotectant trials.

Endospermous non-orthodox seeds appear to offer good potential for cryopreservation. Besides the limited success with embryos of *Strychnos* spp. reported here, considerable success has been achieved for many amaryllid species (Sershen *et al*., 2007), and for the palm, *Phoenix reclinata*, in our laboratory (unpublished observations). Successful cryopreservation of zygotic explants from endospermous seeds may well be a function of the fact that cotyledon excision is unnecessary. This could be highly significant in avoiding, or at least minimizing, excision injury which may well result in a damaging generation of reactive oxygen species (see section 3.4).

When the proliferated callus of *T. dregeana* explants was assessed, the cells were found to be non-embryogenic (Figures 3.42A and B). Thus, two different concentrations of

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BAP were added to the media in an attempt to induce embryogenicity: however, these attempts were unsuccessful.

As a result of this outcome with *T. dregeana* explants, BAP was added to the germination media for *T. emetica* explants prior to, and after, cryopreservation. BAP is a cytokinin commonly used to facilitate shoot formation in explants of many species: e.g. callus tissue from *Ficus religiosa* (Jaiswal and Narayan, 1985) and *Centrosema brasilianum* (Angeloni *et al*., 1992), nodal explants of *Artocarpus heterophyllus* (Rahman and Blake, 1988), *Zingiber officinale* (Balachandran *et al*., 1990) and *Sterculia urens* (Purohit and Dave, 1995), shoot tips from *Gossypium hirsutum* (Agrawal *et al*., 1997), embryonic axes of *Prosopis tamarugo* (Nandwani and Ramawat, 1992) and *Helianthus annus* (Charriere *et al*., 1999) and whole seeds of *Bratonia* sp. (Popov *et al*., 2004). A study by Turner *et al*. (2001) showed that seedlings derived after cytokinin treatments of shoot apices from *Anigiozanthos viridis* ssp. *terraspectans* produce more vigourous shoot apices, which, it was reasoned could promote full germination after axis cryopreservation. However, in the present study, the addition of BAP to the medium did not increase the ability for shoot production by non-cryopreserved explants excised with no vestiges of cotyledons, irrespective of seed storage period and cryoprotectant treatment (Table 3.17). Following cryopreservation by rapid cooling of newly-harvested explants excised with basal cotyledonary segments, the addition of BAP increased the response in terms of the range of cryoprotectant treatments, but not the percentage survival among the treatments (Figure 3.49). In comparison with axes from seeds that had not been stored, survival of explants from stored seeds was poor, being observed only in those treated with a combination of DMSO and sucrose (Figure 3.49). Irrespective of the status of the seeds, however, all survival was in the form of non-embryogenic callus (Figure 3.51).

Explants of *S. gerrardii* were used in an attempt to quantify the concentration of BAP that might stimulate shoot production after cryopreservation. Unexpectedly, the highest survival occurred in the absence of BAP, and a significantly negative impact on survival was obtained when 2mg l^{-1} BAP was used (Figure 3.59). No shoots were produced by

explants exposed to any of the concentrations of BAP used, and surviving explants appeared to be more bulky with a callus-like surface appearance (Figure 3.60).

Future studies should aim at optimizing the cryoprotectant treatments in combination with cooling rates across all three species, to facilitate comparisons and ascertain whether it is possible to standardize the various procedures. In addition, an in-depth analysis of the use of BAP and/or other cytokinins to stimulate shoot development, and auxins to promote callus embryogenicity, should be carried out per species. Additionally, other methods of cooling remain to be investigated, such as ultra-rapid cooling using isopentane (Wesley-Smith *et al*., 1999; 2001a); encapsulation-dehydration (Zhao *et al*., 1999b; Blakesley and Kiernan, 2001); vitrification (Thammasiri, 1999, 2000; Valladares *et al*., 2004) and encapsulation vitrification (Gámez-Pastrana *et al*., 2004; Tanaka *et al*., 2004).

3.4 Quantification of production of reactive oxygen species (ROS)

The different-sized explants (Figure 2.8) of *Trichilia dregeana* were sampled for an epinephrine-adrenochrome colorimetric superoxide assay as described by Beckett *et al.* (2004) to determine whether ROS were produced in response to excision wounding. The biochemical assays indicated a correlation between the ROS production and degree of wounding, with axes excised without any cotyledon attachment showing the highest ROS emission (Univariate ANOVA and *post hoc* Scheffé test, $p < 0.05$), followed, in order, by axes with only basal segments, and axes with 2-mm segments of cotyledon attached (Table 3.18). As is evident from the table, whole (unwounded) embryos (i.e. axis with cotyledons intact) showed negligible evidence of this ROS production (Table 3.18).

High ROS liberation was associated with deficient shoot production by axes (Figure 3.61), with those axes without cotyledon attachments (greatest extent of wounding) all developing roots but no shoots, irrespective of whether the germination medium contained the anti-oxidant AsA or the adsorbent AC (Table 3.18). However, axes with cotyledonary segments showed significantly higher seedling establishment (i.e. both root and shoot production) when cultured on a medium supplemented with AC (Univariate ANOVA and *post hoc* Scheffé test, $p < 0.05$).

Table 3.18: Relationship between epinephrine oxidation, explant size* (inversely related to degree of wounding) and ability for shoot (with root) production on plain WPM and WPM with ascorbic acid (AsA) or activated charcoal (AC). n = 25.

Cotyledon segment	**Oxidized epinephrine** (μmol g^{-1} 15 min^{-1})	**WPM** % shoots	**WPM + AsA** % shoots	**WPM + AC** % shoots
None	5.904	0	0	0
Basal	3.403	48	60	80
2-mm	1.751	64	80	96
Whole embryo	0.004	ND	ND	ND

*refers to amount of cotyledonary tissue attached to axis
ND = not done

Figure 3.61: Seedling development after 10 weeks *in vitro* from explants with no cotyledon (A); basal segment (B); and 2-mm segment (C) when cultured on plain WPM. Bar = 5 mm.

The time-course of ROS production by *T. dregeana* showed that oxidative activity decreased significantly in the more wounded explants after the first 15 min (Univariate ANOVA and *post hoc* Scheffé test, $p < 0.05$) and remained relatively unchanged for the duration of the time trial for all explant sizes (Figure 3.62). ROS activity remained unmeasurably-low in unwounded explants (whole embryos), for the duration of the trial (180 min) (Figure 3.62).

Pea embryonic axes, from seeds imbibed for 24 h, were subjected to the same excision treatments as *T. dregeana*. It was noteworthy that ROS production by pea explants was significantly lower than that occurring with *T. dregeana* explants (Univariate ANOVA, $p < 0.05$). For pea axes, a decline in absorbance also occurred after the initial incubation period (Univariate ANOVA, $p < 0.05$), increasing slightly after 150 min, particularly in the more wounded explants (Figure 3.63). This increase however, was insignificant (Univariate ANOVA, $p > 0.05$). An *in vitro* experiment to assess if excised pea axes produce shoots as well as roots still needs to be determined. Furthermore, it is possible that ROS production measured by this assay is the net effect of both ROS production and ROS quenching by endogenous anti-oxidant systems. As pea is an orthodox seed, anti-oxidants form part of the desiccation tolerance mechanisms. Although imbibed for 24 h, perhaps not all tolerance mechanisms have been lost and pea still has more anti-oxidants than *T. dregeana*, thus a lower net ROS production in pea explants.

Even though absorbance decreased with time after wounding, the results indicate that an oxidative burst occurs during, or immediately after, axis excision as an immediate injury response for both species: further investigations are therefore aimed at measuring ROS production immediately, and at shorter intervals (than the 15 min used in the present study) after excision. The response of the pea axes suggests that more than one oxidative burst could occur over time as a stress response as is also shown in the cells of *Chlorella*, a unicellular alga (Loseva *et al.*, 2002).

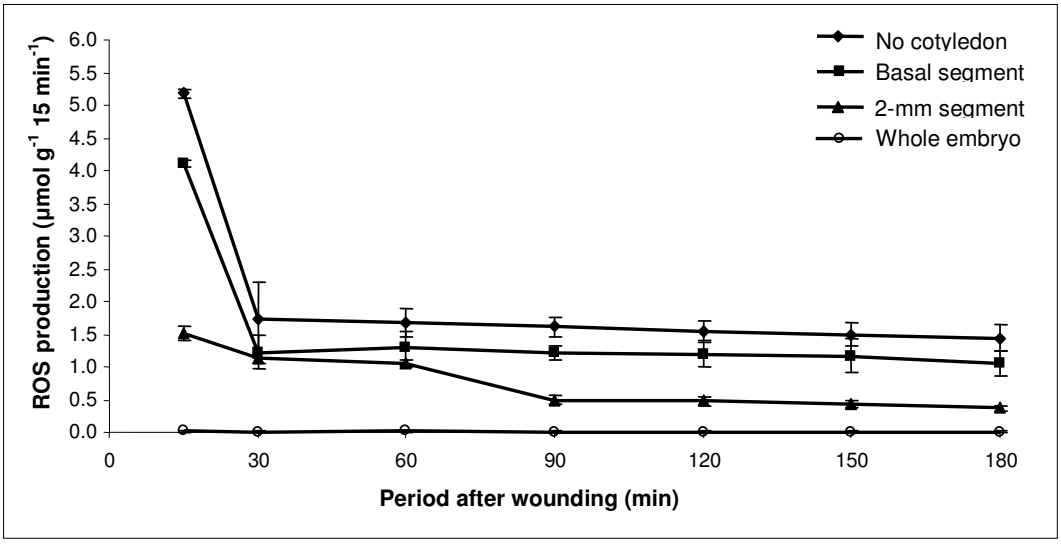

Figure 3.62: The time-course of ROS production in *T. dregeana* explants detected as oxidation of epinephrine to adrenochrome. The same plant material was used throughout, with the solution changed at the start of each time interval. n = 10.

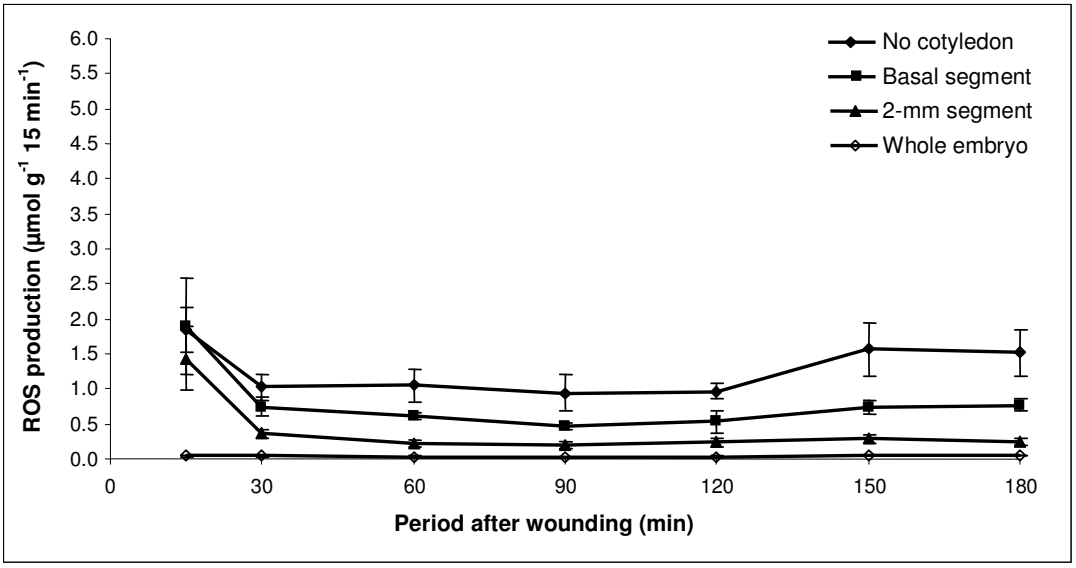

Figure 3.63: The time-course of ROS production in pea explants detected as oxidation of epinephrine to adrenochrome. The same plant material was used throughout, with the solution changed at the start of each time interval. n = 10.

3.4.1 Microscopical analysis

For *T. dregeana* scanning electron microscopical and light microscopical investigations facilitated an appreciation of the nature of the cotyledon attachments to the axis, and the extent and consequences of the lesions formed upon excision. As can be seen from Figure 3.64, complete excision of the sessile cotyledons leaves major wound sites on either side of, and in close proximity to, the shoot apical meristem. Furthermore, proliferation of disorganized callus tissue occurred at the wound sites (Figure 3.65), which in itself might have had deleterious effects on the shoot meristem. It is possible that due to the close proximity of the wound sites to the apical meristem, ROS production as a wounding response, could have adverse and ultimately lethal, effects, thus precluding shoot development.

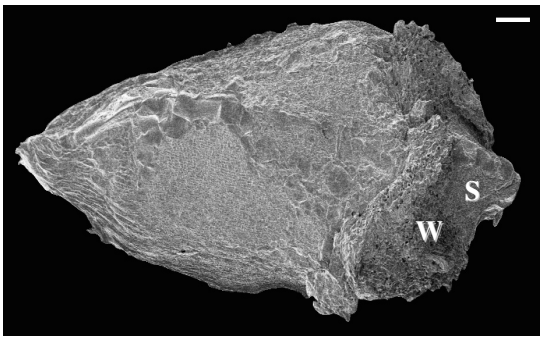

Figure 3.64: Freshly excised axis with no cotyledonary attachment showing the proximity of wound sites (w) to the shoot meristem (s). Bar = 200 µm.

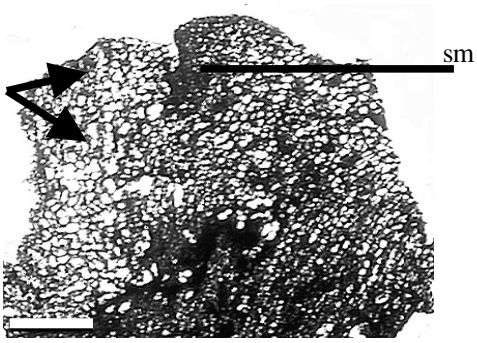

Figure 3.65: Axis without cotyledon attachment after *in vitro* culture for four weeks. Callus production (arrowed area) from wound sites occurred near shoot meristem (sm). Bar = 50 µm.

Ultrastructural studies of the shoot tips of axes immediately after excision showed organized meristematic cells with clearly defined cell walls, (Figure 3.66A) normal disposition of organelles, spherical nuclei and the general appearance commensurate with ongoing metabolism (Figure 3.66B). In contrast, the shoot meristem of 3-d cultured explants from which the cotyledons had been removed flush with the axis surface, had cells with irregular walls, distorted nuclear profiles and irregularly-shaped vacuoles (Figure 3.67A). However, ultrastructurally intact intracellular organelles such as mitochondria and endoplasmic reticulum suggested that a measure of active metabolism was still possible (Figure 3.67B). Nevertheless, in view of the visible ultrastructural abnormalities, and biochemical, or physiological damage which could not be visualized, it is presumed that whatever metabolic capability remained, would have been unbalanced.

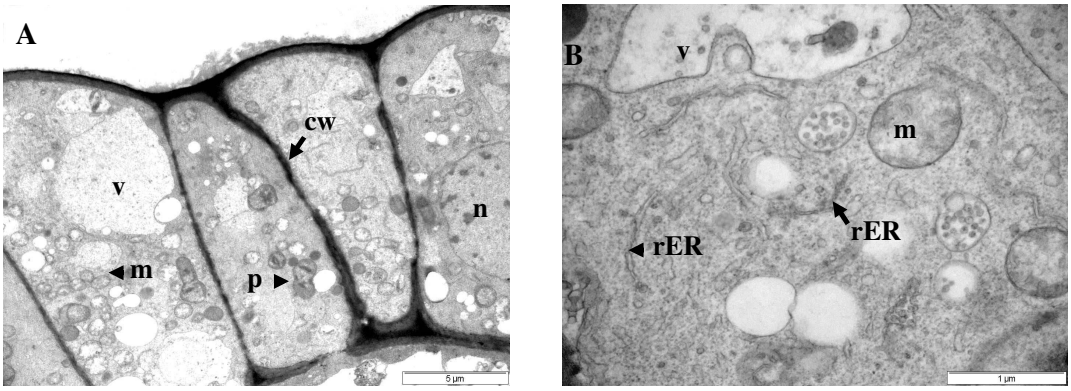

Figure 3.66: Tunica cells of the shoot meristem from explants processed directly after complete cotyledonary excision showing turgid regular-shaped cell walls (A) and an active cytomatrix (B). cw, cell wall; m, mitochondria; n, nucleus; p, plastid; rER, rough ER and v, vacuole.

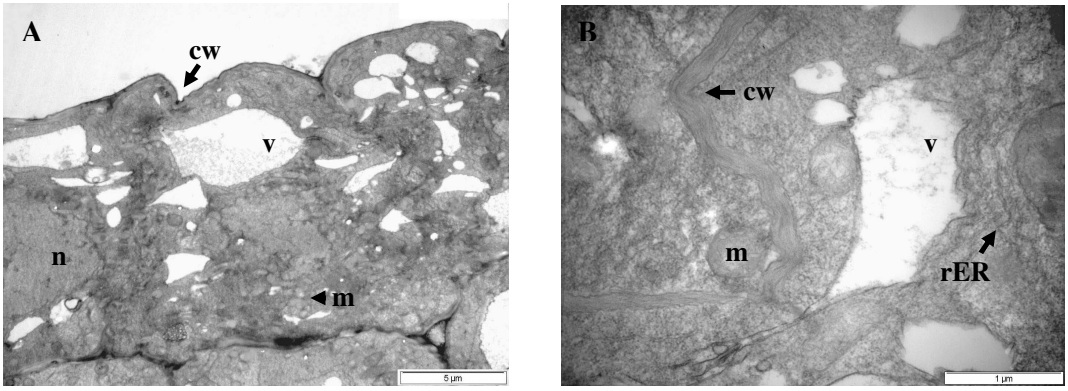

Figure 3.67: Tunica cells of the shoot meristem from explants with no remaining cotyledonary vestiges processed three days after culture showing cell walls and irregular vacuoles (A) although, from their appearance, individual organelles have retained ultra-structural integrity (B). cw, cell wall; m, mitochondria; n, nucleus; rER, rough ER and v, vacuole.

3.4.2 Quantification, and possible consequences of ROS production: concluding remarks

In 2004, Goveia *et al*. reported the lesions on either side of the shoot apex to be the gross cause of damage to the cells of this region, thus compromising shoot development. However, the nature of the causative agent was unknown. Although the initiation and maintenance of the shoot apical meristem is controlled by specific genes (Satoh *et al*., 2003) and wounding has been suggested to inhibit genes controlling the development of meristematic tissue (Souer *et al*., 1996), it is likely that oxidative damage as a result of free radical production is the cause.

A free radical is a highly reactive chemical species containing one or more unpaired electrons and is capable of existing independently (Hendry, 1993). Oxygen is most commonly reduced in mitochondria, chloroplasts and peroxisomes to form superoxide radicals (O_2^-), hydroxyl radicals ($OH^\cdot$) or hydrogen peroxide (H_2O_2) within plant cells. These free radicals are termed reactive oxygen species (ROS) as they are strongly reactive and potentially harmful (Bailly, 2004).

Many ROS are short-lived; this characteristic allows some positive responses within tissues to occur. For example: ROS production in response to pathogen attack has been shown in lichens, French beans and *Chlorella* cells (Minibayeva and Beckett, 2001; Bolwell *et al*., 2002; Loseva *et al*., 2002, respectively) and also plays a role in cell wall loosening to allow for cell elongation and growth, fruit ripening and organ abscission (Fry, 1998; Liszkay *et al*., 2004). In addition, free radicals, as well as H_2O_2, act in signalling, regulating gene expression and cell death (Kovtun *et al*., 2000; Orozco-Cárdenas *et al*., 2001; Pellinen *et al*., 2002).

Reactive oxygen species are also associated with wounding; either triggering wound defence genes (Kovtun *et al*., 2000; Reymond *et al*., 2000; Orozco-Cárdenas *et al*., 2001; Pellinen *et al*., 2002), or stimulating enzymes necessary for synthesis of compounds involved in repair mechanisms (Lagrimini and Rothstein, 1987; Dyer *et al*.,1989). Studies in lichens and wheat roots (Beckett and Minibayeva, 2003; Minibayeva *et al*., 2003, respectively) have demonstrated that an oxidative burst of free radicals is triggered in response to wounding.

If free radicals from oxidative bursts are not scavenged quickly, their build-up is reported to have deleterious consequences (Hendry, 1993; Bailly, 2004). Thus, it is necessary for plants tissues to possess efficient anti-oxidant mechanisms. Edreva (2005) emphasizes the importance of maintaining an equilibrium between ROS production and ROS scavenging, particularly in organelles like the chloroplast. In imbibed orthodox seeds, antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and other anti-oxidants such as 1-cys-peroxiredoxin and conjugated trienes (Abdallah *et al*., 1997; Bailly *et al*., 1998,

2001, 2004; Chandru *et al*., 2003; Türkan *et al*., 2005; Berjak, 2006) function optimally to scavenge ROS. However, in recalcitrant seeds, these anti-oxidant mechanisms may either be impaired or not present, as suggested by Bailly *et al*. (2001) and Türkan *et al*. (2005). Furthermore, Bailly *et al*. (2001, 2004) and Chandru *et al*. (2003) have shown that the antioxidative enzymes produced are also dependent on the maturity of the seed when wounded, ultimately affecting the response to ROS.

Consequently, in response to wounding, ROS accumulate due to inadequate scavenging mechanisms and trigger lipid peroxidation, degradation of nucleic acids and proteins and inactivation of enzymes, ultimately resulting in irreversible cellular damage (Hendry, 1993; Bailly, 2004). Callus production has also been associated with wounding (Stobbe *et al*., 2002) and ROS may be involved in the process, thus possibly accounting for the effects shown in Figure 3.65.

The sustained production of ROS in wounded explants relative to intact seeds (Figure 3.62) suggests that the mechanism to quench ROS in *T. dregeana* axes is ineffective, and that ROS accumulation is implicated in irreversible damage to the shoot meristem. This damage, shown ultrastructually in Figure 3.67, is proposed to prevent shoot development in axes. Generation of ROS may also promote callus formation at the wound sites (Figure 3.65). Furthermore, observations recorded in a previous study (Goveia *et al*., 2004) and presently confirmed (section 3.1.2.2), show that the embryos are under-developed when the seeds are shed, which may render them less able than when fully mature or actually germinating, to counteract oxidative damage. In parallel, Anguelova-Merhar *et al*. (2003) showed that four days after *A. marina* seeds had been harvested, they developed maximum/improved capacity to counteract fungal attack. Thus, it is possible that ROS production in response to fungal presence was able to trigger defence mechanisms in this species (Anguelova-Merhar *et al*., 2003).

The comparison with pea explants (Figure 3.63) shows that ROS are produced in response to wounding in imbibed germinating orthodox seeds, but at a considerably lower level. A second small peak in ROS production (after wounding) was observed in pea explants (Figure 3.63), possibly as a result of increased respiration in response to

wounding, which also has the potential to produce O_2^- (Loseva *et al*., 2002). Similar observations have been made in *Chlorella*, where respiration increased when cells were attacked by pathogens (Loseva *et al*., 2002).

3.4.3 Quenching ROS

In view of the deleterious effects of ROS associated with the shoot apical meristem (shown microscopically and ultrastructurally above), an anti-oxidant treatment was applied to neutralize ROS produced in conjunction with wounding. The rationalization was that upon excision, ROS produced by the explants could be rapidly quenched by the anti-oxidant solution, thereby preventing a build-up and consequent cellular damage.

Ascorbic acid (AsA), is synthesized in germinating seeds (Smirnoff, 1996; De Tullio and Arrigoni, 2003) and is required for the catalytic activity of many enzymes. It has also been suggested to be involved in synthesis of ethylene and gibberellins and in the co-ordination of key enzymes necessary for growth (Smirnoff, 1996; De Tullio and Arrigoni, 2003). In addition, this compound has anti-oxidant properties, thereby providing protection against oxidative damage within cells (Chaitanya *et al*., 2000; Żyracka *et al*., 2005). Therefore, AsA was selected as the anti-oxidant for the treatments.

Activated charcoal (AC) is known to adsorb various organic compounds, including hormones, vitamins, abscisic acid and phenolic metabolites (van Winkle *et al*., 2003). Thus, the addition of this compound to the *in vitro* germination medium could contribute towards seedling development from wounded explants.

When *T. dregeana* axes were immersed in AsA solutions for different periods following excision, explants with no vestiges of cotyledon attached showed a significant decrease in ROS production with soaking period (Univariate ANOVA, $p < 0.05$). Lowest ROS production was observed (Univariate ANOVA and *post hoc* Scheffé test, $p < 0.05$) when explants were soaked in 0.2 mM AsA for 60 min (Figure 3.68). However, this apparent decrease in ROS production may be an experimental artefact. Excised axes were soaked in AsA solution for the appropriate time, the AsA solution discarded, and

the axes then assessed for ROS production. It is possible that ROS produced shortly after excision were leached from the tissue by the AsA solution. The ROS production measured by the epinephrine assay constituted the ROS not leached by the AsA solution.

This putative optimum AsA treatment was then applied to explants with no vestiges of cotyledon attached (as these would be the smallest, and hence most suitable for cryopreservation), which were subsequently germinated on WPM + AC (the best medium determined previously [Table 3.18 and in section 3.1.2]) to assess the effect on shoot development. Ten percent of these explants then showed shoot production when germinated *in vitro* (Figure 3.69). However, although promising, the treatment was far from optimum.

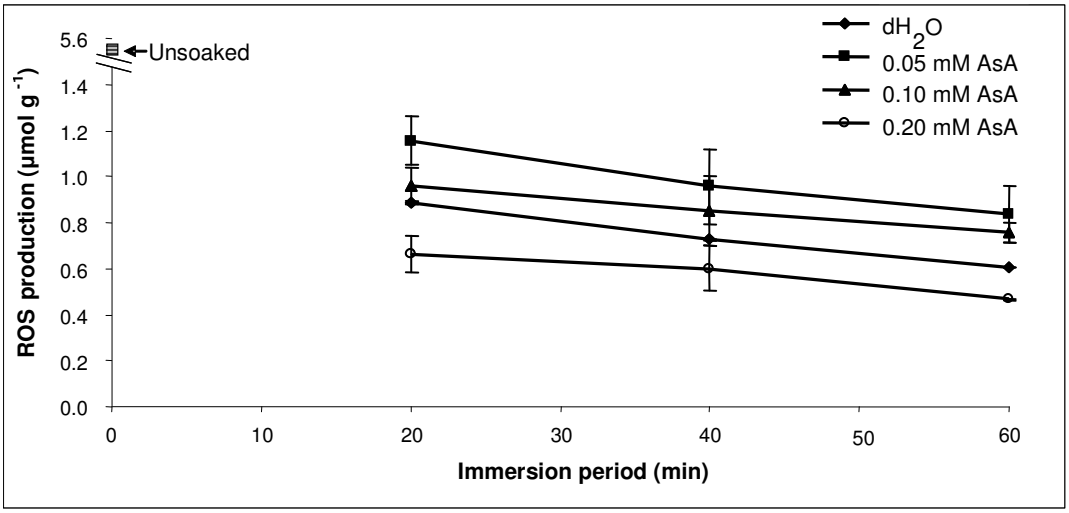

Figure 3.68: The effect of AsA immersion periods immediately after excision on ROS production on *T. dregeana* explants with no cotyledon. Explants were incubated in epinephrine for 15 min to assess ROS production. n = 10.

Figure 3.69: Seedling development showing nascent shoot (ns) production in an explant with no cotyledonary vestiges after soaking for 60 min in 0.2 mM AsA and then cultured on WPM + AC for eight weeks. Bar = 5 mm.

Further studies should test higher AsA concentrations and longer treatment periods, the effects of other anti-oxidants both prior to and directly after excision, as well as excising under the anti-oxidant solution to optimize shoot development in explants with no vestiges of cotyledon attached.

3.4.4 Quenching ROS: concluding remarks

Very limited success of AsA or AC in the germination medium in counteracting the damage suggested to be induced by ROS, suggests that it might occur immediately upon excision, before the axes are respectively immersed in, or cultured with, these compounds *in vitro* (Table 3.18). Axes immersed in AsA immediately after excision showed lower ROS activity (Figure 3.68) and 10% shoot development when cultured *in vitro* (Figure 3.69), indicating that the oxidative burst after excision might be counteracted if immediate ROS production is adequately quenched. While immediate post excision anti-oxidant provision appears to have potential, procedures aimed at promoting anti-oxidant production prior to cotyledon excision, needs to be investigated.

Ascorbic acid is an essential anti-oxidant, being involved in many different stages in the plant life cycle, and is also involved in the synthesis and activation of a spectrum of enzymes (De Tullio and Arrigoni, 2003). Thus, most publications on plant anti-oxidant responses deal particularly with the use of AsA or AsA-activated enzymes. However, a range of other anti-oxidants should also be tested to determine whether it is possible to alleviate ROS production, suggested to be a primary wounding response.

This study indicates that ROS generation associated with wounding may preclude shoot development. Future investigations should aim to identify the specific ROS associated with wounding as well as optimizing an anti-oxidant treatment that would counteract ROS, and apical meristem derangement, thus promoting shoot development.

4. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

This study utilized non-orthodox seeds from the phylogenetically close species *Trichilia dregeana* and *T. emetica* (the exendospermous seeds of which are morphologically similar) and a non-related species *Strychnos gerrardii* (which produces endospermous seeds) to investigate the effect of developmental status on responses of variously-excised explants to dehydration and cryopreservation.

The effect of developmental status on *in vitro* germination of the excised embryonic axes of *T. dregeana* and *T. emetica*, even without the additional stresses of dehydration/ cryopreservation, was clear. Axes from seeds of both species that were stored were better able to withstand excision injury. This may be attributed to the embryonic axes being under-developed at shedding and developing further during seed storage, for the two *Trichilia* species. In contrast, the embryos of *S. gerrardii* may be fully developed when the fruits are shed, but newly-mature seeds (from green fruits) may enter a relatively quiescent phase while endosperm consolidation occurs as the fruit colour changes from green to yellow. In all the species tested, however, short-term storage enhanced *in vitro* germination of embryos or embryonic axes, demonstrating the post-shedding development associated with non-orthodox seeds.

Besides developmental status, another factor that was demonstrated as affecting *in vitro* germination of excised embryonic axes in the exendospermous seeds tested was the degree of wounding to which the axes were subjected, during excision. However, excision is necessary to reduce the explant size for successful dehydration/cryopreservation. Thus further investigations aimed at reducing or optimally, obviating, the deleterious effects of wounding, are necessary. The procedures developed and tested in this study were not successful in avoiding the damage caused by wounding, but demonstrated that such damage is mediated, at least partially, but perhaps principally, by reactive oxygen species (ROS). While the damage may involve ROS and perhaps other toxic substances being released from the wound sites left after excision of the cotyledons, it may be ameliorated by short-term hydrated storage of the seeds prior to axis excision. This may simply be the consequence of further development and extension growth of the shoot apex, thus increasing the distance

between the apical meristem and the wound sites. The optimal storage period for this purpose, as well as the toxins or ROS that may be involved could, however, not be established in the scope of this study, these remaining important lines of further enquiry.

When *in vitro* germination was tested for embryonic axes or embryos of the three species, this was decreased by dehydration of the explants prior to culture, demonstrating the desiccation sensitivity of the explants. Notably, the axis/embryo shoot pole of the explants were shown to be more sensitive to desiccation damage than was the root pole - a phenomenon that has been increasingly observed for many non-orthodox embryonic axes/embryos (e.g. Kioko, 2003; Kioko *et al*., 2006; Perán *et al*., 2006). Short-term storage of *Trichilia* seeds decreased desiccation sensitivity, which is unexpected for recalcitrant seeds. This was thought to be due to an increased resilience of the meristem, which became increasingly organized without germination having being initiated, during storage. This study therefore demonstrated the effect of a further factor (degree of meristem development at shedding) influencing the physiology of post-harvest recalcitrant seeds during storage.

Post-shedding development was also shown to influence positively the amenability of embryonic axes to cryostorage for *Trichilia* spp., while increased development in *S. gerrardii* (as measured by a change in fruit colour from green to yellow) also enhanced the ability of embryos to survive cryopreservation. Although these positive effects were evident for only a small proportion of explants, they were consistent across the species. Thus, as this study set out to investigate, the degree of development appears to have a pivotal effect on the response of non-orthodox explants to cryopreservation. However, only a limited number of cryopreservation protocols could be tested in the course of this study, but the influence of the manipulations to achieve cryopreservation on the response of plant tissues, is widely acknowledged (Berjak and Dumet, 1996; Berjak *et al*., 1996, 1999b; Engelmann, 1999, 2004; Berjak and Pammenter, 2001; Wesley-Smith *et al*., 1999, 2001a). Thus, further trials with more cryostorage regimens may improve the outcomes, and should aim to overcome the underlying causes of the limited success obtained here. An optimization of the developmental status of *in vitro* regeneration methods and of the cryopreservation protocol may lead to the achievement

of organized post-thaw survival, which was not achieved in this study for the *Trichilia* species tested, and was only minimal for *Strychnos gerrardii*.

Further studies should therefore aim to optimize the degree of post-harvest axis/embryo development during storage, as well as utilization of shorter sampling intervals. Attention needs to be directed also towards *in vitro* germination protocols (including the use of appropriate plant growth regulators [e.g. Ryynänen, 1998; Turner *et al*., 2001; Renau-Morata *et al*., 2005; Zhao *et al*., 2005] or new approaches such as the employment of smoke technology [e.g. Light and van Staden, 2004]). Explant preparation techniques including axis/embryo excision, need focused attention, as does the investigation of the effects of a wide array of cryopreservation approaches (including the use of different cooling rates and freezing techniques (reviewed by Berjak and Pammenter, 2007; Wesley-Smith *et al*., 1999; 2004b), cryoprotectants (Meryman and Williams, 1985; Wesley-Smith, 2002; Fuller, 2004), and associated *in vitro* procedures [e.g. culturing explants in different periods of darkness after thawing]).

What should receive priority in the arena of future investigations, is the aspect of ROS production as an immediate injury response, and a means to counteract ROS-mediated effects by the rapid quenching of ROS themselves. To this end, identification of the individual ROS generated, and the potentially ameliorating effects of selected anti-oxidants and when these should be supplied, requires urgent attention. Furthermore, a comparison with hydrated orthodox seeds should also be made.

Ultimately, this study has demonstrated that the successful cryopreservation of the germplasm of the species tested, and others producing recalcitrant seeds, depends on numerous species-specific factors.

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