POST-HARVEST SEED PHYSIOLOGY AND CONSERVATION OF THE GERMPLASM OF *Syzygium cordatum* Hochst.

by

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requirements of the degree of

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Publication 1.

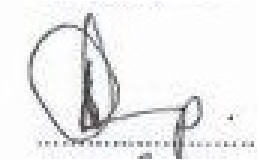
Cheruiyot, C., Wesley-Smith, J., Berjak, P. and Kioko, J.I. (2004). Citric acid as a pre-fixation treatment of phenol laden shoot axes of *Syzygium cordatum*. *Microscopy Society of Southern Africa – Proceedings* **34:** 62.

The experimental work and writing of publication was done by Anastacia Cheruiyot. However, the process of both the experimental work and writing was supervised by Prof. Pat Berjak and Dr Joseph Kioko.

Publication 2.

Cheruiyot, C., Kioko, J.I., Berjak, P. and Wesley-Smith, J. (2007). *In vitro* regeneration of recalcitrant embryonic axes: Effects on the biomass characteristics of resulting plants*. South African Journal of Botan*y **73**: 483.

The experimental work was done by Anastacia Cheruiyot and supervised by Prof. Pat Berjak and Dr Joseph Kioko. The writing was done by Dr Joseph Kioko.

Signed: 

PREFACE

The experimental work described in this thesis was carried out in the School of Life and Environmental Sciences, University of Natal, which later changed to the School of Biological and Conservation Sciences, University of KwaZulu-Natal, from February 2003 to November 2011, under the supervision of Prof. Patricia Berjak and Dr Joseph Kioko. Since January 1st, 2012, the School is now known as School of Life Sciences.

These studies represent original work by the author and have not been submitted in any form to another university. Where use was made of work of other people, it has been duly acknowledged in the text.

Anastacia Chepkorir Cheruiyot

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My darling husband Richard, thanks for the final push! Your contribution at the end made all the difference and for that reason I will be forever grateful.

Above all, thank you to my God! You truly are the best.

This thesis is dedicated to that little child in this world somewhere who marvels at nature and wonders why plants do things the way they do. You are where I was many years ago. May you get to know the God who created them. May your journey of discovery take you to places beyond your imagination and may you gain knowledge to nurture the very plants at which you marvel.

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ABSTRACT

There is global concern about the *ex situ* conservation of the germplasm/genetic resources of recalcitrant-seeded species. While orthodox (desiccation tolerant) seeds afford an ideal means for *ex situ* conservation, this is impossible for recalcitrant seeds which are shed at high water contents, are metabolically active, and are desiccation sensitive, with those of many species losing viablity when only a small proportion of tissue water has been removed. Storing such seeds in the short- to medium-term is possible when parameters to be optimised include the means to obviate dehydration and the most equitable storage temperature – and, if necessary – the best way to curb the activity of seed-associated micro-organisms (usually fungi) during such hydrated storage. Presently, it is generally agreed that the only option for long-term *ex situ* conservation of the germplasm of recalcitrant-seeded species is by cryopreservation (usually in liquid nitrogen) of explants representing the same genetic diversity as do the seeds. To achieve this, the explants of choice are embryonic axes excised from the seeds. However, there are still many problems impeding progress particularly for tropical/sub-tropical species: presently, these need to be resolved on a species-specific basis. To this end, the current investigation was focused on germplasm of the tropical/sub-tropical recalcitrant-seeded species, *Syzygium cordatum* Hoechst. There were two major aspects to the study, viz. optimisation of the 'shelf-life' of intact seeds in the interest of almost immediate planting programmes, and attempting to develop a protocol which would result in successful cryopreservation of zygotic axes excised from the seeds. Chapter One of this Thesis provides an overview of the theoretical basis underlying these two approaches to conservation, as well as a description and significance of the species under study.

Chapter Two describes the study seeking to establish optimal short-term storage conditions for the recalcitrant seeds of *S. cordatum*. Seeds were stored at various relative humidities at three different temperatures (6 ºC, 16 ºC and 25 ºC) for differing periods. Seeds stored at all these temperatures maintained stable water contents. The most mature seeds that were stored in a saturated atmosphere at both 16 ºC and 25 ºC reached their root protrusion stage after three weeks. This, however, occurred in only a small percentage of the seed batches. The majority of the seeds that were stored under saturated atmospheric conditions at 16 ºC and 25 ºC had not reached the stage of radicle elongation before the sixth week of storage, but after this time there was evidence of damage associated with both fungal proliferation and desiccation sensitivity. Seeds stored at 6 ºC and 25 ºC for the longest period had also lost vigour. For seeds stored at 6 ºC and 25 ºC (whether under hydrated or non-hydrated conditions), those stored for the shortest and longest periods produced the smallest seedlings. The seeds stored at 16 ºC appeared to have maintained vigour and seedling size did not change with the period of seed storage prior to sowing. Storage at 6 ºC may have caused stress associated with chilling, while at 25 ºC, seed storage was compromised by fungal proliferation. Those seeds stored in unsaturated atmospheric conditions at 16 ºC exhibited an increase in their germinative index and germination rate after six weeks. This is possibly associated with the ability of seeds, where vigour was not compromised, to counteract fungal proliferation because there was a decrease in the number of seeds showing fungal proliferation. In contaminated seeds, the fungus appeared to proliferate from the surface of the coat, to the cotyledons and eventually to the axes. Seeds generally did harbour fungal inoculum at harvest, but proliferation, was reduced at cool temperatures.

Based on the above observations, storage in sealed plastic bag (non-saturated atmospheric conditions) at 16 ºC was chosen for the short-term maintenance of seeds of *S. cordatum*.

The studies described in Chapter Three sought to establish a protocol for the cryopreservation of embryonic axes of *S. cordatum*. These studies involved the stepwise optimisation of decontamination, regeneration and growth, dehydration, cryoprotection and cooling (freezing) conditions. The most suitable combination of biotechnological manipulations for the preparation of embryonic axes of *S. cordatum* for cryopreservation were: decontamination by exposure to 1% (v/v) $Ca(OCl)_2$ for 5 min; cryoprotection using a 5% solution of dextran and DMSO for 1 h followed by exposure to a 10% solution of these cryoprotectants for another hour; then dehydration in a flash dryer for 75 min; and regeneration in agitated liquid medium containing woody plant medium, 10 g l^{-1} polyvinylpyrrolidone and 75 mg l^{-1} citric acid. A major achievement following this procedure, was the prevention of excessive exudation of phenolic compounds from the explants. Nevertheless, despite optimisation of all these procedures, axes did not survive cryogenic exposure.

One of the objectives of the present study was to develop the means for visualisation of intracellular detail of axis cells of *S. cordatum*. An experiment was thus entrained to investigate the effects of exposing shoot tips to 75 mg l^{-1} citric acid for 10 min before fixation during preparation for transmission electron microscopy. In the absence of any ameliorative treatments, large electron dense polyphenolic precipitates were observed mainly inside vacuoles closely associated with the tonoplast. Less dense, small precipitates were located between the plasmalemma and the cell wall, and organelles were generally not

clearly visible, probably because of leaching of phenolics into the cytoplasm. Thus the effects of various treatments on organelles and the entire cell ultrastructure could not be conclusively determined. When treated with citric acid, cells had no visible polyphenolic precipitates and the apparently intact organelles were clearly visible, so paving the way for electron microscopical examination of this – and perhaps any other – plant tissue containing substantial amounts of phenolic substances.

LIST OF ABBREVIATIONS

%	percent
X^2	chi-square
>	more than
<	less than
1:1	one part to one part
dry or non-sat or unsat	unsaturated storage conditions
hyd or sat	saturated storage conditions
anon	anonymous
ANOVA	one way analysis of variance
$CaCl_2.2H_2O$	calcium chloride
$Ca(OCl)_2$	calcium hypochlorite
cm	centimetres
ºC	degree Celsius
ºC s^{-1}	degree Celsius per second
ºC min^{-1}	degree Celsius per minute
df	degrees of freedom

DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
fmb	fresh mass basis
g	gram
g g^{-1}	grams of water per gram embryo dry weight
g l^{-1}	gram per litre
Gv	germination index
Gp	cumulative germination percent after a specified time
DGs	daily germination speed (this is obtained by dividing the cumulative germination percent by the number of days since sowing [Gp/D])
D	number of days since sowing
ΣDGs	the sum of DGs figures obtained from the daily counts
GSPC	the Global Strategy for Plant Conservation
h	hours
HCl	hydrochloric acid
ICRAF	International Council for Research in Agroforestry
kV	kilovolts
LEAs	late embryogenic abundant proteins
M	molar
µM	micromolar
mM	millimolar
min	minutes
mg	milligrams
mg l^{-1}	milligrams per litre

ml	millilitres
ml l^{-1}	millilitres per litre
mm	millimetres
$MgCl_2.6H_2O$	magnesium chloride
MS	Murashige and Skoog (1962) basal salt and vitamins
N	sample size
nm	nanometres
NaOCl	sodium hypochlorite
P	confidence level
PVP	polyvinylpyrrolidone
R	pearson's product moment correlation index
ROS	reactive oxygen species
SD	standard deviation
Sec	seconds
UN	United Nations
UV	ultra violet
v/v	volume per volume
WC	water content
WPM	McCown's woody plant basal salt mixture (Lloyd and McCown, 1981)
w/v	weight per volume

CHAPTER 1. INTRODUCTION

Over the past few years, biodiversity conservation institutions have placed increasing emphasis on the promotion of fast-growing tree species that have a variety of uses such as fuelwood, timber and fodder, in order to relieve deforestation pressure on indigenous forests. These species are termed 'multipurpose trees' and are often associated with village-level afforestation. The World Agroforestry Centre, formerly the International Centre for Research in Agroforestry (ICRAF) based in Nairobi, has identified over 2000 multipurpose tree species. These species are purposely grown, preserved, or managed, either for commercial purposes, household use, or for land improvement (Hines and Eckman, 1993). One of these multipurpose tree species is *Syzygium cordatum* Hochst.

There are six African species in the genus *Syzygium*. These species occur naturally in the warmer parts of the summer rainfall areas in South Africa and are characterised by their dense evergreen foliage and sweetly scented, conspicuous clusters of white flowers. They produce a large number of fruits (purple when ripe) that are eaten by humans and some animals. Four species that are widely grown in gardens are *S. cordatum*, *S. guineense*, *S. gerrardii* and *S. pondoense.*

Syzygium cordatum (commonly known as waterberry) is a medium sized evergreen tree that grows up to 20 m. According to Pooley (2003), this species

is found in wooded grasslands, forests and along watercourses. The main stem of the tree is usually crooked and the canopy dense with a rounded crown. This plant has characteristic opposite leaves which clutch the stem, with the pairs occurring at right angles to each other. The bluish-green leaves are thick and have a rounded tip and base, which is deeply lobed, and young leaves are reddish.

Syzygium cordatum has a variety of uses: the fruits are eaten and used for wine-making and the leaves for medicine; the wood provides building material and furniture; and products of *S. cordatum* are also used in rituals (Pooley, 2003). As a medicine, the liquid extract of leaves is used to remedy diarrhoea, while pounded leaves, bark and roots are applied to the breasts of nursing mothers to increase milk flow (Msanga, 1998). A bluish-black dye from *S. cordatum*, which is extracted from the bark or fruits by boiling them in water and then 'fixing' it by adding lemon juice and salt (Hines and Eckman, 1993), is used to colour fabrics and leather.

Fruits collected in the forest or from trees retained on rural farmlands are an important source of minerals and vitamins, especially for children, and most fruits are consumed in the forest rather than sold in markets (Hines and Eckman, 1993). This indicates that the importance of these fruits to the communities is nutritional rather than economical. An advantage of the nutritional intake of forest fruits is that different species ripen during different seasons, thus enabling indigenous fruits to continuously compensate for

deficiencies in nutrient intake from green leafy vegetables and cultivated fruits and vegetables (Hines and Eckman, 1993). There is high demand for the products of *S. cordatum* in sub-Saharan Africa, especially for building materials, which puts considerable pressure on natural forest trees, particularly those near villages and towns (Hines and Eckman, 1993). Given the high demand and the value that many people put on indigenous species, it is important to grow the preferred species outside the forest reserves in order to reduce pressure on existing forests (Hines and Eckman, 1993). The World Agroforestry Center, ICRAF, has identified *S. cordatum* as a species that has the potential for cultivation and sustainable use, and also one that requires urgent conservation in Kenya.

Germplasm conservation

The concept of germplasm conservation relates to maintaining the economic, societal and scientific value of the heritable components within and among species (Palmberg-Lerche, 1992; Jackson and Kennedy, 2009). The maintenance of these components has been significantly slow for plant species indigenous to Africa (Okigbo, 1994) due to various factors, which include: climate change (Santuah, 2005); poor management of state-owned forests (Crouch *et al.*, 2008); lack of scientific capacity (Nordling, 2009); lack of financial resources; increased population settlements; and the indiscriminate harvesting of traditionally-utilised species (Jackson and Kennedy, 2009). As these highly utilised species become more scarce, other, less desirable species

are used as substitutes, thus exacerbating the over-exploitation of natural plant resources even further (Mander, 1998).

There is also pressure to collect and conserve the genetic resources of wild species, some of which could be used to improve the productivity of present agricultural crops, which is important in the context of the ever-increasing world population (Gepts, 2006). The protection of plant habitats in which such wild species occur is therefore economically important (Crouch *et al*., 2008).

Many older reviews (e.g. Spears, 1979) predicted that environmental degradation would be a major problem that would have to be remedied by expanding the area dedicated to biotic and wildlife conservation, and by increasing the area of plantation forests. While such remedies have already been implemented, they fail to meet the targets set for biodiversity conservation, thus additional strategies need to be sought to achieve this goal (Paton, 2009).

The United Nations Convention on Biological Diversity was adopted at the 1992 Earth summit in Rio de Janeiro (http://www.cbd.int/convention/text [accessed September 2011]). As directly quoted, this convention was an agreement by a vast majority of world leaders to commit to: “the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources,

including by appropriate access to genetic resources and by appropriate transfer of relevant technologies, taking into account all rights over those resources and technologies, and by appropriate funding (http://www.cbd.int/convention/articles/default.shtml?a=cbd-01 [accessed November 2012])."

Article 7 of this convention describes the responsibility that each member party has towards identification and monitoring of biological diversity. This article states that it is the responsibility of a contracting party to " … identify components of biological diversity important for its conservation and sustainable use …, to monitor … the components of biological diversity identified … paying particular attention to those requiring urgent conservation measures and those which offer the greatest potential for sustainable use (http://www.cbd.int/convention/articles/default.shtml?a=cbd-07 [accessed November 2012])."

In April 2002, the Conference of Parties of the Convention of Biological Diversity adopted The Global Strategy for Plant Conservation (GSPC), which, embodied 16 targets that needed to be achieved by 2010. The targets were then updated in 2010 and extended to 2020 (https://www.cbd.int/decision/cop/?id=12283 [accessed November 2012]). According to the updated goals, five key objectives of the Global Strategy for Plant Conservation are: a) Plant diversity is well understood, documented and recognized; b) Plant diversity is urgently and effectively conserved; c) Plant diversity is used in a sustainable and equitable manner; d) Education and

awareness about plant diversity, its role in sustainable livelihoods and importance to all life on earth is promoted; and e) The capacities and public engagement necessary to implement the Strategy have been developed (http://www.plants2020.net/files/Plants2020/GSPCbrochure/gspc_english.pdf [accessed November 2012]).

This study contributes to objective (b) of the GSPC, i.e. conservation of the diversity of plant germplasm. There are two basic modes of plant germplasm conservation: *in situ* and *ex situ*.

Article 2 of the CBD defines *in situ* conservation as "the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties" (UNCED, 1992). Target 8 of the GSPC is that "… at least 75 per cent of threatened plant species in *ex situ* collections, preferably in the country of origin, and at least 20 per cent available for recovery and restoration programmes (http://www.cbd.int/decision/cop/?id=12283 [accessed November 2012])."

On the other hand, *ex situ* conservation is the conservation of components of biological diversity outside their natural habitats. The GSPC set a target for "… 75% of threatened species to be conserved in accessible *ex situ* collections

and 20% of them be included in recovery and restoration programmes (http://www.plant2020.net/files/plant2020/popular_guide/englishguide.pdf [accessed November 2012])." However, it is unlikely that these targets have been met globally (Berjak, pers. comm.[1]).

Biodiversity conservation programmes generally integrate both *in situ* and *ex situ* conservation methods, but the conservation of genetic variation within a species for immediate breeding purposes uses *ex situ* methods (Chin, 1992).

Ex situ conservation of plant germplasm

Most *ex situ* conservation of plants is by means of seeds, vegetative tissue and cells that are preserved in an artificial environment. For any particular species, it is important that sufficient genetic diversity is conserved in order to allow the species to realise its full evolutionary potential (Chin, 1992; Bartels and Kotze, 2006). The genetic diversity of a plant species is considered sufficient across 20 to 30 individuals for a single population, hundreds of individuals for conserving a gene pool, and several thousand for the maintenance of heterozygosity (Engelmann, 1991). *Ex situ* field conservation has been made almost impossible by a number of factors including: the drastic decrease of land space, the associated high labour costs, the susceptibility of plants to natural disasters, pests and pathogens, and changes in government policies (Engelmann, 1991). These problems facing field genebanks necessitate the

[1] P. Berjak, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban, South Africa.

exploration of other *ex situ* conservation options, principally seed banks and *in vitro* cultures (Chin, 1992).

Seed banking

The capacity of seeds to be stored for a long time under conditions that enable them to maintain viability and vigour provides one of the most efficient methods of conserving plant germplasm (Withers, 1988). Seed banks provide an efficient and cost-effective option for germplasm conservation, not only because seeds occupy relatively little space and require only periodic attention (Bonner, 1990), but also because each seed has the potential to develop into a genetically unique plant, thus maintaining genetic diversity within the species. This means that the full evolutionary potential of the species, as mentioned above, can be realised. Seed storage is used for nearly 90% of the germplasm accessions maintained worldwide (Engels and Engelmann, 1998, reviewed by Börner, 2006).

The key purpose of a seed bank is to collect, conserve and provide or exchange seeds (Chin, 1992). Seed collected could either be from natural forests, or from other *ex situ* sources like seed orchards and other seed banks (Lapido *et al.*, 1992). In seed banks, generally, the seeds collected are assessed so that seeds of only high quality and maximum viability are stored (Chin, 1992). According to that author, stored seeds can either be in active collections that are stored in the short- to medium-term, or base collections that are stored for the long-term.

On the African continent, there are eight seed banking facilities - in Zambia, South Africa, Namibia, Kenya, Nigeria, Burkino Faso, Ethiopia, Tanzania, and Madagascar – but most of stored seeds in Africa are of agricultural value (Berjak and Pammenter, 2004b). Globally, some of the larger seed storage facilities include the Millennium Seed Bank in the UK (http://www.kew.org/index.htm [accessed November 2012]), the Svalbard Global Seed Vault in Norway (http://www.regjeringen.no/en/dep/lmd/campain/svalbard-global-seed-vault.html?id=462220 [accessed November 2012]), and the Australian Tree Seed Centre (http://www.csiro.au/Organisation-Structure/National-Facilities/Australian-Tree-Seed-Centre.aspx [accessed November 2012]); and there are also seed banking facilities in countries such as Costa Rica, India, Japan, China, Russia, Germany and the USA (Börner, 2006).

Seeds stored in a seed bank require appropriate harvesting, drying and handling in order to minimise deterioration. Seeds are usually dried at 15 ºC and a relative humidity of 15% (Chin, 1992) to achieve a moisture content (fmb) of 3-7% (Roos, 1989). These dried seeds are then stored in hermetically sealed containers (Chin, 1992). The sealed containers are maintained at temperatures down to –20 ºC, and tested at regular intervals to monitor viability.

It has become increasingly evident that not all seed types can be stored in the conventional way used by seed banks (Chin and Roberts, 1980) because

seeds of an appreciable number of species have different requirements (Roberts, 1973; reviewed by Berjak and Pammenter, 2004a).

Based on their responses to drying and low temperature, seeds can be divided into two broad categories: orthodox and non-orthodox. Orthodox seeds usually dry down naturally to low moisture levels before harvest; can be further dehydrated after harvest to even lower moisture contents without losing viability; and can be cold-stored for many years (Roberts, 1973; Ellis and Roberts, 1980). Non-orthodox seeds do not conform to the definition of orthodox seeds and include intermediate and recalcitrant seeds. Intermediate seeds can be dehydrated, but not to the extent of orthodox types without losing viability; and may be injured by cold storage. In the dry state, such seeds are also generally relatively short-lived (Ellis *et al.*, 1990; 1991a; Hong and Ellis, 1996). Recalcitrant seeds are shed in the hydrated state, cannot withstand any significant dehydration and cannot be conventionally stored (Roberts, 1973; reviewed by Berjak and Pammenter, 2008). The seeds of *S. cordatum* were first reported by Grabe (1989), to exhibit recalcitrant physiology.

Seed Recalcitrance

According to Roberts *et al.* (1984), recalcitrant seeds are those that cannot tolerate a reduction of their water content below a relatively high value at which they are sensitive to cooling to subzero temperatures (reviewed by Berjak and Pammenter, 2008).

Most recalcitrant-seeded plants occur in environments with few seasonal constraints to immediate seedling recruitment perhaps because the acquisition of desiccation tolerance in those regions would not offer a selective advantage (reviewed by Berjak and Pammenter, 2004a; 2008). With the continuing investigations of a wider range of seeds, it has become evident that more tropical and sub-tropical tree species than previously identified, produce seeds that are recalcitrant (Seme *et al*., 1994; Berjak and Pammenter, 2004a; 2008; Sacandé *et al.*, 2004). Recalcitrance occurs predominantly in tree species (Berjak and Pammenter, 2001; Seed Information Database, http://www.kew.org/data/sid [accessed September 2011]), but has also been found in geophytic amaryllids (Sershen *et al.*, 2008).

A number of mechanisms have been identified as contributing to the acquisition of desiccation tolerance in orthodox seeds. These mechanisms can be either fully present, partially present or absent in seeds (Pammenter and Berjak, 1999). It is suggested that it is the degree of expression of these mechanisms that confer the properties of recalcitrance or orthodoxy to seeds (Berjak and Pammenter, 2001). Those seeds that are highly recalcitrant, e.g. *Avicennia marina* (Farrant *et al.*, 1993) probably express none of the mechanisms implicated in the acquisition of desiccation tolerance, and those that are highly orthodox express all (Berjak and Pammenter, 2001). Between the extremes, however, is a gradation of responses to dehydration that suggests that responses of seeds to desiccation constitute a continuum from extreme

orthodoxy, through the variety of intermediate types, to extreme recalcitrance (Berjak and Pammenter, 2008).

The critical developmental phase that is facilitated by the ability for orthodox seeds to dehydrate to low water (moisture) contents without losing viability, is maturation drying. During maturation drying, most of the processes and mechanisms associated with desiccation tolerance in orthodox seeds have been expressed. Large vacuoles in the cells of orthodox seeds fragment into smaller compartments that are filled with insoluble reserves (Obroucheva and Antipova, 1997); the cells accumulate large amounts of starch outside of their vacuoles (Vertucci and Farrant, 1995) and the cell walls show plasticity, which facilitates folding during dehydration (Webb and Arnott, 1982); the metabolic processes in the seeds 'switch off' (Smith and Berjak, 1995; Berjak and Pammenter, 2008); efficient antioxidant systems that quench free radicals generated during water stress are present and active (Smith and Berjak, 1995); and various protective molecules like late embryogenic abundant proteins (LEAs) (Lalonde and Bewley, 1986; Walters *et al.*, 1997), and sucrose and larger oligosaccharides accumulate in cells (Koster and Leopold, 1988; Hoekstra *et al.*, 2003).

Unlike orthodox seeds, recalcitrant seeds cannot undergo maturation drying (Farrant *et al.,* 1988; Berjak and Pammenter, 2001; 2003; 2008). This means that seeds remain hydrated throughout development, and are actively metabolic before, when, and after they are shed (Pammenter and Berjak,

1999). The metabolic rate of the seeds appears to vary depending on the developmental stage of the seeds, and is related to desiccation sensitivity in that seeds with lower metabolic rates are relatively less sensitive to desiccation (Berjak *et al*., 1993).

Although most of the evidence is anecdotal, it appears that recalcitrant seeds of many species are chilling-sensitive (Roberts, 1973; Chin and Roberts, 1980) and the latter authors record that some, e.g. *Theobroma cacao will* not withstand storage below 15 ºC. However, this is not the case for all recalcitrant seeds, as those occurring in temperate regions are naturally exposed to chilling temperatures, at least at night.

In a seed bank orthodox seeds are generally stored at a moisture content of 7% or less at a temperature of between +5 and –20 ºC (Roos, 1989). This storage practice cannot be used for non-orthodox seeds because of their desiccation (and probable) chilling sensitivity if recalcitrant, with those of the intermediate type being short-lived and, in many cases, also chilling-sensitive, at low water contents (Hong and Ellis, 1996). When conventional storage is modified so that non-orthodox seeds are stored at relatively higher temperatures and high relative humidity (Corbineau and Côme, 1988), rapid fungal proliferation generally occurs, as recalcitrant seeds of many species have been shown to harbour a spectrum of surface and internal fungal contaminants at harvest (Mycock and Berjak, 1990; Calistru *et al.*, 2000). Even if seed-associated mycoflora did not pose a problem, recalcitrant seeds initiate germination in

storage without provision of any extraneous water, becoming increasingly desiccation sensitive as germination progresses (Berjak and Pammenter, 2004a,b). According to those authors, in this condition, the seeds (developing seedlings) rapidly lose vigour and viability in storage.

As an alternative to seed storage, there are several *in vitro* storage options, including short- to medium-term storage, also known as minimal growth storage, which aims at maintaining germplasm in conditions that reduce growth (Engelmann, 1997); and long-term storage by cryopreservation (Gnanapragasam and Vasil, 1990; Engelmann, 2011). As reviewed by Engelmann (2011), many studies on the storage of the germplasm of non-orthodox seeded species suggest that the only feasible method for the conservation of genetic resources of these species producing is by cryopreservation.

Cryopreservation of plant germplasm

Cryopreservation is the conservation of genetic resources as explants/ germplasm in, or over, a cryogen, after the successful application of each of a number of biotechnological procedures, and was first successfully achieved for human spermatozoa as early as 1945 (Parkes, 1945). One of the major advantages availed by cryopreservation is that the frozen material probably maintains genetic stability (Gagliardi *et al.*, 2003; Kioko *et al.*, 2003). Cryostorage can also induce reversible quiescence as in seeds (Tessereau *et*

al., 1994), and minimises exposure to environmental and other hazards (Withers, 1988).

In addition to the genotypes that are usually propagated vegetatively due to having, for instance, sterile seeds, recalcitrant seeds have been identified as best stored by cryopreservation (Engelmann, 1997; 2011). However, as recalcitrant seeds are generally large and cannot be rapidly dehydrated to water contents sufficiently low to avoid lethal freezing damage, selected explants – usually embryonic axes excised from the seeds – are used (Berjak and Pammenter, 2008; Engelmann, 2011). According to the former authors, in cases where embryonic axes do not survive cryostorage, alternative explants – commonly shoot apices or meristems derived from *in vitro*-grown seedlings – are used.

There are various approaches to achieving successful cryopreservation of plant germplasm, as outlined by Engelmann (2000; 2011). These include:

1. The classical approach, which mainly caters for culture systems with small units of homogenous morphology (Engelmann, 1997). The most influential step in this approach is freeze-induced dehydration. Here, cells in the hydrated state are frozen slowly allowing for the formation of ice crystals externally; these serve as centres of ice nucleation, to which water is drawn from within the cell. This procedure is however, not much used for the germplasm of recalcitrant-seeded species probably because, even if the specimens processed are small, they are composed of a variety of tissue-

types which would respond differently to freeze-induced dehydration (Berjak, Pers. comm.[2]).

2. Physical dehydration, which entails dehydrating explants as rapidly as possible in an airflow then plunging them into a cryogen, which is usually liquid nitrogen (LN), which may be sub-cooled to -210 ºC (Wesley-Smith, 2002).
3. Encapsulation-dehydration, which involves encapsulating explants in alginate beads followed by partial dehydration and then plunging them into a cryogen.
4. Vitrification, which consists of treating explants with concentrated cryoprotective substances then desiccating them with very concentrated vitrification solutions, in which they remain during freezing.
5. Encapsulation-vitrification is a cryopreservation method that combines encapsulation-dehydration and vitrification.
6. Pregrowth, which involves culturing explants on media containing cryoprotectants before plunging them into a cryogen.
7. Pregrowth, however, is generally not alone adequate to promote survival of cryogenic temperatures, and is usually followed by dehydration, leading to development of the pregrowth-dehydration combination.

For cryopreservation, plant material can be cooled at three broadly different rates (Engelmann, 2000). Material can be cooled slowly, at less than 0.1 ºC s^{-1}, or undergo cooling at ~3 ºC s^{-1} which is considered as an intermediate rate (Wesley-Smith, 2002), or rapid cooling at rates greater than 100 ºC s^{-1} (Wesley-

[2] P. Berjak, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban, South Africa

Smith *et al.*, 1992). When slow rates of cooling are used they encourage the formation of few, large extracellular ice crystals at cell junctions, which extract intracellular water from cells (Mazur, 1984; Dumet and Benson, 2000), thus (theoretically) avoiding intracellular damage (Wesley-Smith, 2002). The extraction of water from the cytoplasm also results in an increase in cytoplasmic viscosity (Engelmann, 2000), which should protect the cell from freeze injury when tissues are exposed to cryogenic temperatures. This method has generally been used for hydrated tissue that has been cryoprotected (Dumet and Benson, 2000). As explained by Wesley-Smith (2002) this cooling method is effective when explants are exposed to decreasing temperatures in a stepwise manner, before they are plunged into the cryogen.

Slow cooling techniques require sophisticated programmable freezing equipment (Engelmann, 2000), but the use of a device (Mr Frosty® [Nalgene, USA]) which facilitates cooling at 1 ºC min^{-1} in domestic and laboratory freezers, is common (López Lastra *et al.,* 2001)).

To achieve intermediate cooling rates usually involves cryoprotection of tissue followed by partial drying (to water contents below 0.3 g g^{-1} [dry mass basis]) before explants are enclosed in polypropylene cryovials and plunged into liquid nitrogen. The low water content is critical because the presence of too much solution water results in the formation of large intra- and extracellular ice crystals that can cause lethal freezing events (Wesley-Smith, 2002).

Rapid cooling eliminates the formation of large ice crystals and in so doing, promotes the preservation of ultrastructure and avoids cell damage (Wesley-Smith *et al*., 1992; Mycock, 1999). This is achieved by preventing the intra- and extracellular parts of the cell from reaching a thermodynamic equilibrium within the range of temperatures that promote ice crystal growth (Wesley-Smith, 2002). The high cooling rate necessary to prevent equilibrium within this critical temperature range is achieved more readily in small specimens with high surface area to volume ratios because of an efficient heat transfer during convectional cooling (Wesley-Smith, 2002). Thus, small embryonic axes that are cooled at the rate of about 10 000 ºC s^{-1} are more likely to survive the process of freezing than whole seeds, unless the latter are very small – which is rarely the case for recalcitrant or even some intermediate types.

Freezing can result in damage due to either dehydration during slow cooling, or ice crystal formation during cooling at an intermediate rate, or both, resulting in cellular membrane structural damage for different reasons (Wesley-Smith, 2002). For example, ice crystallisation at intermediate freezing rates can physically rupture membranes (Fujikawa and Jitsuyama, 2000). Freezing damage can be remedied by either stabilising membranes and macromolecules using cryoprotectants (Withers, 1988; Benson, 2008) or by increasing cooling rate (Wesley-Smith *et al*., 1992). Rapid cooling not only minimises the time spent by tissue in the range of critical temperatures supporting crystallisation (Wesley-Smith, 2002), but also preserves cytoskeletal integrity (Mycock *et al.*, 1997).

Damage due to ice crystallisation can occur not only during cooling, but also during thawing. The rate of thawing therefore is just as important as the rate of freezing. Lethal ice formation during thawing can be avoided by increasing the rate at which explants are warmed. Rapid thawing is usually achieved by immersion into a liquid at 40 ºC. Different media have been used for thawing, including water (Gnanapragasam and Vasil, 1990; Wesley-Smith, 2002), the liquid version of regeneration medium [that is, growth medium without a solidifying agent] and a solution containing calcium and magnesium ions (Berjak *et al.,* 1999b).

The provision of calcium and magnesium ions in solution during cryopreservation procedures helps maintain cytoskeletal intergrity (Mycock, 1999). During cryopreservation, solute concentrations fluctuate and alter the dynamic solute equilibrium that is associated with the assembly and disassembly of cytoskeletal elements (Mycock, 1999). The use of magnesium and calcium ions at appropriate concentrations, however, buffers the intracellular environment and reduces depolymerisation of microtubules and microfilaments (Mycock, 1999). Most recently, Berjak *et al*. (2011b) have demonstrated the efficacy of strongly-reducing cathodic water (produced by electrolysis of the solution containing Ca^{2+} and Mg^{2+}), which minimises the damaging effects of reactive oxygen species (ROS) generated under stressful conditions, which include warming and rehydration after cryopreservation. (However, those findings considerably post-dated the current work, the approach not then even having been conceptualised).

In the account above, cryoprotection is mentioned as an approach to minimising freeze-damage. Cryoprotectants are a group of compounds, used in various combinations, which lower the supercooling and freezing points of cellular solutions (Kartha, 1985). These compounds include the antioxidant dimethyl sulphoxide (DMSO), sucrose, sorbitol and glycerol. These compounds, when applied singly or in combination, also act as desiccants by acting as compatible solutes, if they penetrate the cells, or by withdrawing water into the extracellular environment in the case of non-penetrating cryoprotectants (see below) (Finkle and Ulrich, 1979; Benson, 1995). Cryoprotectants, however, can have toxic effects on the cells. For instance, at higher concentrations DMSO is cytotoxic and has been suggested to interfere with microfilaments and microtubules (Withers, 1988).

Cryoprotectants are categorised as being either penetrating or non-penetrating. Penetrating compounds, which vary in size, have been proposed to act by penetrating and exerting intracellular colligative effects. This means that the intracellular osmolality is increased, and freeze-related dehydration is better tolerated (Benson, 2008). A further effect is to reduce toxic effects caused by accumulation of electrolytes (Farrant, 1965). Penetrating cryoprotectants effectively depress the freezing point of cellular solutions (Benson, 2008). The larger, non-penetrating compounds have a dehydrating effect on cells, withdrawing water from cells due to an osmotic differential. In this way water that would otherwise be available to form ice crystals is removed thus protecting the cell from lethal ice damage (Benson, 2008). Cryoprotective compounds with low molecular weights such as glycerol and DMSO are

categorised as penetrating, whereas higher molecular weight compounds, (sucrose is the most commonly used) are categorised as non-penetrating (Finkle *et al*., 1985).

The effectiveness of cryoprotective compounds is influenced by various factors including: the chemical nature of the compound, the molecular size and penetrating ability of the compound, the interaction of mixtures of compounds, the rate and temperature of application to the cell, the freezing and thawing rate, and the rate and temperature of the post-thaw dilution (Finkle *et al*., 1985; Mycock *et al*., 1995).

Besides the rate of cooling and cryoprotection, another factor that is intrinsic to successful cryopreservation is the ability of the explant to withstand dehydration. Vertucci and Farrant (1995), hypothesised that desiccation tolerance of vegetative tissue (e.g. resurrection plants) is based on the ability of damaged structures to undergo repair rather than protection of these structures from desiccation damage. Therefore, organisms that tolerate desiccation must be able to prevent or control deleterious reactions induced by the removal of water. Desiccation tolerance is the ability to withstand complete loss of almost all cellular water (Vertucci and Farrant, 1995), some such biological structures tolerating water contents as low as 0.05 g H_2O g^{-1} dry mass (Hoekstra *et al.*, 2003). This ability is found in propagative structures like pollen, orthodox seeds, dormant buds and somatic embryos (Vertucci and Farrant, 1995) and has been found to be associated with the accumulation of di- and oligosaccharides

(Hoekstra *et al.*, 2003) which have been suggested to prevent phase changes in membranes and conformational changes in proteins (Crowe *et al.*, 1998) at water contents ≤ 0.3 g H_2O g^{-1} dry mass (Hoekstra *et al*., 2003).

Desiccation is one of the effective methods of avoiding intracellular freezing damage (Shimonishi *et al*., 2000; Wesley-Smith *et al*., 2001) during cryopreservation, because most free water in the cell is eliminated. In addition, desiccation not only reduces the thermal load of tissue and allows quick passage through the range of critical temperatures that encourage ice growth (Wesley-Smith, 2002), but also lowers the freezing point of the remaining cell solution. Lowering the freezing point reduces the range of critical temperatures that encourage crystallisation (Wesley-Smith *et al*., 1992). Therefore, in order to be frozen without damage, embryos must be partially desiccated to a water content that allows for material to be cooled without lethal ice formation (Berjak *et al.*, 1999a,b).

As mentioned above, the water content of plant material can be reduced by physical dehydration. This can either be slow dehydration in a laminar airflow and over silica gel (Engelmann, 2000), or it can be rapid drying using fast moving air flow in a flash dryer (Berjak *et al*., 1990; Pammenter *et al.*, 2002). The rate of dehydration influences the ability of that plant tissue to survive to particular water contents: the more rapidly tissues can be dehydrated, the lower is the water content at which they will still retain viability (Berjak *et al*., 1990; Pammenter, *et al*., 1991; 1993; Berjak *et al*., 1993; Wesley-Smith, *et al*., 2001).

According to Wesley-Smith *et al*. (2001), all embryonic axes of jackfruit that were flash dried to 0.4 g water g^{-1} dry mass (g g^{-1}) survived, whereas none of those that were dried slowly to the same water content retained viability. Viability retention following flash drying is, however, transient (Pammenter and Berjak, 1999), so axes that have been flash dried in preparation for cryopreservation have to be cooled to cryogenic temperatures immediately.

The lack of desiccation-tolerance mechanisms in recalcitrant seeds directly affects the ability of desiccation-sensitive seeds to be dehydrated without loss of viability. As alluded to previously, recalcitrant seeds remain metabolically active after shedding. There is evidence that their mitochondria remain differentiated (Farrant *et al.*, 1997) and DNA replication and synthesis continue with only the briefest of stasis (Boubriak *et al*., 2000). When dehydrated, recalcitrant seeds/embryos produce high levels of free radicals (Hendry *et al*., 1992), which include unregulated reactive oxygen species (ROS) (Berjak and Pammenter, 2001; Varghese *et al.*, 2009; Whitaker *et al.*, 2010; Berjak *et al.*, 2011b). These ROS are strong oxidants, and if not regulated by effective action of antioxidants, can cause oxidation of macromolecules and membrane components. As recalcitrant seeds/embryos lose solution (freezable) water, metabolism continues but becomes unbalanced. The slower the dehydration, the more time is spent in the water contents that facilitate unbalanced metabolism, and the more ROS are produced and accumulate, resulting in lethal damage (Varghese *et al.*, 2009). This kind of damage is called metabolism-induced or metabolism-linked damage (Berjak *et al*., 1990; Berjak and Pammenter, 2001; Walters *et al.*, 2001), as opposed to dehydration

damage *sensu stricto*, which occurs when structure-associated water is perturbed (Pammenter *et al.*, 1998; Walters *et al.*, 2001). Studies on dehydrated recalcitrant seeds show that there are inadequate DNA repair systems after rehydration (Boubriak *et al.*, 2000) and the antioxidant systems are shown to have failed considerably when the seeds are rehydrated (Varghese *et al*., 2009).

Recalcitrant seeds or embryos of such seeds may be able to accumulate sucrose and oligosaccharides such that an amorphous state called a glass could be formed at sufficiently low water contents. However, as glasses do not form above water contents of ~0.3 g g^{-1} [dry mass basis] (Leopold *et al*., 1994), these would be totally ineffective, as when slowly dried, recalcitrant seeds/embryos lose viability at water contents around 0.8 g g^{-1} (Pammenter *et al.*, 1993). It is possible that when sufficiently-rapidly dried, intracellular glasses may form in recalcitrant embryos or embryonic axes – which could explain the basis of their survival subsequent to cryogen exposure. In contrast, recalcitrant material not having the capacity for intracelluar glass formation, no matter how rapidly dried, might typify those species for which it has hitherto proved impossible to cryopreserve embryos or embryonic axes (Berjak, Pers. Comm.[2])

Damage due to drying can either be metabolism-derived or related to non-freezable water (Vertucci and Farrant, 1995; Pammenter *et al.*, 1998; Berjak and Pammenter, 2003). Metabolism-derived damage usually occurs during slow drying and is associated with uncontrolled metabolism as discussed

above. Desiccation damage *sensu stricto*, which is not tolerated by recalcitrant seeds, embryos or axes, is related to the removal of structure-associated (non-freezable) water (Pammenter *et al.*, 1991; 1993), i.e. water which is associated with cell membranes and macromolecular surfaces (Pammenter *et al.,* 1998). It is important to note that the amount of water that can be lost through rapid drying without lethal damage depends on the developmental stage of the seeds. For *Landolphia kirkii*, Berjak *et al*. (1992) showed that immature axes had a lower non-freezable water level and could tolerate rapid drying to lower water contents than axes of mature seeds. However, this could have been related to the lipid-rich nature of the cells of the immature embryonic axes (Berjak, Pers. Comm.[2]).

Some plant tissues survive when dried down quickly because this allows for low water contents to be reached with minimal or non-lethal levels of metabolic damage (Berjak *et al*., 1993; Pammenter *et al.*, 2002; Wesley-Smith, 2002; Kioko *et al.*, 2003), and allows the maintenance of ultrastructural integrity (Kioko *et al.*, 2006). The reverse also holds true: dehydrated seeds/embryos need to be rehydrated rapidly to minimize the time spent at water contents that can induce metabolism derived damage (Perán *et al.*, 2004).

In general, the important factors that affect the response of recalcitrant seeds to dehydration are: drying rate, dehydration temperature, developmental status of seeds, and rehydration rate and conditions (Song *et al.*, 2003).

The ability of embryos to be frozen without consequent loss of vigour or viability is influenced by various factors that operate in combination. These factors include the size and stage of development of the embryos, their water content and ability to withstand dehydration, and procedurally, the nature and concentration of cryoprotectants used, and the method and rate of cooling and thawing (reviewed by Berjak *et al*., 1999b). In the present study, these variables needed to be optimised for *S. cordatum* zygotic embryos.

Objectives of this study

The production of non-orthodox seeds, either recalcitrant (Roberts, 1973) or intermediate (Ellis *et al*., 1990), has a number of implications in the context of *ex situ* germplasm conservation, because the seeds are short lived, unstorable by conventional methods (Farrant *et al*., 1988; Berjak and Pammenter, 2001), and are heavily predated in their natural environment (Berjak, Pers. Comm.[2]). This means that seedling recruitment is diminished leading to the erosion of the plants as resources and of the genepool (Berjak, 2000). There is, therefore, an urgent need to gain knowledge about the reproductive biology of all, or at least as many as possible, of our indigenous species so as to categorise the seed types, determine their growth requirements and develop protocols for their *ex situ* storage, in order to conserve planting stock. This knowledge will help ensure that sufficient quantities of high quality seeds are available for re-introduction to forests (and other) systems (Singhal and Khanna, 1992), which has become ever more urgent in the face of climate change in southern Africa (Berjak *et al.*, 2011a).

The present investigation aimed at studying the post-harvest physiology and developing protocols for short- to -medium term storage, for whole seeds of *S. cordatum* in order to extend their 'shelf-life'. The extended 'shelf-life' would allow the seeds to remain viable from the time of harvest to the time of planting. The extension of 'shelf life', however, is not appropriate for long-term germplasm conservation. Hence, the research also aimed at developing cryopreservation protocols for the zygotic germplasm of *S. cordatum*.

CHAPTER 2. EFFECTS OF SEED STORAGE TEMPERATURE AND RELATIVE HUMIDITY ON THE VIABILITY AND VIGOUR OF *Syzygium cordatum*

2.1 Introduction

Seed moisture (water) content and storage temperature are two important factors that affect the storability of both orthodox (Vertucci and Roos, 1993; Walters *et al.*,1998) and non-orthodox (Hor *et al.*, 1984; Corbineau and Côme, 1986; Ellis *et al.*, 1991b) seeds.

Recalcitrant seeds are metabolically active when shed (Pammenter and Berjak, 1999) and are best stored in conditions that maintain them as hydrated as when they are harvested, and at the lowest temperature that does not negatively affect vigour and viability (Berjak and Pammenter, 2008). Accompanying hydrated storage, however, is fungal proliferation which affects seed quality in storage, rapidly lowering both vigour and viability (Calistru *et al.*, 2000; Hong *et al.*, 2005; Berjak and Pammenter, 2008).

There are currently no reports on the medium- to long-term storage of the seeds of *S. cordatum*. However seeds of the related species, *S. cuminii,* were damaged by drying below a water content of 63% (fmb)[3], with survival decreasing to 58% and 14% at 50% and 21% water content, respectively, with none surviving when water content had declined to ~10% (IPGRI/DFSC, 1999).

[3] In keeping with data reporting in the current work, the water contents given for *S. cuminii* (as % fresh mass) converted to a dry mass basis are: 1.90 g g^{-1}, 1.00 g g^{-1}, 0.27 g g^{-1} and 0.10 g g^{-1}.

The seeds showed typical characteristics of a high degree of recalcitrance (Mittal *et al.*, 1999). *Syzygium cuminii* seeds stored in open containers remained viable for less than two weeks when stored at room temperature in India (Kandya, 1987), but maintained viability for up to 12 weeks when stored at 16 ºC (IPGRI/DFSC, 1999). It is likely that the seeds stored hydrated at room temperature proceeded further along the germination pathway than those stored at 16 ºC, with metabolic processes being slower at the lower temperature. It is known that as germinative metabolism progresses in recalcitrant seeds the period for which they can be stored becomes increasingly shorter (Berjak and Pammenter, 2008). The differences in survival of the *S. cuminii* seeds at the two temperatures (Kandya, 1987; IPGRI/DFSC, 1999) and at declining water contents (IPGRI/DFSC, 1999) indicate the importance of these parameters on maintenance of germinability of seeds of *S. cuminii* and related species.

The present study investigated the storage behaviour of a closely related species, *S. cordatum*. Various combinations of seed water content and storage temperature were used to determine the storability of the seeds, survival being assessed by germination testing. This study lends itself to contributing towards achieving the targets set by the Global Strategy for Plant Conservation for *ex situ* conservation of a multipurpose species (Jackson and Kennedy, 2009).

Observation showed that fruits of *S. cordatum* often showed signs of insect infestation, which would facilitate fungal invasion and rapid deterioration of the

seeds. Therefore the present study also aimed at characterising the type and severity of insect attack of harvested fruits and associated manifestation of fungal proliferation during seed storage.

2.2 Materials and Methods

2.2.1 Seed procurement and handling

Fruits of *Syzygium cordatum* were collected from locations in Durban, South Africa (29°58′07″S; 30°56′52″E), and around Eldoret (00°30'N; 35°17'E) and Kitale (01°00'N; 35°00'E) in Kenya, by the researcher or instructed experienced lay-people and transported to the laboratory in Durban, South Africa within 3-4 days.

In order to prevent possible damage as it was not known whether the seeds were chilling sensitive (Lyons, 1973), fruits collected in Kenya were packed in sterilised, insulated cooler boxes and transported to the laboratory in Durban in the 'live animal' hold of an aircraft, because the temperatures in the cargo section of the aircraft may drop to levels that are lethal to chilling-sensitive seeds. It was imperative that fruits, rather than seeds, be transported because the presence of the pulp reduces the rate at which seeds lose water and may also prevent precocious germination (Berjak and Pammenter, 2004a).

Once in the laboratory, the fruit pulp (pericarp) was removed immediately, seeds were cleaned, surface-sterilised by soaking in 1% NaOCl for 10 min (Baiyeri and Mbah, 2006) and dusted with Benomyl [methyl 1-(butyl carbamoyl)

benzimidazol-2-ylcarbamate] (Dupont, Benlate WP), a fungicide that is widely effective (Boyer and South, 1987).

2.2.2 Seed storage treatment and sampling

The longevity of the seeds stored at shedding water content was determined by conducting a factorial experiment based on storage relative humidity conditions and storage temperatures. Storage relative humidity conditions were: storage in sealed plastic bags in which seed water content would equilibrate with the enclosed atmosphere (termed non-hydrated), and storage in a hydrated atmosphere, where seeds were placed in a monolayer on a mesh that was situated ~100 mm above water-hydrated paper towel in a 5 litre sealed bucket. The effects of storage at three temperatures, viz.: 6, 16 and 25 ºC, were tested at regular intervals, samples of 20 seeds per treatment were drawn from storage and the germination rate and capacity, seed water content, seedling biomass allocation, and the extent of fungal infection were determined.

2.2.3 Water content determination

Water contents of cotyledons and embryonic axes, separately, were determined gravimetrically. The seed parts were weighed on a 5-place balance, then dried in an oven at 80 ºC, re-weighed at intervals, and dried further until there was no change in mass. The water content was expressed as g H_2O g^{-1} dry mass (g g^{-1}).

2.2.4 Vigour and Viability assessment

Twenty seeds per treatment were sown in moist vermiculite (buried just deep enough to cover the seed), and maintained in a germination room (16 h light, 8 h dark) at 25 ºC. Germination was assessed weekly. The criterion for germination was selected as radicle protrusion. Germinability was expressed both as total percent germination (totality) and as germination energy. Germination energy is an expression of the germination percentage reached when the mean daily germination speed has reached its peak (Willan, 1985). Vigour was expressed as a germination index, which is a calculated value that combines both germination rate and total germination. The germination index was calculated using a formula described by Djavanshir and Pourbeik (1976).

$$Gv = (\Sigma DGs/N)\ Gp/10,$$

where

Gv = germination index

Gp = cumulative germination percent after a specified time

DGs = daily germination rate/speed (this was obtained by dividing the cumulative germination percent by the number of days after sowing [Gp/D])

D = number of days after sowing

ΣDGs = the sum of DGs figures obtained from the daily counts

N = number of daily counts, starting from the date of the first germination.

2.2.5 Biomass allocation

The number of weeks taken for roots to protrude and for shoots to develop was recorded. The lengths of the roots and shoots were measured every week for three months. Thereafter, the biomass of leaves, roots, and shoots was determined by drying the various seedling parts in an oven at 80 ºC to constant weight and recording the final mass.

2.2.6 Statistical analysis

Data collected were analysed using the SPSS statistical programme. Statistical significance of parametric data was tested using analysis of variance (ANOVA), at a 95% confidence level. Correlation between some sets of treatments and results were tested using a Pearson's product moment test.

2.2.7 Assessment of contamination status: fluorescence microscopy (adapted from Rohringer *et al*., 1977)

Ten seeds stored at 16 ºC for either 0, 4 or 10 weeks were fixed in a solution of ethanol:dichloromethane (3:1 v/v) containing 0.15% trichloroacetic acid for 24 h. The seeds were washed twice for (15 min) each time with 50% ethanol. The seeds were then washed twice (15 min each) with 0.05 M sodium hydroxide. Thereafter seeds were rinsed thrice in sterile water. The washed seeds were soaked in Tris/HCl buffer (pH 5.8) for 30 min. Then the seeds were stained for 5 min with freshly prepared 0.1% Uvitex 2BT 130 (Ciba-Geigy Corp., Basel,

Switzerland) in buffer and rinsed thoroughly with sterile water. The stained seeds were then immersed in 25% aqueous glycerol for 30 min and stored in 50% aqueous glycerol (v/v) and trace quantities of lactophenol. Seed components were then viewed using a Nikon E-400 microscope equipped with epifluorescence optics. The filter combination was UV-2A, at 330-380 nm excitation bands and a barrier filter at 420 nm was used to visualize fungal structures that were expressed as light blue fluorescence.

2.3 Results and Discussion

2.3.1 Seed characteristics and seed-associated fungi

During harvest, fruits at various maturation stages, as indicated by their outer pericarp colour, were collected (Fig. 2.1a and 2.1b) but only fruits that were fully purple (Fig. 2.1c) were chosen for sampling. Fruits of many plant species producing recalcitrant seeds are not all abscised at the same developmental stage, but whatever the developmental stage, recalcitrant seeds initiate germination sooner or later at the water content at which they are shed (Berjak, 2006).

All the seeds sampled were polyembryonic, with 92 ± 0.012% containing four embryos (Fig. 2.1c). However, twin seedlings rarely occurred when these seeds were set to germinate. When twin seedlings occurred, the germination count from that seed was considered to be one.

Polyembryogeny has been observed in other species of *Syzigium* (e.g. *S. paniculatum* [Thurlby *et al*., 2011]), and has a bearing on the conservation status of the species under study. This is because, in one of the mechanisms by which polyembryogeny arises, apomixis, additional asexual embryos develop from the diploid maternal tissue surrounding the zygotic embryo (Richards, 2003). If the zygotic embryo is outcompeted during development by the asexual embryos, this results in the production of embryos (and hence seeds) which have acquired their entire genome from the mother plant, which

steadily reduces the genetic diversity in the population, with possible eventual formation of 'seed clones' (Janzen, 1977; Richards, 2003).

Although there have been no studies yet to determine how polyembryogeny arises in *S. cordatum*, current approaches to the *ex situ* conservation of this species would need to be re-assessed if apomixis were to be the dominant mode of induction of polyembrogeny.

During 2004, two fruiting seasons were recorded in KwaZulu-Natal. The first, initiated in December 2003 continued to February, and the second occurred between April and June. During the second fruiting season, fruits collected were infested with weevils, scale insects and larvae of three lepidopteran species: *Cryptophlebia leucotreta* (Tortricidae: Olethreutinae), *Lolaus* spp. (Lycaenidae) and *Deudodrix (Virachola) dinochares* (Lycaenidae) (Fig. 2.2c).

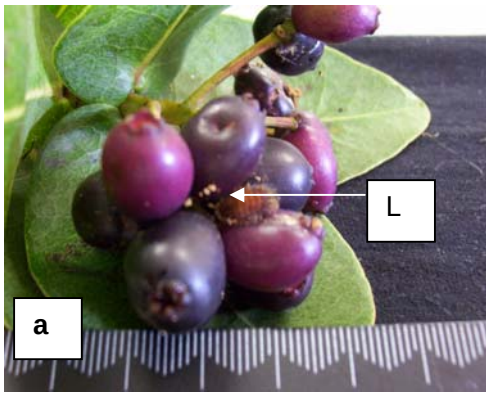
Larvae of *C. leucotreta* are borers affecting a variety of fruits. They have been recorded to attack fruits of species of *Citrus*, *Crassula*, and *Quercus,* and *Prunus persica*, and fungal galls on *Acacia karroo* and *Eugenia* sp. *Deudorix (Virachola) dinochares* (common name: apricot playboy) are found in abundance in KwaZulu-Natal and other parts of southern Africa (Pennington, 1994). The larvae of *C. leucotreta* feed on a variety of other trees too, including *Syzygium cordatum*, *Combretum zeyheri*, *Burkea africana* and *Swartzia madagascariensis* (Pennington, 1994). According to Pooley (2003), larvae of *Deudodrix dinochares* feed on the leaves of *S. cordatum*, but there is no record of their feeding on seeds. Larvae of *Lolaus* spp., on the other hand, have not

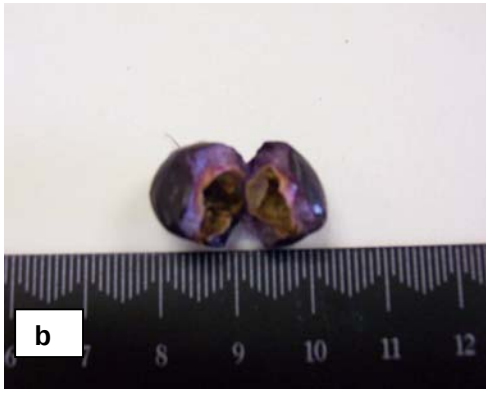
been recorded to feed on *S. cordatum*, feeding mainly on the trees of the genus *Tapinanthus* (Pennington, 1994).

Figure 2.1. (a) Fruits showing the variety of maturation stages during harvest. (b) A selection of fruits representing the maturation stages available during harvest. (c) The two purple fruits (F) represent the two maturation stages that were selected for the storage trials. The green cotyledons and excised axes show the polyembryonic nature of the seeds. Labels. F, fruits; S, seed; C, cotyledons; A, axes.

Insect infestation has a negative effect on seed quality. These larvae and weevils impact negatively on seed storage and germplasm conservation of the species, as they feed on the natural propagating units of the plant – the seeds (Figs. 2.2, a and b). In addition to feeding on seeds, these animals facilitate fungal growth on seeds in storage. Insect infestation is often controlled by enclosing dry (quiescent) seeds in a carbon-dioxide-rich environment (Chin, 1992). However, recalcitrant seeds (which are metabolically active) and cannot

be dehydrated, are likely to lose viability when consistently exposed to carbon dioxide during storage.





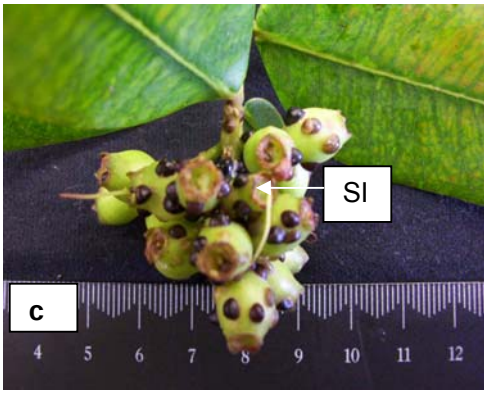


Figure 2.2. (a) A lepidopteran larva boring into the fruit of *S. cordatum*. (b) A fruit where the seed has been consumed by larvae. (c) Scale insects attached to immature fruits are illustrated.

Labels. L, lepidopteran larvae; SI, scale insects.

2.3.2 Effects of storage conditions and storage period on the water content of cotyledons and embryonic axes

Generally, embryonic axes had higher and more varied water contents than did cotyledons, with variation occurring more widely in seeds stored under hydrated conditions. There was no significant change in water content in axes at 6 ºC and 16 ºC for seeds stored under either hydrated or non-hydrated storage conditions, except for axes of seeds stored at 16 ºC under hydrated conditions

(Fig. 2.3 a-d). However, a significant decline in cotyledon and axis water contents for seeds stored under hydrated at 25 ºC was observed after two weeks in storage (Fig. 2.3 e). A similar drop in water content was observed for seeds of *S. cuminii* stored at 25 ºC (IPGRI/DFSC, 2002). In the present study the decline in water contents observed coincided with root protrusion and fungal proliferation on the seeds in this storage condition. The reason for the decline is not clear, but it is possible that the storage container was imperfectly sealed, and thus hydrated conditions were not maintained. The subsequent increase in water contents is suggested to be the consequence of fungal respiration.

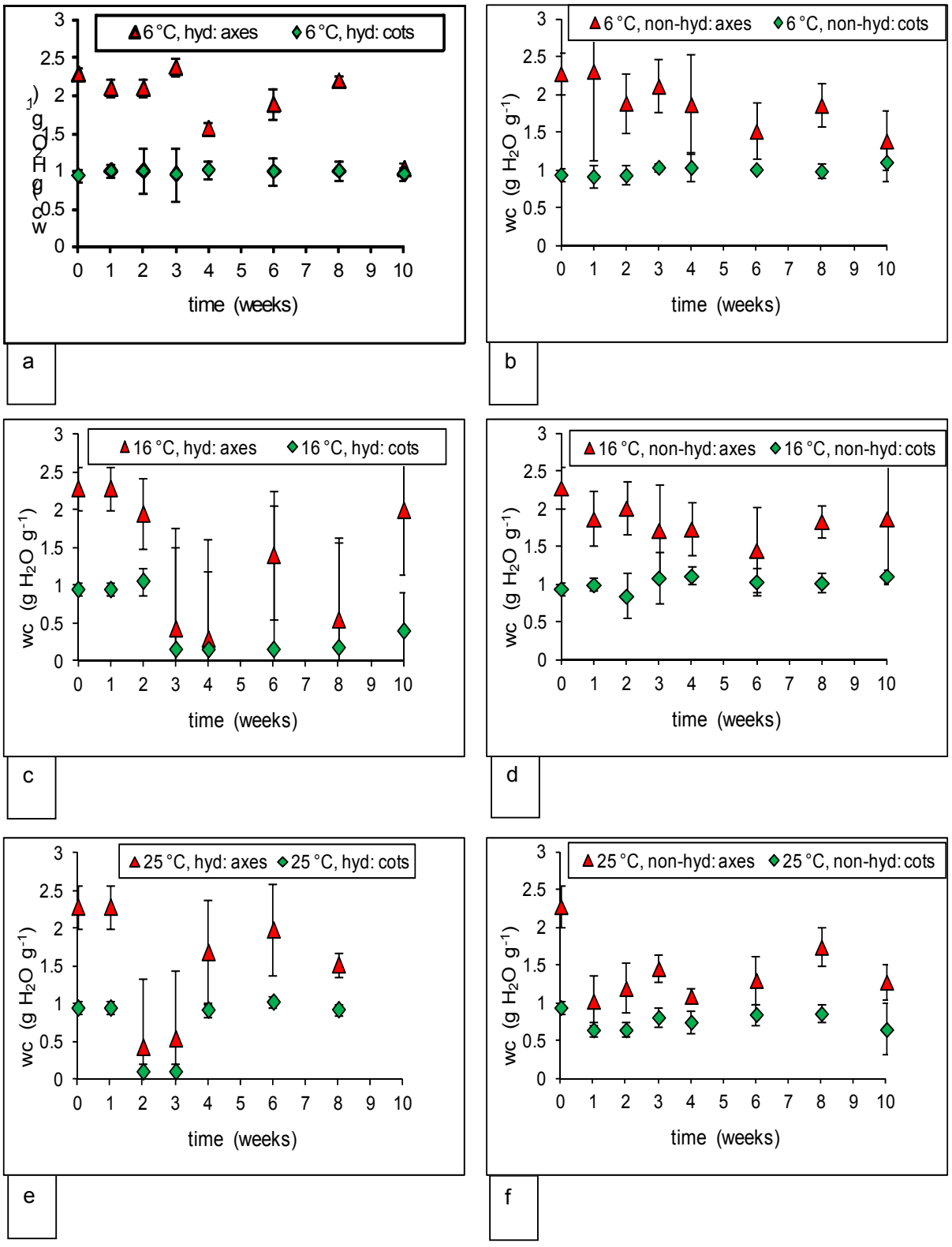


Figure 2.3. Changes in the water contents (g g^{-1} dmb) of *S. cordatum* embryonic axes and cotyledons following seed storage at either hydrating or non-hydrating conditions at 6 ºC, 16 ºC and 25 ºC

2.3.3 Effects of storage conditions and storage period on vigour and viability

Seed deterioration during storage can be assessed by a reduction in viability (Smith and Berjak, 1995). In this investigation, germination was considered to have occurred when the radicle emerged, and vigour was measured using germination energy and germination index.

Germination of *S. cordatum* seeds is hypogeal (Msanga, 1998). During the initial stages of germination the seed coat cracks then the radicle emerges and develops into a tap-root. As germination progresses, the epicotyl elongates and causes the cotyledons to separate. These separated cotyledons remain in the germination medium while the epicotyl elongates to protrude from the medium. In this investigation, seeds had to be removed from the medium for germination to be assessed. According to Msanga (1998), the germinability of freshly shed *S. cordatum* seeds was 80% four weeks after sowing and increased to 90% after five weeks. In the present study, 90% of the seeds sown immediately after harvest germinated after five weeks of sowing. Seeds from all storage conditions exhibited a decline in germination index and totality after the first week of storage, then an increase after the second week and another decline after the third week of storage. Thereafter there was a variation of responses by the seeds in the different storage conditions (Figs 2.4 and 2.6) with seeds stored in hydrated conditions at 16 ºC maintaining a steady germination index after being in storage for three weeks (Fig. 2.4). This pattern may indicate that seeds become increasingly sensitive to storage conditions after six weeks in storage.

Studies by Farrant *et al.* (1988) showed that in recalcitrant seeds of the species studied, subcellular germination events are initiated almost immediately after shedding and continue while seeds are in storage. These events include the organisation of mitochondria, increased protein synthesis, endomembrane development, and the initiation of cell vacuolation and cell division. Thereafter root protrusion occurs. The initial subcellular changes in recalcitrant seeds occur without the need for additional water. However, once extensive vacuolation and cell division have been initiated, seeds require additional water in order to complete the germination process successfully (Farrant *et al.*, 1989). Those authors presented evidence showing that seeds stored to the point of, but not including, cell division and vacuolation, show enhanced rates of germination relative to newly-harvested seeds. If, however, seeds are held in storage – even under conditions precluding water loss – germination rates and the total percentage germination are reduced because additional water is required for active growth by division and vacuolation (Farrant *et al.*, 1989). As the required water content increases, but there is no source of exogenous water, desiccation stress effectively increases to a point where seed viability is lost (Farrant *et al.,* 1988). When seeds are stored under conditions that maintain their shedding water content (hydrated storage), germinative metabolism is initiated and the longevity of seeds in storage depends on the time it takes for germination to occur. In the case of seed storage under non-hydrated conditions, recalcitrant seeds lose vigour and viability faster than seeds stored in a hydrated atmosphere (Farrant *et al*., 1989). Non-hydrated storage has been shown to result in subcellular damage accumulating

considerably faster, accompanied by a more rapid decline in germinability when compared with seeds sampled from hydrated storage – i.e. when stored in a hydrated atmosphere (Farrant *et al*., 1989).

Based on the observation by Farrant *et al*. (1989) that seeds entraining germinative metabolism during storage show apparently enhanced germination rates, the increase of germination energy shown in the sixth week in storage in this study may indicate that similar preparation for germination before cell division and vacuolation – i.e. prior to root protrusion – occur in the sixth week of storage for seeds of *S. cordatum* (Fig. 2.5). In another study (IPGRI/DFSC, 2002), seeds of *S. cuminii* exhibited a similar response to storage – viability declined to unacceptable levels after six weeks in hydrated storage. Short-term storage of seeds for conservation purposes, using the 6 ºC and 25 ºC, was therefore not recommended beyond six weeks because vigour loss, which is usually the first indication of seed deterioration before viability loss (Smith and Berjak, 1995; Walters *et al*., 1998) was evident. The loss of vigour in seeds stored at 6 ºC can be associated with chilling damage and with fungal damage at 25 ºC (fungal damage is discussed below in section 2.3.6). It is noteworthy that seeds stored at 16 ºC were the only set of seeds that maintained a high level of vigour and viability even after six weeks in storage.

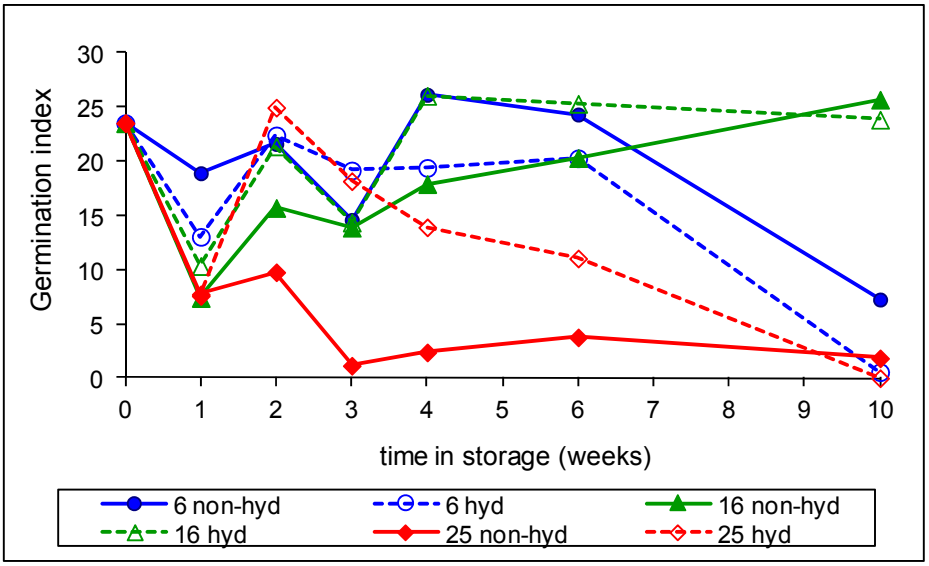


Figure 2.4. Changes in the germination index of seeds stored under varying storage conditions for different periods.

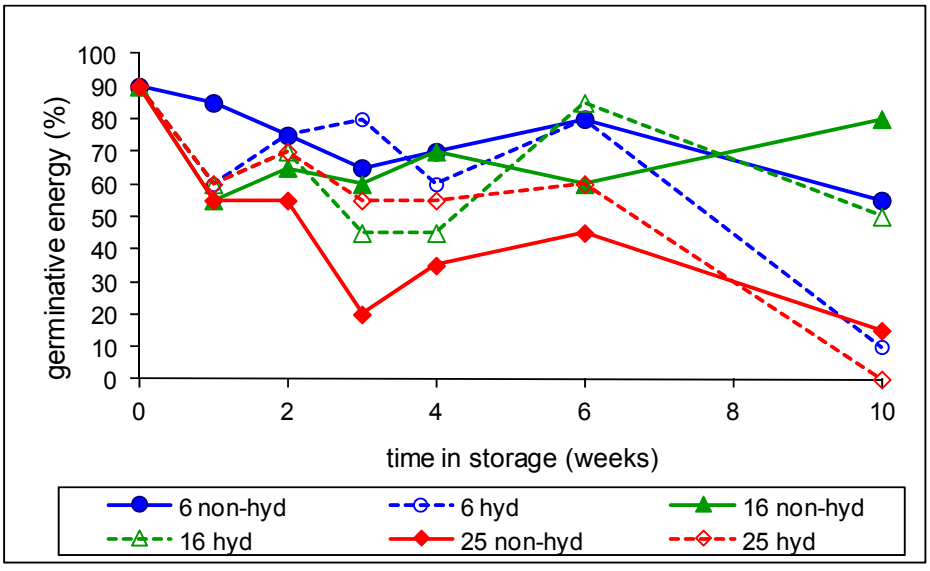


Figure 2.5. Changes in the germination rate (germination energy) of seeds stored under varying storage conditions for different periods.

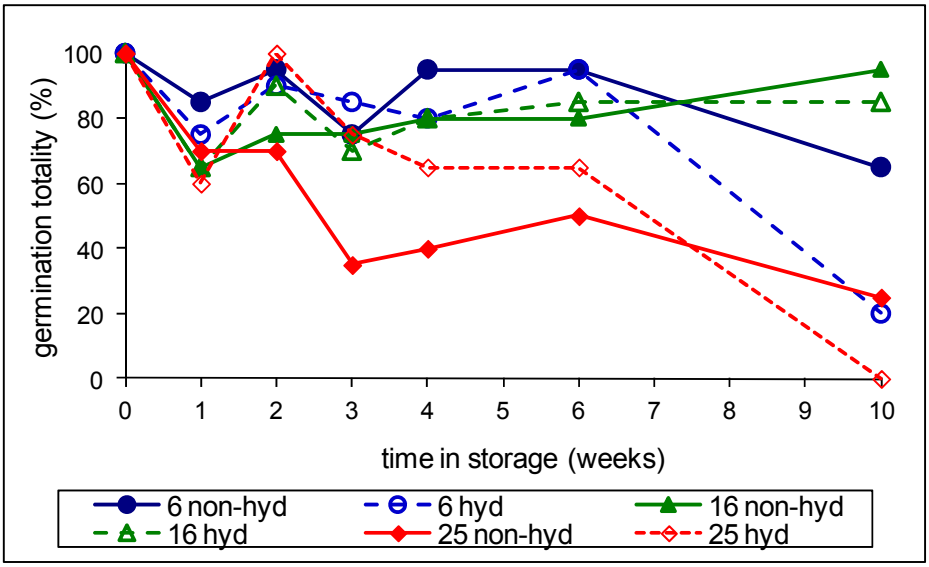


Figure 2.6. Changes in the germination totality of seeds stored under varying storage conditions for different periods.

It can be postulated that the recalcitrant seeds of *S. cordatum* used in these storage experiments had acquired the ability for full germinability and establishment (i.e. radicle protrusion and shoot emergence) prior to storage. This is based on the apparent lack of a lag between the two processes, by the seeds sown immediately after harvest (Table 2.1[control]). In the present study, seeds in 61.8% of the storage treatments showed root protrusion and shoot development in the same week, while those in 35.3% of the storage treatments exhibited a 1-week lag; and only 2.9% of the treatments resulted in seeds exhibiting a lag longer than 1 week (Table. 2.1). For four out of the six storage conditions tested, this time lag between root protrusion and shoot development occurred on the 10th week in storage when seeds had incurred damage due to either water stress, fungal proliferation or both. For the highly recalcitrant seeds of *Avicennia marina*, Farrant *et al.* (1993) showed that seeds acquire the ability

for root protrusion about two weeks before they acquire the capacity for seedling establishment. Those authors suggested that a lag exists between root protrusion and full germinability in immature seeds so that root protrusion coincides with the end of histodifferentiation and the acquisition of full germinability occurs midway through the phase of growth and reserve accumulation.

Table 2.1. The number of weeks taken for radicle emergence and shoot development to occur for seeds stored under either hydrated or non-hydrated conditions at either 6 ºC, 16 ºC or 25 ºC for different periods.

Storage temperature (ºC)	Storage type	Storage time (Weeks)	Number of weeks taken for roots to emerge (av. root length [cm] ± SD)	Number of weeks taken for shoots to develop (av. shoot length [cm] ± SD)
Control		0	4 (3.1 ± 1.88)	4 (2.5 ± 2.39)
6	Non-hydrated	1	3 (0.88 ± 0.96)	3 (0.12 ± 0.51)
6	Non-hydrated	2	2 (0.16 ± 0.69)	3 (1.30 ± 2.05)
6	Non-hydrated	3	2 (0.46 ± 0.91)	2 (0.02 ± 0.09)
6	Non-hydrated	4	2 (1.54 ± 1.26)	2 (0.19 ± 0.53)

6	Non-hydrated	10	3 (0.16 ± 0.52)	4 (0.02 ± 0.09)
6	Hydrated	1	3 (0.76 ± 1.20)	3 (0.015 ± 0.07)
6	Hydrated	2	3 (2.6 ± 1.73)	3 (1.7 ± 1.54)
6	Hydrated	3	2 (0.50 ± 0.99)	3 (0.77 ± 1.78)
6	Hydrated	4	2 (1.7 ± 1.86)	3 (0.82 ± 1.69)
6	Hydrated	6	4 (4.4 ± 3.26)	4 (2.5 ± 2.82)
6	Hydrated	10	3 (0.12 ± 0.54)	4 (0.055 ± 0.25)
16	Non-hydrated	1	3 (0.05 ± 0.22)	4 (0.20 ± 0.72)
16	Non-hydrated	2	3 (1.5 ± 1.50)	3 (0.33 ± 0.81)
16	Non-hydrated	3	2 (0.41 ± 1.03)	3 (0.81 ± 1.35)
16	Non-hydrated	4	2 (1.4 ± 1.46)	2 (0.22 ± 0.67)
16	Non-hydrated	10	2 (1.4 ± 1.65)	2 (0.35 ± 0.57)
16	Hydrated	1	3 (0.41 ± 0.74)	3 (0.025 ± 0.11)
16	Hydrated	2	3 (2.4 ± 2.35)	3 (0.74 ± 1.52)
16	Hydrated	3	2 (1.3 ± 1.54)	2 (0.12 ± 0.37)

16	Hydrated	4	1 (1.2 ± 1.42)	2 (1.2 ± 1.69)
16	Hydrated	10	2 (1.6 ± 1.86)	2 (0.14 ± 0.43)
25	Non-hydrated	1	3 (0.025 ± 0.11)	4 (0.29 ± 0.88)
25	Non-hydrated	2	4 (1.0 ± 1.45)	4 (0.015 ± 0.07)
25	Non-hydrated	3	3 (0.13 ± 0.56)	6 (0.77 ± 1.88)
25	Non-hydrated	4	6 (0.89 ± 2.18)	6 (0.64 ± 1.61)
25	Non-hydrated	6	4 (0.51 ± 1.58)	4 (0.41 ± 1.26)
25	Non-hydrated	10	2 (0.39 ± 0.95)	3 (0.070 ± 0.24)
25	Hydrated	1	4 (0.40 ± 0.78)	4 (0.030 ± 0.13)
25	Hydrated	2	3 (2.5 ± 2.25)	3 (1.1 ± 1.98)
25	Hydrated	3	2 (1.3 ± 1.35)	2 (0.1 ± 0.29)
25	Hydrated	4	1 (0.19 ± 0.51)	2 (0.16 ± 0.43)
25	Hydrated	6	1 (0.15 ± 0.67)	2
25	Hydrated	10	0	0

Seeds of *S. cordatum* germinated in storage (Fig. 2.8). This is a characteristic typical of recalcitrant seeds of tropical and subtropical species (Farrant *et al.,* 1989). From Fig. 2.7, it is evident that temperature affected the percentage germination of seeds stored in hydrated conditions: seeds stored at 6 ºC did not germinate, while less seeds stored in hydrated conditions at 16 ºC germinated during storage than did those stored in hydrated conditions at 25 ºC. These results indicate that the germinative process is significantly slowed down at storage in lower temperatures (such as 6 ºC), although germinability is retained once seeds are removed from storage and sown on vermiculite.

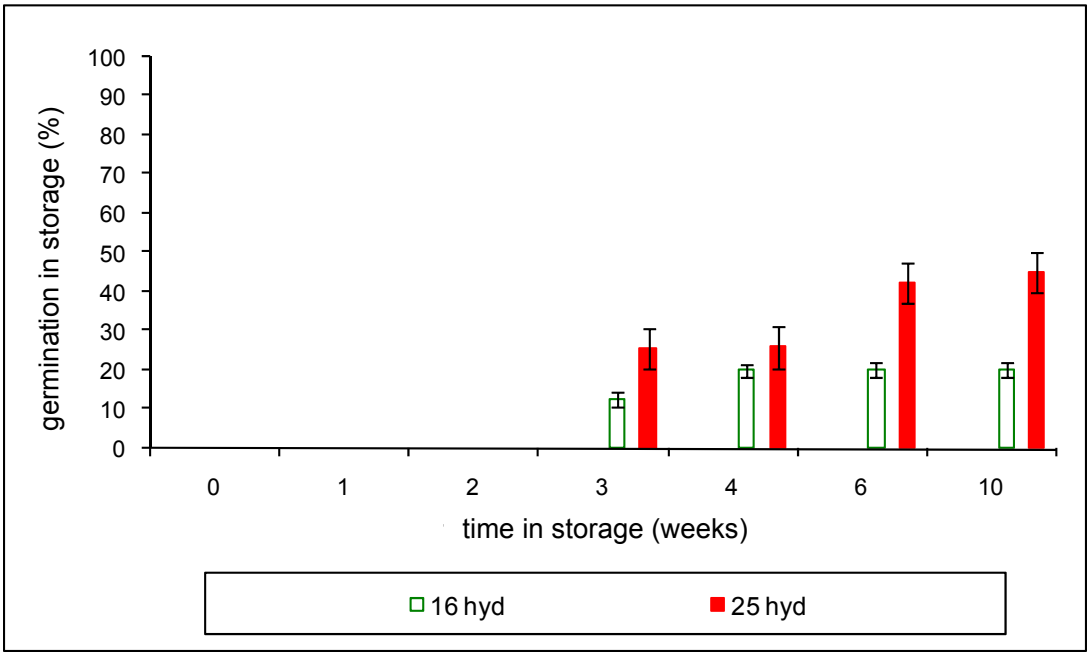


Figure 2.7. Percentage of seeds that germinated in storage. Germination was observed for seeds from only the two treatments indicated in the legend.

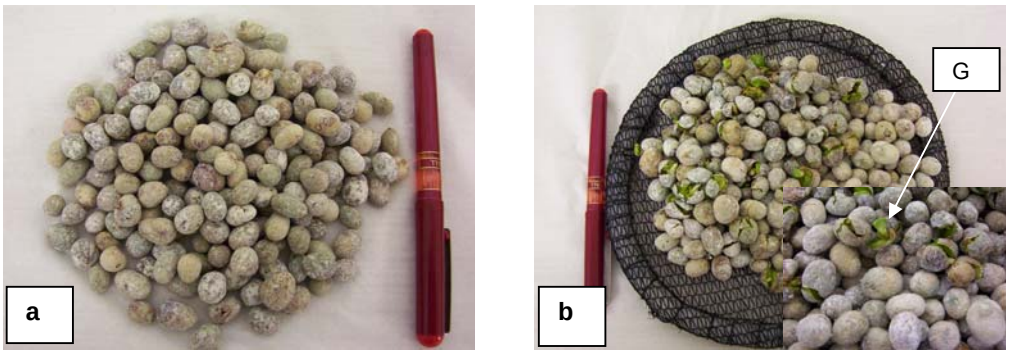


Figure 2.8. (a) Seeds stored in non-hydrated conditions for 10 weeks at 16 ºC. (b) Seeds stored under hydrated conditions for 10 weeks at 16 ºC.

Label: G = seeds exhibiting germination in storage.

2.3.4 Effects of storage conditions and storage period on plant growth

In order to evaluate the overall quality of *S. cordatum* seedlings produced from seeds that had been stored under different relative humidities at different temperatures, a number of morphological attributes were measured. These attributes were: plant length, root dry mass and shoot dry mass. Based on Pearson's product moment correlation, there was a significant positive correlation ($r = 0.729$, $N = 88$, $P < 0.05$) between plantlet biomass and plantlet length in *S. cordatum* (Fig. 2.9). This positive correlation is an indication of healthy seedlings (Butola and Samant, 2007), with no stunted root and/or stems and no lateral branching.

The biomass and plantlet lengths of seedlings from seeds stored under both hydrated and non-hydrated conditions at 6 ºC and in hydrated conditions at

25 ºC increased significantly after the first week in storage and thereafter decreased as storage time increased (Figs. 2.10 and 2.11). This significant increase in biomass was, however, not evident for seeds stored under both hydrated and non-hydrated conditions at 16 ºC and under non-hydrated conditions at 25 ºC. There have been other reports that indicate the effect of seed condition on seedling characteristics: seedling biomass of *Ardisia elliptica* declined when seeds were stored for longer than two months (Pascarella and Horvitz, 1999); in the late 1950s and early 1960s, Baron and Fowler (1964) reported that seed size, seed age and seed storage conditions affected the seedling size of sugar pine. There have also been numerous studies that have linked seed storage conditions with subsequent seedling vigour and performance (e.g. for *Haloxylon salicornicum* [Clor *et al*., 1976]; for *Vicia sativa* [Kalsa *et al*., 2011]; and for coffee, *Coffea arabica* [Rosa *et al*., 2011]).

The differences in seedling performance among seeds from the various storage treatments may be related to the vigour of the seeds at sowing, as reviewed by Ellis (1992). For seeds stored at 25 ºC under non-hydrated conditions in this study, vigour was lower than that of seeds stored under all the other conditions, from two weeks in storage onwards (see Figs 2.4 – 2.5). Thus, although there was no significant decrease in seedling biomass for seeds retrieved from this treatment after increasing storage periods, there was merely a maintenance of very low biomass relative to that of seeds from other storage treatments. On the other hand, seeds stored at 16 °C, whether under hydrated or non-hydrated conditions, maintained a relatively high and consistent vigour throughout the

storage period, while the vigour of seeds in other treatments decreased over time (Figs 2.4 – 2.5).

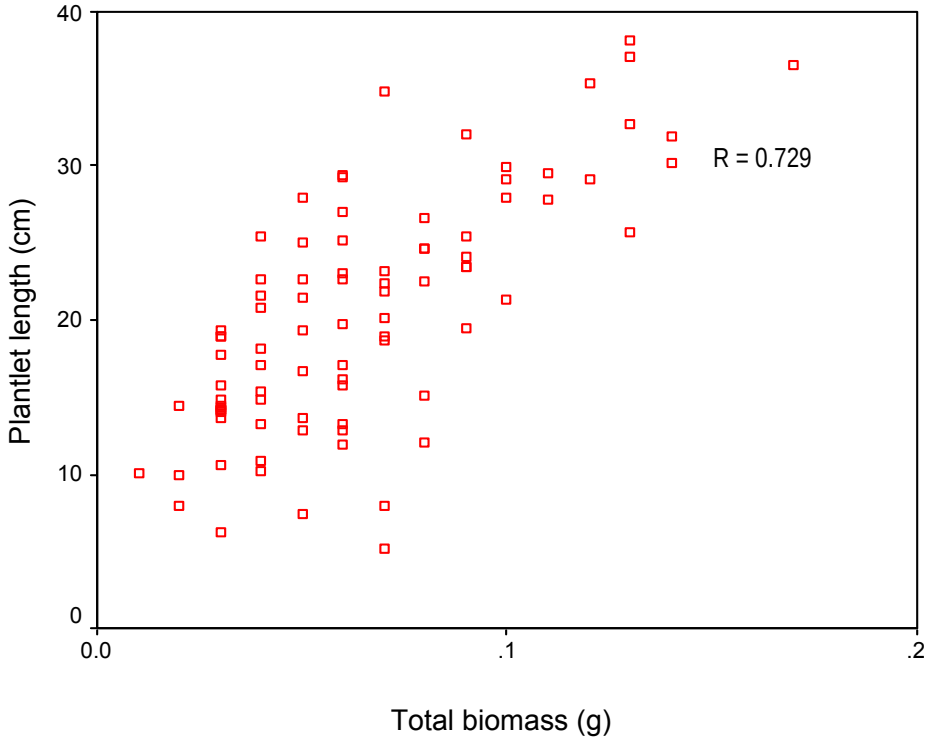


Figure 2.9. Correlation between plantlet biomass and plantlet length at three months after sowing.

Physicochemical damage to recalcitrant zygotic germplasm has been shown to adversely affect the growth/ morphology of seedlings obtained from the damaged explants (e.g. Sershen *et al*., 2010, 2011). Therefore the basis of reduced vigour (and hence a reduction in biomass accumulation in subsequent seedlings) may be the sustained water stress and/ or fungal proliferation at 25 °C (at which temperature metabolism would have been highest), and probably a degree of chilling damage in seeds stored at 6 °C.

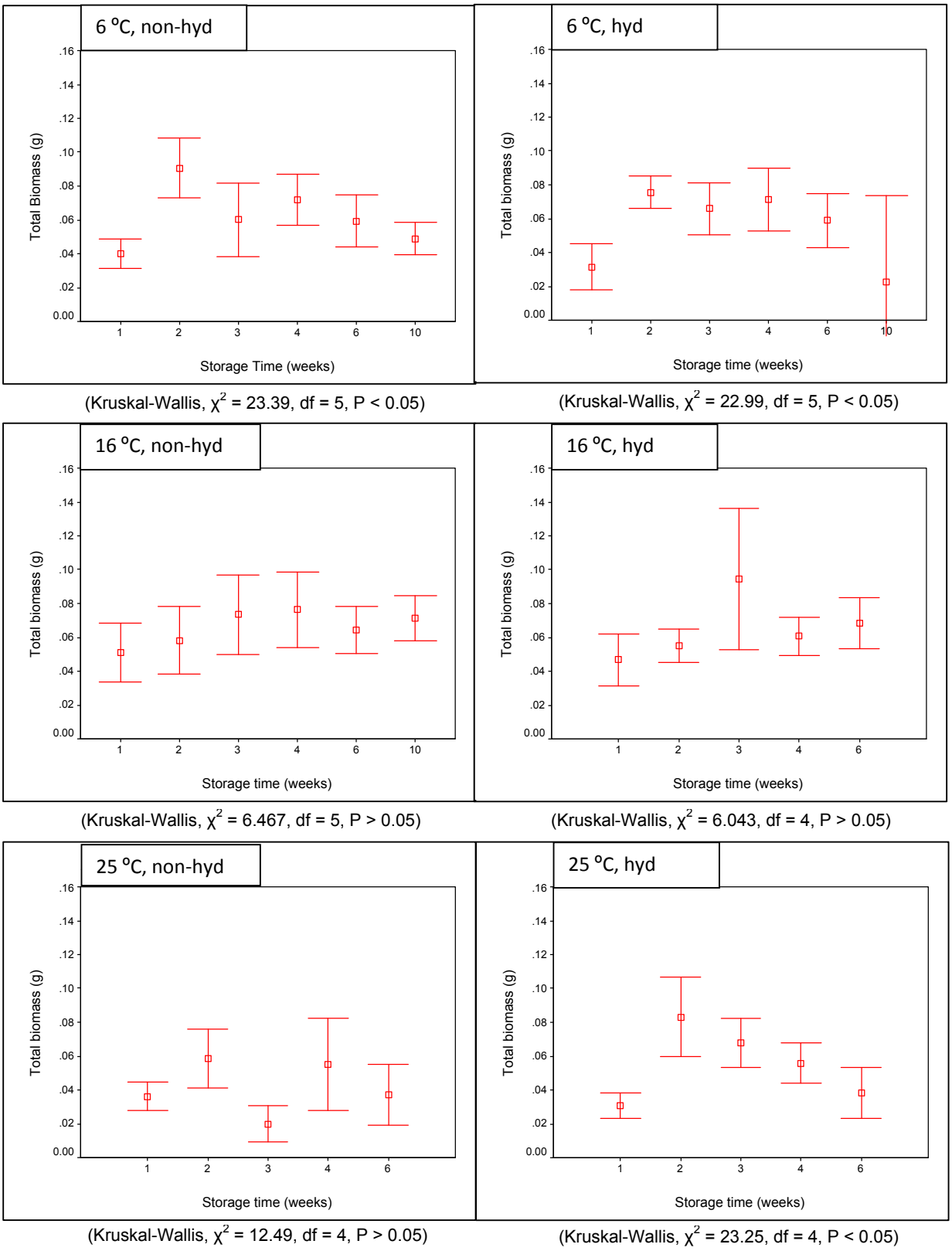
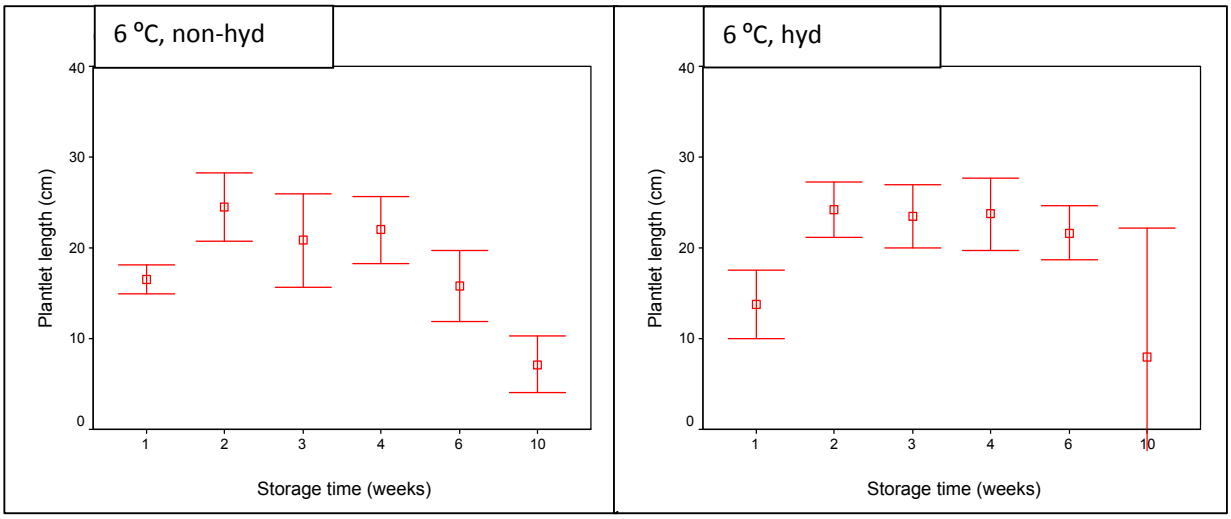
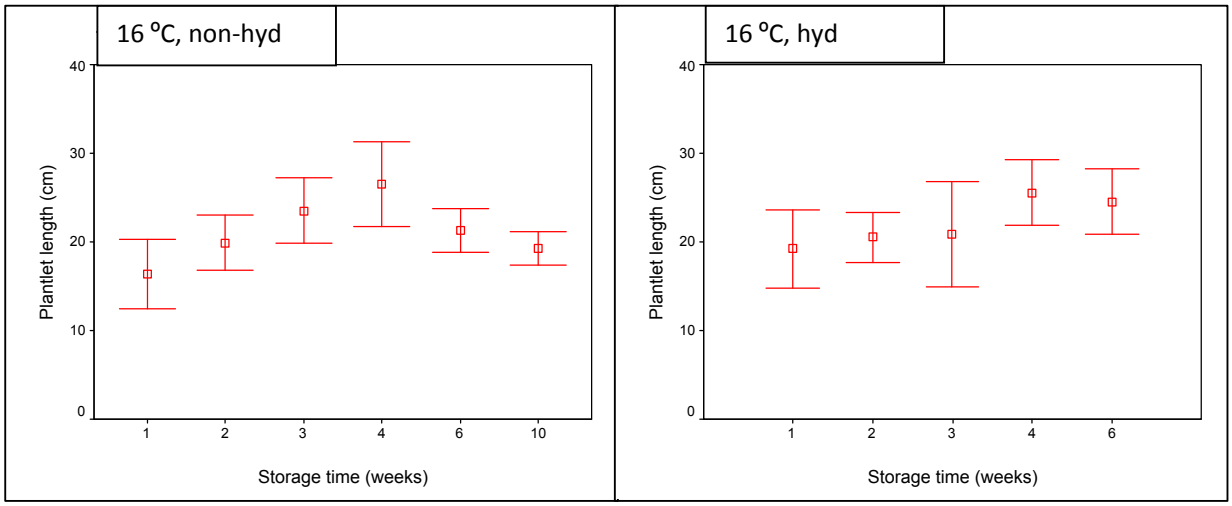


Figure 2.10. Total biomass accumulated by three-month-old seedlings of seeds that had been stored under various conditions for different periods.



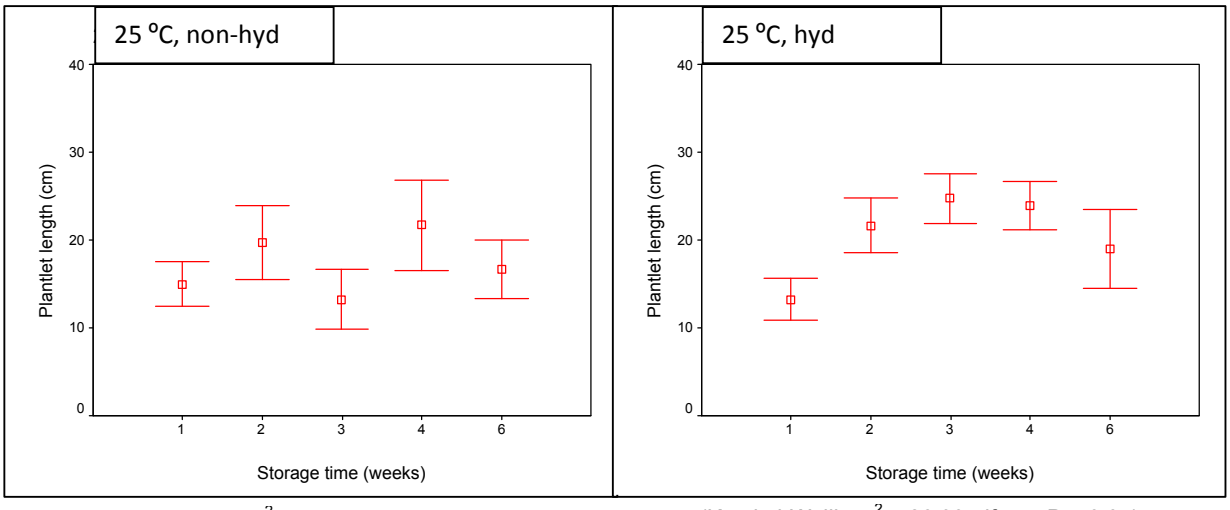
(Kruskal-Wallis, χ^2 = 44.47, df = 5, P < 0.05)

(Kruskal-Wallis, χ^2 = 23.93, df = 5, P < 0.05)



(Kruskal-Wallis, χ^2 = 18.06, df = 5, P < 0.05)

(Kruskal-Wallis, χ^2 = 8.177, df = 4, P > 0.05)



(Kruskal-Wallis, χ^2 = 10.18, df = 4, P > 0.05)

(Kruskal-Wallis, χ^2 = 26.30, df = 4, P < 0.05)

Figure 2.11. Total plantlet length of three-month-old seedlings of seeds that had been stored under various conditions for different periods.

2.3.5 Shoot:root ratio

The shoot:root ratio has been described as the ratio of the amount of plant tissues that have growth functions to the amount of those that have supportive functions (Allaby, 1998). The shoot fixes carbon dioxide from the atmosphere and loses water via transpiration, while the roots extract nutrients and water from the soil. Thus, from a water balance perspective, a certain amount of transpiring tissue/ foliage needs an appropriate amount (surface area or dry mass) of roots to absorb soil water and offset transpirational losses. A low shoot:root ratio therefore implies that the plant has better drought avoidance potential (Bernier *et al*., 1995). On the other hand, a higher shoot:root ratio may be an advantage for seedling establishment when effective competition for light is advantageous (Ogawa, 2005). In this study, the shoot:root ratio for seedlings of *S. cordatum* was approximately 2, and did not change appreciably with the storage period of the seeds (Fig 2.12). After 10 weeks of storage, however, seedlings from seeds stored at 25 ºC (hydrated) had a noticeably higher shoot:root ratio than those from seeds stored under other conditions. This may be the morphological result of a greater extent of germination and growth in seeds stored hydrated at 25 ºC.

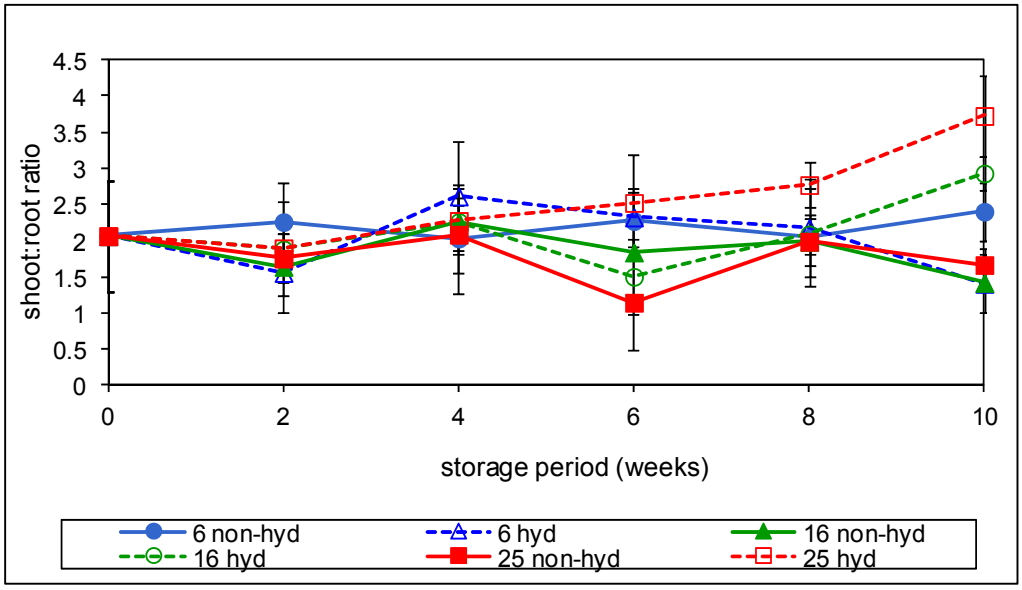


Figure 2.12. Shoot:root ratio of three-month-old seedlings raised from seeds stored for different periods at 6 ºC, 16 ºC and 25 ºC.

2.3.6 Effects of storage conditions on microbial contamination

Microbial contamination was observed for all storage conditions tested in this study, except the non-hydrated storage at 6 ºC (Figs. 2.13 and 2.15), thus indicating that surface sterilization of seeds before storage was not sufficient to eliminate fungal infection in seeds of *S. cordatum*.

The initial occurrence and level of contamination differed among storage conditions (Fig. 2.13). No contamination was observed on seeds stored in non-hydrated conditions at 6 ºC. Under hydrated conditions, contamination at 6 ºC occurred about a week later than it did at 16 ºC and 25 ºC. At 16 ºC, the seeds stored in hydrated conditions got contaminated after two weeks in storage while

those in non-hydrated storage conditions were contaminated two weeks later, after four weeks in storage. Seeds stored at 25 ºC, whether under hydrated or non-hydrated conditions, had consistently more contamination than seeds stored under other storage conditions. By 10 weeks in storage, all seeds stored in hydrated conditions at 25 ºC were contaminated.

Recalcitrant seeds have been shown to be particularly vulnerable to fungal infection in storage (Anguelova-Merhar *et al.*, 2003), with the level of contamination generally increasing during hydrated storage while viability drops concurrently (e.g. studies on surface-sterilzed seeds of *Trichilia dregeana* by Berjak *et al*., 2004). Similarly, for *Avicennia marina*, fluorescence microscopical studies revealed that there was progressive deterioration of the internal tissue of seeds due to fungal infection during hydrated storage (Anguelova-Merhar *et al*., 2003).

In this study, a proportion of seeds from all treatments, except those stored at 6 ºC in hydrated conditions for three weeks, got contaminated when they were sown in vermiculite (Fig. 2.14). There was a varied level of contamination. However, there was a general trend towards increase of contamination with storage time for all seeds stored in all storage conditions except those stored in non-hydrated conditions at 16 ºC. Seeds stored in non-hydrated conditions at 16 ºC exhibited a reduction in fungal infection with storage time. This reduction in fungal infection coincided with an increase in vigour with time in storage (Fig. 2.4).

The increased contamination of seeds after sowing as time in storage increased may have been due to a deeper manifestation of microbial contaminants in the seed. For example, there was no visible evidence of fungal infection during storage on seeds stored non-hydrated at 6 ºC. However when these seeds stored for 10 weeks were sown on vermiculite, 60% of the seeds were infected with fungi (Fig. 2.14). This suggests that seeds had fungal inocula at harvest and proliferation was inhibited by chilling. This result also highlights the need for a more reliable method of assessing fungal contamination of recalcitrant seeds, e.g. by molecular detection and diagnosis (Mukherjee *et al*., 2006).

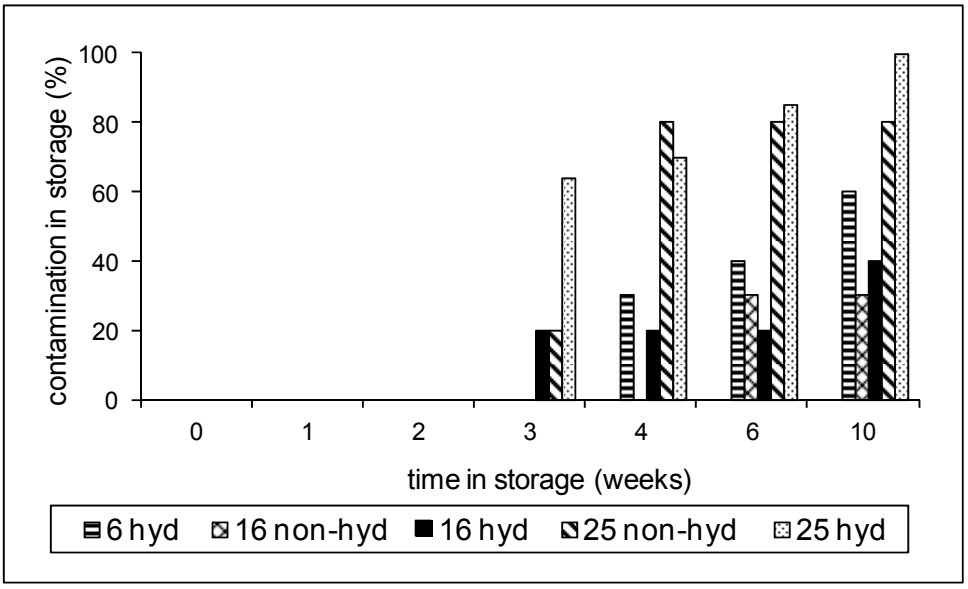


Figure 2.13. Level of contamination on seeds during storage in varying conditions for different periods. No contamination was recorded for seeds stored non-hydrated at 6 ºC.

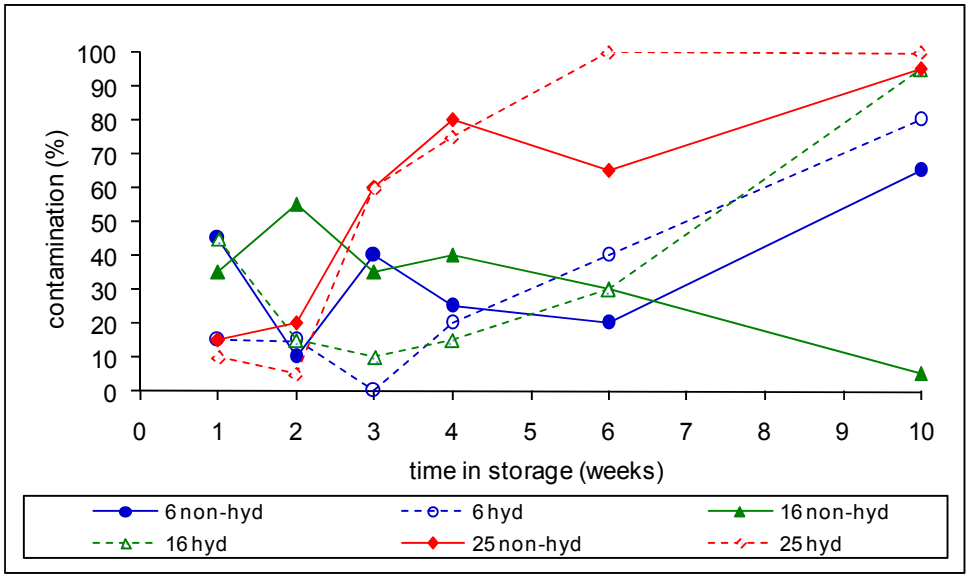


Figure 2.14. Level of contamination on seeds when sown on vermiculite after they had been stored in varying conditions for different periods

Figure 2.15. Contaminated seeds. F = fungal mycelium

By using a fluorescence microscope with a filter combination of UV-2A, at 330-380 nm excitation bands, it emerged that immediately after harvest, there was visible evidence of fungal structures that were expressed as blue fluorescence

(Fig. 2.16 a), as has been reported to be the case with many recalcitrant seeds at large (Berjak *et al.,* 2004). After four weeks in storage the seed coat surface had few, scattered, fungal mycelia (Fig. 2.16 d). By ten weeks in storage there was a dense mycelium covering most of the seed (Fig. 2.16 g); there were hyphae in the cotyledon (Fig. 2.16h) and fungal mycelia had invaded the embryonic axes (Fig. 2.16 i).

The presence of fungi on/ in the seed tissues of *S. cordatum* in this study is not unexpected, as recalcitrant seeds have been shown to generally harbour a wide spectrum of mycoflora, even when freshly harvested (e.g. Anguelova-Merhar *et al*., 2003; Berjak *et al*., 2004). It appears that, in this study, there was a relatively small amount of inoculum, located either in, or just beneath, the seed coat. Since the seeds were necessarily stored without desiccation and at temperatures that can facilitate microbial growth, the fungal inoculum progressively proliferated throughout the seed tissue during storage. Thus, in this study, there was a steeply increasing rate of fungal contamination of seeds during storage, even though the seeds had been surface-sterilized and coated with a fungicide prior to storage (see section 2.3.6). The increasing prevalence of fungi in the internal tissues of the seeds with storage time implies that the fungi may be the primary cause of viability loss. This is in contrast to some cases, such as that observed for *Trichila emetica* (Kioko *et al*., 2006) where there is negligible fungal contamination in stored recalcitrant seeds if the fungi are eliminated from the seed surface prior to storage. For *S. cordatum*, longer shelf life may be attained if the seeds are treated with an effective systemic fungicide.

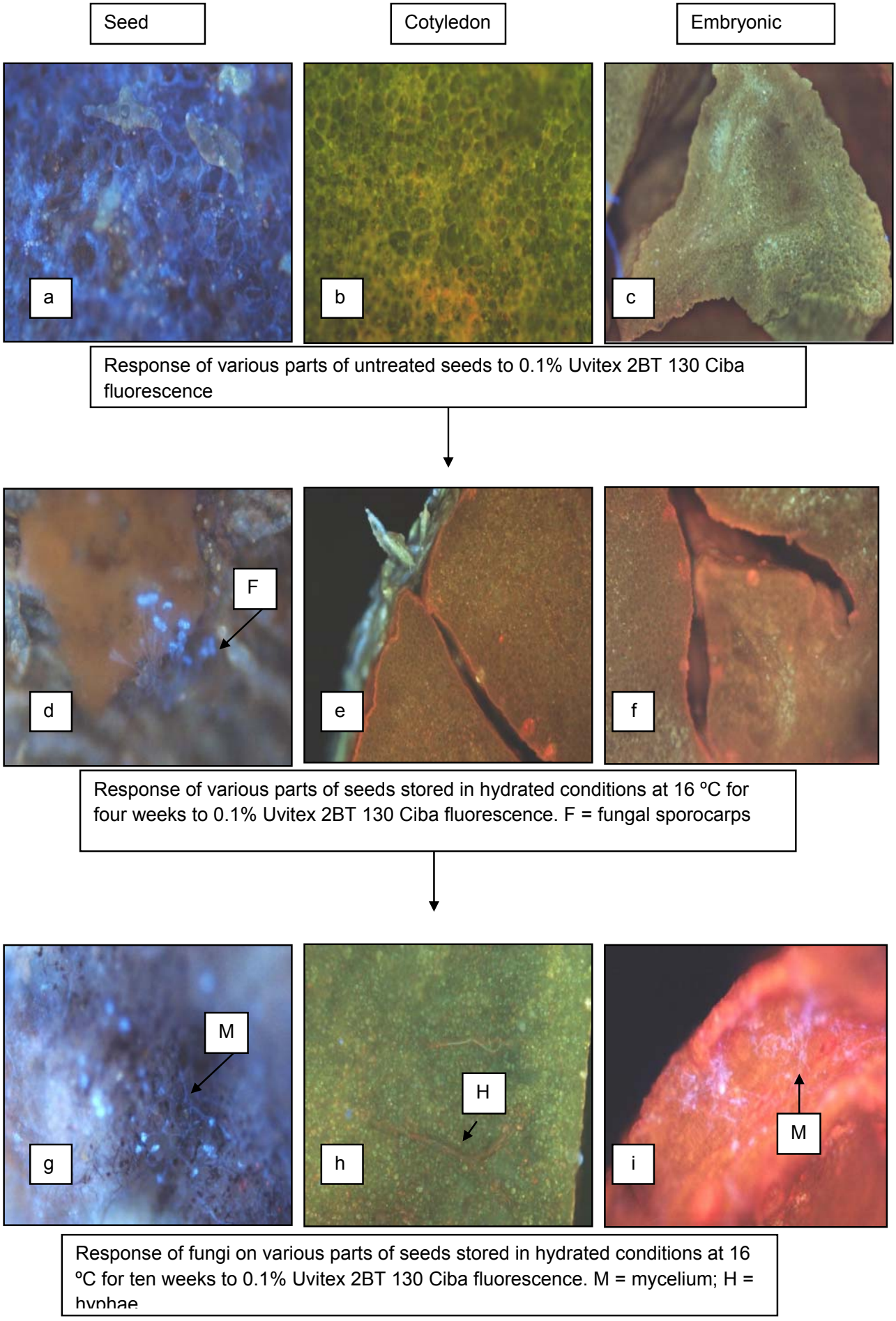


Figure 2.16 Response of fungi to different parts of seeds stored in hydrated conditions at 16 ºC for various periods to 0.1% Uvitex 2BT 130 Ciba fluorescence.

2.4 Conclusion: Short-term storage of *Syzygium cordatum* seeds

This chapter describes a study seeking to establish optimal short-term storage conditions for the recalcitrant seeds of *Syzygium cordatum*. Seeds were stored at two levels of relative humidity, at three different temperatures (6 ºC, 16 ºC and 25 ºC) for different periods. Seeds stored at 6 ºC, 16 ºC and 25 ºC maintained stable water contents throughout the storage period. The most mature seeds that were stored in a hydrated atmosphere at both 16 ºC and 25 ºC reached their root protrusion stage after three weeks in storage. This, however, occurred in only a small percentage of the seed population. The majority of the seeds that were stored under hydrated atmospheric conditions at 16 ºC and 25 ºC did not exhibit the characteristics associated with the final stages of germination (radicle protrusion) for at least six weeks in storage, but after this time there was loss of viability and vigour, which may have resulted from damage associated with both contamination and sustained water stress in the seeds.

The seed storage conditions affected the growth/ morphology of the resulting seedlings. At 6 ºC and 25 ºC, seeds stored for the shortest and longest periods produced the smallest seedlings. Seedling size/ vigour has been shown to be related to seed quality (e.g. Cook and Smart, 2004; Bladé and Vallejo, 2008), and storing the seeds at these temperatures may have affected seed quality, i.e. storage at 6 ºC may have caused damage associated with chilling, while storage at 25 ºC was highly conducive to fungal proliferation. For seeds stored

at 16 ºC, seedling size did not change significantly with the period of seed storage. This was the storage temperature at which seeds maintained the highest level of vigour, an important component of seed quality (Sanchez *et al*., 1993). Recent studies (e.g. Sershen *et al*., 2010; Sershen *et al*., 2011) have also demonstrated that the stress undergone by some recalcitrant seeds can affect subsequent growth of seedlings recovered from those seeds.

From the results of this study, the shelf-life of the seeds of *S. cordatum* can be extended up to 10 weeks by storage in non-hydrated conditions at about 16 ºC, which provides an opportunity for planned field-planting for either conservation or utilization purposes.

CHAPTER 3. TOWARDS THE CRYOPRESERVATION OF *Syzygium cordatum* EMBRYONIC AXES

3.1 Introduction

For the cryopreservation of the germplasm of recalcitrant-seeded species, embryonic axes are excised from the seeds and used as the preferred explants for storage, as they offer the same genetic variability availed by intact seeds (Normah *et al.*, 1986, Engelmann, 2011). Axes, which are generally small, also provide a favourable surface area:volume ratio which faciliates rapid rates of dehydration and cooling (Berjak and Pammenter, 2001). The use of embryonic axes, however, requires the optimisation of several procedures (Reed, 1996; Berjak *et al*., 1999a,b) including decontamination, development of suitable growth/ germination media, exposure to cryoprotectants (although not always used) which stablilise cells during cooling, and dehydration to appropriate water contents at the best rate able to be achieved to avoid metabolism-induced dehydration damage or dehydration damage *sensu stricto*. Development of cryopreservation procedures also involves the optimisation of cooling and warming (thawing) rates and the composition of the rehydration medium (Mycock, 1999). These factors, in the way they relate to the cryoconservation of plant germplasm in South Africa, have been reviewed by Berjak *et al*. (2011a).

For *S. cordatum*, one of the key factors that may affect successful cryopreservation of embryonic axes is that they contain appreciable amounts of phenolic compounds (Premsager, 2009). Phenolics are substances that accumulate in different plant tissues and cells during ontogenesis, and in

response to various environmental stimuli (Hutzler *et al.,* 1998). Phenolics are located in various areas of the cell; in the wall (Hutzler *et al*., 1998; Gorshkova *et al*., 2000), in vacuoles, and associated with the plasma membrane (Einhellig, 1995) and nucleic acids (Hutzler *et al.,* 1998). According to Einhellg (1995), phenolics cause depolarisation of the cell membrane by influencing membrane ATPase activity and can alter the influx and retention of ions. Phenolics can also inactivate enzymes, precipitate proteins and form cross-links with nucleic acids (Koonjul *et al.*, 1999), which consequently interfere with cellular function. A particular problem in the context of cryopreservation and *in vitro* procedures for explants which contain or produce substantial amounts of phenolics, is that these compounds leach into growth media, and severely compromise onwards development of explants (Zhou *et al*., 2000).

In order to contribute to the long-term storage of *S. cordatum* this study sought to establish a protocol for the cryopreservation of the zygotic embryonic axes by the serial optimisation of procedures for decontamination, regeneration and growth, cryoprotection, dehydration and cooling. The effect of the various procedures in the context of phenolics production was also a focus of this study.

3.2 Materials and methods

3.2.1 Contamination status and contaminant control

In order to evaluate the best means of decontamination of excised embryonic axes intended for cryopreservation, embryonic axes were excised and divided into two sets. One set was surface-sterilized through soaking in any one of:

- Sterile water (control),
- 1% (v/v) NaOCl for 5 min,
- 1% (v/v) NaOCl for 10 min,
- 1% (w/v) calcium hypochlorite [$Ca(OCl)_2$] for 5 min
- 70% (v/v) ethanol for 10 min,

The axes were then rinsed thrice in sterile distilled water and thereafter plated onto MS (Murashige and Skoog, 1962) medium. In each case, 4 replicates of 5 axes, were used.

The other set of embryonic axes was surface-sterilized with each of the above mentioned sterilants, rinsed and plated onto MS medium containing a mixture of 2.5 ml l^{-1} Previcur® (propamocarb-HCl, AgrEvo, Pietermaritzburg, South Africa), 0.2 ml l^{-1} Early Impact (triazole and benzamidazole, Zeneca Agrochemicals, S. Africa), and 50 g l^{-1} kanamycin.

The decontaminated embryonic axes were cultured for 3 weeks in a growth chamber at 20 ºC/26 ºC (night/day) in an 8 h dark/16 h light photoperiod.

The best decontamination procedure was assessed by its efficacy in eradicating contaminants, and the lack of harmful effects on the survival of embryonic axes.

3.2.2 *In vitro* growth

Surface-sterilised embryonic axes were aseptically plated (5 embryonic axes per 90 mm Petri dish) on growth medium.

To establish a suitable growth medium, 20 embryonic axes, in 5 sets of four, were plated onto media (pH 5.6-5.8) all containing 30 g l^{-1} sucrose and 8 g l^{-1} agar [uniLAB® Saarchem (Pty) Ltd], but incorporating other components as listed below:

- Murashige and Skoog (1962) basal salt and vitamins
- Murashige and Skoog (1962) basal salt and vitamins combined with 2.5 ml l^{-1} Previcur®, 0.2 ml l^{-1} Early Impact and 50 g l^{-1} kanamycin
- Murashige and Skoog (1962) basal salt and vitamins, and 4 g l^{-1} activated charcoal,
- Murashige and Skoog (1962) basal salt and vitamins, and 100 mg l^{-1} ascorbic acid
- McCown's woody plant basal salt mixture (Lloyd and McCown, 1981)

- McCown's woody plant basal salt mixture (Lloyd and McCown, 1981) combined with 2.5 ml l^{-1} Previcur®, 0.2 ml l^{-1} Early Impact and 50 g l^{-1} kanamycin
- McCown's woody plant basal salt mixture (Lloyd and McCown, 1981) and 4 g l^{-1} activated charcoal,
- McCown's woody plant basal salt mixture (Lloyd and McCown, 1981) and 100 mg l^{-1} ascorbic acid, and
- agitated liquid medium containing McCown's woody plant basal salt mixture (Lloyd and McCown, 1981), 10 g l^{-1} polyvinylpyrrolidone (PVP; Sigma, St Louis, MO, USA) and 75 mg l^{-1} citric acid. The axes were placed on filter paper on a short pipe in the medium (Fig. 3.1.). The filter paper was wetted by the medium during the agitation process.

The embryonic axes were cultured for 3 weeks in a growth chamber at 20 ºC/26 ºC (night/day) in an 8 h dark/16 h light photoperiod.

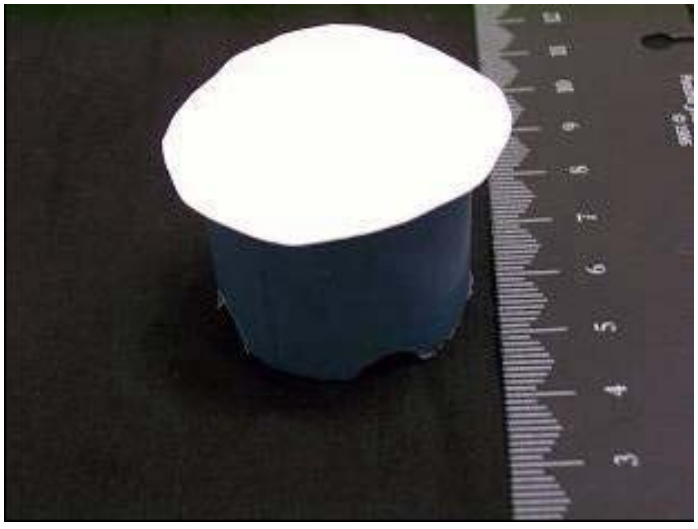


Figure 3.1. Filter paper placed on short pipe, which was then immersed in liquid medium so that the surface of the medium touched the filter paper and wetted it. Embryonic axes were located on the wetted filter paper.

The growth of the embryonic axes on the different media was assessed every five days for 30 d. The parameters assessed were: germination capacity, seedling length (shoot+root length), root collar diameter which was measured using a vernier caliper, number of leaves, presence or absence of visible contamination and explant colour. All responses which indicated that the embryonic axes had resumed growth i*n vitro*, such as growth of the radicle beyond 5 mm or shoot formation, were considered as 'survival'.

3.2.3 Dehydration

Embryonic axes were dehydrated for different lengths of time using a flash drier (Pammenter *et al.*, 2002), an apparatus with which dry air is rapidly passed through a monolayer of embryonic axes placed on a mesh, thus achieving rapid dehydration. Twenty axes were flash dried for each of the following times: 0 (control), 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 120 min.

3.2.4 Water content determination

Water content of embryonic axes was determined gravimetrically. Twenty embryonic axes were weighed on a 5-place balance, dried in an oven at 80 ºC for 48 h to ensure total loss of water. The water content was expressed as g H_2O g^{-1} dry mass.

3.2.5 Cryoprotection

Both low molecular weight and high molecular weight cryoprotectants were used (Benson, 1995; 2008). The following low molecular weight cryoprotectants were tested: sucrose, glycerol and dimethyl sulphoxide (DMSO), while the high molecular weight cryoprotectants used were dextran and polyvinylpyrrolidone (PVP). Axes (20 axes in four replicates of 5 axes each) were exposed to 5% solutions of cryoprotectant for 1 h and then transferred to a 10% solution of the same cryoprotectant for another hour. Thereafter the viability of the axes and water contents were determined.

3.2.6 Cooling (adapted from Wesley-Smith, 2002)

Naked embryonic axes were plunged into nitrogen slush at -210 ºC (liquid nitrogen sub-cooled under vacuum in order to achieve ultra-fast cooling the rate being reported as >10 000 ºC s^{-1}; Wesley-Smith, 2002) which minimises the time during which ice crystallisation, and consequently, potentially lethal damage could occur.

3.2.7 Thawing (adapted from Mycock, 1999)

The frozen embryonic axes were thawed by rapid immersion in a solution containing 1 μM $CaCl_2.2H_2O$ and 1 mM $MgCl_2.6H_2O$ at 40 ºC and then transferred to a similar solution at room temperature for 40 min.

3.2.8 Photography

Photographs were taken on film using a stereo microscope (Wild M4A [Heerbrugg, Switzerland]) or a Sony MVC-FD92 digital still camera.

3.2.9 Transmission Electron Microscopy

Embryonic axes were processed for transmission electron microscopy after either exposing them to 75 mg l^{-1} citric acid or not. The extreme tip of the radicle was then fixed in 2.5% (v/v) glutaraldehyde in 100 mM phosphate buffer at pH 7.0, containing 0.5% (w/v) caffeine. The specimens were then post-fixed for 1 h in 0.5% (w/v) aqueous osmium tetroxide, dehydrated through a series of increasing concentrations of acetone then placed in low viscosity epoxy resin (Spurr, 1969) which was polymerised for 16 h at 70 ºC. Ultra-thin sections were cut with glass knives using a Reichert Ultracut E ultramicrotome (Leica, Austria), and collected on copper grids. Grids were then floated on droplets of saturated uranyl acetate, for 10 min, then washed with-gas free water for 30 s, followed by floating grids on droplets of lead citrate for 10 min and then washed with gas-free water for another 30 s. Sections from at least three blocks per treatment were viewed with a JEOL JEM 1010 transmission electron microscope at 80 kV. Images that represented the general appearance of cells from citric acid treated and untreated embryos were captured on Kodak 4489 film, which was specifically manufactured for electron microscopy.

3.2.10 Statistical analysis

Data collected were analysed using the SPSS statistical programme. All non-parametric data were analysed using the Kruskal-Wallis test for K-independent variables at a 95% confidence level. Statistical significance of parametric data was tested using analysis of variance (ANOVA), also at a 95% confidence level. Correlation between some sets of treatments and results were tested using Spearman's Rank test because the data was non-parametric.

3.3 Results and discussion

3.3.1 Decontamination of embryonic axes of *Syzygium cordatum*

As reviewed by George (1996), there are three types of contamination in plant tissue culture: acute contamination which happens almost immediately after establishment of cultures and is associated with ineffective surface sterilisation; contamination that becomes evident after culture establishment and is associated with endogenous micro-organisms; and chronic contamination which is manifested after a long period in seemingly sterile cultures.

Recalcitrant seeds across species have been found to harbour a spectrum of fungi, the inoculum of which is frequently internally located (Mycock and Berjak, 1990). Furthermore, the embryonic axes could be exposed to a variety of contaminants during the different procedural stages when they are being processed – before, during, and after cryopreservation. Fungi therefore need to be effectively eliminated in the first instance, and steps must be taken to decontaminate explants subsequently, when axes are handled under non-sterile conditions. (From experience in our laboratory, however, it should be noted that elimination of systemic infection from embryonic axes is among the most difficult of tasks).

The establishment of an optimal contamination-control procedure may require appropriate combinations of sterilant-types, concentrations and exposure times. Experience has shown that when fungi are eliminated, bacterial contaminants

predominate on embryonic axes and surrounding media, which necessitates the administration of a 'cocktail' of systemic fungicides and antibiotics (Berjak and Pammenter, 2004b).

Further considerations in controlling contamination are the means of sterilant administration. Depending on the type and position of the contaminant, the sterilants can be either administered by surface-sterilisation (for superficially-borne contamination) or by addition to the *in vitro* culture media (for endogenous micro-organisms).

3.3.1.1 Effects of sterilants on contamination

In order to optimise a decontamination procedure for *S. cordatum* (or any other species) successfully, it is important that the sterilant, however effective it may prove to be in eliminating contaminating micro-organisms, should have minimal adverse effects on the viability of embryonic axes. Sodium hypochlorite at concentrations of between 2% and 5% has been used successfully to eliminate fungal contaminants on many plants (e.g. Abdel-Mallek *et al.*, 1995).

In this study, all embryonic axes that were not exposed to any surface sterilisation exhibited contamination (Fig. 3.2), indicating that a surface sterilisation treatment was required to precede *in vitro* culturing. In this regard, the kanamycin and fungicide mixture in the media, on their own, did not provide sufficient protection from contaminants (Fig. 3.2). These results suggest the presence of acute and possibly endogenous contamination (George, 1996) on and in embryonic axes of *S. cordatum*.

Both 1% $Ca(OCl)_2$ on its own, and 70% ethanol when combined with the 'cocktail' of kanamycin and systemic fungicide, eliminated visible contamination from the embryonic axes. Embryonic axes subjected to all the other decontamination treatments continued to exhibit fungal contamination (Fig. 3.2).

The Plant Protection Research Institute in Pretoria, South Africa identified the fungal contaminants as *Penicillium* cf. *hirsutum* Dierckx, *Penicillium olsonii* Bain. & Sartory and *Penicillium* cf. *crustonum* Thom (Fig. 3.4). *Penicillium* spp. have been reported as common seed-borne pathogens that can be transmitted to axes and consequently to seedlings (Pongpanich, 1990). Mycock and Berjak (1990) found that species of *Penicillium*, in combination with *Aspergillus*, *Fusarium* and *Alternaria*, were found in the embryonic axes of recalcitrant seeds of a variety of species during storage.

In this study, it was noteworthy that there was variation in the effectiveness of different sterilants when embryonic axes were cultured on the same medium (MS) (Fig. 3.2) and when the same sterilant was used for embryonic axes which were cultured on different growth media (Fig. 3.3). The variation in contamination responses to the same surface sterilisation procedure indicates the importance of considering nutrient media as a key factor that may affect the effectiveness of a decontaminant (Fig. 3.3). This perhaps implies that particular nutrient media or additives to media may enable explants to elaborate endogenous anti-fungal compounds, and others not.

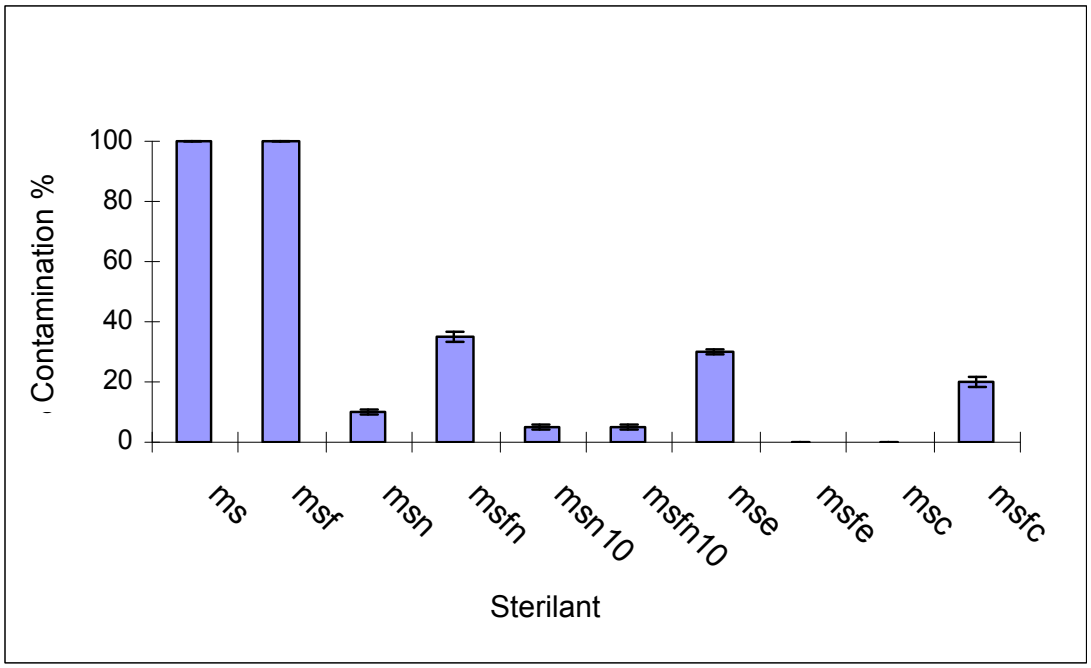


Figure 3.2. Percentage of contaminated axes from freshly harvested seeds of *S. cordatum* after decontamination with various sterilants, as listed below: n=20

ms =	axes not surface sterilized and plated on MS
msf =	axes not surface sterilised and plated on MS with fungicide
msn =	1% Na0Cl (5 min)+ MS
msfn =	1% NaOCl (5 min) + MS with fungicide
msn10 =	1% NaOCl (10) + MS
msfn10 =	1% NaOCl (10) + MS with fungicide
mse =	70% ethanol + MS
msfe =	70% ethanol + MS with fungicide
msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicide

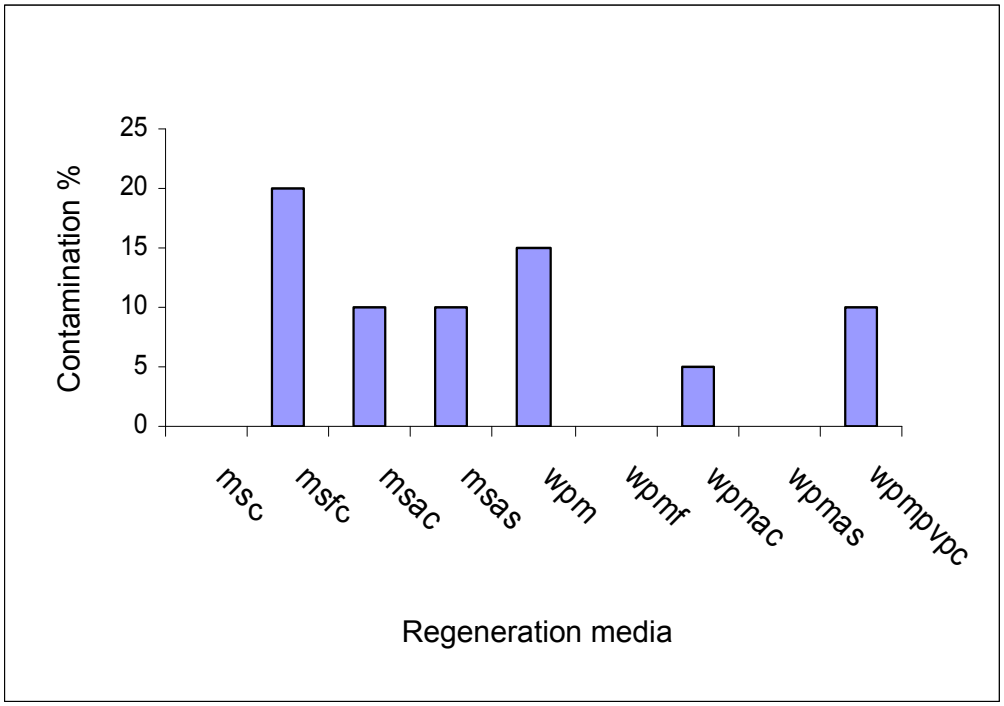


Figure 3.3. Percentage of *S. cordatum* embryonic axes showing contamination. Axes were surface sterlilised with 1% calcium hypochlorite and thereafter plated onto different growth media combinations as listed below: n=20

msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicide
msac =	1% $Ca(OCl)_2$ + MS with activated charcoal
msas =	1% $Ca(OCl)_2$ + MS with ascorbic acid
msca =	1% $Ca(OCl)_2$ + MS with citric acid
wpm =	1% $Ca(OCl)_2$ + WPM
wpmf =	1% $Ca(OCl)_2$ + WPM with fungicide
wpmac =	1% $Ca(OCl)_2$ + WPM with activated charcoal
wpmas =	1% $Ca(OCl)_2$ + WPM with ascorbic acid
wpmpvpc =	1% $Ca(OCl)_2$ + WPM with PVP + citric acid

Figure 3.4. Contaminants on embryonic axes of freshly harvested seeds of *S. cordatum*. The axes had been surface-sterilized by soaking in 1% NaOCl for 5 minutes and plated onto MS medium containing fungicides

3.3.1.2 Effects of sterilants on in vitro *germination*

Chemicals used for reducing contamination have been reported to have phytotoxic effects on seeds (Dumroese *et al.*, 1988). However, for *S. cordatum,* exposure to the decontamination treatments did not have deleterious effects on the germination capability of embryonic axes (Fig. 3.5). Untreated embryonic axes did not germinate after 20 days in culture, and fungi proliferated. This indicates that without surface sterilisation, embryonic axes were overrun by the microbial infection and were not vigorous enough to overcome contaminants (Fig. 3.5).

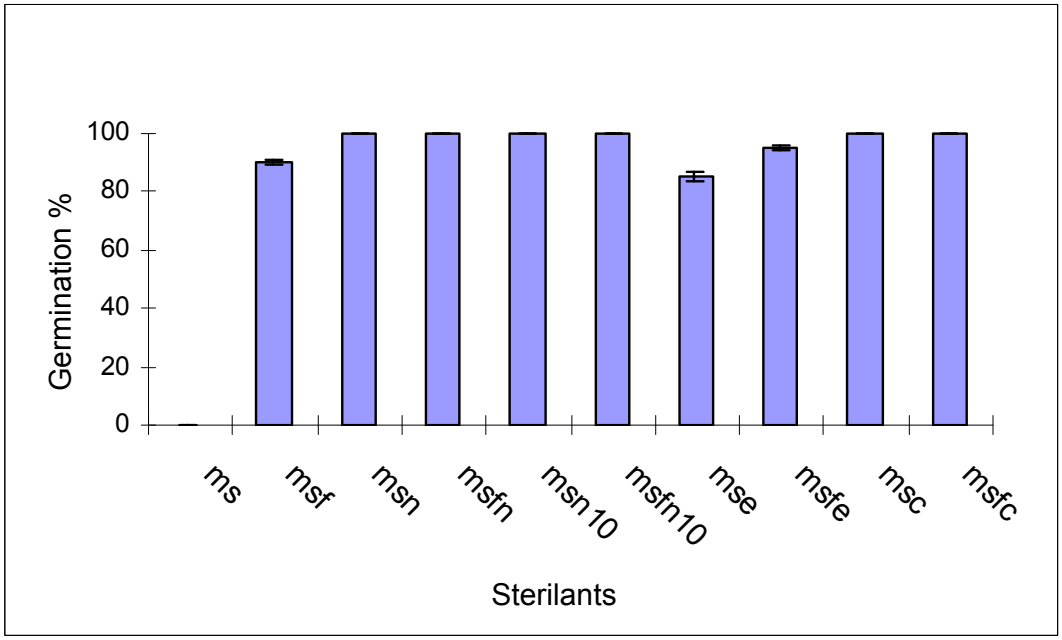


Figure 3.5. The effect of various sterilants (as listed below) on the viability of embryonic axes of freshly harvested seeds of *S. cordatum;* n=20

ms =	axes not surface sterilised and plated on MS without fungicide
msf =	axes not surface sterilised and plated on MS with fungicide
msn =	1% Na0Cl (5 min) + MS
msfn =	1% NaOCl (5 min)+ MS with fungicide
msn10 =	1% NaOCl (10 min) + MS
msfn10 =	1% NaOCl (10 min) + MS with fungicide
mse =	70% ethanol + MS
msfe =	70% ethanol + MS with fungicide
msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicide

3.3.1.3 Effects of sterilants on seedling length, root collar diameter and seedling leaf production

Seedling length, root collar diameter and leaf production have been used as an indicator of plant growth and health as in the case of duckweed (http://mobot.org/jwcross/duckweed/duckweed-measuring-growth.htm [accessed October 2011]). These are non-destructive techniques for measuring plant growth and development.

In this study, embryonic axes exposed to 1% $Ca(OCl)_2$ grew to more than double the length of those from all the other treatments (ANOVA, $F_{9,189}$ = 46.504, $P < 0.05$). The mean length of seedlings was 21 mm (Fig. 3.6) and an average of 12.25 leaves formed per seedling (Fig. 3.8) after 3 weeks in culture. Such an association between enhanced growth and successful decontamination using 1% NaOCl has also been observed for other species, e.g. for the seeds of African breadfruit (Baiyeri and Mbah, 2006).

Noteworthy in this study, however, was the fact that exposure to culture media containing kanamycin and systemic fungicides resulted in callused seedlings with miniature leaves (Fig. 3.9) and thickened stems (Fig. 3.7). A similar stunting effect of this combination of antimicrobial agents was reported for *Telfairia occidentalis* (fluted pumkin) by Ajayi *et al*. (2006). Kanamycin, an aminoglycoside antibiotic widely used as a selective agent in plant genetic transformation, has also been shown to inhibit the growth of wild (non-transformed) plant cells (Zhang *et al*., 2001) and to inhibit plantlet regeneration (Eapen and George, 1990), even though triazolic fungicides, such as Early Impact, have been shown to be beneficial to plant growth (e.g. Jaleel *et al.*,

2008). Thus, a less deleterious antibiotic treatment is necessary for the control of bacterial contamination in these *S. cordatum* embryonic axes. Different antibiotics, thermotherapy, or other formulations such as the Plant Preservative Mixtures (PPM[4]) may be options in this regard.

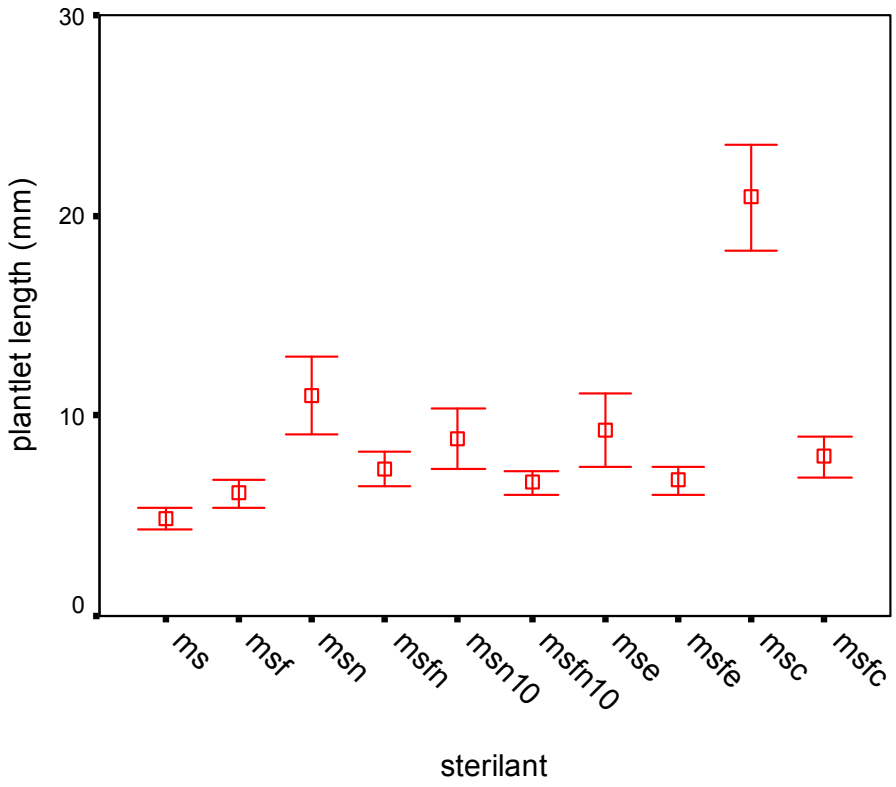


Figure 3.6. Average length of seedlings from embryonic axes of *S. cordatum* that were exposed to various sterilants, as listed below; n=20

ms =	axes not surface sterilised and plated on MS without fungicide
msf =	axes not surface sterilised and plated on MS with fungicide
msn =	1% NaOCl (5 min) + MS
msfn =	1% NaOCl (5 min) + MS with fungicide
msn10 =	1% NaOCl (10) + MS

[4] Plant Cell Technology, Washington, DC, USA (http://www.ppm4plant-tc.com/)

msfn10 =	1% NaOCl (10) + MS with fungicide
mse =	70% ethanol + MS
msfe =	70% ethanol + MS with fungicide
msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicide

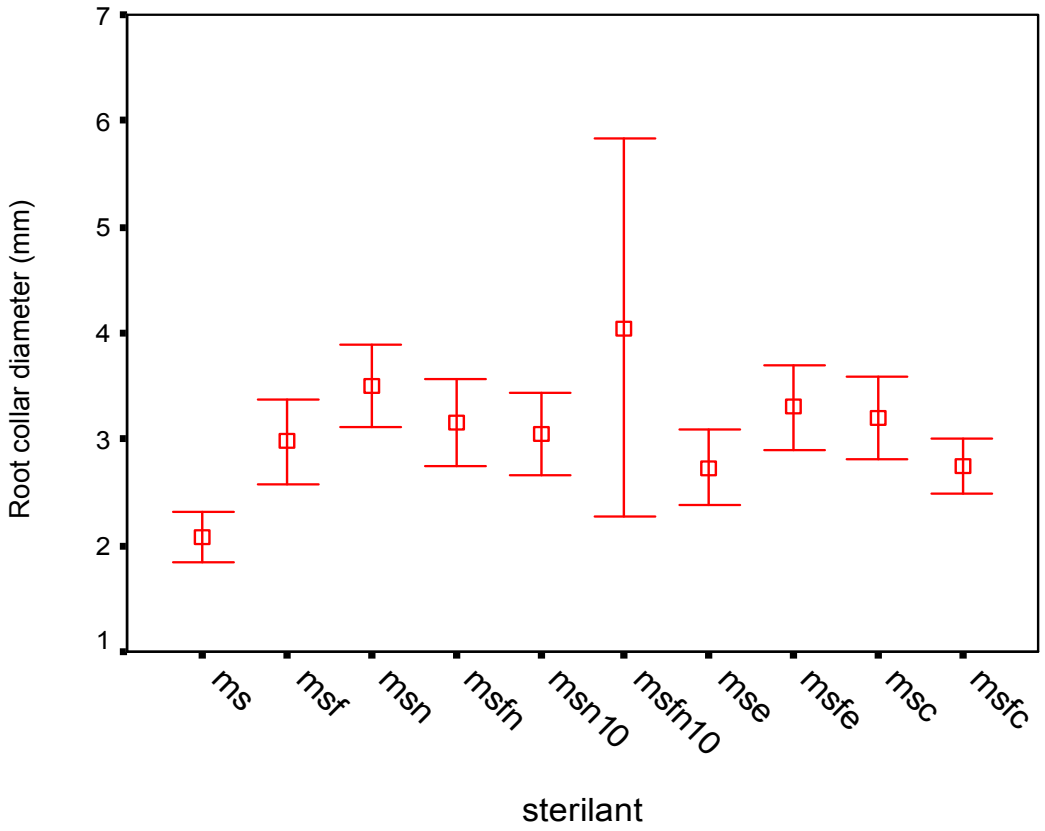


Figure 3.7. Average root collar diameters of seedlings from embryonic axes of *S. cordatum* that were exposed to various sterilants; n=20.

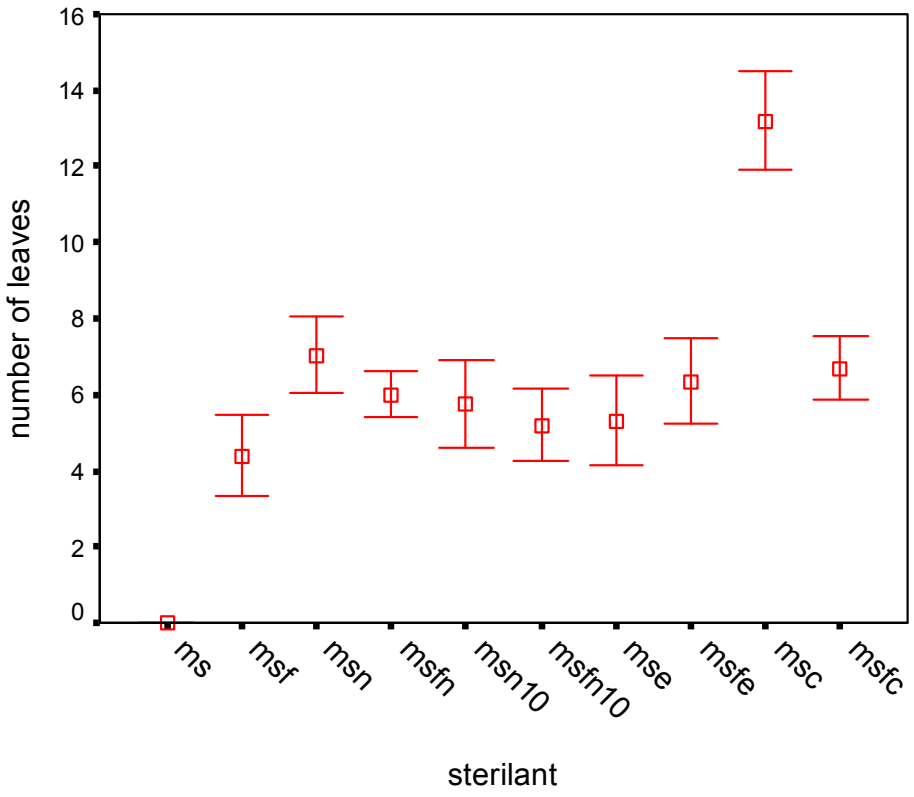


Figure 3.8. Average number of leaves on seedlings from embryonic axes of *S. cordatum* that were exposed to various sterilants; n=20.

ms =	axes not surface sterilised and plated on MS without fungicide
msf =	axes not surface sterilised and plated on MS with fungicide
msn =	1% NaOCl (5 min) + MS
msfn =	1% NaOCl (5 min) + MS with fungicide
msn10 =	1% NaOCl (10) + MS
msfn10 =	1% NaOCl (10) + MS with fungicide
mse =	70% ethanol + MS
msfe =	70% ethanol + MS with fungicide
msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicide

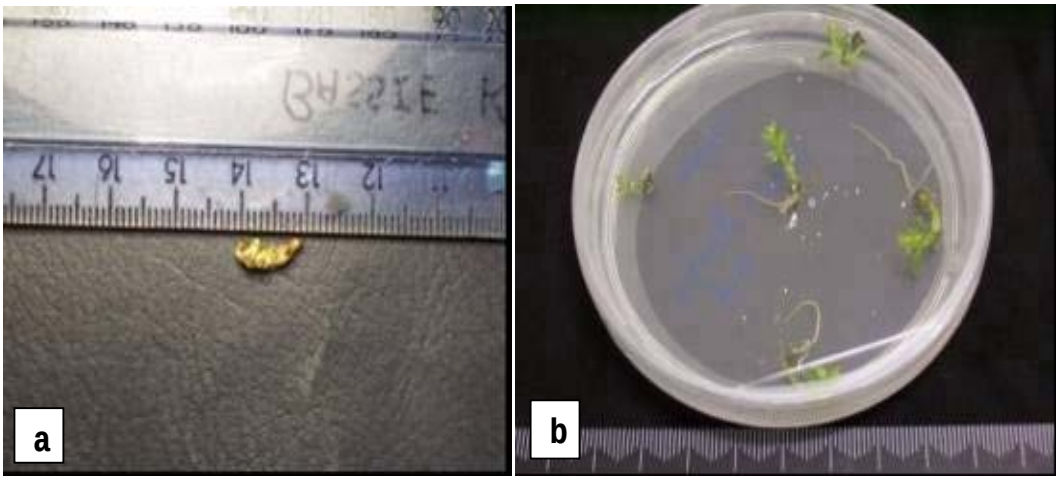


Figure 3.9. a) Stunted seedling of an embryonic axes that was exposed to the 'cocktail' of kanamycin and systemic fungicide. b) Seedlings from axes surface sterilised with calcium hypochlorite.

3.3.1.4 Effects of sterilants on phenolics production

Some fungicides, such as triazolic fungicides, e.g. the Early Impact used in this study, have been reported to confer protective action on plants through the accumulation of phenolic compounds (Ronchi *et al.*, 1997; Jaleel *et al.*, 2008). All the sterilants tested were associated with a response in terms of phenolics exuded from excised axes of *S. cordatum* (Fig. 3.10). This response varied from sterilant to sterilant (Fig. 3.11). The production of phenolics in response to sterilants, however, was less than that for non-sterilised axes (Fig. 3.11). Cell necrosis due to fungal deterioration in non-sterilized axes may have resulted in the higher degree of accumulation of brownish phenolic substance (Santiago *et al*., 2000) than in sterilized embryonic axes.

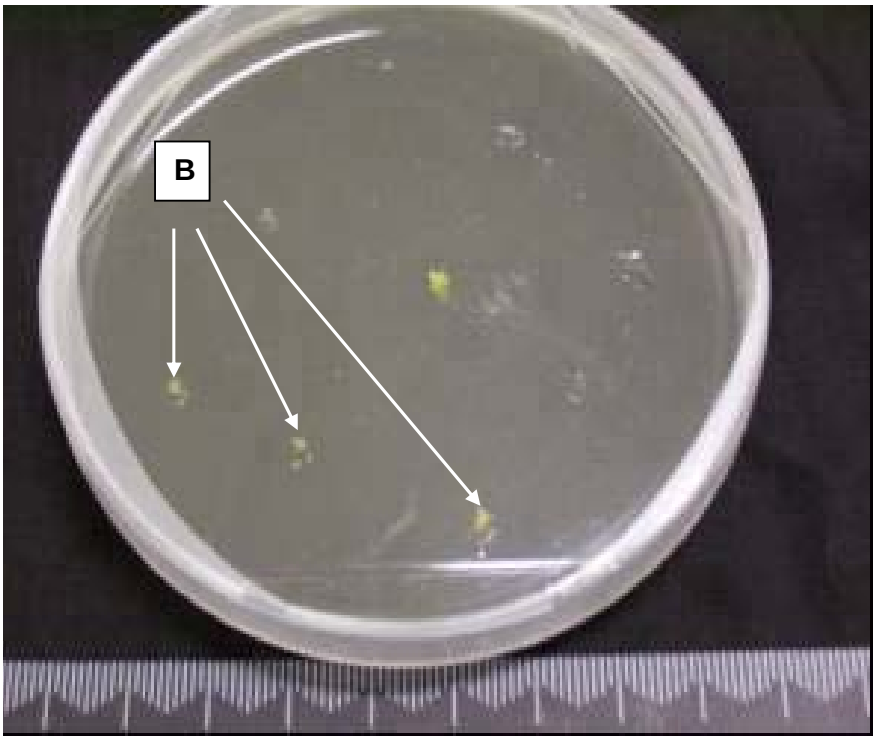


Figure 3.10. A normally growing embryonic axis surrounded by others with browning associated with phenolics after exposure to sterilants. B = browning axes.

When contamination, germination capacity, seedling vigour and phenolics exudation are taken into consideration, surface sterilisation of embryonic axes with 1% $Ca(OCl)_2$ was the most suitable decontamination treatment for embryonic axes of *S. cordatum*. This treatment eradicated all observable contamination, had no adverse effect on the germination capacity of the axes, resulted in significantly longer seedlings with a higher number of leaves than those of other treatments tested and was not associated with any significant response in terms of phenolics exudation by axes.

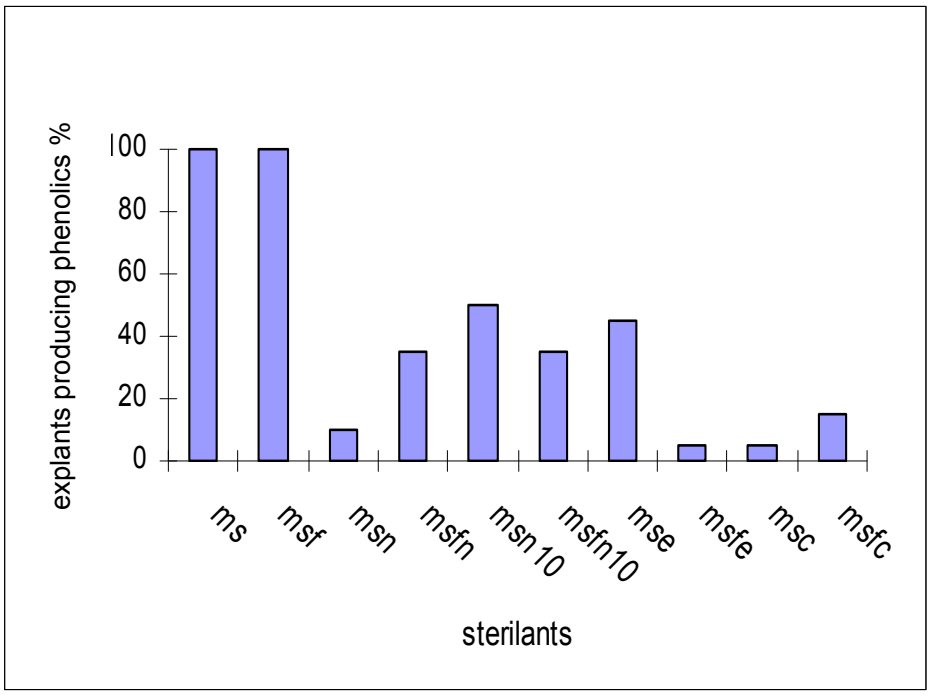


Figure 3.11. The effect of various sterilants on the exudation of phenolics by embryonic axes of recently harvested seeds of *S. cordatum;* n=20.

ms =	axes not surface sterilised and plated on MS without fungicide
msf =	axes not surface sterilised and plated on MS with fungicide
msn =	1% NaOCl (5 min) + MS
msfn =	1% NaOCl (5 min) + MS with fungicide
msn10 =	1% NaOCl (10) + MS
msfn10 =	1% NaOCl (10) + MS with fungicide
mse =	70% ethanol + MS
msfe =	70% ethanol + MS with fungicide
msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicide

3.3.2 Growth media

Before cryopreservation of embryonic axes can be undertaken, it is essential for suitable culture conditions to be established to ensure successful *in vitro* germination of axes (Berjak and Pammenter, 2004b). This is essential because excised axes have been separated from cotyledonary (or endospermic) food reserves and therefore must be grown on nutrient medium. The growth medium must afford all the components necessary for germination and early seedling development by providing component ratios and concentrations that most nearly match the optimum requirement for the cells and tissue to grow and differentiate (Gamborg, 1984). Additionally, all the environmental parameters in the growth room have to be optimised to suit both the recovery of explants immediately after cryopreservation, and subsequent onwards development.

Two basal salt compositions were tested; Murashige and Skoog (MS; 1962) and Lloyd and McCown's (1981) Woody Plant Medium (WPM). The MS salt composition is widely used for plant *in vitro* procedures (Gamborg, 1984), whereas WPM was initially used to protect explants of woody plants from chloride ion susceptibility but is now commonly used in culture procedures for woody plants (George, 1996).

Activated charcoal, PVP, citric acid and ascorbic acid were added to media in attempts to minimise the deleterious effects of phenolic exudates on the embryonic axes.

3.3.2.1 Effects of media on in vitro *development*

While some phenolic acids have been reported to inhibit hydraulic conductivity and nutrient uptake by roots thus inhibiting growth in plants (Inderjit *et al.*, 2002), none of the media tested substantially affected the germination capacity of embryonic axes of *S. cordatum* (Fig. 3.12) as they all supported over 80% germination. However, there were some differences, in that 100% germination was not obtained on MS media containing activated charcoal or ascorbic acid, and on WPM incorporating activated charcoal. It is possible that adsorption of a medium component(s) by activated charcoal could have limited its/their availability to the axes, while ascorbic acid incorporated into the MS medium might have quenched reactive oxygen species with a signalling role during germination (Müller *et al.*, 2009; Kranner *et al.*, 2010). Nevertheless, provision of ascorbic acid did effectively counteract phenolics-associated browning (see Figs 3.16 & 3.17).

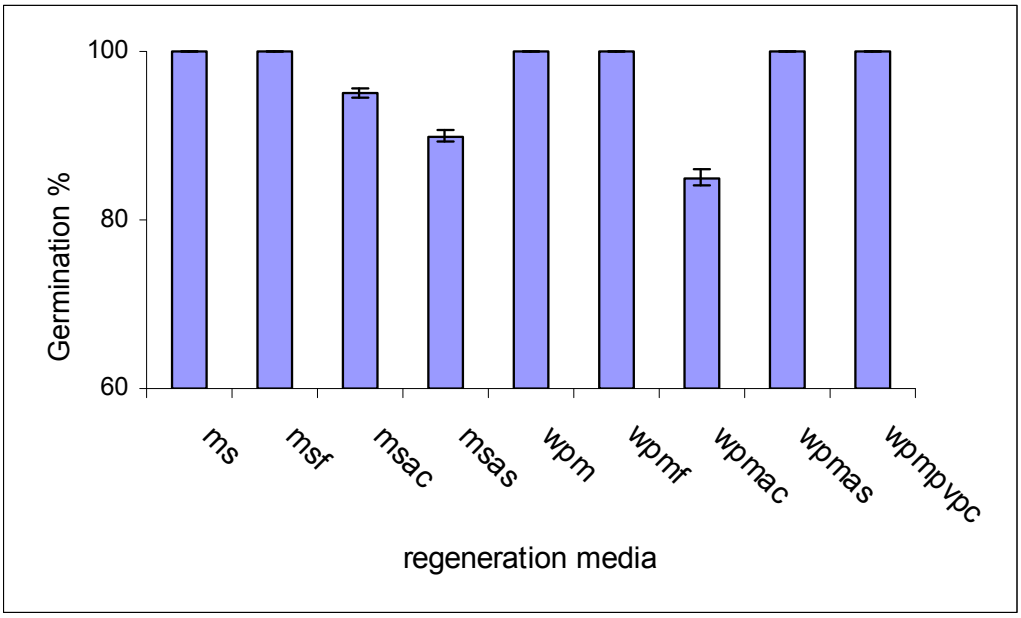


Figure 3.12. The effect of various regeneration media on embryonic axes of freshly harvested seeds of *S. cordatum;* n=20.

msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicides
msac =	1% $Ca(OCl)_2$ + MS with activated charcoal
msas =	1% $Ca(OCl)_2$ + MS with ascorbic acid
msca =	1% $Ca(OCl)_2$ + MS with citric acid
wpm =	1% $Ca(OCl)_2$ + WPM
wpmf =	1% $Ca(OCl)_2$ + WPM with fungicide
wpmac =	1% $Ca(OCl)_2$ + WPM with activated charcoal
wpmas =	1% $Ca(OCl)_2$ + WPM with ascorbic acid
wpmpvpc =	1% $Ca(OCl)_2$ + WPM with PVP + citric acid

3.3.2.2 Effects of medium composition on seedling length, root collar diameter and seedling leaf production

As reported for the sterilisation trials, stunted growth and callusing associated with the kanamycin and fungicide mixture were again evident (Fig. 3.9). Seedlings exposed to kanamycin and fungicide being significantly shorter than those grown on media not incorporating these components (ANOVA, $F_{8.161}$ = 9.42, $P < 0.05$). The lengths of seedlings exposed to all the other treatments were not significantly different (Fig. 3.13). The root collar diameters of plantlets from all treatments, however, varied significantly (ANOVA, $F_{8.161}$ = 9.23, $P < 0.05$). Those of seedlings exposed to a combination of WPM and ascorbic acid (wpmas) had stems that were significantly thicker than the other seedlings

except those exposed to plain MS and to the combination of WPM, PVP and citric acid (Fig. 3.14). The shoots of seedlings exposed to a combination of MS and activated charcoal were significantly thinner than all those of any other treatment (Fig. 3.14). There was a marked difference in the number of leaves produced by seedlings from the different treatments (ANOVA, $F_{8.161} = 37.58$, $P < 0.05$). Those exposed to plain MS and to the combination of WPM, PVP and citric acid had approximately double the number of leaves compared with all the other treatments (Fig. 3.15). This variation in seedling length, root collar diameter and leaf production, is presumed to be an indication of a variation in nutrient availability from the different culture media.

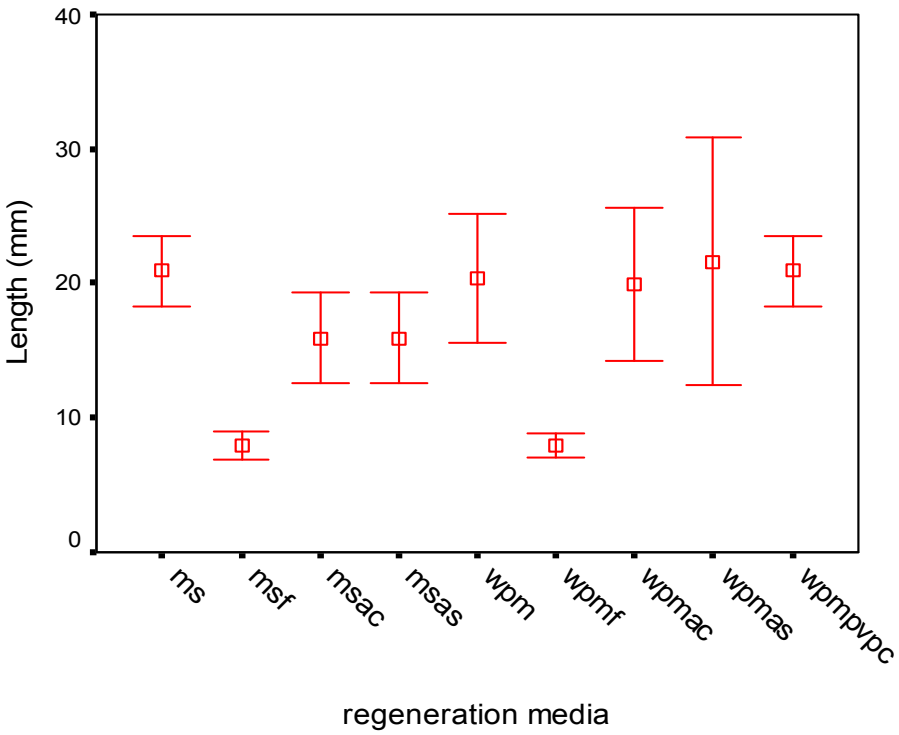


Figure 3.13. The effect of the various media on the length of seedlings developed from embryonic axes of freshly harvested seeds of *S. cordatum;* n=20.

msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicides
msac =	1% $Ca(OCl)_2$ + MS with activated charcoal
msas =	1% $Ca(OCl)_2$ + MS with ascorbic acid
msca =	1% $Ca(OCl)_2$ + MS with citric acid
wpm =	1% $Ca(OCl)_2$ + WPM
wpmf =	1% $Ca(OCl)_2$ + WPM with fungicide
wpmac =	1% $Ca(OCl)_2$ + WPM with activated charcoal
wpmas =	1% $Ca(OCl)_2$ + WPM with ascorbic acid
wpmpvpc =	1% $Ca(OCl)_2$ + WPM with PVP + citric acid

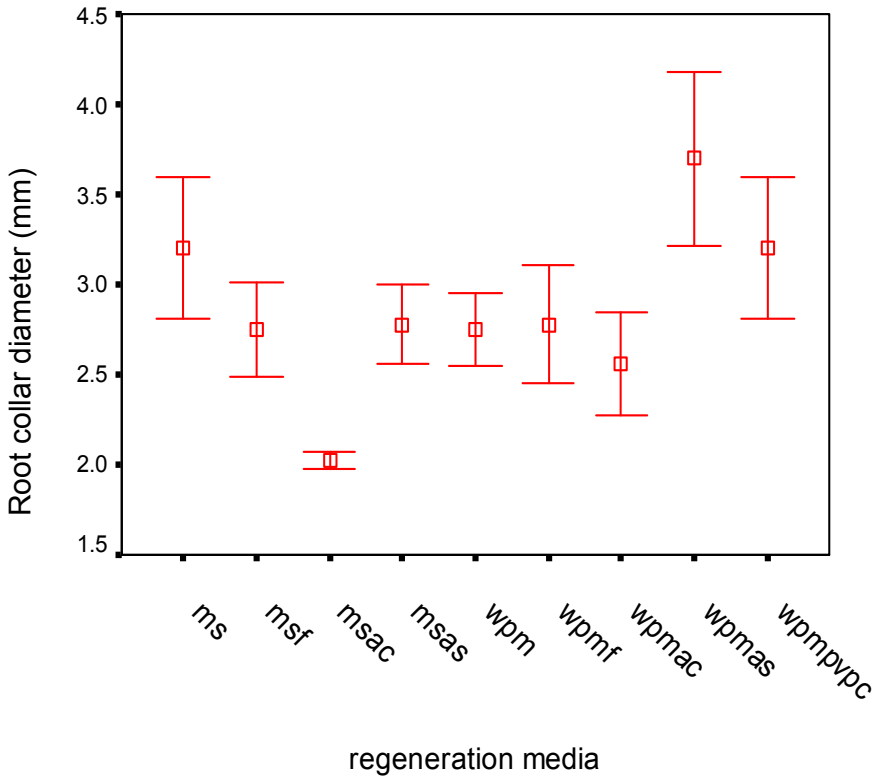


Figure 3.14. The effect of various media on the root collar diameter of seedlings of embryonic axes of freshly harvested seeds of *S. cordatum;* n=20.

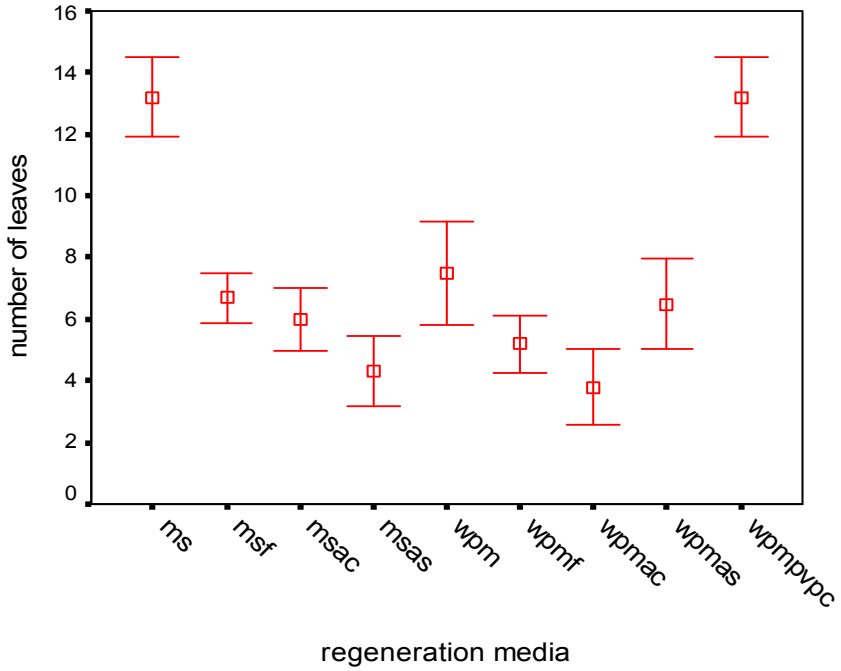


Figure 3.15. The effect of various media on the number of leaves produced by seedlings developed from embryonic axes of freshly harvested seeds of *S. cordatum*; n=20.

msc =	1% $Ca(OCl)_2$ + MS
msfc =	1% $Ca(OCl)_2$ + MS with fungicides
msac =	1% $Ca(OCl)_2$ + MS with activated charcoal
msas =	1% $Ca(OCl)_2$ + MS with ascorbic acid
msca =	1% $Ca(OCl)_2$ + MS with citric acid
wpm =	1% $Ca(OCl)_2$ + WPM
wpmf =	1% $Ca(OCl)_2$ + WPM with fungicide
wpmac =	1% $Ca(OCl)_2$ + WPM with activated charcoal
wpmas =	1% $Ca(OCl)_2$ + WPM with ascorbic acid
wpmpvpc =	1% $Ca(OCl)_2$ + WPM with PVP + citric acid

3.3.2.3 Effects of regeneration media on exudation of phenolics

Although plain MS was among the suitable media in terms of its effect on germination capacity and seedling growth, it (in addition to MS with fungicide, MS with activated charcoal, WPM, WPM with fungicide and WPM with activated charcoal) did not completely prevent the exudation of some phenolics by embryonic axes (Fig. 3.16). However, observable browning associated with exudation of phenolics by the excised embryonic axes was counteracted on MS medium containing ascorbic acid (Figs 3.16 & 3.17) and those based on WPM containing ascorbic acid or a combination of PVP and citric acid (Figs 3.16 & 3.18). The antioxidant activity of ascorbic acid and citric acids could have effectively prevented polymerisation of phenolics (George, 1996; Rozes *et al*., 2003) in cultures of embryonic axes of *S. cordatum*. Additionally, phenolics may have been adsorbed by PVP, which may have prevented their oxidation.

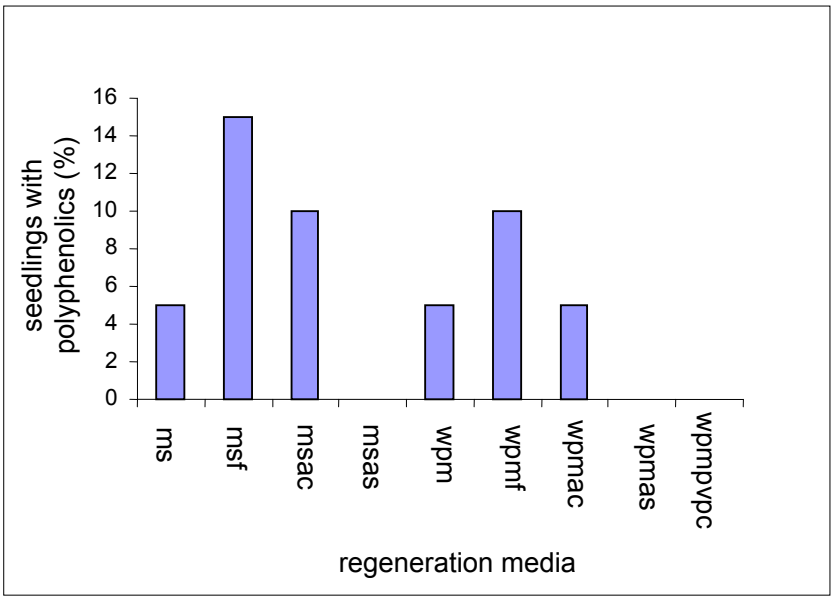


Figure 3.16. Exudation of phenolics in relation to the various media. Embryonic axes were excised from freshly harvested seeds of *S. cordatum;* n=20.

ms =	MS
msf =	MS with fungicide
msac =	MS with activated charcoal
msas =	MS with ascorbic acid
wpm =	WPM
wpmf =	WPM with fungicide
wpmac =	WPM with activated charcoal
wpmas =	WPM with ascorbic acid
wpmpvpc =	WPM with PVP + citric acid

Figure 3.17. Seedlings that were germinated on MS medium containing ascorbic acid

Figure 3.18. Seedlings that were germinated on WPM incorporating PVP and citric acid.

From the results presented above, it was concluded that the most suitable growth medium for embryonic axes of *S. cordatum* excised from freshly harvested seeds is WPM incorporating PVP and citric acid. However, an *in vitro* culture medium is just one step in attaining successful cryopreservation. As reviewed by Wesely-Smith (2002), in order to achieve cryopreservation of embryonic axes of recalcitrant-seeded species, explants need to be dehydrated to a water content that will obviate – or at least minimise – often lethal freezing injury.

3.3.3 Dehydration

As mentioned earlier (Section 3.1), desiccation-sensitive seeds/axes are vulnerable to two types or levels of damage upon dehydration. The first occurs when the tissue slowly loses solution water, and is termed metabolism-linked/-

induced desiccation damage. The consequences of this water loss (as was discussed in Chapter 1) usually result in the loss of viability at water contents below 0.8 g g^{-1} (Pammenter *et al.*, 1993, 1998; Walters *et al.*, 2001). However, recalcitrant material will transiently survive considerably more removal of solution water if dehydration can be rapidly achieved – i.e. by flash drying (Pammenter *et al.*, 2002).

The second type/level of dehydration damage involves the removal of structure-associated water (Pammenter *et al*., 1991; Walters *et al.*, 2001). The water content at which matrix-bound (structure-associated, non-freezable) water would be removed has been termed by Finch-Savage (1992) as the critical moisture (water) content in recalcitrant seeds. Pammenter *et al.* (1993) showed that the water content at the point where non-freezable water would be perturbed is similar for both desiccation-sensitive and -tolerant seeds, and is generally below 0.3 g g^{-1}. Those authors hypothesised that the amount of non-freezable water is not a critical factor in conferring desiccation tolerance or sensitivity, and that desiccation-tolerant seeds withstand the loss of a considerable proportion of this water. In contrast, removal of non-freezable water is lethal to recalcitrant seeds/embryonic axes (Pammenter *et al*., 1991). This kind of damage is termed desiccation damage *sensu stricto* (Pammenter, *et al*., 1998; Walters *et al.*, 2001).

3.3.3.1 Effects of dehydration on water content and germination

Embryonic axes of *S. cordatum* were flash dried for various periods and their germination capacity and water content determined. The germination capacity of the axes was not affected by lowering the water content from 4.5 g g^{-1} to 1.8

g g^{-1} (Fig. 3.19). Below 1.5 g g^{-1}, however, the germination capacity of the axes decreased with decreasing water contents (Fig. 3.19).

The effect on germination by lowering water content to below 1.8 g g^{-1}, which occurred between 45 and 60 min of flash drying, indicates that *S. cordatum* seeds are highly desiccation-sensitive. It is inferred that, at such a relatively high water content, there would have been ongoing metabolism, including respiration. However, under conditions of water stress the metabolism becomes deranged, with one of the consequences being the 'escape' of free radicals/reactive oxygen species (ROS) from respiratory and photosynthetic pathways (as reviewed by Berjak and Pammenter, 2008) leading to metabolism-linked desiccation damage. Important to note however is that axes dried for 75 min achieved a water content of 1.2 g g^{-1} whilst more than 50% of the axes survived.

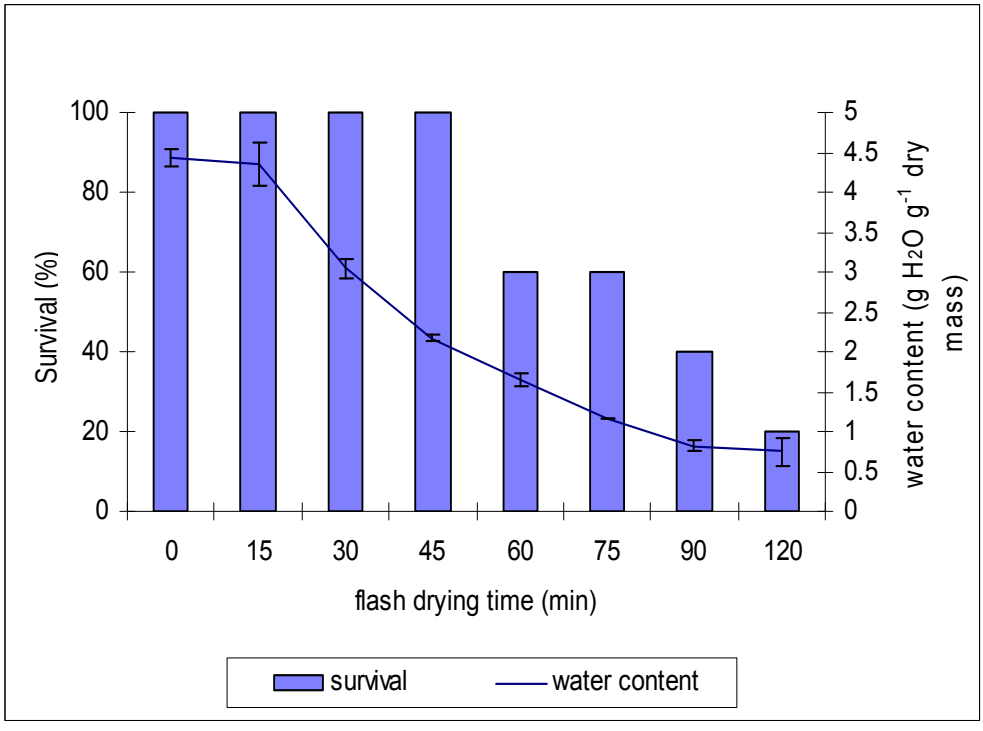


Figure 3.19. The effect of flash drying embryonic axes from recently harvested seeds of *S. cordatum* for various times on survival and water content

3.3.3.2 Effects of dehydration and regeneration media on germination and exudation of phenolics

Having established a regeneration medium that was suitable for growth of non-dehydrated embryonic axes of *S. cordatum* (Section 3.3.2), the next step was to ascertain whether the procedure would be appropriate for dehydrated embryonic axes. The embryonic axes were flash dried for 75 min to achieve a water content of 1.2 g g^{-1} and plated out on a selection of growth media, selected for their ability to inhibit exudation of phenolics.

There was 100% survival of dehydrated embryonic axes regenerated on a combination of McCown's woody plant basal salt mixture (Lloyd and McCown, 1981), 10 g l^{-1} PVP and 75 mg l^{-1} citric acid, while fewer than 60% of axes survived on the other media (Fig. 3.20). The WPM with PVP + citric acid medium was also the only medium that apparently prevented the exudation of phenolics by the axes (Fig. 3.21). Citric acid and other antioxidants have been known to prevent the polymerisation of phenolics by inhibiting their oxidation (George, 1996). The polyamide, PVP, adsorbs phenols through hydrogen bonding and prevents their oxidation and polymerisation. Also, PVP combines with oxidised and polymerised phenolics and prevents further oxidation by phenolase enzymes. The use of PVP was also successful in binding polyphenolics during desiccation of (polyphenolics-rich) *Myrothamnus flabellifolius* (Koonjul *et al.*, 1999). Medium containing PVP was, therefore, the choice for the regeneration of excised axes of *S. cordatum* after they had been dehydrated.

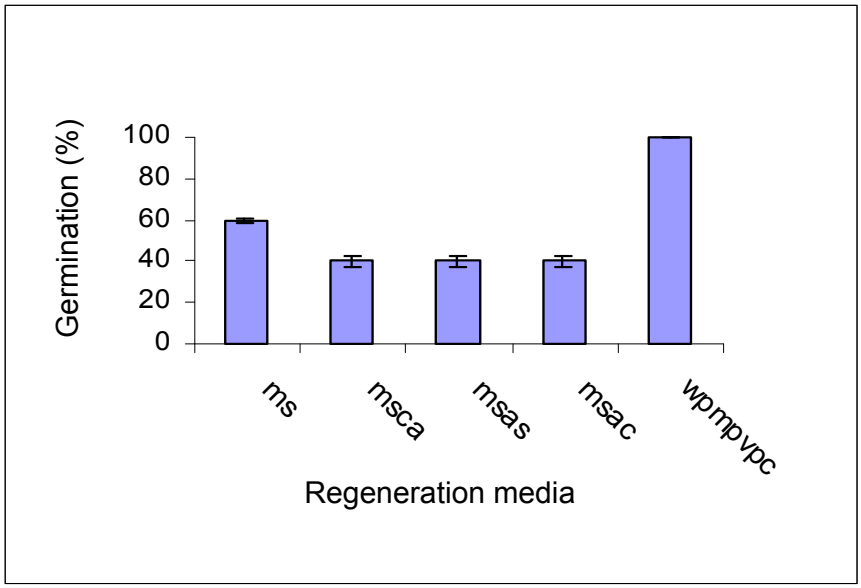


Figure 3.20. The effect of various growth media on regeneration (indicated by germination) of embryonic axes after flash drying to 1.2. g g^{-1}. Axes were excised from newly-harvested seeds of *S. cordatum*.

ms =	MS
msca =	MS with citric acid
msas =	MS with ascorbic acid
msac =	MS with activated charcoal
wpmpvpc =	WPM with PVP + citric acid

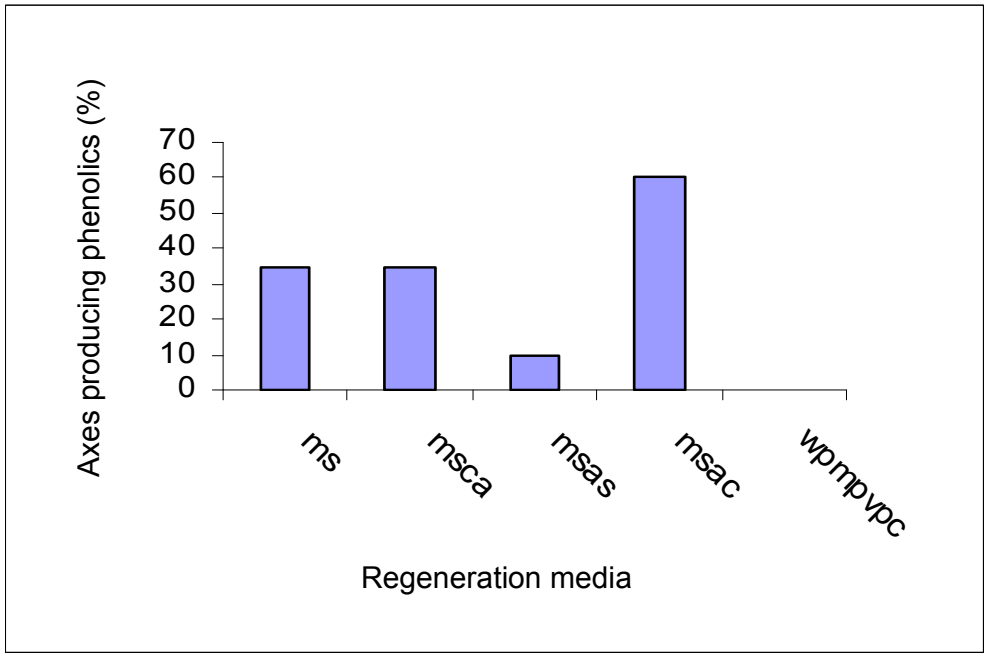


Figure 3.21. The percentage of flash dried axes (water content 1.2 g g^{-1}) showing exudation of phenolics when cultured on various growth media. Embryonic axes were excised from recently harvested seeds of *S. cordatum*.

ms =	MS
msca =	MS with citric acid
msas =	MS with ascorbic acid
msac =	MS with activated charcoal
wpmpvpc =	WPM with PVP + citric acid

3.3.4 Cryoprotection

A cryoprotectant is any additive that is provided to cells prior to cryopreservation in order to achieve a higher survival after thawing (Fuller, 2004). Some cryoprotectants, particularly the non-penetrating types, offer the advantage of dehydrating cells, therefore increasing intracellular viscosity, while penetrating cryoprotectants counteract increases in electrolyte concentration to damaging levels (Farrant, 1965). In these ways, cryoprotectants reduce possible damage which could occur during slow or rapid cooling.

The main damaging event associated with rapid cooling could occur when intracellular ice crystals propagate and cause physical membrane damage, gas bubble formation and organelle disruption (Fuller, 2004). Rapid cooling, however, has also been found to be successful, when it minimises the time an explant spends within the temperature range in which lethal ice crystallization occurs (Wesley-Smith *et al*., 1992). With few exceptions (Varghese *et al*., 2009; Kistnasamy *et al*., 2011), very rapid cooling has been found to be superior to slower cooling in our laboratory (Wesley-Smith, 2002).

3.3.4.1 Effects of low molecular weight cryoprotectants followed by flash drying on water content, germination of, and exudation of phenolics by embryonic axes of S. cordatum

Low molecular weight, penetrating, cryoprotectants have two effects on cells. First, they reduce the rate of diffusion of water out of the cell during slow

cooling, thereby counteracting ice crystal damage. Secondly, they provide colligative support by reducing the amount of cell volume change thereby avoiding the excessive accumulation of toxic electrolytes (Farrant, 1965). Basically, they reduce the concentration of electrolytes at any temperature during the freezing process (Farrant 1965; Taylor *et al.*, 1974).

All the low molecular weight cryoprotectants tested in this study significantly reduced axis water contents even before they were further dehydrated in a flash dryer, with the combination of sucrose and DMSO facilitating the greatest degree of dehydration and DMSO alone having the least dehydrating effect (Fig. 3.22). A similar dehydrating effect of cryoprotectants was described for freshly excised embryonic axes of *Zizania texana* (Walters *et al*., 2002).

However, the combination of cryoprotection and dehydration for 75 min significantly reduced the viability of the axes (Fig. 3.23). There was a reduction in viability by drying alone. Dehydrating for 75 min was observed to achieve low water content below 1.5 g g^{-1} whilst maintaining a survival of over 50% of the axes tested (Fig. 3.19). The reduction in viability after a combination of cryoprotection and dehydration for 75 min was possibly a result more of dehydration than of cryoprotection *per se*. The decreases in viability coincided with a marked increase in the numbers of axes exhibiting polyphenolic exudation (Fig. 3.24). Berjak and Pammenter (2004b), observed that cryoprotectants can have a deleterious effect on embryonic axes of tropical recalcitrant-seeded species, and this effect was also observed for recently harvested axes of *S. cordatum*.

Embryonic axes that were exposed to glycerol and then dehydrated for 60 min maintained high viability compared to those treated with the other cryoprotectants tested in this study (Fig. 3.23). Embryonic axes exposed to DMSO with 60 min dehydration and glycerol with 60 min dehydration however had water contents above the established critical water content of 1.2 g g^{-1} (Fig. 3.22). The higher survival of embryonic axes exposed to glycerol with 60 min dehydration could be due to the low toxicity of glycerol on exposure to living cells (Fuller, 2004).

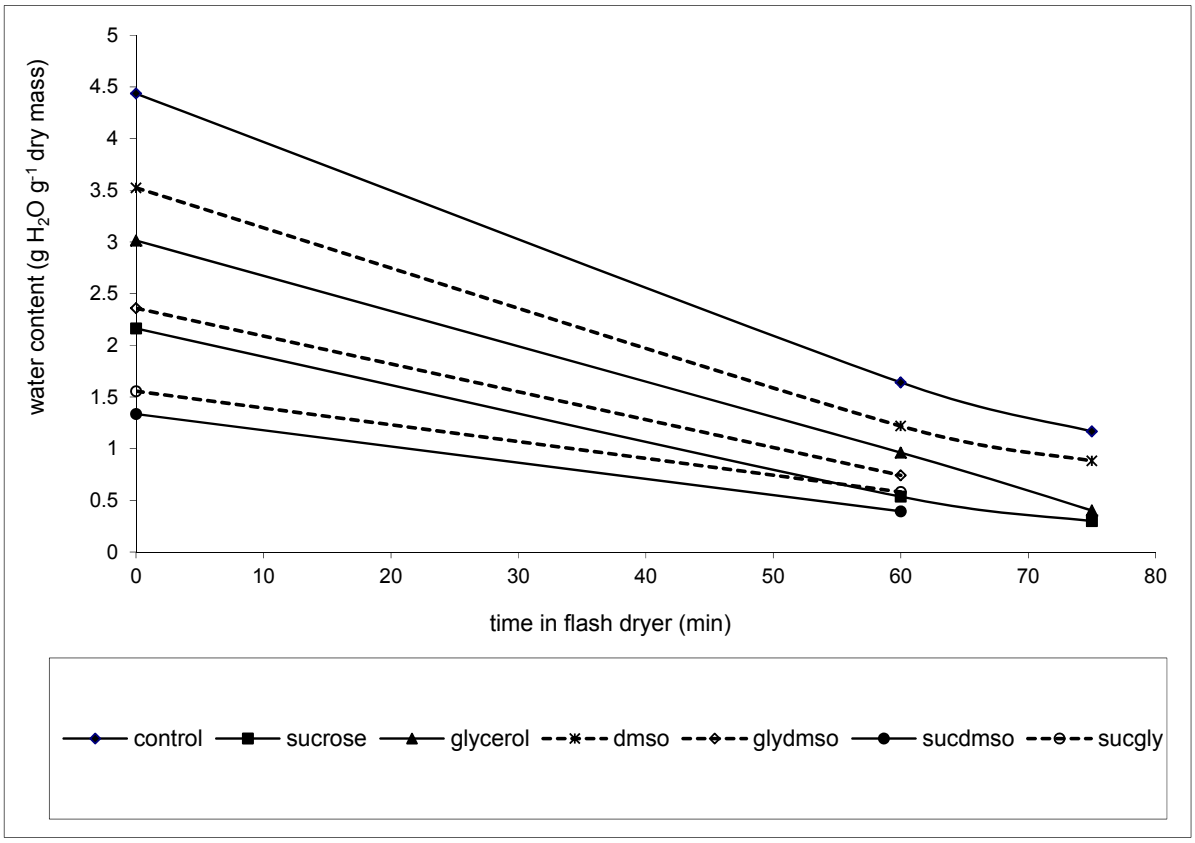


Figure 3.22. The effect of a combination of dehydration upto 75 min after exposure to low molecular weight cryoprotectants on the water contents of embryonic axes of recently harvested seeds of *S. cordatum*; n=20. glydmso = glycerol with DMSO; sucdmso = sucrose with DMSO; sucgly = sucrose with glycerol

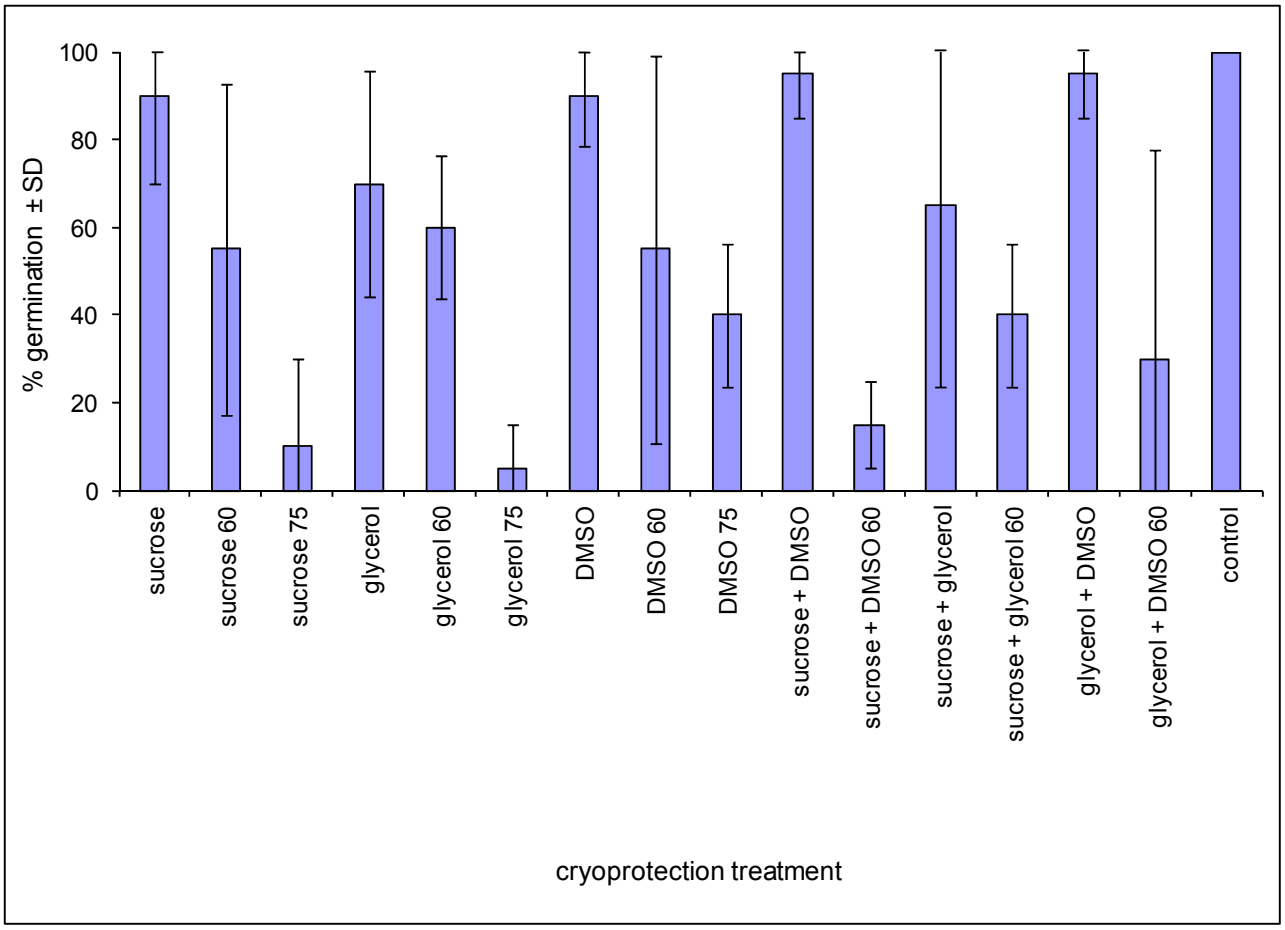


Figure 3.23. The effect of a combination of dehydration for 60 min and 75 min following exposure to low molecular weight cryoprotectants on the germination of embryonic axes excised from seeds of *S. cordatum*; n=20.

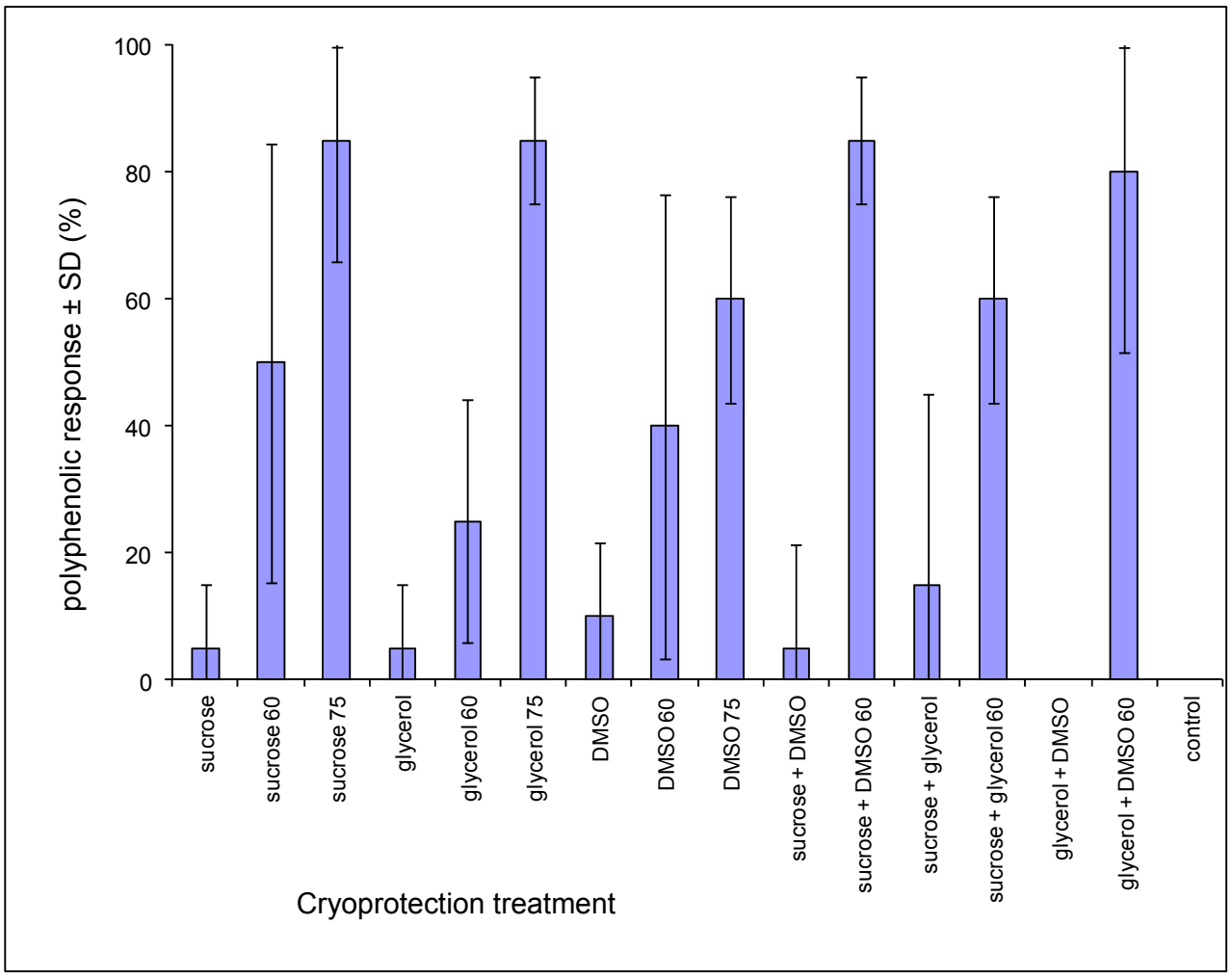


Figure 3.24. The effect of a combination of dehydration for 75 min after exposure to low molecular weight cryoprotectants on the exudation of phenolics by embryonic axes of recently harvested seeds of *S. cordatum*. n=20

3.3.4.2 Effects of dehydration and high molecular weight cryoprotectants on water content, germination and phenolic response of embryonic axes of S. cordatum

Both the high molecular weight cryoprotectants and their combinations with DMSO had a dehydrating effect on embryonic axes (Fig. 3.25). Exposure to PVP and DMSO and further dehydration for 60 to 75 min had an increasingly

deleterious effect on the viability (Fig. 3.26) but a decreasing effect on exudation of phenolics by embryonic axes (Fig. 3.27). The embryonic axes that were exposed to dextran and further dehydrated for 75 min had a water content of about 0.3 g g^{-1} (Fig. 3.25) but viability was still above 50% (Fig. 3.26). Exposure to 5% w/v dextran solution as a polymeric cryoprotecting agent has been recorded as having the effect of reducing toxicity of permeating cryoprotectants such as DMSO when used in combination (Fuller, 2004). This protective effect of dextran may also have contributed to the embryonic axes exuding less phenolics which led to higher viability at the reduced water content. In view of the response of this species to dextran, further investigations on the use of this cryoprotectant for preparation of axes for cryopreservation needs to be done.

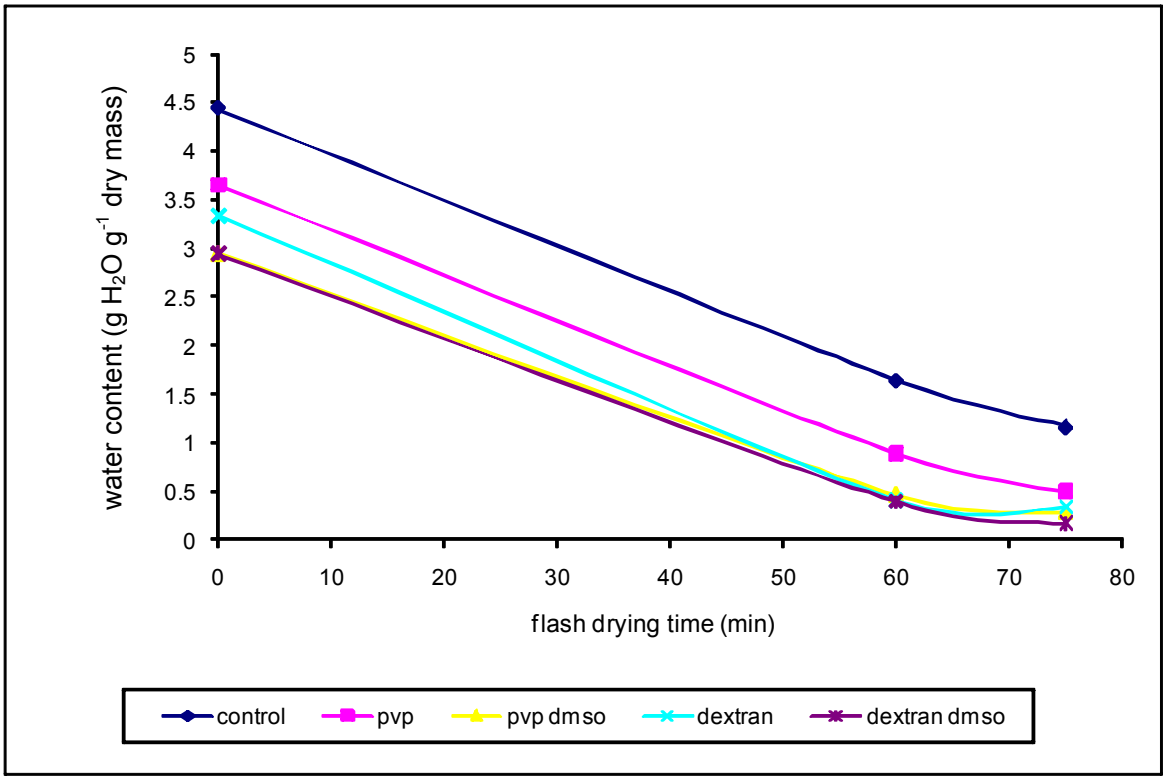


Figure 3.25. The effect of a combination of dehydration upto 75 min after exposure to high molecular weight cryoprotectants on the water contents of embryonic axes of recently harvested seeds of *S. cordatum*; n=20.

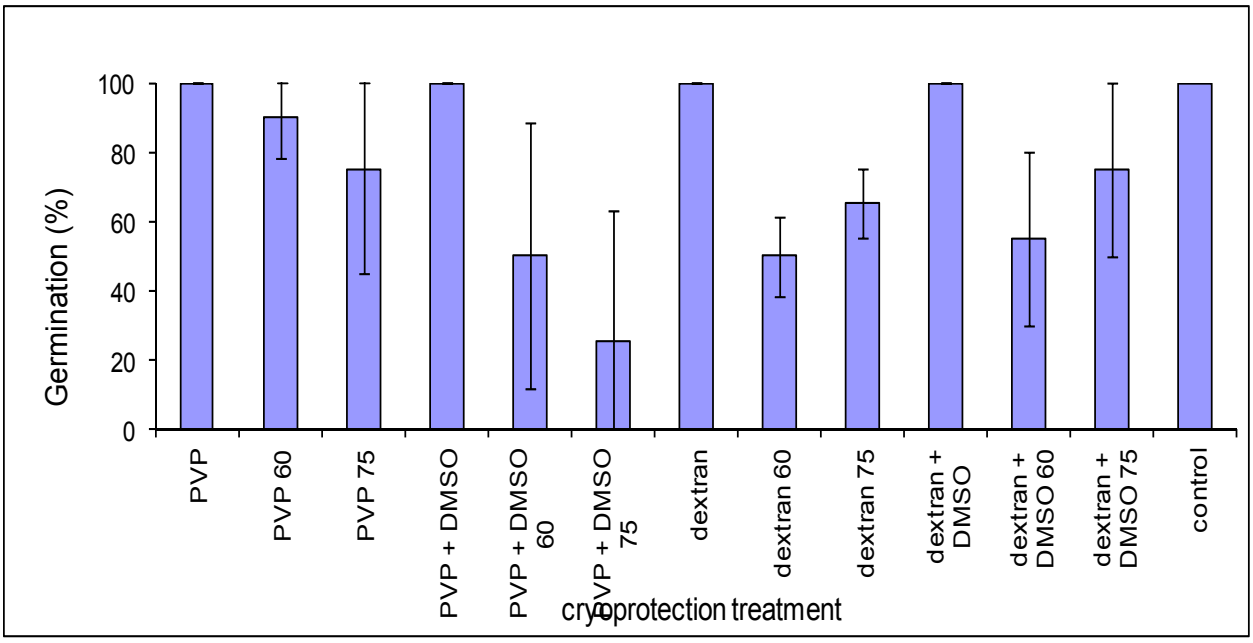


Figure 3.26. The effect of a combination of dehydration for 60 min and 75 min following exposure to high molecular weight cryoprotectants on the germination of embryonic axes of recently harvested seeds of *S. cordatum;* n=20.

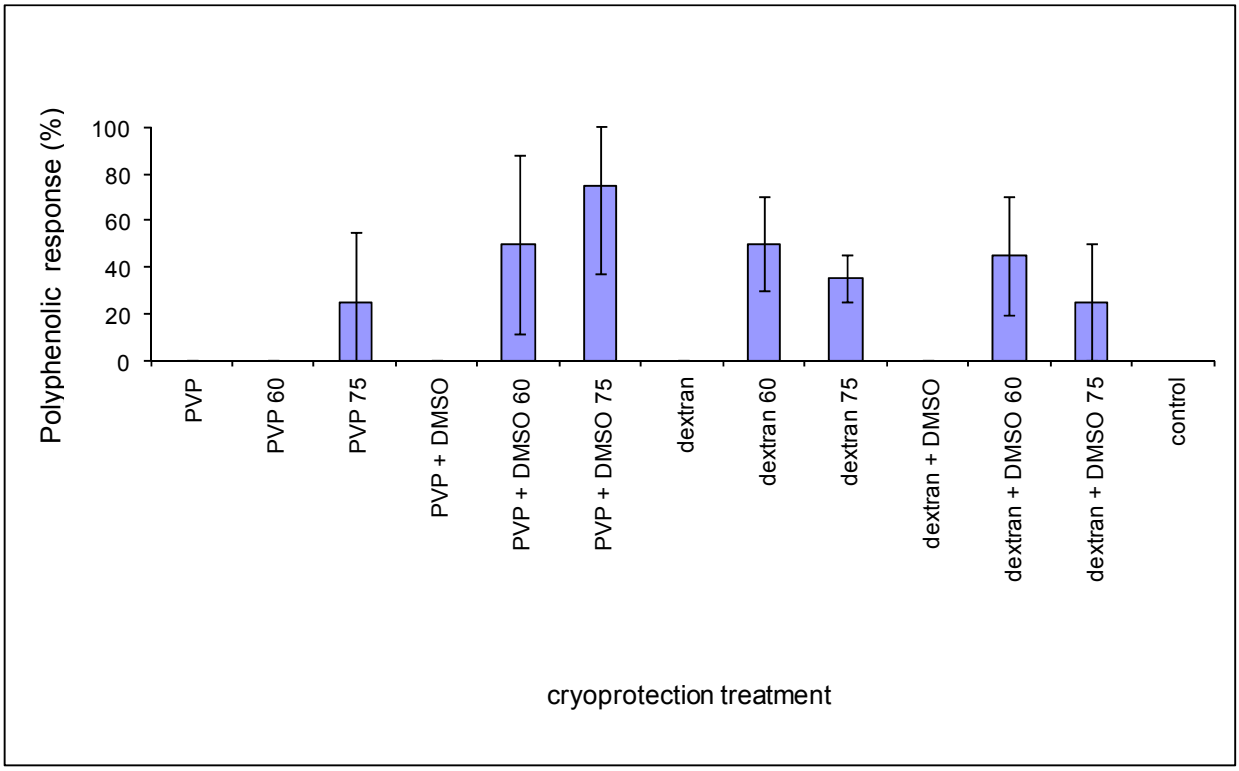


Figure 3.27. The effect of a combination of dehydration for 60 min and 75 min following exposure to high molecular weight cryoprotectants on the exudation of phenolics by embryonic axes from recently harvested seeds of *S. cordatum*; n=20.

3.3.5 Cryopreservation

This critical water content of embryonic axes of *S. cordatum* is significantly above 0.15 g g^{-1} (see section 3.3.3.1) which has been estimated to be the water content that is suitable for cryopreservation via slow cooling (Wesley-Smith *et al*., 1992). Axes were therefore rapidly dehydrated for 60-75 min, which brought the shedding water content from *ca.* 4.44 g g^{-1} down to between 0.17 – 1.64 g g^{-1}, depending on the cryoprotectant used. At these water contents the axes were cooled at rapid rates by plunging them into either liquid nitrogen at -196 ºC or nitrogen slush at -210 ºC. Exposure of axes to liquid nitrogen resulted in no survival, regardless of the cooling rate used (Table 3.1).

Table 3.1. Survival of *S. cordatum* embryonic axes after cryoprotection, dehydration, and cryoprerservation. Axes were cryopreserved by plunging into nitrogen slush; n=20; nt= not tested.

Cryoprotectant	**dehydration period**	**average water content (g/g)**	**% survival**	
			dehydrated only	**dehydrated + cryopreserved**
None	0	4.44	100	0
	60	1.64	38	0
	75	1.17	22	0
Sucrose	0	2.16	90	0
	60 min	0.54	55	0
	75 min	0.30	10	0

Glycerol	0	3.01	70	0
	60 min	0.96	60	0
	75 min	0.40	5	0
DMSO	0	3.52	90	0
	60 min	1.22	55	0
	75 min	0.88	40	0
sucrose+ DMSO	0	1.34	95	0
	60 min	0.39	15	0
	75 min	nt	nt	nt
sucrose+glycerol	0	1.56	65	0
	60 min	0.58	40	0
	75 min	nt	nt	0
glyverol+DMSO	0	2.36	95	0
	60 min	0.74	30	0
	75 min	nt	nt	nt
PVP	0	3.65	100	0
	60 min	0.89	90	0
	75 min	0.50	75	0
PVP+DMSO	0	2.95	100	0
	60 min	0.46	50	0
	75 min	0.28	25	0
Dextran	0	3.33	100	0
	60 min	0.41	50	0
	75 min	0.34	65	0
Dextran+DMSO	0	2.94	100	0
	60 min	0.40	55	0
	75 min	0.17	75	0

By using two cooling methods (plunging into liquid nitrogen or nitrogen slush) and 10 cryoprotective treatments and two dehydration periods each, this study tested 40 treatments for the cryopreservation of embryonic axes of *S. cordatum* but none of the treatments resulted in any post-thaw survival. The apparent difficulty in achieving successful cryopreservation of embryonic axes has been reviewed by Berjak *et al.* (1999a; 1999b; 2011b), and may be due to the relatively large size and highly heterogeneous morphology and physiology of recalcitrant-seed embryonic axes. The wounding associated with excision of embryonic axes from cotyledonary tissue has particularly been shown to lead to the production of lethal ROS in the embryonic axes (e.g. Goveia *et al*., 2004), and there have been several studies on how to quench the ROS, with the use of cathodic water being particularly effective for embryonic axes of tropical recalcitrant seeds (Berjak *et al.*, 2011a). Treatments to counteract the effects of wounding and/ or ROS, which might contribute to the lack of survival after cryopreservation, should be explored for the axes of *S. cordatum*. It is also recommended that other kinds of explants, other than embryonic axes, should be investigated for use in the cryoprervation of *S. cordatum* germplasm. In this regard, recent successful cryopreservation of the shoot-tips of *T. emetica* (Varghese *et al.,* 2009) present an example of the use of vegetative explants for the cryopreservation of the germplasm of tropical recalcitrant-seeded tree species. Thus, the work done in this study on cryopreservation of *S. cordatum* may be considered as a necessary first step towards the long-term conservation of the germplasm of this species, but the objective remains to be achieved.

3.3.6 Transmission electron microscopy[#]

Ultrastructural examination can reveal much in the context of the effects of each step undertaken when establishing cryopreservation protocols for axes from recalcitrant seeds. However, the considerable intracellular content of phenolic compounds in zygotic embryos of *S. cordatum* presented a problem in the ultrastructural study of shoot tips using transmission electron microscopy.

The polymerised phenolics reacted with osmium tetroxide and resulted in substantial electron-dense deposits that obscured visualisation of ultrastructural features. According to Minorsky (2001), plants have vacuoles that are dedicated to accumulation of phenolics. Additionally, these vacuoles, also have an acidic interior and an affinity for basic dyes which are precipitated by the phenols. The vacuolar contents also tend to be more viscous than their surrounding cytoplasm (Minorsky, 2001). Further, major problem, however, is that phenolic compounds tend to leach out of the vacuoles during fixation for electron microscopy (Fig. 3.28). These leached compounds auto-fix the surrounding cytoplasm and lead to an obscured visibility of the ultrastructure (Fig. 3.29). Caffeine has been reported to overcome this problem by precipitating epicatechin-like phenolics, thus maintaining the phenolic compounds within the vacuolar compartments (Mueller and Greenwood, 1978).

[#] The results presented in this Section were published in the Proceedings for the Microscopy Society of southern Africa, volume 34, 2004. pg 65.

In the case of *S. cordatum* embryonic axes, the addition of caffeine was inadequate to improve resolution of the ultrastructure (Fig. 3.29 and Fig. 3.30).

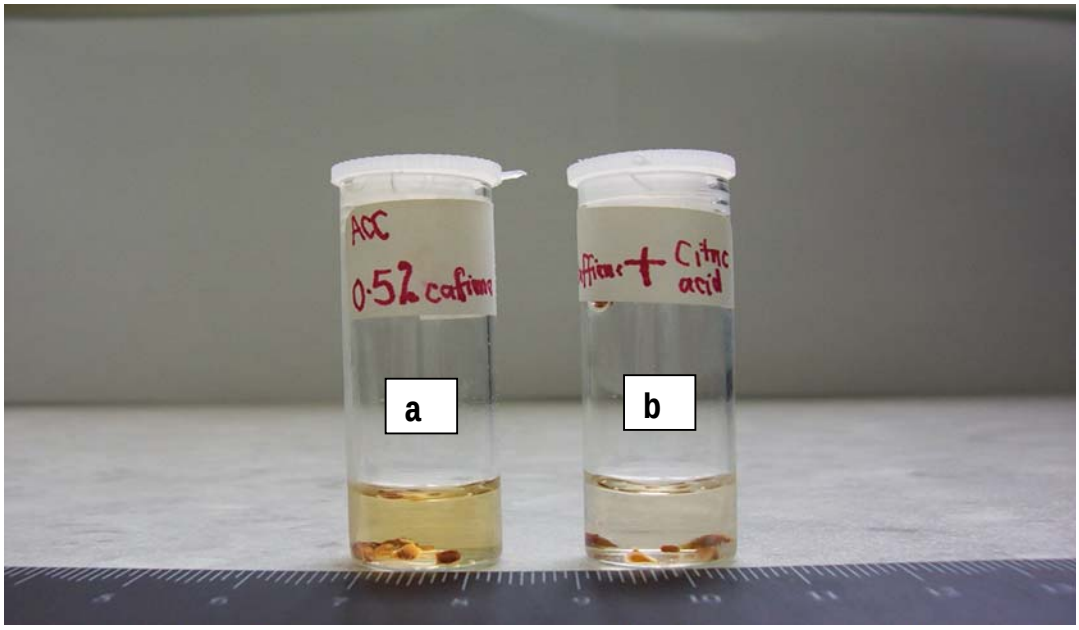


Figure 3.28. Embryonic axes in glutaraldehyde incorporating caffeine, (a) without citric acid and (b) with 75 mg l^{-1} citric acid.

Citric acid is an antioxidant which has the ability to inhibit the polymerisation of phenolics (George, 1996). An investigation was, therefore, set up to test the effectiveness of citric acid in solving the problems. Axes of *S. cordatum* were thus exposed to citric acid before they were fixed in preparation for electron microscopy. In brief, the results showed that when treated with 75 mg l^{-1} citric acid, cells had no visible polyphenolic precipitates and the apparently intact organelles were clearly visible, so paving the way for electron microscopical examination of this – and perhaps any other – plant tissue containing substantial amounts of phenolic substances.

The results from this experiment were presented in the paper below (see below) at the 2004 Conference of the *Microscopy Society of Southern Africa – Proceedings* (**34:** 62).

CITRIC ACID AS A PRE-FIXATION TREATMENT OF PHENOL-LADEN SHOOT AXES OF *Syzygium cordatum*

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Recalcitrant seeds are shed in the hydrated state, cannot withstand dehydration and are often sensitive to low temperature. *Syzygium cordatum* is a highly utilised indigenous species with recalcitrant seeds that contain large deposits of phenols. The increasing scarcity of this species demands the urgent conservation of its genetic resources. Seed storage provides the best option for germplasm conservation, not only because the seeds occupy little space and require only periodic attention, but also because each seed has the potential to develop into a genetically unique plant, thus maintaining genetic diversity within the species. Conventional long-term seed storage techniques involve limiting metabolic processes by storing seeds at low water content and low temperature. This storage practice is not suitable for recalcitrant seeds because they are sensitive to low water content and temperature. Many studies on the storage of recalcitrant seeds suggest that cryopreservation is the best method for conserving their genetic resources.

Ultrastructural studies are an important tool for determining the effects of each step undertaken when establishing successful cryopreservation protocols for recalcitrant seeds. However, high levels of phenols in zygotic embryos of *Syzygium cordatum* presented a problem in the ultrastructural study of shoot tips using transmission electron microscopy. The polymerised phenolics reacted with osmium tetroxide and resulted in electron dense deposits that obscured the visibility of cell ultrastructural features. Caffeine was added to avoid polymerisation and leaching of phenolics outside the vacuole compartment and to precipitate epicatechin-like phenolics[2], but this was not enough to improve visibility of the ultrastructure. Citric acid and other antioxidants have been reported to be effective in preventing the polymerisation of phenolics by inhibiting the oxidation of phenols[1]. An experiment was therefore set up to investigate the effect of exposing shoot tips to 75 mg l^{-1} citric acid for 10 min before fixing them in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer containing 0.5% (w/v) caffeine. These shoot tips were then post-fixed in 0.5% (w/v) osmium tetroxide, dehydrated in a series of increasing concentrations of acetone and embedded in Spurr's resin. Thereafter they were prepared for TEM using standard sectioning and contrasting procedures.

In the absence of citric acid, large electron dense polyphenol-caffeine precipitates were observed mainly inside the vacuoles, closely associated with the periphery of the tonoplast (Fig.3.29). Less dense, small precipitates were located between the plasmalemma and the cell wall, and organelles were not clearly visible (Fig. 3.30) thus the effects of various treatments on organelles and the entire cell ultrastructure could not be conclusively determined. Citric acid treated cells, however, had no visible phenolic precipitates and had apparently intact organelles that were clearly visible (Fig. 3.31). The absence of visible polyphenol-caffeine precipitates after treating shoots of *Syzygium*

cordatum with citric acid will enable the ultrastructural study of this species and those of other phenol-laden plant material

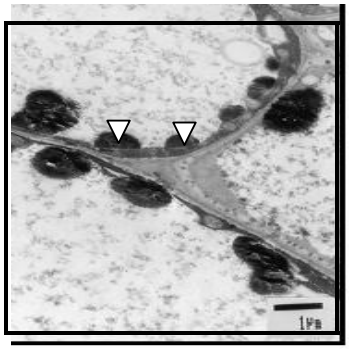

Fig. 3.29. Cells that were not treated with citric acid showing large precipitates of phenolics (arrows).

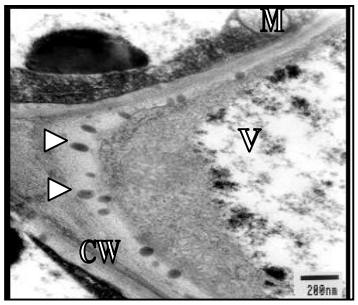

Fig. 3.30. Cells that were not treated with citric acid showing small precipitates of phenolics (arrows).

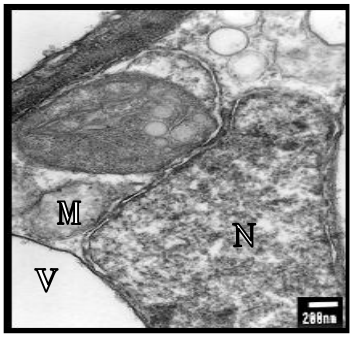

Fig. 3.31. Cells that were treated with citric acid.

3.4 Conclusion: Long-term storage of *Syzygium cordatum*

This study optimised some of the steps which should facilitate cryopreservation of *S. cordatum* embryonic axes. The steps were: surface-sterilisation by exposure to 1% (v/v) $Ca(OCl)_2$ for 5 min; cryoprotection in a 1:1 mixture of 5% dextran and 5% DMSO for 1 h followed by cryoprotection in 10% dextran and 10% DMSO for another hour, then dehydration in a flash dryer for 75 min and regeneration in agitated liquid medium containing McCown's woody plant basal salt mixture (Lloyd and McCown, 1981), 10 g l^{-1} polyvinylpyrrolidone (PVP) and 75 mg l^{-1} citric acid.

The tested procedures for cooling towards cryopreservation did not result in any post-thaw survival. In this respect, the study has contributed in establishing those parameters that do not facilitate successful freezing of the embryonic axes of this species. A number of investigations and reviews (e.g. Berjak *et al*., 1999a; 1999b; Sershen *et al*., 2010; Pammenter *et al*., 2011) have highlighted the challenges that must be overcome, almost on a species-basis, to achieve successful cryopreservation of zygotic axes. Additionally, where there have been protracted difficulties to the successful cryopreservation of embryonic axes due mainly to the physiology and anatomy of the axes, there have been studies aimed at establishing protocols for the cryopreservation of explants alternative to zygotic axes, e.g. for *Theobroma cacao* and *Barringtonia racemosa* (Naidoo, 2008); *Trichilia emetic*a, *T. dregeana* and *Protorhus*

longifolia (Naidoo *et al*., 2011) and *Ekebergia capensis* (Hajari *et al*., 2011). Recent successful cryopreservation of shoot tips of *Syzygium francissi* by the encapsulation-dehydration method (Shatnawi *et al.*, 2004), may indicate the possibility of successfully using an alternative approach to the cryopreservation of *Syzygium* spp, via vegetative explants.

CHAPTER 4. CONCLUDING COMMENTS

Recalcitrant seeds are shed in the hydrated state, and are sensitive to dehydration, especially when water is lost slowly, and often also to low temperature. *Syzygium cordatum* is a highly utilised indigenous species producing recalcitrant seeds that contain large deposits of phenolic compounds. As for any heavily-utilised species, the increasing scarcity demands urgent conservation of the available genetic resources. Seed storage provides the most efficient means for germplasm conservation, not only because the seeds occupy relatively little space and require only periodic attention, but also because each seed has the potential to develop into a genetically unique plant, thus maintaining genetic diversity within the species. Seeds are conventionally stored in the long-term at low water contents and low temperatures. However, this approach is not suitable for recalcitrant seeds given their inherent sensitivity to those conditions. Many studies on the storage of recalcitrant seeds suggest that cryopreservation is the best method for conserving their genetic resources.

This research investigated the post-harvest responses of *S. cordatum* seeds, aiming to develop protocols for short-to-medium term storage for whole seeds, in order to extend their ‘shelf-life’, which would allow viability retention from the

time of harvest to the time of planting. The extension of this 'shelf life', however, is not sufficient for long-term germplasm conservation. For this reason, this research also aimed to develop cryopreservation protocols for the zygotic germplasm of the *S. cordatum*.

It was established that seeds stored in sealed plastic bags (non-saturated atmospheric conditions) at 16 ºC would retain nearly full germinability for up to 10 weeks, with storage at the cooler 6 ºC or the warmer 25 ºC resulting in full viability loss within this period. For cryopreservation, it was necessary to establish appropriate protocols for *in vitro* growth, dehydration, and cooling. The most suitable *in vitro* growth protocol for the embryonic axes involved surface-sterilisation with 1% (v/v) $Ca(OCl)_2$ for 5 min, and regeneration in an agitated liquid medium containing McCown's woody plant basal salt mixture (Lloyd and McCown, 1981), 10 g l^{-1} polyvinylpyrrolidone (PVP) and 75 mg l^{-1} citric acid. Embryonic axes could be dehydrated to water contents in the region of 0.3 g g^{-1} while maintaining 50% viability, provided they were first cryoprotected with a 5% solution of dextran and DMSO for 1 h, followed by exposure to a 10% solution of these cryoprotectants for another hour, before being flash dried for 75 minutes.

No post-thaw survival was obtained following exposure to liquid nitrogen using the cooling techniques tested, and therefore there are avenues for further

study, including investigating both a different array of freezing techniques, and investigating the physiological and ultrastructural basis for the responses obtained. Towards the latter, this study established that exposing shoot tips to 75mg l^{-1} citric acid for 10 min before fixation (in preparation for transmission electron microscopy) prevents the polymerisation of phenolics within the cells. Such polymerisation renders most of the cell impenetrable to electrons, and makes transmission electron microscopy not feasible. Thus, the establishment that citric acid overcomes the polymerisation problem represents a major breakthrough achieved in this study.

This study also recommends that other types of explants, alternative to zygotic axes, be investigated for the explants approach to the cryopreservation of the germplasm of *S. cordatum*.

Having established a tangible, applicable method for achieving a shelf-life of at least 10 weeks for *S. cordatum* seeds, and having established several parameters that would contribute to the cryopreservation of the zygotic axes of the species, this study lends itself to contributing towards achieving the targets set by the Global Strategy for Plant Conservation for *ex situ* conservation of an endangered species.

REFERENCES

Abdel-Mallek, A. Y., Hemida, S. K. and Bagy, M. M. K. (1995). Studies on fungi associated with tomato fruits and effectiveness of some commercial fungicides against three pathogens. *Mycopathologia* **130:** 109-116.

Ajayi, S.A., Berjak, P., Kioko, J.I., Dulloo, M.E. and Vodouhe, R.S. (2006) Observations on *in vitro* behaviour of the zygotic axes of fluted pumpkin. *African Journal of Biotechnology* **5**, 1397-1401.

Allaby, M. (1998). Root-shoot ratio. *A Dictionary of Plant Science*. Oxford University Press, Oxford, United Kingdom. Available from http://www.encyclopedia.com [Accessed February 23, 2011].

Anguelova-Merhar, V.S., Calistru, C. and Berjak, P. (2003). A study of some biochemical and histopathological responses of wet-stored recalcitrant seeds of *Avicennia marina* infected by *Fusarium moniliforme*. **92:** 401-408.

Baiyeri, K.P. and Mbah, B.N. (2006). Surface sterilization and duration of seed storage influenced emergence and seedling quality of African breadfruit (*Treculia africana* Decne). *African Journal of Biotechnology* **5:** 1393-1396.

Baron, F.J. and Fowler, C.W. (1964). Sugar pine seedlings, a reflection of seed handling? *Tree Planters' Notes* **66**: 22-25.

Bartels, P. and Kotze, A. (2006). Wildlife biomaterial banking in Africa for now and the future. *Journal of Environmental Monitoring* **8:** 779-781.

Benson E.E. (1995) Cryopreservation of shoot-tips and meristems. In: *Methods in Molecular Biology Vol XX: Cryopreservation and Freeze Drying Protocols.* (Eds.) M. McClennan and J. Day). Humana Press Inc. Totowa, NJ. pp 121-132.

Benson, E.E. (2008). Cryopreservation of Phytodiversity: A Critical Appraisal of Theory & Practice. *Critical Reviews in Plant Sciences* **27**: 141-219.

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

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

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

Berjak, P. and Pammenter, N. W. (2003). Biology of recalcitrant seeds. In Murphy, D. J and Murray, B. G., eds. *Encyclopedia of Applied Plant Science.* Elsevier Ltd, London, United Kingdom. pp. 56-62.

Berjak, P. and Pammenter, N. W. (2004a). Recalcitrant seeds. In Benech-Arnold, R.L. and Sánchez, R.A., eds. *Seed Physiology: Applications to Agriculture.* Haworth Press, New Jersey, United Stated of America. pp. 305-345.

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

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

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

Berjak, P., Vertucci, C. W. and Pammenter, N. W. (1992). Homoiohydrous (recalcitrant) seeds: developmental status, desiccation sensitivity and the state of water in axes of *Landolphia kirkii* Dyer. *Planta* **186:** 249-261.

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

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

Berjak, P., Kioko, J.I., Walker, M., Mycock, D.J., Wesley-Smith, J., Watt, P. and Pammenter, N.W. (1999b). Cryopreservation- an elusive goal? In:

Marzalina, M., Khoo, K.C., Jayanthi, N., Tsan, F.Y. and Krishanpillay, B. (Eds.) *Recalcitrant Seeds*. Forestry Research Institute of Malaysia. pp 96 – 109.

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

Berjak, P., Bartels, P., Benson, E.E., Harding, K., Mycock, D.J., Pammenter, N.W., Sershen, and Wesley-Smith, J. (2011a). Cryoconservation of South African plant genetic diversity. *In Vitro Cellular & Developmental Biology – Plant*, **47:** 65-81.

Berjak, P., Sershen, Varghese, B. and Pammenter, N.W. (2011b). Cathodic amelioration of oxidative stress accompanying procedures necessary for cryopreservation of embryonic axes of recalcitrant-seeded species. *Seed Science Research* **21:** 187-203.

Bernier, P.Y., Lamhamedi. M.S. and Simpson, D.G. (1995). Shoot:root ratio is of limited use in evaluating the quality of container conifer stock. *Tree Planters' Notes* **46:** 102-106.

Bladé, C. and Vallejo, V.R. (2008). Seed mass effects on performance of *Pinus halepensis* Mill. seedlings sown after fire. *Forest Ecology and Management* **255**: 2362–2372

Bonner, F. T. (1990). Storage of seeds: potential and limitations for germplasm conservation. *Forest Ecology and Management* **35:** 35-43.

Börner, A. (2006). Preservation of plant genetic resources in the biotechnology era. *Biotechnology Journal* **1:** 1393-1404.

Boubriak, I., Dini, M., Berjak, P. and Osborne, D.J. (2000). Desiccation and survival in the recalcitrant seeds of *Avicennia marina*: DNA replication, DNA repair and protein synthesis. *Seed Science Research* **10:** 307-315

Boyer, J. N. and South, D. B. (1987). Excessive seedling height, high shoot-to-root ratio, and benomyl root dip reduce survival of stored loblolly pine seedlings. *Tree Planter's Notes* **38:** 19-22.

Butola, J.S. and Samant, S.S. (2007). Effect of different growth environments on seed germination and growth performance of seedlings of Ashwagandha (*Withania somnifera* Dunal). *Indian Journal of Forestry* **30:** 529-534.

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

Chin, H.F. (1992). Seed banks – Conserving the past for the future. Conference 23rd ISTA Congress, Buenos Aires, Argentina

Chin, H. F. and Roberts, E. H.(1980). *Recalcitrant crop seeds*. Tropical Press Sdn.Bdh, Kuala Lumpur, Malaysia.

Clor, M. A., Al-Ani, T.A. and Charchafchy, D.F. (1976). Germinability and Seedling Vigor of *Haloxylon salicornicum* as affected by storage and seed size. *Journal of Range Management* **29**:60-62.

Cook, C.G. and Smart, J.R. (2004). Seed quality evaluation of eight kenaf cultivars. *Industrial Crops and Products* **3**: 213-216.

Corbineau, F. and Côme, D. (1986). Experiments on the storage of seeds and seedlings of *Symphonia globulifera* L.f. (Guttiferae). *Seed Science and Technology* **14:** 585-591.

Corbineau, F. and Côme, D. (1988). Storage of recalcitrant seeds of four tropical species. *Seed Science and Technology* **16:** 97-103.

Crouch, N.R., Douwes, E., Wolfson, M.M., Smith, G.F. and Edwards, T.J. (2008). South Africa's bioprospecting, access and benefit-sharing legislation: current realities, future complications, and a proposed alternative. *South African Journal of Science* **104:** 355-366.

Crowe, J.H., Carpenter, J.F. and Crowe, L.M. (1998). The role of vitrification in anhydrobiosis. *Annual Review of Physiology* **60:** 73-103.

Djavanshir, K. and Pourbeik, H. (1976). Germination value: a new formula. *Silvae Genetica* **25:** 79-83.

Dumet, D. and Benson, E.C. (2000). The use of physical and biochemical studies to elucidate and reduce cryopreservation-induced damage in hydrated/desiccated plant germplasm. In Engelmann, F. and Takagi, H., eds.

Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application, Tsukuba, Japan and International Plant Genetic Resources Institute, Rome, Italy. pp. 385-387.

Dumroese, R. K., James, R. L., Wenny, D. L. and Gilligan, C. J. (1988). Douglas-fir seed treatments: effects on seed germination and seedborne organisms. In Landis, T. D., eds. *Proceedings of Combined Meeting of the Western Forest Nursery Associations, Vernon, British Colombia, 1988.* pp. 155-160.

Eapen, S. and George, L. (1990). Influence of phytohormones, carbohydrates, aminoacids, growth supplements and antibiotics on somatic embryogenesis and plant differentiation in finger millet. *Plant cell, Tissue and Organ Culture* **22**: 87–93.

Einhellig, F.A. (1995). Mechanisms of action of allelochemicals in allelopathy. In Inderjit, Dakshini, K.M.M., and Einhellig, F.A., eds. *Allelopathy: Organisms, Processes and Applications*. American Chemical Society, Washington DC, United. pp. 96-116.

Ellis, R.H. (1992). Seed and seedling vigour in relation to crop growth and yield. *Plant Growth Regulation* **11**: 249-255.

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

Ellis, R. E., Hong, T. D. and Roberts, E. H. (1990). An intermediate category of seed behaviour? I. Coffee. *Journal of Experimental Botany* **41:** 1167– 1174.

Ellis, R. E., Hong, T. D. and Roberts, E. H. (1991a). An intermediate category of seed behaviour? II. Effects of provenance, immaturity, and imbibition on desiccation-tolerance in coffee. *Journal of Experimental Botany* **42:** 653 – 657.

Ellis, R. E., Hong, T. D. and Roberts, E. H. (1991b). Effect of storage temperature and moisture on the germination of papaya seeds. *Seed Science Research* **1:** 69-72.

Engelmann, F. (1991). *In vitro* conservation of tropical plant germplasm – a review. *Euphytica* **57:** 227-243.

Engelmann, F. (1997). Importance of desiccation for the cryopreservation of recalcitrant seed and vegetatively propagated species. *Plant Genetic Resources Newsletter* **112:** 9-18.

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

Engelmann, F. (2011). Germplasm collection, storage and conservation. In Altman, A., Hasegawa, P.M. (eds) *Plant biotechnology and agriculture*. Academic Press, Oxford. pp. 255-268.

Engels, J. and Engelmann, F. (1998). Introductory statement. *Seed Science Research* **8:** 1-2.

Farrant, J. (1965). Mechanisms of cell damage during freezing and thawing and its prevention. *Nature* **205:** 1284-1287.

Farrant, J. M., Berjak, P. and Pammenter, N.W. (1993). Studies on the development of the desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* (Forssk.) Vierh.: the acquistion of germinability and response to storage and dehydration. *Annals of Botany* **71:** 405-410.

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

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

Farrant, J. M., Pammenter, N. W. and Berjak, P. (1993). Seed development in relation to desiccation tolerance: a comparison between desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation-tolerant types. *Seed Science Research* **3:** 1-13.

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

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

Finkle B.J. and Ulrich, J.M. (1979). Effects of cryoprotectants in combination on the survival of frozen sugarcane cells. Plant Physiology **63**: 598–604.

Finkle, B. J., Zavala, M. E. and Ulrich, J. M. (1985). Cryoprotective compounds in the viable freezing of plant tissue. In Kartha, K.K., eds. *Cryopreservation of Plant Cells and Organs.* CRC Press, Florida, United States of America. pp. 75-113.

Fujikawa, S. and Jitsuyama, Y. (2000). Ultrastructural aspects of freezing adaptation of cells by vitrification. - In: Engelmann, F.,Takagi, H. (ed.): *Cryopreservation of Tropical Plant Germplasm.* pp. 36-42. IPGRI

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

Gagliardi, R.F., Pacheco, G.P., Carneiro, L.A., Valls, J.F.M., Vieira M.L.C. and Mansur, E. (2003). Cryopreservation of *Arachis* species by vitrification of *in vitro*-grown shoot apices and genetic stability of recovered plants. *Cryoletters* **24**: 103-110.

Gamborg, O.L. (1984). Plant cell cultures: nutrition and media. In Vasil, I.K., eds. *Cell Culture and Somatic Cell Genetics of Plant*. *Vol **1:** Laboratory*

Procedures and their Application. Academic Press, London, United Kingdom. pp. 18-26.

George, E. F. (1996). *Plant Propagation by Tissue Culture*. Exegetics Ltd. England. Exegetics Ltd., Edington, United Kingdom.

Gepts, P. (2006). Plant genetic resources conservation and utilization: the accomplishments and future of a societal insurance policy. *Crop Science* **46:** 2278-2292.

Gnanapragasam, S. and Vasil, I. K. (1990). Plant regeneration from cryopreserved embryonic cell suspension of a commercial sugarcane hybrid (*Saccharum* sp.). *Plant Cell Reports* **9:** 419-423.

Gorshkova, T.A., Salnikov, V.V., Pogodina, N.M., Chemikosova, S.B., Yablokova, E.V., Ulanov, A.V., Ageeva, M.V., Van Dam, J.E.G. and Lozovaya, V.V. (2000). Composition and distribution of cell wall phenolic compounds in flax (*Linum usitatissinum* L.) stem tissues. *Annals of Botany* **85:** 477-486.

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

Grabe, D. F. (1989). Report of the seed moisture 1986 – 1989 working group on recalcitrant seeds. *Seed Science and Technology* **17** (Supplement): 87-93.

Hajari, E., Berjak, P., Pammenter, N.W. and Watt, M.P. (2011). A novel means for cryopreservation of germplasm of the reclacitrant-seeded species, *Ekebergia capensis*. *CryoLetters* **32**: 308-316.

Hendry, G. A. F., Finch-Savage, W. E., Thorpe, P. C., Atherton, N. M., Buckland, S. M., Nilsson, K. A. and Seel, W. E. (1992). Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. *New Phytologist* **122:** 273-279.

Hines, D. A. and Eckman, K. (1993). Indigenous multipurpose trees of Tanzania: uses and economic benefits to the people. Available from http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/x5327e/x5327e00.htm [Accessed on 23 January 2005].

Hoekstra, F.A., Golovina, E.A. and Nijsse, J. (2003). What do we really know about desiccation tolerance mechanisms? In Nicholás, G., Bradford, K.J., Côme, D. and Pritchard, H.W., eds. *The Biology of Seeds: Recent Research Advances: Proceedings of the Seventh International Workshop on Seeds, Salamanca, Spain, 2002.* CAB International. pp 259-270.

Hong, T. D. and Ellis, R. H. (1996). A protocol to determine seed storage behaviour. In Engels, J. M. M. and Toll, J., eds. *IPGRI Technical Bulletin No. 1*. International Plant Genetic Resources Institute , Italy, Rome.

Hong, T. D., Ellis, R. H., Tam, B.N. and Le Tam, V.T. (2005). Effects of desiccation on seed germination, cracking, fungal infection and survival in storage of *Sterculia foetida* L. *Seed Science and Technology* **33:** 705-712.

Hor, Y. L., Chin, H. F. and Zain Karim, M. (1984). The effect of seed moisture and storage temperature on the storability of cocoa (*Theobroma cacao*) seeds. *Seed Science and Technology* **12:** 415-420.

Hutzler, P., Fischbach, R., Heller, W., Jungblut, T.P., Reuber, S., Schmitz, R., Veit, M., Weissenbock, G. and Schnitzler, J. (1998). Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. *Journal of Experimental Botany* **49:** 953-965.

Inderjit, Streibig, J.C. and Olofsdotter, M. (2002). Joint action of phenolic acid mixtures and its significance in allelopathy research. *Physiologia Plantarum* **114:** 422-428.

IPGRI/DFSC (1999). The project on handling and storage of recalcitrant and intermediate tropical forest tree seeds. Newsletter, 5. International Plant Genetic Resources Institute (IPGRI), Rome.

IPGRI/DFSC (2002). The project on handling and storage of recalcitrant and intermediate tropical forest tree seeds. Newsletter, 8. International Plant Genetic Resources Institute (IPGRI), Rome.

Jackson, P.W. and Kennedy, K. (2009). The global strategy for plant conservation: a challenge and opportunity for the international community. *Trends in Plant Science* **14:** 578-580.

Jaleel, C.A., Gopi, R. and Panneerselvam, R. (2008). Biochemical alterations in white yam (*Dioscorea rotundata* Poir.) under triazole fungicides: impact on tuber quality. *Czech Journal of Food Science* **26:** 298-307.

Janzen, D. (1977). What are dandelions and aphids? *The American Naturalist* **111:** 586-589.

Kalsa, K.K., Tomer, R.P.S. and Abebie, B. (2011). Effects of storage duration and hydro-priming on seed germination and vigour of Common vetch. *Journal of Science and Development* **1**: 65-73.

Kandya, A. K. (1987). Forest seed in India: Research and problems. In Kamra, S. K., eds. *Proceedings of the International Symposium on Forest Seed Problems in Africa, Harare, Zimbabwe, 1987.* Sweden, Umea: Swedish Agriculture University. pp.124-142.

Kartha K.K. (1985). Cryopreservation of Plant Cells and Organs. CRC Press Inc., Boca Raton, Florida.

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

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

Kioko, J., Berjak, P., Pammenter, N., Lamb, J. and Gumede, Z. (2003). Is the genetic fidelity maintained following the cryopreservation of the seeds of the endangered African pepper bark (*Warburgia salutaris*)? In Nicols, G., Bradford, K. J., Come, D. and Pritchard, H. W., eds. *The Biology of Seeds: recent research advances: Proceedings of the Seventh International Workshop on Seeds, Salamanca, Spain, 2002*. CABI publishing. pp. 327-335.

Kistnasamy, P., Berjak, P. And Pammenter, N.W. (2011). The effects of desiccation and exposure to cryogenic temperatures of embryonic axes of *Landolphia kirkii*. *Cryoletters* **32:** 28-39.

Koonjul, P.K., Brandt, W.F., Farrant, J.M. and Lindsey, G.G. (1999). Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. *Nucleic Acids Research* **27**: 915–916.

Koster, K. L. and Leopold, C. A. (1988). Sugars and desiccation tolerance in seeds. *Plant Physiology* **88:** 829-832.

Kranner, I., Roach, T., Beckett, R.P., Whitaker, C. and Minibayeva, F. (2010). Early production of reactive oxygen species during seed germination and early seedling growth in *Pisum sativum*, *Journal of Plant Physiology* **167:** 805-811.

Lalonde, L. and Bewley, J. D. (1986). Patterns of protein synthesis during the germination of pea axes, and the effects of an interrupting desiccation period. *Planta* **167:** 504-510.

Lapido, D. O., Esegu, F. and Oduwaiye, E. A. (1992). Production of quality tree seeds. In Wolf, H., eds. *Seed procurement and legal regulations for forest reproductive material in tropical and subtropical countries*. GTZ-Forestry Seed Centre, Muguga, Kenya pp. 59-75.

Leopold, C. A., Sun, Q. W. and Bernal-Lugo, I. (1994). The glassy state in seeds: analysis and function. *Seed Science Research* **4:** 267-274.

Lloyd, D.G. and McCown, B.H. (1981). Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot-tip culture. *Proceedings International Plant Propagation society* **30:** 421-427.

López Lastra C.C., Hajek A.E.and Humber R.A. (2001). Effects of two cryopreservation techniques on viability and pathogenicity of entomophthoralean fungi. Canadian Journal of Botany **79:** 861-864.

Lyons, J. M. (1973). Chilling injury in plants. *Annual Review on Plant Physiology.* **24:** 445-466.

Mander, M. (1998). *Marketing of indigenous medicinal plants in South Africa. A case study in KwaZulu – Natal*. FAO, Rome, Italy.

Mazur, P. (1984). Freezing of living cells: mechanisms and applications. American Journal of *Cell Physiology* **16:**125-142.

Minorsky, P.V. (2001). Phenolic vacuoles and auxin transport. *Plant Physiology* **127:** 1570-1571.

Mittal, R. K., Hansen, H. J. and Thomsen, K. (1999). Effect of seed treatments and storage temperature on storability of *Syzygium cuminii* seeds. In Marzalina, M., Khoo, K. C., Jayanthi, N., Tsan, F. Y. and Krishnapillay, B., eds. *"Recalcitrant Seeds": Proceedings of the International Union of Forest Research Organization (IUFRO): Recalcitrant* Seeds. Kualar Lumpur, Malaysia, 1999. Forest Research Institute. pp. 227-234.

Msanga, H. P. (1998). *Seed germination of indigenous trees in Tanzania: including notes on seed processing, storage and plant uses*. Canadian Forest Service, Edmonton, Canada.

Mueller, W. C. and Greenwood, A.W. (1978). The ultra structure of phenolic storing cells fixed with caffeine. *Journal of Experimental Botany* **29:** 757-764.

Mukherjee, M., Watt, D.A. and Berjak, P. (2006). Molecular detection and diagnosis of fungal contaminants of recalcitrant seeds: *Quercus robur* L. acorns as a model system. *Seed Science and Technology* **34:** 415-427.

Müller, K., Linkies, A., Vreeburg, R.A., Fry, S.C., Krieger-Liszkay, A. and Leubner-Metzger, G. (2009). *In vivo* wall loosening by hydroxyl radicals during

cress seed germination and elongation growth. *Plant Physiology* **150:** 1855-1865.

Murashige, T. and Skoog, E. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15:** 473 – 497.

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

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

Mycock, D., Wesley-Smith, J. and Berjak, P. (1995). Cryopreservation of somatic embryos of four species with and without cryoprotectant treatment. *Annals of Botany* **75:** 31-336.

Mycock, D. J., Berjak, P., Pammenter, N. W. and Vertucci, C. W. (1997). Cryopreservation of somatic embryoids of *Phoenix dactylifera*. In Ellis, R. H, Murdoch, A. J. and Hong, T.D. eds. *Basic and Applied Aspects of Seed Biology* Kluwer Academic Publishers, London, England. pp 75-82.

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

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

Nordling, L. (2009). Africa analysis: a level playing field for biodiversity. *Science and Develoment Network.* Available from http://www.scidev.net/sub-saharan-africa/opinions 1/5/2010. [Accessed on May 1, 2010].

Normah, M. N., Chin, H. F. and Hor, Y. L. (1986). Desiccation and cryostorage of embryonic axes of *Hevea brasiliensis* Muell. –Arg. *Pertanika* **9:** 299-303.

Obroucheva, N. V. and Antipova, O. V. (1997). Physiology of the initiation of seed germination. *Russian Journal of Plant Physiology* **44:** 250-264, Translated from *Fiziologiya Rastenii* **44:** 287-302.

Ogawa, K. (2005). Relationships between mean shoot and root masses and density in an overcrowded population of hinoki (*Chamaecyparis obtusa* (Sieb. et Zucc.) Endl.) seedlings. *Forest Ecology and Management* **213:** 391–398

Okigbo, B. N. (1994). Conservation and use of plant germplasm in African traditional agriculture and land use systems. In Putter, A., eds. *Safeguarding the genetic basis of Africa's traditional crops.* CTA, the Netherlands/IPGRI, Rome, Italy. pp.15-38.

Palmberg-Lerche, C. (1992). Conservation of genetic resources as an integral part of forest management and tree improvement. In Wolf, H., eds. *Seed Procurement and Legal Regulations for Forest Reproductive Material in Tropical and Subtropical Countries*. GTZ-Forestry Seed Centre, Muguga, Kenya. pp.169-178.

Pammenter, N. W. and Berjak, P. (1999). A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Science Research* **9:** 13-38.

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

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

Pammenter, N.W., Berjak, P., Wesley-Smith, J., Vander Willigen, C. (2002). Experimental aspects of drying and recovery. pp. 93-110 *in* Black, M, Pritchard, H.W. (eds) *Desiccation and survival in plants: drying without dying*. CABI, Wallingford, UK.

Pammenter, N. W., Greggians, V., Kioko, J. I., Wesley-Smith, J., Berjak, P. and Finch-Savage, W.E. (1998). The time factor during dehydration of non-

orthodox (recalcitrant) seeds: effects of differential drying rates on viability retention of recalcitrant seeds of *Ekebergia capensis*. *Seed Science Research* **8:** 463-471.

Pammenter, N.W., Berjak, P., Goveia, M., Sershen, Kioko, J.I., Whitaker, C. and Beckett, R.P. (2011). Topography determines the impact of reactive oxygen species on shoot apical meristems of recalcitrant embryos of tropical species during processing for cryopreservation. *Acta Horticulturae* **908**: 83-92.

Parkes, A.S. (1945). Preservation of human spermatozoa at low temperatures. *British Medical Journal* **2**:212–213.

Pascarella, J.B. and Horvitz, C.C. (1999). Seed and seedling ecology of the invasive non-indigenous shrub *Ardisia elliptica* Thunb. (Myrsinaceae) in South Florida. In Cannizzaro, P.J., eds. *Proceedings of 25th Annual Conference – Ecosystems Restoration and Creation, Tampa, Florida, United States of America, 1999.* pp. 76-88.

Paton, A. (2009). Biodiversity informatics and the plant conservation baseline. *Trends in Plant Science* **14:** 629-637.

Pennington, K. (1994). *Pennington's Butterflies of Southern Africa*. In Henning, G. A., Pringle, E. L. I. and Ball, J. B., eds. Struik Winchester Publishers, Cape Town, South Africa. pp.156-166.

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

Pongpanich, K. (1990). Fungi associated with forest tree seeds in Thailand. In *Proceedings of the International Union of Forest Research Organization (IUFRO) Workshop on Pests and Diseases of Forest Plantations, Bangkok, Thailand, 1990.* RAPA Publication. pp. 114-121.

Pooley, E. (2003). *The complete field guide to trees of Natal, Zululand and Transkei*. Natal floral publications trust. Durban, South Africa. pp. 372-375.

Premsagar, V. (2009). Optimising Methods for Embryonic Axis Fixation and Micropropagation of *Syzygium cordatum* Hochst. Unpublished Thesis, University of KwaZulu-Natal, Durban.

Reed, B.M. (1996). Pretreatment strategies for the cryopreservation of plant tissues. In Normah, M.N., Narimah, M.K., Clyde, M.M., eds. In vitro *Conservation of Plant Genetic Resources.* Plant Biotechnology Laboratory, Faculty of Sciences, Universiti of Kebangsaan, Malaysia. pp.73-87.

Richards, A.J. (2003). Apomixis in flowering plants: an overview. *Philosophical Transactions of the Royal Society* **358:** 1085-1093.

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

Roberts, E. H., King, M. W. and Ellis, R. H. (1984). Recalcitrant seeds: their recognition and storage. In Holden, J. H. W. and Williams, J. T. Allen and Onwin, eds. *Crop genetic resources: Conservation and Evaluation*. George Allen and Unwin, London, United Kingdom. pp. 39-52.

Rohringer, R., Kim, W. K., Samborski, D. J., and Howes, N. K. (1977). Calcofluor: An optical brightener for fluorescence microscopy of fungal plant parasites in leaves. *Phytopathology* **67:** 808-810.

Ronchi, A., Farina, G., Gozzo, F. and Tonelli, C. (1997). Effects of a triazolic fungicide on maize plant metabolism: modifications of transcript abundance in resistance-related pathways. *Plant Science* **130:** 51-62.

Roos, E. E. (1989). Long-term seed storage. *Plant Breeding Reviews* **7:** 129-158.

Rosa, S.D.V.F., da Carvalho, A.M., McDonald, M.B., Von Pinho, E.R.V., Silva, A.P. and Veiga, A.D. (2011). The effect of storage conditions on coffee seed and seedling quality. *Seed Science and Technology* **39**: 151-164

Rozes, N., Arola, L. And Bordons, A. (2003). Effect of phenolic compounds on the co-metabolism of citric acid and sugars by *Oenococcus oeni* from wine. *Letters in Applied Microbiology* **36:** 337-341.

Sacandé, M., Jøker, D., Dulloo, M.E., Thomsen, K.A. (eds) (2004) *Comparative storage biology of tropical tree seeds*. International Plant Genetic Resources Institute, Rome, Italy.

Sanchez, V. M., Sundstrom, F. J., McClure, G. N. and Lang, N. S. (1993). Fruit maturity, storage and postharvest maturation treatments affect bell pepper (*Capsicum annuum* L.) seed quality. *Scientia Horticulturae* **54:** 191-201

Santiago, L.J.M., Louro, R.P. and De Oliveira, D.E. (2000). Compartmentation of phenolic compounds and phenylalanine ammonia-lyase in leaves of *Phyllanthus tenellus* Roxb. and their induction by copper sulphate. *Annals of Botany* **86:** 1023-1032.

Santuah, N. (2005). Climate change 'threatens to evict African plants'. *Science and Development Network.* Available from *http://www.scidev/dossiers/index.cfm*. [Accessed November 3, 2011].

Seme, E. N., Chahira, P. W. and Kemel, J. K. (1994). *Ex situ* conservation of African crop germplasm. In Putter, A., eds. *Safeguarding the Genetic Basis of Africa's Traditional Crops.* CTA, the Netherlands and International Plant Genetic Resources Institute, Rome, Italy. pp. 101-103.

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

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

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

Shatnawi, M., Johnson, K. and Torpy, F. (2004). *In vitro* propagation and cryostorage of *Syzygium francissi (Myrtaceae)* by the encapsulation-dehydration method. *In Vitro Cellular and Developmental Biology – Plant* **40:** 403-407.

Shimonishi, K., Ishikawa, M., Suzuki, S. and Oosawa, K. (2000). Cryopreservation of melon somatic embryos by desiccation method. In Engelmann, F. and Takagi, H., eds. *Cryopreservation of tropical plant germplasm. Current research progress and application.* Current Research Progress and Application, Tsukuba, Japan and International Plant Genetic Resources Institute, Rome, Italy. pp. 167-171.

Singhal, R. M. and Khanna, P. (1992). Quality seed production and tree improvement in India, In *Seed procurement and legal regulations for forest reproductive material in tropical and subtropical countries*. Edited by Wolf, H. GTZ-Forestry Seed Centre Muguga. pp. 141-146.

Smith, M. T. and Berjak, P. (1995). Deteriorative changes associated with the loss of viability of stored desiccation-tolerant and desiccation-sensitive seeds. In Kigel, J. and Galili, G., eds. *Seed development and germination*. Marcel Dekker Inc. New York, United States of America. pp. 701-746.

Song, S-Q., Berjak, P., Pammenter, N.W., Ntuli, T.M. and Fu, J.R. (2003). Seed recalcitrance: a current assessment. *Acta Botanica Sinica* **45:** 638-643.

Spears, J.S. (1979). Can the wet forest survive? *Commowealth Forestry Review* **58:** 165-180.

Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electromicroscopy. *Journal of Ultrastructure Research* **26:** 31-43.

Taylor, R., Adams, G. D. J., Boardman, C. F. B. and Wallis, R.G. (1974). Cryoprotection – permeant vs nonpermeant additives. Cryobiology **11:** 430-438.

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

Thurlby, K. A. G., Connelly, C., Wilson, P.G. and Rossetto, M. (2011). Development of microsatellite loci for *Syzygium paniculatum* (Myrtaceae), a rare polyembryonic rainforest tree. *Conservation Genetics Resources* **3:** 205-208.

UNCED (1992). Convention on Biological Diversity. United Nations Conference on Environment and Development, Geneva.

Varghese D., Berjak P. and Pammenter, N.W. (2009). Cryopreservation of shoot tips of *Trichilia emetica*, a tropical recalcitrant-seeded species. *Cryo-Letters* **30**: 280-90.

Vertucci, C. W. and Farrant, J. M. (1995). Acquisition and loss of desiccation tolerance. In Kigel, J. and Galili, G., eds. *Seed Development and Germination*. Marcel Dekker Inc., New York, United Sates of America. pp. 237-271.

Vertucci, C.W. and Roos, E.E. (1993) Theoretical basis of protocols forseed storage II: the influence of temperature on optimal moisture levels. *Seed Science Research* **3**: 201–213.

Walters, C., Rao, K. N. and Hu, X. (1998). Optimizing seed water content to improve longevity in *ex situ* genebanks. *Seed Science Research* **8** (supplement 1)**:** 15-22.

Walters, C., Ried, J. L. and Walker-Simmons, M. K. (1997). Heat-soluble proteins extracted from wheat embryos have tightly bound sugars and unusual hydration properties. *Seed Science Research* **7:** 125-134.

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

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

Webb, M.A. and Arnott, H.J. (1982). Cell wall conformation in dry seeds in relation to the preservation of structural integrity during desiccation. *American Journal of Botany* **69**: 1657-1668

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

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

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

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

Willan, R. L. (1985). A guide to forest seed handling: with special reference to the tropics. *FAO Forestry Paper No. 20/2.* Rome, Italy. pp. 379.

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

Zhang, B.H. Liu, F., Liu, Z.H., Wang, H.M. and Yao, C.B (2001) Effects of kanamycin on tissue culture and somatic embryogenesis in cotton. *Plant Growth Regulation* **33:** 137–149.

Zhou, J., Zhou, J., Zeng, H., Wang, G. and Zhu, Z. (2000). Advance of studies on browning and antibrowning techniques in the tissue culture of horticultural plants. *Acta Horticulturae Sinica* **27:** 481-486.