Aspects of post-harvest seed physiology and cryopreservation of the germplasm of three medicinal plants indigenous to Kenya and South Africa.

by

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Submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

School of Life and Environmental Sciences
Faculty of Science
University of Natal, Durban.
2002.

Preface

The experimental work described in this thesis was carried out in the School of Life and Environmental Sciences of the University of Natal, Durban, under the supervision of Profs Patricia Berjak and Norman W. Pammenter.

These studies are the original work of the author, and have not been submitted in any form for any degree or diploma in any other tertiary institution. Where use was made of the work of others, it has been duly acknowledged in the text.

Dedication

I dedicate this work to my mother, and to the memory of Tata, my father.

Acknowledgements

I would like to sincerely thank my supervisors, Profs Patricia Berjak and Norman Pammenter, for their invaluable support, encouragement and guidance throughout, and beyond, the course of this study. I am truly grateful.

I am also indebted to other present and past members of the School of Life and Environmental Sciences who made this work possible. To name a few: Prof. Paula Watt for advice in tissue culture; Dr. Jenny Lamb, Ms. Zandile Gumede and Ms. Eitimad Abdel Rahman for help with DNA extraction and PCR-RAPD work; Dr. James Wesley-Smith and Mrs Priscilla Maartens for training and assistance in electron microscopy; Ms. Denise Berjak for enthusiastic, untiring help in the dark-room; and members of the Plant Cell Biology Research Group (including the late Mr. Mjabu Mwelase) for good cheer, support, and countless hours of help in the field and in the lab.

To my friends, thanks for the encouragement, support, and always providing a bright side of life.

To my family in Kenya, it saddens me deeply that I had to be away from all of you for all this time. Nothing can replace the time lost, and this work is only small consolation.

To Eunice, thank you for being there, for understanding, for love.

I would like to acknowledge and express gratitude for the financial support from the International Plant Genetic Resources Institute (IPGRI, Rome), and the University of Natal Research Fund.

Lastly but not least, I would like to thank God for giving me life, and for carrying me during those times I could not walk by myself.

Abstract

The current state of global biodiversity is one of sustained and increasing decline especially in developing countries such as South Africa, where, medicinal plants face a particular threat due the herbal medicine trade, and because *in situ* conservation measures have not stemmed the exploitation of these plants (Chapter 1). Furthermore, seed storage, which offers an efficient *ex situ* conservation technique, cannot presently be applied to many medicinal plants, either because these species produce short-lived, recalcitrant seeds, or the post-shedding behaviour of the seeds is altogether unknown.

This study investigated three medicinal plant species indigenous to Kenya and South Africa: *Trichilia dregeana* and *T. emetica*, of which no population inventories exist and no wild populations were encountered locally during the course of this study; and *Warburgia salutaris*, one of the most highly-utilised medicinal plants in Africa, and which is currently endangered and virtually extinct in the wild in some countries such as South Africa. Aspects of post-shedding seed physiology (Chapter 2) and the responses of the germplasm of the three species to cryopreservation (Chapter 3) were studied using viability and ultrastructural assessment, with the aim of establishing methods for both short-term and the long-term preservation, via appropriate seed storage and cryopreservation, respectively. The effect of cryopreservation on genetic fidelity, a crucial aspect of germplasm conservation, was assessed by polymerase chain reaction (PCR) based random amplified polymorphic DNA (RAPDs), using *W. salutaris* as a case-study (Chapter 4).

The seeds of all three species were found to exhibit non-orthodox behaviour. On relatively slow-drying, seeds of *T. dregeana* and *T. emetica* lost viability and ultrastructural integrity at axis water contents of 0.55 g g^{-1} (achieved over 6 d) and 0.42 g g^{-1} (after 3 d) respectively, while flash-drying of embryonic axes facilitated their tolerance of water contents as low as 0.16 g g^{-1} (*T. dregeana*, flash-dried for 4 h) and 0.26 (*T. emetica*, flash-dried for 90 min). Seeds of *W. salutaris* were relatively more tolerant to desiccation, remaining viable at axis water contents below 0.1 g g^{-1} when desiccated for 6

d in activated silica gel. However, excised embryonic axes flash-dried to similar water contents over 90 min lost viability and were ultrastructurally damaged beyond functionality.

In terms of storability of the seeds, those of *T. dregeana* could be stored in the fully hydrated state for at least 5 months, provided that the quality was high and microbial contamination was curtailed at onset of storage, while those *T. emetica* remained in hydrated storage for about 60 d, before all seeds germinated in storage. Seeds of *W. salutaris*, even though relatively tolerant to desiccation, were not practically storable at reduced water content, losing viability within 49 d when stored at an axis water content of 0.1 g g^{-1}. The seeds of all three species were sensitive to chilling, suffering extensive subcellular derangement, accompanied by loss of viability, when stored at 6 °C.

Thus, *T. dregeana* and *T. emetica* are typically recalcitrant, while those of *W. salutaris* are suggested to fit within the intermediate category of seed behaviour. For either recalcitrant or intermediate seeds, the only feasible method of long-term germpalsm preservation may be cryopreservation.

Subsequent studies established that whole seeds of *W. salutaris* could be successfully cryopreserved following dehydration in activated silica gel. However, whole seeds of *T. dregeana* and *T. emetica* were unsuitable for cryopreservation, and excised embryonic axes were utilised. For these, *in vitro* germination methods, as well as cryoprotection, dehydration, freezing and thawing protocols were established. Post-thaw survival of the axes of both species was shown to depend on cryoprotection, rapid dehydration and cooling (freezing) in cryovials. Embryonic axes of *T. dregeana* regenerated only as callus after cryopreservation, while those of *T. emetica* generated into apparently normal plantlets. Thawing/rehydration in a 1:1 solution of 1 µ*M* $CaCl_2.2H_2O$ and 1 m*M* $MgCl_2.6H_2O$ increased the percentage of axes surviving freezing, and that of *T. emetica* axes developing shoots. The effect of the extent of seed/axis development on onward growth after cryopreservation was apparent for seeds of *W. salutaris* and excised axes of *T. emetica*.

The seeds of *W. salutaris* surviving after cryopreservation germinated into seedlings which appeared similar to those from non-cryopreserved seeds, both morphologically and in terms of growth rate. Analysis using PCR-RAPDs revealed that there were no differences in both nucleotide diversity or divergence, among populations of seedlings from seeds which had been sown fresh, or those which had either been dehydrated only, or dehydrated and cryopreserved. Thus, neither dehydration alone, nor dehydration followed by cryopreservation, was associated with any discernible genomic change.

The above results are reported and discussed in detail in Chapters 2 to 4, and recommendations and future prospects outlined in Chapter 5.

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CHAPTER 1:

Biodiversity, conservation and seed storage: a review.

1.1 Current value, status and trends in Global Biodiversity

The concept of biological diversity (or in its short form, biodiversity) is used to refer to the total variability of life on earth (Lovejoy, 1980; Wilson and Peters, 1988; Chauvet and Olivier, 1993), and includes taxonomic diversity (the sum of the taxa of all living organisms on earth), ecological diversity (the linkages and relationships between the organisms and their environments), and genetic diversity (the variation within and between the populations of species) (Lovejoy, 1996). The actual total biodiversity on earth is not accurately known, but the taxonomic diversity is estimated to include some 320 000 species of plants, 1 500 000 species of fungi, 9 100 000 species of animals, 600 000 species of protists and 1 000 000 species of bacteria (Groomridge, 1992; Heywood, 1995).

The values placed on biodiversity are strongly linked to human influences and the underlying social and economic driving forces; and depend on the knowledge of the scientific rôle of particular factors. Today, humans are dependent on fundamental biological systems for their sustenance, health, well-being and enjoyment of life: all of humanity's food and many medicines and industrial products are derived from wild and domesticated components of biological diversity. Biotic resources also serve recreation and tourism, and underpin the ecosystems which provide humanity with many amenities or services (Jeffries, 1997).

Economically, the value of biological diversity has been demonstrated for a number of biological systems. For example, the financial benefit of water supplied to Melbourne, Australia, from forested catchments was already valued at US$250 million per year in 1982 by Read, Sturgess and Associates, while eight years ago pharmaceutical prospecting in the tropical forests of Costa Rica was valued at US$ 4.81 million per product (Aylward, 1993). A summary of examples of historical, current and foreseeable importance of biodiversity to humans is presented in Table 1.1.

The value of biological diversity and the attendant exploitation has led to losses through deleterious human impacts such as over-exploitation, habitat destruction, introductions and exterminations, ecosystem cascades, and pollution (Jeffries, 1997). While it is difficult to develop a time-course of the quantity of global biodiversity, the "Living Planet Index (LPI)", developed by the World Wildlife Fund (WWF) [WWF, 2002] provides valuable insight. The LPI is an aggregate of three different indicators of the state of natural ecosystems around the world: the area of natural forest cover; the populations of freshwater species; and the populations of marine species. By monitoring these indicators, it is possible to discern how fast biodiversity is disappearing from the Earth.

The WWF report, released in 2002 showed that, between 1970 and 2000, the LPI had fallen by 37%, at a rate of approximately 1.2% per year. Particularly, according to the earlier LPI (WWF, 1999), the natural forest cover fell by 10%; and in Africa, Asia, and Latin America; this amounted to a loss of some 150 000 km^2 per year, equivalent to half of the size of Burkina Faso or one-eighth of the size of S. Africa. This decline included a steeper, but unquantified loss of biodiversity and forest quality. For example, global extinction rates are estimated to have risen from one species per day in 1970, to one species every 12 minutes in 1992 (Wynberg, 1993). If this trend is maintained, about 25% of the global species diversity could face extinction in the next 50 years, and all species on earth would be lost within 300 years.

Even though species richness is the most commonly used currency for quantifying biodiversity, the genetic diversity within a species holds important implications for conservation programmes (e.g. Miller *et al.*, 2001). Generally, mammals, birds, and reptiles have similar overall levels of genetic diversity, whereas amphibians have higher, and fishes lower, levels (Ward *et al.*, 1992). In plants, a large number of mating systems produces a richer variety of population genetic structures than in animals, with wind-pollinated plants exhibiting the highest levels of heterozygosity followed by animal-pollinated plants and then self-pollinated plants (Hamrick and Godt, 1990).

Table 1.1 Some examples of historic, current and foreseeable importance of biodiversity to humans. (after Jeffries, 1997).

Importance	Historic	Current	Foreseeable
Ecosystem services	Pollination of crop plants by bees	Wetlands to clear pollution; effective against many pollutants, especially nutrient over-enrichment	Soft coastal engineering to counteract sea level rises from global warming
Medicine	Penicillin; antibiotic derived from fungus	Horse Shoe crab blood used in bioassays	Rainforest plants; new drugs to combat existing and future diseases
Biotechnology	Rattans, Asian forest palms used for fibres for many building artefacts	Bacterial genes introduced to crops to confer resistance to insects	Metal digesting bacteria; potential use to clear contamination and extract valuable metals
Environmental monitoring	Losses of bird species as evidence of DDT impact	European forests damaged by acid precipitation	Species change and habitat changes in response to global warming
Food	Irish potato famine due to over-reliance on one vulnerable cultivar	The weedy tomato wild gene improved domestic cultivars	High value exotic forest fruits
Recreation	Victorian fern craze; wide-spread hobby	Birdwatching; Major recreational activity in developed world	Eco-tourism; growth area for global tourism
Pets and domestic animals	Exotic zoo animals as gifts and status symbols	Value of current pet-trade	Potential for use of historic breeds as new and useful attributes are discovered.
Political and social	Symbolic function in Celtic religion; animals as companions to St Cuthbert	Eco activists, e.g. Greenpeace and Earth First; highly visible political impact	Local ownership and control of resources in developing world.

Within each group, however, levels of genetic diversity vary considerably because different life-history patterns produce different levels of gene flow and different population sizes, with larger populations harbouring more genetic variability than smaller ones.

One of the biggest means of the creation and sustaining of genetic diversity of populations is domestication, which leads to a proliferation of forms – cultivars in plants and breeds in animals (Jeffries, 1997). However, only a small proportion of taxonomical biodiversity is domesticated. For example, of the 320 000 species of vascular plants, only 500 are domesticated, with 90% of national *per capita* supplies of food plants coming from only 103 species (Prescott-Allen and Prescott-Allen, 1990). These domesticated taxa have been subjected to much selection and improvement, often utilising genotypes from non-domesticated relatives (e.g. Samuel *et al.*, 1986). On the other hand, in non-domesticated taxa, reduced populations lead to a concomitant narrowing of genetic diversity.

The decline of ecological diversity as evidenced by the drop in the Living Planet Index, the steady decrease of species numbers and the accompanying narrowing of genetic diversity demonstrate that the current trend in the global biodiversity is one of sustained decline. Reduced populations and narrowed genetic diversity increase the risk of extinction, as they restrict the response of the population to changed environmental conditions. This is either because the loss of alleles reduces heritabilities, or because only a few pleiotropic alleles in the reduced population will alter genetic correlations among characters (Barbault and Sastrapradja, 1995). The factors driving this trend, as well as the possible means of checking it, are greatly dependent on specific circumstances in different parts of the world.

1.2 Biodiversity and the medicinal plant trade in Africa

Africa is home to a large number of Centres of Biodiversity, each of which is considered as an evolutionary heartland, and perhaps a last refuge, of specific taxa (Jeffries, 1997). That author reports that, in terms of plant life, the International Union for the Conservation of Nature (IUCN) recognises 32 Centres of Plant Diversity in Africa. These areas contain large numbers of endemics and important gene pools of plants, many of which are of value to humans or potentially useful. The use to which such plants are put depends generally on the ethnoecology of the local human population (Martin, 1995).

Many people in Africa rely heavily on the use of traditional medicines for their primary source of healthcare, as is the case in many developing countries, and the main constituent of traditional African medicines is plants (Kokwaro, 1976; Cunningham, 2000). With 70 – 80% of Africa's population relying on traditional medicines (Cunningham, 1988), the importance of medicinal plants is enormous, and medicinal plants are now attracting serious attention, as evidenced by the recommendation by WHO that proven traditional remedies should be incorporated within national drug policies (Wondergem *et al.*, 1989). There have also been moves towards greater professionalism within traditional African medicine (Last and Chavunduka, 1986), and also by the increased commercialisation of pharmaceutical production using traditional medicinal plants with known efficacy.

However, inadequate attention has been paid to the conservation aspects of medicinal plant resources. These resources are declining, with varying factors driving the decline including the fact that management practices applied in traditional African societies have been abandoned for more market-oriented practices. For example, historically, the collection and gathering of medicinal plants was restricted to Traditional Medical Practitioners (TMPs) whose harvesting methods were sustainable. There were taboos against collection of plants at certain periods (Good, 1987) and also intentional resource conservation controls that maintained the abundance of medicinal species. For example, the widespread practice of conserving edible wild fruit-bearing trees for their fruits or

Centre, the total value of the imports of medicinal plants in Organization for Economic Cooperation and Development (OECD) countries, Japan and the USA increased from US $ 335 million in 1976 to US $ 551 million in 1980 (Husain, 1991). In terms of some individual countries, already more than a decade ago, Germany imported 75-80 tonnes of *Griffonia simplicifolia* seeds from Ghana annually (Abbiw, 1990), while recently, France was reported as importing, from Cameroon alone, 575 tonnes of *Voacanga africana* seed, 220 tonnes of the bark of *Prunus africana*, and 15 tonnes of *Pausinystalia johimbe* bark (Bioresources Development and Conservation Programme, 2000). Some of the medicinal plants, and the respective active ingredients, exported from Africa to European countries are listed in Table 1.3.

Unfortunately, the high demand for medicinal plants, as indicated by the examples above, is not accompanied by conservation measures or sustainable exploitation. This is a world-wide trend: for example, Rifai and Kartawinata (1991) reported from Indonesia that in order to satisfy the demand of one big Swiss pharmaceutical company, which had requested eight tonnes of seeds of *Voacanga grandifloria* (an endangered species with very light seeds), all available seeds in the forest would have to be harvested, leaving nothing for regeneration. Furthermore, the low prices paid for the plants do not cover replacement or resource management costs, and as such, there is a continuous decline of medicinal plant species in developing countries generally, including those in Africa.

A number of medicinal plant species stand out as having declined dramatically as a result of exploitation and trade. In South Africa, *Mondia whitei* has disappeared from the Durban area, where it had been recorded before 1900, while *Siphonochilus natalensis* has disappeared from its only known localities in the Inanda and Umhloti localities (Cunningham, 2000). By 1938, all that could be found of *Warburgia salutaris* in Natal and Zululand was described as "poor coppices, every year cut right to the bottom" (Gerstner, 1938), and this species is now endangered (Scott-Shaw, 1999).

Table 1.2 The quantities of the herbal medicines sold annually in the largest quantity (in standard 50 kg maize bags) by 54 herb traders in the KwaZulu Natal region, South Africa (Cunningham, 1988).

Species	Part used	Quantity sold (50-kg bags)
Scilla natalensis	Bulb	774
Eucomis autumnalis	Bulb	581
Alepidia amatymbica	Root	519
Adenia gummifera	Stem	459
Albizia adianthifolia	Bark	424
Cilvia miniata	Bulb	397
Clivia nobilis	Bulb	397
Pentanisia prunelloides	root	343
Senecio serratuloides	leaves/stem	340
Gunnera perpensa	Root	340
Rapanea melanophloeos	Bark	327
Dioscorea sylvatica	whole plant	326
Warburgia salutaris	Bark	315
Bersama sp.	Bark	295
unidentified sp.	Root	288
Kalanchoe crenata	leaves/stem	284
Boweia volubilis	Bulb	257
Trichilia emetica (& T. dregeana)	Bark	252
Turbina oblongata	Root	249
Rhoicissus tridentata	Root	244
Bulbine latifolia	Bulb	240
Ocotea bullata	Bark	234
Stangeria eriopus	root	233
Cryptocarya sp.	Bark	228
Anemone fanninii	Root	227
Eucomis sp. cf. bicolor	Bulb	224
Rhus chirindensis	Bark	222
Helinus integrifolius	Stem	222
Schotia brachypetala	Bark	220
Vernonia neocorymbosa	leaves/stem	216
Dioscorea dregeana	whole plant	212
Ornithogalum longibracteatum	Bulb	208
Erythrophleum lasianthum	Bark	201
Solanum aculeastrum	Fruit	198
Curtisia dentata	Bark	197

Table 1.3 Some details of African indigenous medicinal plants that are harvested as a source of active ingredients for export purposes. All the species are also used for traditional medicine in the source-countries. (from Cunningham, 2000).

Species	**Part used**	**Active ingredients**	**Source-country**
Corynanthe pacyceras	Bark	Corynanthine Corynathidine Yohimbine	Ghana
Griffonia simplicifolia	Seed	BS11 lectin	Cameroon Côte d'Ivoire Ghana
Harpagophytum procumbens	Root	Glucoiridoids	Namibia
Harpagophytum zeyeris	Root	Glucoiridoids	Namibia
Hunteria eburnea	Bark	Eburine and other alkaliods	Ghana
Jateoriza palmata	Root	Palmitrin Colambamine	Tanzania
Physostigma venenosum	Fruit	Physiostigmine	Côte d'Ivoire
Prunus africana	Bark	Sterols Triterpines n-doconasol	Cameroon Kenya Madagascar
Rauvolfia vomitiora	Root	Reserpine Yohimbine	Congo, DR Mozambique
Voacanga africana	Seed	Voacamine	Cameroon Côte d'Ivoire Ghana
Voacanga thouarsii	Seed	Voacamine	Cameroon

The situation is similar in other countries where *W. salutaris* is found: in Kenya, some of the largest *W. salutaris* trees were reported to be ring-barked and dying in 1991 (Kokwaro, 1991), while in Zimbabwe, all that remained of wild populations of this species a decade ago were a few coppice shoots (Cunningham, 2000). Other medicinal plant species that are now endangered or vulnerable to extinction include *Ocotea bullata, Bowiea volubilis, Haworthia limifolia, Scilla natalensis, Albizia suluensis* and *Begonia homonyma* (Scott-Shaw, 1999; Table 1.4).

Table 1.4 The conservation status of some traditional medicinal plant species in selected African countries (after Cunningham, 2000).

a) COTE D'IVOIRE

Extinct in the wild

- *Monanthotaxis capea* (Annonaceae) - aromatic leaves used for washing for cosmetic purposes.

Vulnerable and declining

- *Garcinia afzelii* (Clusiaceae) - favoured and important source of chewing sticks in Ghana, Côte d'Ivoire and Nigeria
- *Okoubaka aubrevillei* (Oknemataceae) - used symbolically to ward off evil spirits. Potent allelopathic effect on most surrounding plants. Endemic family to Guineo-Congolian region. Potential source of new and interesting organic compounds.

b) ZIMBABWE

Endangered

- *Warburgia salutaris* (Canellaceae) - only known at present from a few small coppice shoots found in the Mhangura Forest, Eastern Highlands, Zimbabwe where it was heavily exploited for commercial purposes (national trade to urban centres and rural TMPs) as well as by guerillas sheltering in the forests during the war, as access to pharmaceutical medicines was very limited. Bark is used as a panacea for all ills, and specifically for headaches, abdominal pains, an abortifacient and to treat venereal disease. Widely acknowledged to be scarce, and probably the most expensive traditional medicine sold in Zimbabwe.

Vulnerable and declining

- *Alepidea amatymbica* (Apiaceae) - very limited distribution in Zimbabwe (a few localities in the eastern Highlands, yet sold in small quantity at many markets, where it is widely acknowledged to be becoming scarce. Although this species is heavily exploited in South Africa, leading to local disappearance of this resource in some cases, it is far more widespread here than in Zimbabwe.

c) SWAZILAND

Vulnerable and declining

- *Warburgia salutaris* (Canellaceae) - used for coughs, colds, upset stomach and as a snuff for headaches.
- *Alepidea amatymbica* (Apiaceae) - used for coughs and colds.
- *Siphonochilus aethiopicus* (Zingiberaceae) - used for coughs and colds, as well as for protection against lightning.

All of the above species are heavily exploited for local use as well as in response to the urban demand in South Africa. According to local herbalists, *Siphonochilus aethiopicus* has disappeared from known localities outside Malolotja Nature Reserve, Swaziland.

d) SOUTH AFRICA

Extinct in the wild

- *Siphonochilus natalensis* (Zingiberaceae)
- *Siphonochilus aethiopicus*

- **Endangered**
- *Warburgia salutaris* (Canellaceae) - used for coughs, colds, as a snuff for headaches (powdered bark mixed with bark from *Erythrophleum lasianthum* (Fabaceae).

Vulnerable and declining

- *Dioscorea sylvatica* (Dioscoreaceae) - tuber used as a douche for swollen udders of cattle, for chest complaints and for magical purposes.
- *Bersama tysoniana* (Melianthaceae) - bark used by diviners together with saponin- rich species such as *Helinus integrifolius* in an *ubulawu* mix to enable them to interpret dreams clearly.
- *Ocotea bullata* (Lauraceae) - used for symbolic purposes to make a person "smell and become unpopular".
- *Ocotea kenyensis* (Lauraceae) - use as above.
- *Curtisia dentata* (Cornaceae) - red-coloured bark used for magical purposes. Use unknown and kept very secret.
- *Pleurostylia capensis* (Celastraceae) - use unpublished.
- *Faurea macnaughtonii* (Proteaceae) - bark used to treat menstrual pains, also for tuberculosis.
- *Loxostylis alata* (Anacardiaceae) - use not recorded.
- *Mystacidium millarii* and *capense* (Orchidaceae). Commonly not distinguished by herbalists as different. Both species (and many other epiphytes) used for symbolic purposes.
- *Ledebouria hypoxidoides* (Liliaceae) - bulbs used to prepare enemas.

As a successor to sustainable traditional management practices, one of the main strategies used in protecting medicinal plants from over-exploitation is legislation. However, this has been largely ineffective in controlling the use of medicinal plants in Africa. Under colonial administration, religious therapy systems practised by traditional healers were equated with witchcraft and legislated against almost everywhere in Africa (Gerster, 1938; Staugard, 1985; Cunningham, 1990). In South Africa, there were also attempts to prohibit the sale of traditional medicines within urban areas such as the efforts made by the Natal Pharmaceutical Society in the 1930s in Durban (Cunningham, 2000). However, measures had only the undesirable effect of driving the commerce in medicinal plants underground.

Forest legislation in most African countries generally recognizes the importance of customary usage rights (including the gathering of medicinal plants) and conservation land or certain plant species are set aside for strict protection (Schmithusen, 1986). In South Africa, for example, forestry legislation was promulgated in1914 for the protection of economically important timber species such as *Ocotea bullata,* and specially protected status has been given since 1974 to all species within the families Liliaceae, Amaryllidiaceae and Orchidaceae due to their prominence in the herbal medicine trade. The KwaZulu-Natal Nature Conservation Bill, as amended in 1999, added *Warburgia salutaris*, *Encephalartos cerinus* and *Ocotea bullata* to this list. This legislation prevents the exploitation and sale of specially protected species, the exploitation of indigenous plant species in State forests, and hawking of the specified medicinal plants.

At best, the legislation described above has merely slowed down the rate of harvesting. Extensive exploitation within forest reserves still occurs in South Africa. In KwaZulu-Natal where legislation prohibits protected indigenous plants (many of which are medicinal) from being imported into, or exported from, the province, it is known that these plants are gathered from outside its borders and illegally transported into markets where they are sold (Hannweg, 1995). Other studies have reported that legislation has failed to achieve the objective of protecting or conserving medicinal plants (e.g. Cunningham, 1988).

Other measures that have been used for conservation include *in situ* approaches such as the establishment of core conservation areas (CCAs) [Cunningham, 2000] and ethnobotanical reserves, which often concentrate on a "holding action" to maintain the *status quo* without providing viable alternatives to collecting customary plants; and *ex situ* approaches such as establishment of plantations or conservation stands. However, these measures, besides being costly, remain dependent on legal regulation to be effective, and there is neither a guarantee of the safety of a plant in even the best-protected nature reserve, nor information on the extent to which reserves conserve populations of indigenous plant species in countries like S. Africa (Siegfried, 1989).

On the other hand, one of the most cost-effective and efficient means of the conservation of plant germplasm is seed storage, used in nearly 90% of the germplasm accessions maintained worldwide (Engels and Engelmann, 1988).

1.3 The Application of seed storage in the conservation of plant germplasm

Seed storage offers the safest and the least costly means of plant germplasm conservation (Withers, 1988). Stored seeds do not replace wild populations, but act as an "insurance policy", especially if a taxon is facing extinction. Seed banks are very efficient and cost-effective because the seed occupies very little space and requires only periodic attention. Furthermore, each seed represents a potential, genetically unique plant, and therefore genetic diversity within a given population or species can be conserved via seeds.

The period for which seeds can be stored without losing viability depends on storage temperature, relative humidity and seed moisture (Ellis and Roberts, 1980). The storage life of seed also varies greatly with species, with orthodox seeds being storable for long, predictable periods under defined conditions including low temperatures and seed water contents (Roberts, 1973). Unless debilitated by xerotolerant storage fungi, seeds stored under such conditions should maintain high viability and vigour at least from harvest

until the next growing season (Berjak *et al.*, 1989), or for many decades at –18°C (IBPGR, 1976).

Because of their amenability to long-term storage, orthodox seeds have been widely utilised in, and form the basis of, the establishment of seed-banks and gene-banks. Around the world there are scattered Tree Seed Centres, which aim, *inter alia*, to keep representative seed samples as germplasm base collections. The Southern African Development Community (SADC) Plant Genetic Resources Centre (SPGRC, outside Lusaka, Zambia) for example, collects and maintains triplicate seed samples of indigenous plant populations, with one sample located at a National Plant Genetic Resources Centre in the country where it was collected, a second sample kept as a base collection at the SPGRC headquarters in Zambia, and a third sample kept as a safety collection outside the region (SPGRC, 2002). In Kenya, the Kenya Forestry Seed Centre maintains seed samples of over 250 species found in that country (Albrecht, 1993), while similar facilities exist other African countries such as Burkina Faso (Bancé, 1993); in other tropical countries such Australia (Australian Tree Seed Centre, online), and Latin America (Tropical Agriculture Research and Higher Learning Center [CATIE] Tree Seed Bank in Costa Rica [CATIE, online]); and in Europe (e.g. DANIDA Forest Seed Centre, Denmark (DANIDA, online).

The importance of seeds in plant germplasm conservation is evidenced by what might be described as the most significant conservation initiative ever undertaken (Time Magazine, Jan 17, 2000): the Millennium Seed Bank (MSB) of the Royal Botanic Gardens, Kew, at Wakehurst Place, in Ardingly, West Sussex, England, built at a cost of US$ 130 million. The MSB aims to collect and conserve the germplasm of 10%, of the world's seed-bearing flora (over 24 000 species), and to conserve seeds of the entire UK native seed-bearing flora (Royal Botanic Gardens, online).

The processes involved in seed banking as described above include seed collection, drying, viability testing, and storage at low temperatures (-20°C in the case of the MSB [Royal Botanic Gardens, online]). However, this procedure is applicable only to orthodox

seeds (Roberts, 1973), which acquire desiccation tolerance during development (Pammenter and Berjak, 1999). Non-orthodox seeds, described as either recalcitrant (Roberts, 1973) or intermediate (Ellis *et al.*, 1990), are not storable under the same regimen as orthodox seeds. For recalcitrant seeds, this is because the seeds remain desiccation-sensitive during development and after shedding, may be chilling-sensitive, and are short-lived (Farrant *et al.*, 1988; Berjak and Pammenter, 2001); whereas intermediate seeds, although relatively tolerant to desiccation, lose viability relatively rapidly if stored at the lowered water contents (Ellis *et al.*, 1991), and some will not tolerate low temperatures, especially when hydrated (Hong and Ellis, 1996).

The limitation of conventional seed banking practices to orthodox seeds poses problems in the conservation of many African medicinal plants, such as those of the species investigated in this study, because they produce non-orthodox seeds. For many such species, the seeds can be stored optimally only at relatively high temperatures, e.g. 15 °C for *Hopea odorata* and *Mangifera indica* (Corbineau and Côme, 1988). This encourages lethal fungal proliferation, as recalcitrant seeds have been shown to harbour a spectrum of surface and internal fungal contaminants at harvest (Mycock and Berjak 1990; Calistru *et al.*, 2000). In an effort to control this contamination, fungicides have been used. Coating seeds of *Hevea brasiliensis* with 0.3% (w/w) Benlate® (active ingredient: methyl 1-(butylcarbamoyl)-2-benzizimidazolecarbamate; Dupont, Wilmington, Delaware, USA) has been found to extend the longevity from 3 to 12 months (Chin, 1988). Such a treatment is, however, ineffective if the mycoflora is internal, as is often the case with recalcitrant seeds.

A further problem if fungal proliferation is controlled is that the seeds germinate at the relatively high storage temperatures since they are sufficiently hydrated, and, since the conditions in storage are not appropriate for subsequent seedling growth, they ultimately die. In an attempt to overcome precocious germination, growth-inhibiting hormones have been applied to seeds prior to storage. While some studies have reported no improvement in longevity of seeds after treatment with abscisic acid (e.g. Mumford and Brett, 1982), Goddard (1995) reported that the application of ABA in an alginate gel coating had a

significant effect in extending the storage lifespan of the seeds of *Avicennia marina*. However, subsequent studies by Motete *et al.* (1997) indicated that the extension of the lifespan of these seeds could be achieved by application of the gel alone, with the beneficial effects being ascribed to its fungicidal/fungistatic effects. This extension was in the order of only a few weeks and, although significant for these short-lived seeds, would not be applicable for long-term storage.

The only method currently considered feasible for the long-term conservation of non-orthodox seed germplasm is cryopreservation (Stanwood, 1985; Berjak *et al.*, 1999).

1.3.1 The Application of cryopreservation in the conservation of non-orthodox seed germplasm

Cryopreservation describes the storage of living biological material at ultra-low temperatures, normally at, or near, -196°C, the temperature of liquid nitrogen (Withers, 1988). It may be applied to either sexually-produced or vegetative propagules, depending on the purpose of conservation. For wide genetic representability in storage, cryostorage of sexual propagules is most appropriate (Krøgstrup *et al.*, 1992). Successful protocols have been developed for achieving, for example, the cryopreservation of whole seeds and embryos of *Azadirachta indica* (Berjak and Dumet, 1996), and of excised embryonic axes of *Camellia sinensis* (Wesley-Smith *et al.*, 1992); peach (Deboucaud, *et* al., 1996) and *Quercus robur* (Berjak *et al.*, 1999). In general, unless intact seeds can survive substantial dehydration (which is rarely the case with non-orthodox seeds), successful freezing demands the use of excised embryonic axes.

However, seeds are not always available, and, in fact, for the species under investigation in the present study (*T. dregeana*, *T. emetica* and *W. salutaris*), seed production was highly unpredictable from year to year. Although the use of axes excised from seeds is imperative in terms of conservation of genetic diversity, the genetic recombinations associated with seed production may be undesirable in breeding programmes, while new genotypes may also need to be conserved before plants reach reproductive maturity. In such cases the use of vegetative propagules for conservation is obligatory.

This can be done by the cryopreservation of somatic embryos, as has been achieved for several species (Dumet *et al.*, 1993; Mycock and Berjak, 1993; Mycock *et al.*, 1995); or cell suspension cultures, used successfully for cryopreservation and subsequent regeneration of *Citrus deliciosa* (Aguilar *et al.*, 1993). Shoot-tips have also been successfully cryopreserved, e.g. for chicory, *Cichorium intybus* (Demeulemeester *et al.*, 1993), and *Solanum tuberosum* (Harding and Benson, 1994). Other examples of the successful cryopreservation of plant germplasm are listed in Table 1.5.

Regardless of the material used, successful cryopreservation, depends on several parameters. These include:

(a) The development of appropriate *in vitro* systems, depending on the material to be cryopreserved. In most instances where germplasm of species producing recalcitrant seeds is to be conserved via sexual propagules, excised embryonic axes would be the explants of choice. This is because whole seeds are relatively large, cannot be suitably rehydated (see later), and would cool far too slowly during the freezing step of cryopreservation, with consequent lethal damage. The embryonic axes of most species, constituting only an insignificant fraction of the seed mass/volume, are more amenable to successful freezing. Thus, an optimal *in vitro* germination medium is essential to assess the viability of excised axes prior to, and following, cryopreservation, and to produce plants from the cryopreserved explants. For the cryopreservation of vegetative material, e.g. shoot tips or somatic embryos, appropriate micropropagation techniques are a prerequisite in order to regenerate and multiply plants from the cryopreserved material.

Table 1.5. Examples of plant species in which cryopreservation techniques have been developed in the last five years. csc, cell suspension cultures; m, meristems; se, somatic embryos; sh, shoot tips; ea, embryonic axes; c, callus; p, protoplasts; s, seeds.

Species	Explant frozen	Reference
Allium sativum (garlic)	sh	Makowska *et al.* (1999).
Asparagus officinalis	csc	Suzuki *et al.* (1997).
Armoracia rusticana (horseradish)	sh	Phunchindawan *et al.* (1997).
Artocarpus heterophyllus (jackfruit)	ea	Thammasiri (1999).
Brassica napus (oilseed rape)	se	Li *et al.* (1999).
Aesculus hippocastanum (horse chestnut)	ea	Wesley-Smith *et al.* (2001b).
Beta vulgaris	sh	Vandenbussche *et al.* (1999).
Chrysanthemum cinerariaefolium	sh	Hitmi *et al.* (1999).
Colocasia esculenta (taro)	sh	Takagi *et al.* (1997).
Cornus florida	p	Craddock *et al.* (2000).
Dendrobium candidum	s	Wang *et al.* (1997).
	sh	Mandal *et al.* (1996).
Doritis pulcherrima	s	Thammasiri (2000).
Hevea brasiliensis (rubber)	c	Engelmann *et al.* (1997).
Holostemma annulare	sh	Decruse *et al.* (1999).
Ipomoea batata (sweet potato)	se	Blakesley *et al.* (1997).
Iris nigricans	se	Shibli (2000).
Malus pumila (apple)	sh	Wu *et al.* (1999).
Manihot esculantum (cassava)	sh	Escobar *et al.* (1997).
Medicago sativa (alfalfa)	c	Vandenbussche and Deproft (1998).
Mentha picata (mint)	sh	Hirai and Sakai (2000).
Moricandia arvensis	csc	Craig *et al.* (1997).
Musa sp. (banana)	sh	Thinh *et al.* (1999).
Olea europaea Var. Arbequina	sh	Martinez *et al.* (1999).
Oryza sativa (rice)	c	Watanabe *et al.* (1999).
Picea glauca	se	Bomal and Tremblay (2000).
Pinus sylvestris (Scots pine)	c	Haggman *et al.* (1999).
Pinus patula	se	Ford *et al.* (2000).
Populus alba	sh	Lambardi *et al.* (2000).
Prunus domestica	sh	Decarlo *et al.* (2000).
Prunus ferlenain	sh	Helliot and Deboucaud (1997).
Solanum tuberosum (potato)	sh	Grospietsch *et al.* (1999).
Saccharum officinarum (sugarcane)	sh	González-Arnao *et al.* (1998).
Wasabia japonica (wasabi)	s	Potts and Lumpkin (1997).
Zizania palustris	ea	Touchell and Walters (2000).

(b) Establishment of appropriate freezing protocols, which must take into account the following:

(i) a suitable **water content** for successful cryopreservation. Embryonic axes of recalcitrant seeds are hydrated and metabolically active, as are other potential explants. However, exposure of tissue with too high a moisture level to sub-freezing temperatures may result in lethal damage. At the cellular level, the damage is either mechanical, in which case intracellular ice crystals are formed, resulting in cell membrane and organelle damage; or biochemical, which occurs as a result of differential precipitation of electrolytes and subsequent shifts in pH and plasmolysis (Meryman, 1966). When ice is formed in the intercellular spaces, the growing ice nucleation sites draw water out of solution, making the (extracellular) solution more concentrated and reducing its water potential. Water then moves from the cells to the extracellular space, leading to cell dehydration. To avoid freezing injury, the moisture content must be as low as is possible without jeopardizing the viability of the tissue, thus the intracellular osmotic potential will be high. The high concentration of solutes lowers the freezing point of the intracellular fluid, and this can lessen the danger of freezing injury. The necessary dehydration/procedures must be optimised to avoid – or at least minimise – the deleterious effects outlined above.

(ii) The **rate of freezing.** The damage caused by freezing is envisaged to depend on the rate of cooling (Mycock *et al.*, 1991; Wesley-Smith *et al.*, 1992, 2001b). With rapid cooling, there is insufficient time for ice crystals to form, and the solution ideally becomes vitrified to form a glass, avoiding the crystallised state, or, if crystals do form, the time factor restricts their growth to only non-injurious dimensions (Wesley-Smith, 2002). Hence, the damage caused by freezing should be avoided by employing rapid cooling (Wesley-Smith *et al.*, 1992).

(iii) **Cryoprotection.** In order to reduce freezing injury in hydrated tissues, cryoprotectants have been widely used. These substances depress both the freezing and super-cooling points of water, and thus reduce the amount of water removed as result of dehydration due to ice formation (Kartha, 1985). Even though cryoprotectants may be toxic to plant tissues depending on the type and

concentration and the plant species (Kartha, 1985) they have been used successfully in particular cases. A glycerol/sucrose cryoprotectant solution has been suggested to facilitate vitrification in hydrated pea axes, without harming the cells and tissues (Mycock *et al.*, 1991), and has also been successfully used to facilitate cryopreservation of somatic embryos of *Phoenix dactylifera* (Mycock *et al.*, 1995).

These factors, which are mentioned only cursorily above, are elaborated in detail in chapters 2-5.

1.4 Species under investigation in this study

1.4.1 *Trichilia dregeana* Sond.

Trichilia dregeana (Meliaceae), known commonly as the Forest Mahogany (English) or *umKuhlu* (Zulu), is an evergreen forest tree with a very dense crown, found in high-rainfall coastal forests on the eastern coast of Africa, from the Eastern Cape Province in South Africa, to Swaziland, and northwards to tropical Africa (Albrecht, 1993; Pooley, 1993). It flowers in October-December to produce whitish-green, velvety, sweet-scented flowers in dense bunches amongst the leaves (Fig. 1.1). Fruit development is completed within about 6 months, with maturity in March/May. The fruits are roundish capsules up to 30 mm in diameter, ripening brown and splitting into three segments to reveal six black seeds, each almost completely covered by a scarlet aril (Fig. 1.1).

Throughout its ecological range, *Trichilia dregeana* is a multi-purpose tree, the uses of which have been documented since the 1800s, when wood from this tree was used to repair ships entering Durban harbour (Pooley, 1993). The wood is also used for fuel, timber and furniture production, the leaves for fodder, and the bark and roots as herbal medicine against stomach complaints and headache (Coates-Palgrave, 1981). In Zimbabwe, the bark is used as a purgative, for procuring abortions and as fish poison (Gelfand *et al.*, 1985), while the leaves are used in the treatment of syphilis in Nigeria (Desta, 1993).

Chemical studies have yielded five limonoids from the seeds (Mulholland and Taylor, 1980), and aqueous leaf extracts show some antimicrobial activity (Desta, 1993).

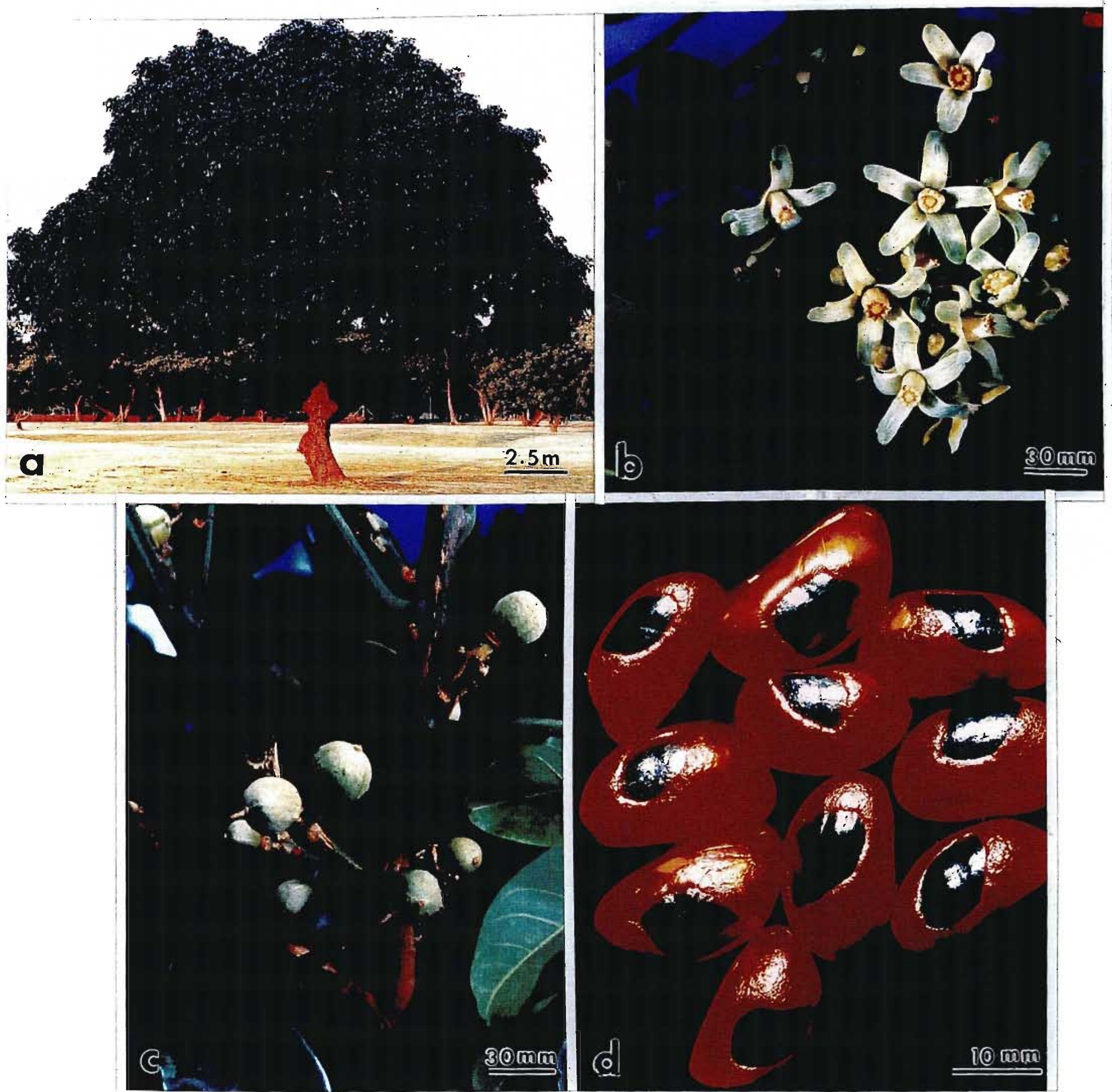

Fig. 1.1 *Trichilia dregeana* tree (a), showing flowers (b), roundish fruits with an indented tip and no neck (c), and mature seeds with a black testa almost completely covered by a scarlet aril (d).

The tree is cultivated as a beautiful ornamental plant in parks, avenues, and gardens (Albrecht, 1993), and has been widely cultivated as a street tree in Durban, South Africa. The flowers provide forage for bees and butterflies, with several species of butterfly breeding on this tree, including the white barred *Charaxes* (Pooley, 1993). The seeds are eaten by birds and people, and used to produce oil for soap and cosmetics. Since they contain up to 65% lipid by weight (FAO, 1983), the species is a potential oil-crop.

Even though no inventory is available, it appears that the wild populations of this species are dwindling. The observations and enquiries made during this study did not yield any wild populations of this species locally, with the only available samples being those planted alongside city avenues and parks for ornamental purposes.

The seeds of this species were originally reported to be recalcitrant and chilling-sensitive Choinsky (1990).

1.4.2 *Trichilia emetica* Vahl.

Trichilia emetica is the second of only two species of *Trichilia* found in S. Africa. It is commonly known as the Natal Mahogany (English) and has the same Zulu name as *T. dregeana* (umKuhlu). The physical resemblance between *T. emetica* and *T. dregeana* is striking, and has led to many workers using the species names interchangeably. However, the two species can be distinguished by the morphology of leaves and fruits. The leaves of *T. emetica* have brownish hairs beneath (especially on the veins), and have a rounded tip where they are widest. In contrast, the leaves of *T. dregeana* are almost hairless and have a pointed tip, being widest in the middle and tapering towards the tip. The fruits of *T. emetica* are roundish, 10-25 mm in diameter, and have a distinct neck (Fig. 1.2) and a pointed/beaked tip, unlike those of *T. dregeana*, which have no neck, and have an indented tip (Fig. 1.1).

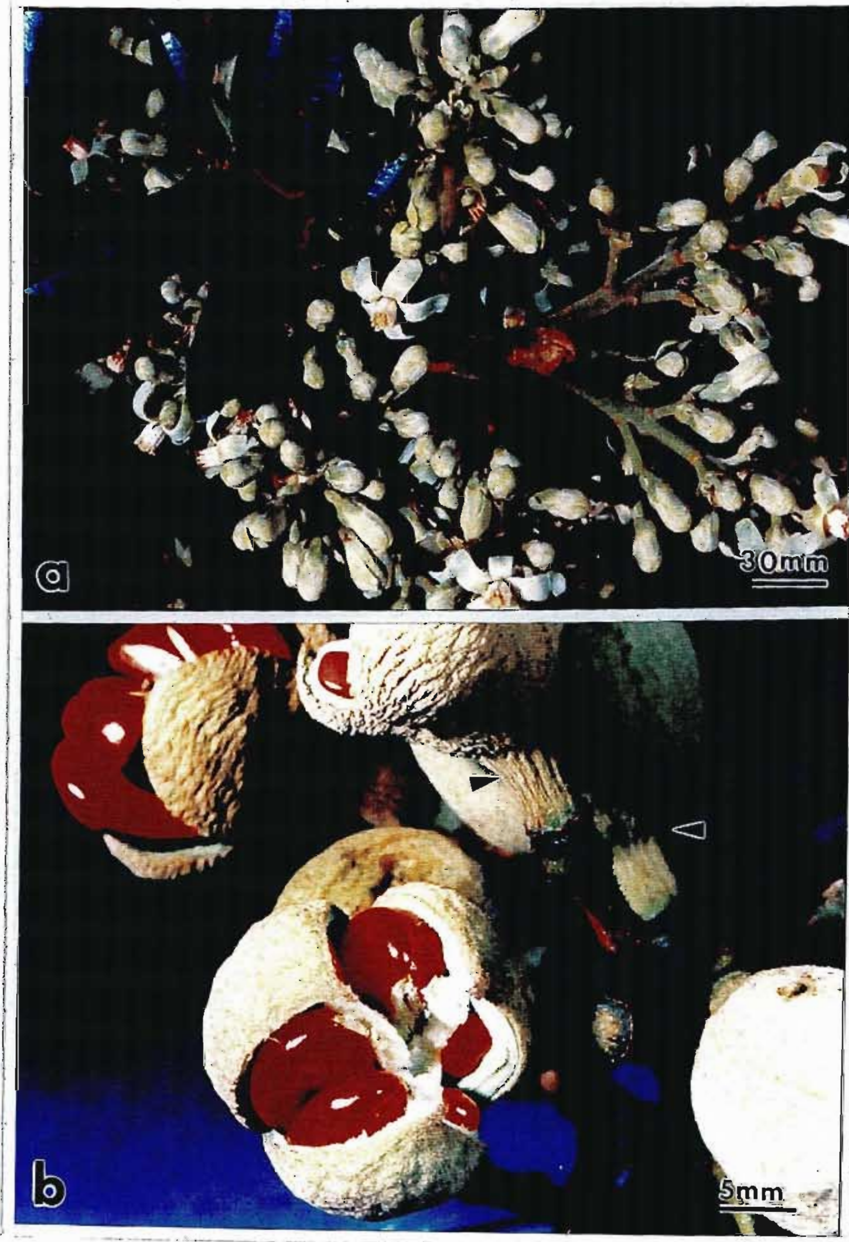

Fig. 1.2. (a) Flowers of *T. emetica*. Note that the leaves of *T. dregeana* (Fig. 1.1b, c), are widest in the middle and taper sharply towards the base, unlike those of *T. emetica*, shown here. (b), Fruits of *T. emetica*, showing a narrow neck (arrow-heads) connecting the fruit to the stalk. The mature seeds closely resemble those of *T. dregeana* (Fig. 1.1d)

T. emetica occupies largely the same ecological area as *T. dregeana*, being found in forests along the Natal coast, and northwards to tropical Africa. Seed production culminates in mature seeds in January and February. The fruits are eaten by baboons, monkeys and antelope, and the seeds by birds (Pooley, 1993). That author reports that sunbirds feed on the nectar, monkeys eat the flowers, and the leaves are browsed by game.

The seeds are soaked in water and the 'soup' eaten with spinach by local populations, while oil extracted from the seeds is used cosmetically, and in some countries (e.g. Tanzania) the seeds are a major commercial resource as they provide the raw material for soap factories (Msanga[1], pers. comm.). This is based on the high lipid content of the seeds, which is between 58 and 68%, comprising 55% oleic and 45% palmitic esters (Watt and Breyer-Brandwijk, 1962). Those authors report that in herbal medicine, the powdered bark is used as an emetic (hence *emetica*) and as enemas; and infusions of the bark or leaf are used for lumbago, rectal ulceration in children, and dysentery. Enemas made from the bark are administered for kidney ailments, as stomach and blood cleansers, and also for intestinal worms by the Vhavenda in northern S. Africa (Mabogo, 1990).

The active ingredients isolated from the bark include a large number of limonoids and trichilin A (van Wyk *et al.*, 1997). Such compounds have been reported to have insect anti-feedant properties as well as anti-microbial and anti-inflammatory activity (Bruneton, 1995). Other studies have indicated that the purgative effects of the bark may be due to the resin content (Jamieson, 1916), while the rootbark contains a bitter principle, related to calicedrin, which is reputed to have anti-malarial properties (Oliver-Bever, 1986).

Besides its medicinal uses, the bark is also used to produce a pinkish dye, and the wood for carvings, musical instruments, household implements, furniture, boats and canoes

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[1] H.P. Msanga, National Tree Seed Centre, Morogoro, Tanzania.

(Pooley, 1993). The tree is also a popular ornamental species, valued for shade and planted around homesteads and public places.

Trichilia emetica is not considered threatened, although it is highly utilised for herbal medicine (more than 12 tonnes of bark are traded annually in Durban alone [Table 1.2]). As was the case with *T. dregeana*, no wild populations of *T. emetica* were encountered in the course of this study.

There is little published information on the seed behaviour of this species, but the seeds have been categorised as recalcitrant (Maghembe and Msanga, 1989; Msanga, 1998) and unstorable (Albrecht, 1993).

1.4.3 *Warburgia salutaris* (Bertol.f.) Chiov

W. salutaris (Cannellaceae) is the only species of the genus *Warburgia* in S. Africa, and is a synonym with *W. ugandennis* and *W. breyeri* (Pooley, 1993). Known commonly as the pepper-bark tree (English) or *isiBaha* (Zulu), it is a medium-sized tree (4 – 8 m) in Southern Africa (Pooley, 1993), becoming larger towards tropical Africa, where it attains heights of up to 42 m (Albrecht, 1993).

The fruits, produced during October – December, are shiny-green, slightly pear-shaped, and covered with gland dots. Tasting a scrap of fruit, leaf, or bark gives a spicy peppery sensation, from which the species gets its English name. The bark has been shown to contain numerous sesquiterpenoid dialdehydes such as warburganal, muzugadial, polygodial, mukaadial and ugadensidial (Warthen *et al.*, 1983; Taniguchi and Kubo, 1993), as well as mannitol (Watt and Breyer-Brandwijk, 1962). Warbuganal is a potent anti-feedant against the African army worm, *Spodoptera eempta*, is cytotoxic, and has haemolytic and molluscicidal properties (Hutchings, 1996). Warburganal, muzugadial and polygodial show broad antimicrobial activity against various yeasts and a variety of other fungi, and are highly active particularly against *Saccharomyces cerevisae*, *Candida utilis* and *Sclerotinia libertiana* (Taniguchi and Kubo, 1993). According to those authors, polygodial showed the

most potent antimicrobial activity against these fungi, displaying activity comparable to that of amphotericin B, and was found to enhance significantly the antifungal activities of actinomycin D and rifampicin and also to synergise the antifungal activity of maesanin against *Candida utilis*.

As a result of these properties, *W. salutaris* is one of the most widely-used medicinal plants in Africa. It is indicated, in the many areas of Africa where it grows, for respiratory problems such as coughs, colds, and chest complaints; as well as numerous other complaints such as influenza, rheumatism, venereal disease, malaria, toothache, gastric ulcers, and constipation (Watt and Breyer-Brandwijk 1962; Hutchings and van Staden, 1994; Hutchings, 1996; Johns *et al.*, 1990).

The high demand for the bark of *W. salutaris* has resulted in over-exploitation, and, even though the species is specially protected by law in S. Africa, it remains highly endangered (Scott-Shaw, 1999).

There have been efforts to propagate the plant cuttings (e.g. Fig 1.3) in an attempt to supplement the remnants of wild populations (Esterhuyse, 1996; Scott-Shaw, 1999; Symmonds and Crouch, 2000), but such are hampered by a lack of seeds from the resulting plants. Most fruits produced abort early, and the remaining ones are parasitised by fungi and insect larvae and predated on by birds and primates, with the result that in some countries (e.g. S. Africa) mature seed production in the wild is unknown (Scott-Shaw, 1999). That author also reports that no seedlings have been observed in the wild in S. Africa, and that of the only 11 sub-populations known around the country, only two exceed 20 individuals. Due to poor seed production, these populations are thought to consist of sucker-produced ramets, consisting of just one clone each.

When seeds are available, there is little information on the post-shedding physiology of the seeds, which are considered recalcitrant (Albrech, 1993; Msanga, 1998).

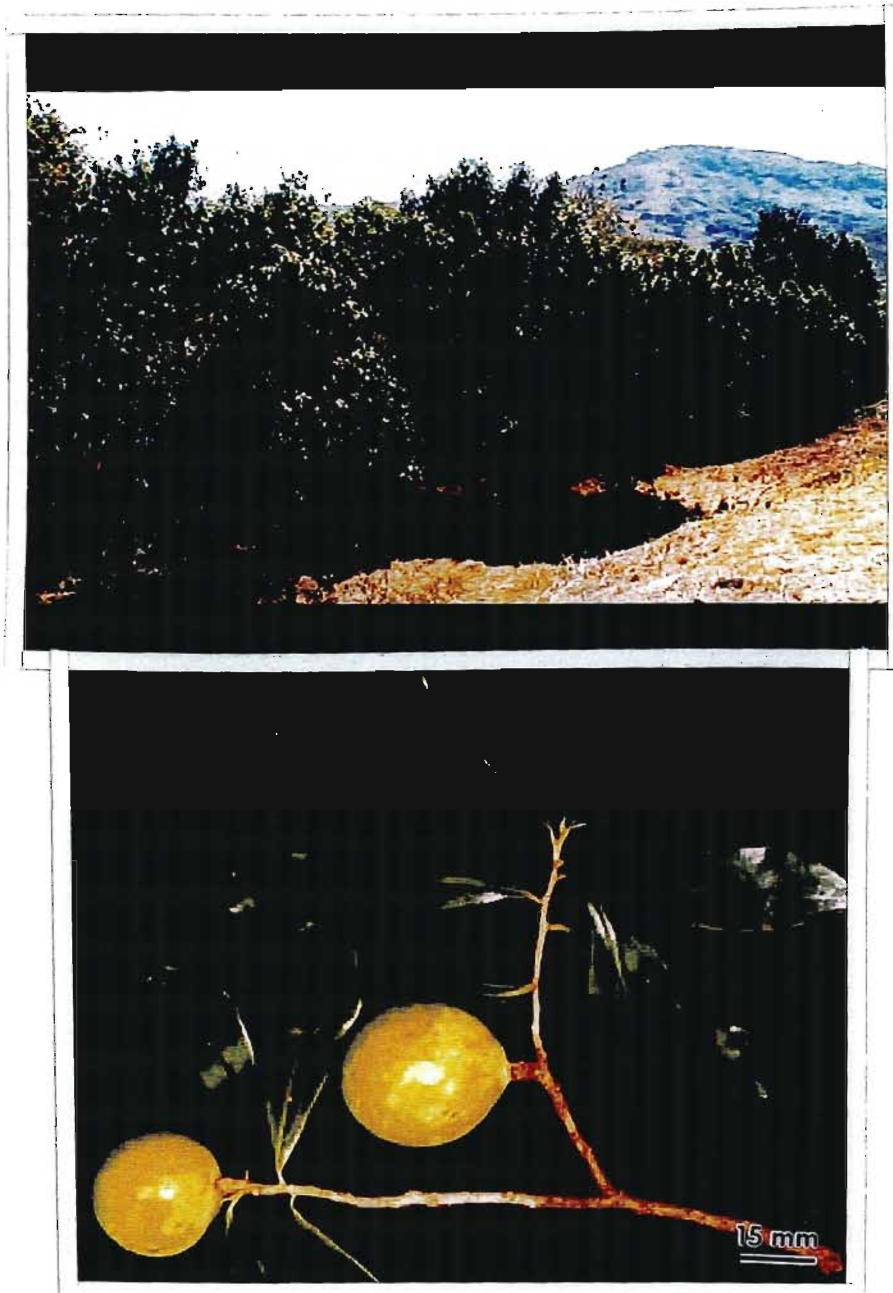

Fig 1.3. (a) Trees of *W. salutaris*, obtained from cuttings planted at the Silverglen medicinal plants nursery, Chatsworth, Durban. Even though these trees are planted in protected sanctuary, seed production is still effectively precluded by predation of young fruits by primates, birds and insects, and it was not possible to obtain sufficient seeds of high quality from these trees, during this study. (b) Fruits of *W. salutaris*.

1.5 Objectives of this study

The objectives of this study were two-fold: to elucidate aspects of the post-shedding physiology of the seeds of three medicinal plants indigenous to South Africa - *Trichilia dregeana, T. emetica* and *Warburgia salutaris* - and to establish protocols for cryopreservation of the germplasm of these species. This is expected to provide viable means of long-term storage and conservation of the germplasm of these threatened species, which is at the moment impossible, via cryopreservation.

CHAPTER 2:

Aspects of post-shedding physiology of the seeds of *Trichilia emetica, T. dregeana* and *Warburgia salutaris.*[#]

2.1 Introduction

Based on their post-harvest behaviour, seeds have been classified as being either orthodox or recalcitrant (Roberts, 1973), and this categorisation has persisted, augmented by a third, later-described category of seeds referred to as intermediate (Ellis *et al.*, 1990).

As defined by Roberts (1973) orthodox seeds are characterised by the ability to withstand a high degree of desiccation and to remain viable for predictably long periods under conditions of low temperature and low water contents. Seeds which are recalcitrant, on the other hand, are damaged by dehydration below a relatively high water content, are often chilling-sensitive, and are generally short-lived and unstorable for useful periods (Roberts, 1973; Chin and Roberts, 1980). Seeds categorised as showing intermediate post-harvest behaviour are relatively desiccation-tolerant, but do not remain viable for long periods at reduced water contents. Such seeds, particularly if they are of tropical origin, may also be chilling-sensitive (Ellis *et al.*, 1990; Hong and Ellis, 1996).

The post-shedding behaviour of the seeds of many species may not, however, clearly fit into the discrete categories described above, and a continuum of seed behaviour has therefore been suggested (Pammenter and Berjak, 1999). Even within the recalcitrant category, there are marked differences in the post-harvest responses of seeds of individual species, leading to the recognition of highly-, moderately- and minimally-recalcitrant seeds (Farrant *et al.*, 1988; Berjak *et al.*, 1989).

[#] Parts of this chapter were incorporated in, and published, in: *Cryo-Letters* **19**, 5-13 (1998);

and reported in: M. Marzalina, K.C. Khoo, N. Jayanthi, F.Y. Tsan and B. Krishanpillay (eds) *Recalcitrant Seeds: IUFRO Seed Symposium 1998*. FRIM, Kuala Lumpur, Malaysia. pp 96-109 and pp 265- 371 (1999)

Among the plants producing recalcitrant seeds are many tree species, particularly those of tropical origin. Examples are jackfruit (*Artocarpus heterophyllus;* Moraceae [Wesley-Smith *et al.*, 2001a), mangroves such as *Avicennia marina* (Avicenniaceae [Farrant *et al.*, 1989) and *Bruguiera gymnorrhiza* (Rhizophoraceae [Farnsworth, 2000]), riverine species such as Brazil nut (*Bertholletia excelsa;* Lecythidaceae [Cunha *et al.*, 1996)), moist forest hardwoods such as species of *Shorea* spp. (Diptercarpeceae [Krishna and Naithani, 1998; Tsan, 2000) and *Trichilia* spp. (Meliaceae [Maghembe and Msanga, 1988; Choinsky, 1990]), important food sources such as cocoa (*Theobroma cacao*; Sterculiaceae [Li and Sun, 1999]), mango (*Magifera indica*; Anacardiaceae[Lizada, 1991]) and avocado (*Persea americana*; Lauraceae [Raja *et al.*, 2001), major commercial species such rubber (*Hevea brasilienseis*; Euphorbiaceae [Berjak, 1989]); and medicinal species such as the mangosteens (*Garcinia* spp., Clusiaceae [Normah *et al.*, 1997]).

Among temperate species, those producing recalcitrant seeds include horse chestnut (*Aesculus hippocastanum*; Hippocastanaceae [Bonner, 1990]), sycamore and sugar maple (*Acer* spp.; Aceraceae [Bonner, 1990]), the pedunculate / English oak (*Quercus robur*; Fagaceae [Finch-Savage *et al.*, 1996]), tea (*Camellia sinensis*; Theaceae [Berjak *et al.*, 1993]), and the trifoliate orange (*Poncirus trifoliata*; Rutaceae [Purohit and Doijode, 1998]).

Although the species listed above are dicotyledonous, seed recalcitrance is also encountered in monocotyledonous species, e.g. coconut (*Cocos nucifera*; Arecaceae [Chin, 1978]), dwarf paintbrush (*Scadoxus membranaceus*; Amaryllidaceae [Farrant *et al.*, 1989), and wild rice (*Zizania* spp.; Poaceae [Probert and Longley, 1989]). Among the gymnosperms, species which produce recalcitrant seeds are monkey puzzle (*Araucaria* spp.; Araucariaceae [Tompsett, 1984]), Henkel's Yellow-wood (*Podocarpus henkelii*[Mbambezeli and Reynolds, 2002]) and *P. latifolius*; Podocarpaceae [Albrecht, 1993]).

The number of plants known to produce recalcitrant seeds has steadily grown (Tweddle *et al.*, 2002) with the interest in regional indigenous plant species and biodiversity around the

world, and there is increasing evidence that orthodox seed behaviour might not be the norm, as has previously widely been held (Berjak and Pammenter, 2003).

2.2 A review of seed recalcitrance

The biological basis for recalcitrant seed behaviour was described as being largely unknown, and perhaps not the same for all species, according to Chin (1988). However, several factors have since been identified as distinguishing the physiology of recalcitrant seeds from that of orthodox types, as follows:

2.2.1 Degree of development

Recalcitrant seed behaviour is intimately tied to development. One major characteristic of the development of recalcitrant seeds is that, unlike orthodox types, they do not undergo maturation drying as a final phase in development (Farrant *et al.*, 1992; Tompsett and Pritchard, 1993; Lin and Chen, 1995; Pammenter and Berjak, 1999). Recalcitrant seeds are therefore shed at relatively high water contents (0.3 to 4.0 g g^{-1}, depending on the species, provenance and season) compared with 0.15 g g^{-1} or less, for orthodox seeds (Pammenter and Berjak, 1999). Before, or after, shedding at these high water contents, recalcitrant seeds of most species cannot withstand even relatively little dehydration without loss of vigour or viability, while orthodox seeds can withstand dehydration to water contents as low as 0.053 g H_2O per g dry matter, g g^{-1} (Hong and Ellis, 1996). Seeds characterised as intermediate, according to those authors, lose viability when desiccated below water contents of 0.14 - 0.11 g g^{-1}. However, regardless of the relationship between viability and declining water content, it is not satisfactory, and may be misleading, to describe a 'critical water content' below which seeds lose viability, as the response to desiccation depends on several factors such as the rate and temperature of drying (e.g. Ntuli *et al.*, 1997; Pammenter *et al.*, 1998).

Recalcitrant seeds not only remain hydrated, but are metabolically active up to, and after, shedding (e.g. Farrant *et al.*, 1988; Berjak *et al.*, 1989). It has been hypothesised that this is because these seeds lack, or have an abbreviated, postvascular separation phase (Kermode and Finch-Savage, 2002). Germination-associated events are initiated shortly after, and

occasionally even prior to, shedding (Farrant *et al.*, 1986; Farrant *et al.*, 1988; Motete *et al.*, 1997) and, as these events progress, the seeds become increasingly more sensitive to desiccation since they require additional water to complete the process (Farrant *et al.*, 1989; Pammenter *et al.*, 1991; Berjak *et al.*, 1992, 1993).

The lack of maturation drying, sensitivity to desiccation, and the continuing actively metabolic condition of recalcitrant seeds contribute to their inability to maintain viability for practically-useful storage periods, even if water content is not allowed to decline.

2.2.2. Intracellular physical characteristics

The intracellular physical characteristics of recalcitrant seeds have been suggested to differ considerably from those of orthodox seeds generally in three major aspects: degree of vacuolation and reserve deposition; conformation of the DNA, chromatin and nuclear architecture; and the reaction of the cytoskeleton during dehydration. The relative state of intracellular differentiation is a further aspect, which is considered separately, below.

2.2.2.1 Vacuolation and reserve deposition

A necessary requirement for plant cells to tolerate desiccation is the ability to withstand the mechanical stresses that accompany the reduction in volume during dehydration (Iljin, 1957). This ability is thought to be attained either by the sub-division of large fluid-filled vacuoles into smaller ones, or by the filling of the large vacuoles by insoluble reserve material (Farrant *et al.*, 1997). Those authors studied the subcellular characteristics in relation to development and dehydration in of three different seed types: *Avicennia marina* (a tropical, wetland species characterised by highly recalcitrant seeds), *Aesculus hippocastanum* (a temperate, moderately recalcitrant seed species), and *Phaseolus vulgaris*, which produces orthodox seeds. The results showed that the degree of vacuolation in axis cells at maturity was directly related to the desiccation-sensitivity of the seeds. In *A. marina* embryonic axes, in which the meristematic root primordia lost viability when the water content was reduced below 0.5 g g^{-1}, vacuolar volume accounted for 60 – 90% of the cell volume. There was no appreciable level of insoluble reserves accumulated either in the embryonic axes or cotyledons, with storage reserves being mainly in the form of soluble

sugars. In the temperate species (*A. hippocastanum*), in which viability was retained by mature embryonic axes dried to a water content of between 0.42 – 0.25 g g^{-1}, fluid-filled vacuoles occupied a very small fraction of cell volume at maturity, and there was considerable accumulation of insoluble reserves. The situation in *P. vulgaris* was in stark contrast to that in *A. marina*: the (orthodox) seeds of the former species tolerated water contents as low as 0.08 g g^{-1}, while vacuoles occupied an insignificant proportion of the reserve-rich volume of axis cells. In the cotyledons, any remaining vacuoles were filled with an amorphous insoluble material, presumably protein. Those authors, therefore, demonstrated that vacuolar volume in the mature seed organs was directly correlated, and insoluble reserve accumulation inversely correlated, with the degree of desiccation sensitivity, as suggested by Iljin (1957).

2.2.2.2 Conformation of the DNA, chromatin and nuclear architecture

Dehydration affects the conformation of not only the cytomatrical skeletal elements (see below), but the integrity of the nuclear architecture as well, including the nuceloskeleton and the genetic material. It has been suggested that the maintenance of the integrity of nuclear architecture during dehydration and in the dry state, and its re-establishment on rehydration, is an *a priori* requirement for desiccation tolerance (Pammenter and Berjak, 1999).

In desiccation-sensitive tissue, DNA becomes highly degraded during dehydration, as a result of non-repaired double-strand breaks, contributing significantly to viability loss (Osborne and Broubriak, 1994). Those authors showed, in contrast, that the DNA in desiccation-tolerant tissue is stable to dehydration, and the competence of the DNA repair mechanisms withstands desiccation.

One of the factors thought to contribute to the ability of the genetic material of desiccation-tolerant tissue to withstand dehydration is the conformation of the DNA, alternative forms of which are the A-, B- and Z-forms (Saenger *et al.*, 1986). Whereas the B (right-handed) helix is favoured in the fully hydrated state, the loss of moisture during the maturation drying of orthodox seeds has been suggested to lead to the formation of supercoils or anisomorphic structures and changes from the B form to an A (right-handed) or a Z (left-

handed) helix (Osborne and Broubriak, 1994). Accompanying a reduction in water activity, maturation drying is characterised by an inactivation of mRNA transcription (Brunori, 1967), and this is correlated with DNA methylation, which may accompany the conversion of B- to Z-DNA (Wolfe, 1995). The formation of Z-DNA is also favoured by a high cation (Na^+, Mg^{2+}, K^+ and Zn^{2+}) concentration, which must accompany dehydration (Osborne and Broubriak, 1994).

Z-DNA has been shown to occur naturally in the nuclei of a number of eukaryotic organisms, including wheat (Wolfe, 1995). Although the dehydrated state is not a requirement for the existence of A- or Z-DNA, the increased incidence of supercoils (approx. 50% more) in dehydrated bacterial cells (Setlaw, 1992) implies that these conformations of DNA are a property of, or an adaptation to, low moisture contents.

Besides the conformation of the DNA itself, the structure of chromatin is also linked to the ability of tissues to tolerate dehydration. In desiccation-tolerant tissues, dehydration results in the chromatin being highly condensed, a state in which there is no DNA replication (Sargent *et al.*, 1981). Upon rehydration, the chromatin remains in the highly condensed state in the early stages, and becomes re-dispersed at water contents at which DNA replication resumes. This reversible, orderly chromatin compaction appears to be associated with desiccation tolerance, while its re-dispersion characterizes the desiccation-sensitive state, a situation seen in resurrection plants as well (Hallam and Luff, 1980).

The phenomenon of organised, reversible chromatin condensation is integral to nuclear division and particular proteins have been associated with it, including topoisomerase II and the SMC family of non-histone proteins (Hirano *et al.*, 1995). It has been suggested that molecules related to those that are active in chromosome condensation during mitosis might well contribute to the similar phenomenon observed in developing, desiccation-tolerant seeds (Pammenter and Berjak, 1999). Those authors posit that a protein or proteins conforming to the definition of LEAs might also be involved in protection of the chromatin in the dry state. Immunolocalisation studies have shown LEAs to be associated with nuclei

of maize embryo cells, particularly in the aleurone, scutellum and embryonic leaves (Asghar *et al.*, 1994).

The disposition of the chromatin is also significantly affected by the status of the nuceloskeleton; a supporting framework based on lamins, which are type V intermediate filaments (McNulty and Saunders, 1992; Minguez and Moreno Díaz de la Espina, 1993). These are organised into a nuclear lamina (a mesh underlying the inner surface of the nuclear envelope) and an extensive network which ramifies throughout the nucleus; and have been suggested to support and localise the chromatin in discrete domains (Spector, 1993; Wolfe, 1995).

In desiccation-sensitive tissues, the nucleoskeleton is irreversibly disassembled by deleterious dehydration (Merhar *et al.,* 2002). On the other hand, disassembly of the nuclear lamins in desiccation-tolerant tissues, related to ion concentration and phosphorylation, must be reversible (Wolfe, 1995). According to that author, nuclear lamins disassemble when phosphorylated to a level four times that of the fibrous form, and re-assemble upon dephosphorylation .

From the foregoing, it appears that the stability of DNA, chromatin and nuclear architecture in the dehydrated state is a pre-requisite for desiccation tolerance in seeds

2.2.2.3 Reaction of the cytoskeleton to dehydration

The internal organisation of organelles in plant cells is maintained by a dynamic cytoskeleton, which includes microtubules (polymers of α- and β- tubulin units), microfilaments (composed of F-actin, the polymerised form of G-actin) and intermediate filaments (comprising several diverse proteins). During dehydration, the cytoskeleton dissociates (Mycock *et al*, 2000) and, in desiccation-tolerant tissues, is presumed to re-assemble in an orderly manner upon rehydration (e.g. Bartolo and Carter, 1991). In recalcitrant tissues, however, the capability to reassemble both microfilaments and microtubules appears to be lost following dehydration beyond a certain extent, leading to a loss in intracellular support and structural organisation (Pammenter and Berjak, 1999;

Mycock *et al.*, 2000). This structural damage affects not only the spatial organisation of organelles and membranes, but must also impact on certain cytomatrical (cytoplasmic) enzyme systems which exist as multi-enzyme particles in plant cells (Hrazdina and Jensen, 1992), formed by the binding of key or anchor enzymes to the microfilaments of the cytoskeleton (Masters, 1984).

Thus failure of the cytoskeleton to re-assemble following dehydration to deleteriously low water contents would have physiological, as well as structural, consequences in the cells of desiccation-sensitive seed tissues.

2.2.3 Synthesis of protective molecules

2.2.3.1 Late Embryogenic Accumulating / Abundant proteins (LEAs)

Maturation drying, characteristic of orthodox seeds, is preceded by the synthesis of a set of hydrophilic proteins termed Late Embryogenic Accumulating / Abundant proteins [LEAs] (Galau *et al.*, 1986). These proteins are involved in the acquisition and maintenance of desiccation tolerance, possibly because their amphipathic nature facilitates interaction with a wide range of macromolecules, thus preventing denaturation of the latter during the imposed stresses of dehydration (Dure *et al.*, 1989), or by acting as chaperones (Russouw *et al.*, 1997). More recently, it has been suggested that these (and possibly other) proteins may be integral to the highly viscous, glassy condition occurring in desiccated orthodox seeds (Buitink, 2000).

Although LEA proteins are implicated in desiccation tolerance, and are ubiquitous in both dicotyledonous and monocotyledonous orthodox seeds (Thomas 1993), they are not altogether absent among recalcitrant seed species. Dehydrins, which are a sub-set of LEAs have been shown to be present in the recalcitrant seeds of *Acer saccharinum*, *A. pseudoplatanus*, *Zizania palustris*, and *Z. latifolia* (Gee *et al.*, 1994), *Quercus robur* (Finch-Savage and Blake, 1994), *Aesculus hippocastanum*, *Camellia sinsnsis*, *Araucaria angustifolia*, and *Castanea sativa*, (Farrant *et al.*, 1996). However, Farrant and co-workers (1996) could not detect LEAs in mature embryonic axes of the recalcitrant seeds of *Avicennia marina*, *Barringtonia racemosa*, *Brugueria exaristata*, *B. gymnorrhiza*, *Ceriops*

tagal, Rhizophora apiculata, R. mucronata and *R. stylosa*, although the axes of *B. racemosa* produced LEAs in response to drying. Thus, the absence or presence of LEAs may not account for recalcitrance or orthodoxy. These (and perhaps other proteins) may, however, act synergistically with soluble oligosaccharides to confer desiccation tolerance in orthodox species (Fu *et al.*, 1995).

2.2.3.2 Sucrose, certain oligosaccharides and sugar alcohols

The rôle of carbohydrates in desiccation tolerance in seeds has been considered very significant for some years. At low water contents, the highly viscous glass they form in the cytomatrix curtails intracellular movement and the possibility of unregulated metabolism and its deleterious consequences, as well as the consequences of free radical formation. It is suggested that the lifespan of orthodox seeds under defined storage conditions is intimately associated with the relative stability of the glassy state and the attendant reduction of molecular mobility (Buitink *et al.*, 2000). In this regard, however, Buitink (2000) has questioned the central rôle of sugars in intracellular glass formation, her evidence strongly suggesting that proteins (perhaps LEAs) are the basis of intracellular vitrification upon desiccation of orthodox seeds. This notwithstanding, the following paragraphs review what has been conceptualised about the glassy state in the context of the carbohydrates.

The carbohydrates suggested to be involved in desiccation-tolerance / seed longevity are sucrose and oligosaccahrides, usually raffinose and/or stachyose (Koster and Leopold, 1988; Leprince *et al.*, 1990a; Blackman *et al.*, 1992) and galactosyl cyclitols (a sugar alcohol) (Horbowicz and Obendorf, 1994; Obendorf, 1997). There is no doubt that sugars do accumulate during the maturation stages of orthodox seeds and are associated with the onset of desiccation tolerance (Sun and Leoplold, 1993; Sun *et al.*, 1994). For example, accumulation of stachyose is temporally associated with acquisition of desiccation tolerance in developing soybean embryos (Blackman *et al.*, 1992), while a similar relationship has been established for raffinose in cereal seeds (Black *et al.*, 1996). Conversely, the onset of germination, accompanied by the loss of desiccation tolerance, has been associated with the decline in the amounts of these sugars in the seeds in soybean and maize (Koster and Leopold, 1988).

It is possible that the accumulation of non-reducing sugars is a mechanism that (i) removes the availability of reducing sugars, which are respiratory substrates; (ii) counteracts Maillard reactions (non-enzymatic sugar-protein interactions [Ikan, 1996]) during desiccation; (iii) retains and make respiratory substrates readily available when the seeds are rehydrated.

During dehydration, the mixture of desiccation-tolerance associated oligosaccharides must be incorporated in the highly viscous super-saturated intracellular solution and, since raffinose and stachyose inhibit the crystallisation of sucrose (Koster, 1991; Grases *et al.*, 1994), the mixture had long been thought to constitute a glassy state. The storability of orthodox seeds has been shown to be inversely proportional to the mass ratio of sucrose to soluble oligosaccharides (Horbowicz and Obendorf, 1994; Steadman *et al.*, 1996). Whether or not the sugars, or proteins, or both are critical to vitrification, there is general agreement that this phenomenon does occur during the late stages of desiccation. Furthermore, it is the formation and the persistence of the glassy state which is central to its rôle in the desiccated state. In this glassy environment, any reaction that could proceed freely in the originally fluid (hydrated) state of the cell contents, becomes severely hampered (Leopold *et al.*, 1994), imposing a stasis on intracellular activity. Therefore, processes such as those which produce free radicals are reduced, and this plays a major part in protecting membranes and avoiding the denaturation of macromolecules (Pammenter and Berjak, 1994). A further, and intrinsically vital rôle of high intracellular viscosity, is that it prevents the close apposition between membranes, thus preventing them from making contact (Bryant *et al.*, 2001). The ideas formulated by those authors also challenge the 'Water Replacement Hypothesis' (Clegg *et al.*, 1982), for which there is very little *in vitro* evidence. Once an orthodox seed is dry and ametabolic therefore, the glassy state may play a major rôle in the prolonged longevity of the seed under appropriate storage conditions.

The association of desiccation tolerance and prolonged longevity with the accumulation of appropriate concentrations and proportions of sucrose and other oligosaccharides may imply that these are lacking in desiccation-sensitive, short-lived recalcitrant seeds. However, the recalcitrant seeds of several species have been shown to produce sucrose and

oligosaccharides (Berjak *et al.*, 1989; Farrant *et al.*, 1993; Finch-Savage and Blake, 1994; Lin and Huang, 1994; Steadman *et al.*, 1996), which might accumulate in mass ratios conducive to glass formation (Horbowicz and Obendorf, 1994). This, perhaps, supports the argument of Buitink (2000) that sugars are supplementary to the process of vitrification. Those arguments notwithstanding, glass formation would occur only at appropriately low water contents, and recalcitrant seeds would have lost viability before such water contents could be attained.

2.2.3.3 Deployment of amphipathic molecules

It has been shown that certain endogenous amphipathic molecules, such as flavinols, migrate into the membranes of desiccation-tolerant pollen and seed embryos as water is lost and are partitioned into the lipid bilayer (Hoekstra *et al.*, 1997; Golovina *et al.*, 1998). Those authors suggest that such partitioning substantially lowers the water content at which the membrane lipids undergo a change from the liquid crystalline to the gel phase, and therefore contributes to the maintenance of the integrity of membranes in the dry state in desiccation-tolerant organisms. The phase changes of the membranes are reversible, restoring them to a functional condition upon rehydration (Hoekstra *et al.*, 1992; 1997). Similarly, the movement of amphipathic molecules into the membranes is suggested to be reversed on rehydration, which might account for the transient leakage that is invariably observed when dry desiccation-tolerant material (both pollen and seeds) is imbibed (Pammenter and Berjak, 1999).

In recalcitrant seeds, the migratory endogenous amphipaths may be lacking or non-functional. Thus, dehydration may result in non-reversible membrane phase changes (Vertucci and Farrant, 1995); or a partial reversibility in which only the membrane lipids regain their liquid crystalline phase while the proteins remain irreversibly altered, as observed in dehydrated cells of recalcitrant *Camellia sinensis* seeds (Sowa *et al.*, 1991).

2.2.3.4 Presence and operation of anti-oxidant systems

Desiccation damage has been ascribed, at least in part, to cytotoxic oxidative products produced during dehydration. The first wave of such products occurs in the intermediate water contents (0.45 – 0.7 g g^{-1}, corresponding to type 4 water [water potential of –1.1 to -3 MPa], Vertucci and Farrant, 1995). In orthodox seeds and desiccation-tolerant vegetative tissues, these products are removed by the operation of either or both non-enzymatic and enzymatic anti-oxidant systems. For example, in *Craterostigma plantagineum* (a desiccation-tolerant resurrection plant), an inhibitor of lipoxygenase (the activity of which results in lipid hydroperoxide formation) accumulates in the leaves during desiccation (Bianchi *et al.*, 1992). In the other resurrection plants, *C. wilmsii* and *Xerophyta viscosa*, the activity of ascorbate peroxidase increases during dehydration and that of superoxide dismutase (SOD) and glutathione reductase increases during rehydration (Sherwin and Farrant, 1996).

Anti-oxidant systems are apparently also present in desiccation-sensitive tissues (Hendry *et al.*, 1992; Finch-Savage, *et al.*, 1993; Bagnoli *et al.*, 1998; Tommasi *et al.*, 1999). However, they are apparently ineffectual in terms of protecting against desiccation damage, possibly because they become impaired under conditions of water stress (Smith and Berjak, 1995). This may account for the rapid accumulation of free radicals that accompanies the dehydration of the axes of recalcitrant seed species such as *Quercus robur*, *Castanea sativa* and *Aesculus hippocastanum* (Finch-Savage *et al.*, 1994). In *Q. robur*, the accumulation of free radicals is accompanied by decreasing activity of anti-oxidant enzymes and declining α-tocopherol content (Hendry *et al.*, 1992; Finch-Savage *et al.*, 1993).

Generation of the superoxide anion (a free radical) accompanying membrane damage has also been reported for the highly-recalcitrant, tropical species, *Shorea robusta* (Chaitanya and Naithani, 1994), while dehydration of the seeds of the aquatic grass, *Zizania palustris*, was accompanied by hydroperoxide formation at the various dehydration temperatures tested (Ntuli *et al.*, 1997).

Thus, it appears that one of the major causes of desiccation damage in desiccation-sensitive seed tissues is the inability of such tissues to effect adequate protection against the injury caused by oxidative events deriving from uncoordinated metabolism during dehydration.

2.2.3.5 The possible rôle of oleosins

One of the consequences of deterioration in seeds is that the lipid bodies in the cells coalesce (Smith and Berjak, 1995). Such coalescence has been observed in both deteriorating, stored orthodox seeds (Smith and Berjak, 1995) and in desiccation-damaged recalcitrant seeds (Leprince *et al.*, 1998). In non-deteriorated orthodox seeds, such coalescence is thought to be prevented by the presence of a unique group of proteins termed oleosins (Huang, 1992). Oleosins have a central hydrophobic domain, which interacts with the periphery of the lipid bodies in plant cells, and an amphipathic N-terminal domain which, with the C-terminal domain, interacts with the aqueous cytomatrix, thus providing a boundary between individual lipid bodies and their aqueous surroundings, and separating lipid bodies from one another.

In the hydrated state, therefore, the oleosins ensure the existence of lipid bodies as discrete entities in the aqueous cytomatrix. It has been suggested that in desiccation-tolerant seeds the oleosins prevent these bodies from coalescing on dehydration (Leprince *et al.*, 1998). Those authors found that oleosins were lacking, or deficient, in several non-orthodox seeds studied: no oleosins appeared to occur in the highly desiccation-sensitive seeds of *Theobroma cacao*, and in both *Quercus rubra* (a temperate recalcitrant) and *Azadirachta indica* (putatively intermediate) the oleosin:oil body ratio was very small. Dehydration of the seeds of the species mentioned above did not have deleterious effects on the oil bodies, but on rehydration, the stability of these bodies was compromised in the oil-rich *T. cacao* and *A. indica*. Thus, it has been suggested that oleosins may be particularly important in stabilising the oil bodies of seeds in which these bodies are very large (Leprince *et al.*, 1998).

2.2.4 The presence and operation of repair mechanisms during rehydration

The ability of orthodox seeds and other desiccation-tolerant tissues to withstand desiccation does not depend only on the avoidance of such damage prior to, or during, desiccation, but also on the ability of these tissues to repair desiccation damage on rehydration. Evidence of such repair has long been documented (e.g. Berjak and Villiers, 1972). Using ultrastructural studies during early germination of maize embryos from seeds stored under unfavourable conditions, those authors showed that an accumulation of aberrant membrane formations (particularly in mitochondria) occurred during dehydration and that, provided the deterioration was not too severe, these were repaired in the imbibed seeds in the lag phase preceding germination. Severely damaged organelles were removed into the lytic vacuoles.

The observation that in seed lots that have been maltreated, the germination lag increases and rate of germination decreases even though final germination may remain, suggests the operation of repair processes in seeds of many species during the lag phase. One of the enzymes thought to be involved in repair mechanisms, that has been studied in a number of species, is L-isoaspartyl methyltransferase, which initiates the conversion of abnormal L-isoaspartyl residues in proteins to the normal form. Kester *et al.* (1997) showed that the activity of this enzyme decreased in artificially aged tomato seeds as germination rate and viability declined, while Mudgett *et al.* (1997) have made similar observations in seeds of barley and detected the activity of this enzyme in seeds of 45 species from 23 families representing most of the divisions of the plant kingdom.

More evidence of repair during rehydration has been come from DNA studies. Boubriak *et al.* (1997) demonstrated that rapid DNA repair occurs during early imbibition of dry cereal grains. Those authors reported that if repair during imbibition was blocked, then re-drying of the embryos caused severe DNA degradation. DNA repair has also been demonstrated in imbibed, but non-germinating, seeds of dormant cell lines of *Avena fatua* (Elder and Osborne, 1993)

The ability for DNA repair in embryos on re-hydration of seeds from the dry state, ensuring a transcriptionally-competent genome, may be an essential component of the suite of mechanisms that facilitates desiccation tolerance. Such ability may be absent or inadequate in desiccation-sensitive tissues. Non-lethal dehydration of recalcitrant seeds results in an increase in germination lag and a decrease in the rate of germination (e.g. Wesley-Smith *et al.*, 1995). This might imply presence of some degree of repair or, possibly, the accumulation of damage. However, later work by Boubriak *et al.* (2000) on hypocotyl tips of mature *Avicennia marina* seeds showed that after the loss of 22% of the water initially present, DNA damage cannot be repaired, even when water is made freely available, and that DNA replication does not fully recover on rehydration after only 8% water loss.

The absence or ineffectiveness of intracellular repair systems in desiccation-sensitive tissues must contribute significantly to marked damage on re-hydration, possibly a consequence of both the drying process and the inrush of water on rehydration (imbibitional damage). The contribution of imbibitional damage has not been detailed; however, our recent (unpublished) observations have shown that direct immersion of partially-dried recalcitrant axes, or their direct imbibition on moist filter paper, is far superior to their rehydration by equilibration in a saturated atmosphere. This observation, made for three unrelated species does not imply that there is no imbibition damage, but is interpreted as the outcome of deleterious reactions at 'intermediate' water contents (see below) during slow rehydration, reaching lethal levels. This could be the consequence of either the absence, but more probably the inadequacy, of intracellular repair mechanisms.

2.2.5 'Switching off' of metabolism

The rates and types of metabolic processes in seeds are related to the level of hydration within the tissues (Vertucci, 1993; Vertucci and Farrant, 1995). In dehydrated orthodox seeds, low levels of electron transport have been recorded (Vertucci, 1989a; 1993) and, at water contents above about 0.25 g g^{-1}, measurable mitochondrial respiration occurs, as metabolic rates increase with hydration (Vertucci, 1989a). While full metabolism is achieved at high water contents, the metabolism that occurs at 'intermediate' water contents is suggested to be unregulated and potentially injurious, the injury being significantly

mediated by free radicals (Hendry, 1993; Vertucci and Farrant, 1995). The water contents considered to be 'intermediate' correspond to type 3 to type 4 water (Vertucci, 1990), equivalent to water potentials of -14 to -1.5 MPa, or water contents in the range 0.3 to 1.0 g g^{-1} (Vertucci and Farrant, 1995).

Although deleterious oxidative damage occurs at 'intermediate' water contents, the tissues of orthodox seeds must pass through these water contents during maturation drying and again when they are rehydrated. It has been suggested that these seeds are able to avoid damage by 'switching off' cellular metabolism (Pammenter and Berjak, 1999), a phenomenon also encountered in xerotolerant resurrection plants (Bewley, 1979; Farrant *et al.*, 1999), and by the activity of various anti-oxidants. The situation in desiccation-sensitive seeds is different, as these are metabolically active throughout development and, even if the metabolic rate slow down at shedding, such seeds remain demonstrably metabolically active. Additionally, recalcitrant seeds appear to lack adequate mechanisms that allow orthodox seeds to counteract the free-radical mediated damage at 'intermediate' water contents.

2.2.6 Intracellular de-differentiation

One of the characteristics that enables desiccation-tolerant tissues to withstand high degrees of dehydration is the de-differentiation of intracellular organelles accompanying or preceding tissue dehydration. This phenomenon characterises maturing orthodox seeds (Farrant *et al.*, 1997), and has been documented for the resurrection plants, *Xerophyta viscosa* (Sherwin and Farrant, 1996) and *X. scabrida* (Tuba *et al.*, 1996). These *Xerophyta* spp. are poikilochlorophyllous and show slow recovery rates on rehydration, during which the photosynthetic apparatus is re-assembled.

Comparison of the ultrastructure in mature seeds of *Avicennia marina* and *Aesculus hippocastanum* (which are recalcitrant) with those of orthodox *Phaseolus vulgaris* showed the persistence of highly differentiated mitochondria in both recalcitrant species, while those of *P. vulgaris* were almost entirely de-differentiated, even before the onset of maturation drying (Farrant *et al.*, 1997). The retention, in desiccation-sensitive tissues, of organelles in

highly differentiated states – and, indeed, of overall intracellular organisation typical of hydrated tissue – during dehydration, contributes to the vulnerability of membranes and cytoskeletal elements to desiccation damage (Pammenter and Berjak, 1999).

2.2.7 The rate and temperature of drying

The response of plant tissues to dehydration has been shown to depend on the rate at which water is removed from the tissues. In orthodox seeds and desiccation-tolerant vegetative tissues, better desiccation tolerance is achieved with slower drying (Bewley and Black, 1994; Oliver and Bewley, 1997; Corbineau *et al.*, 2000). This is presumed to be because slow drying allows sufficient time for the induction and operation of protection mechanisms (Pammenter *et al.*, 2000). In some bryophytes the rate of drying affects the recovery time rather than the water content tolerated: rapidly-dried material recovers more slowly on rehydration. It has been suggested that this is because rapid drying precludes protection processes and more time is required for repair on rehydration (Oliver and Bewley, 1997).

In contrast to desiccation-tolerant tissues, recalcitrant material does not appear to possess effective repair mechanisms (see above) that would operate during slow dehydration (see above). Instead, the extended period at intermediate water contents favours the accumulation of the products of harmful oxidative processes which have been suggested to take place at such water contents as a result of the loss of control of metabolism and/or the failure of anti-oxidant systems (Côme and Corbineau, 1996). Rapid dehydration, on the other hand, minimizes the period during which deleterious aqueous-based reactions occur (Berjak and Pammenter, 1997; Pritchard and Manger, 1998; Walters *et al.*, 2001), especially when the specimens are small and/or lose water readily. In the case of occasional recalcitrant species, however, slower dehydration has proved to be less injurious than rapid rates of water loss (Liang and Sun, 2000). The reasons for this are presently not clear, but may be the consequence of the volume of tissue being dehydrated and exactly what is meant by 'slow' and 'rapid'.

It has also been suggested that slow dehydration of metabolically active recalcitrant seeds affords them time to progress further towards germination, during which they become increasingly desiccation-sensitive, thus losing viability at higher water contents (Farrant *et al.*, 1985; Berjak *et al.*, 1989, 1992, 1993). However, Pammenter and Berjak (1999) caution that this would be the case only if the drying rate were sufficiently slow not to curtail ongoing germination. The harmful consequences of slow drying may therefore be more strongly associated with the unregulated metabolism at 'intermediate' water contents, than with increasing desiccation-sensitivity as a result of the progression of germination.

The possibility of using rapid drying to facilitate transient survival of recalcitrant seed material to lower water contents has led to the development of 'flash-drying', a technique by which excised embryonic axes are dehydrated very rapidly in a stream of dry air (Berjak *et al.*, 1990). Excised embryonic axes are necessarily used for flash-drying because most recalcitrant seeds are too large to dry rapidly. In seeds that are amenable to rapid drying, higher rates of dehydration have been shown to have a similar effect to that when excised axes are used (Farrant *et al.*, 1985; Pritchard, 1991; Pammenter *et al.*, 1998). This effect is, however, less pronounced, probably because the axis in an intact seed cannot dry as rapidly as when it is excised. Although it was suggested that the removal of the axis from cotyledonary material may influence the response of the isolated axis to dehydration (Finch-Savage, 1992), the work of Pammenter *et al.* (1998) has shown that this is not the case .

Even though rapid drying generally enables recalcitrant seed material to tolerate dehydration to lower water contents than if slowly-dried, these seeds will still not survive dehydration beyond a lower limit, which is always higher than the water content to which orthodox or intermediate seeds can be dried (Pammenter and Berjak, 1999). This limit is usually close to, but not lower than, the water content below which the remaining water is structure-associated and non-freezable (Pammenter *et al.*, 1993). At this lower limit of water content, the damage that occurs upon further dehydration would be desiccation damage *sensu stricto*, which is caused by the removal of the water required to maintain the integrity of intracellular structures (Pammenter *et al.*, 1998; Walters *et al.*, 2001).

Another factor that affects the water content to which recalcitrant seeds can be dehydrated without losing viability, is the temperature at which dehydration occurs. This has been termed "*Zizania*-like behaviour" (Pammenter and Berjak, 1994), following the findings of Kovach and Bradford (1992) that the seeds of wild rice (*Zizania palustris*), classified as recalcitrant, could (probably transiently) tolerate water contents as low as 0.06 – 0.08 g g^{-1}, if dehydration was carried out at a temperature of about 25 °C. Subsequent studies by Ntuli *et al.* (1997) showed that *Z. palustris* seeds tolerated dehydration better when dried at 25 °C, than at either 10 °C or 37 °C.

It is therefore misleading to specify a 'critical water content' beyond which the seeds of a particular species cannot tolerate desiccation, without taking into account the dehydration rate and temperature at which the water is removed. It has also been established that the ability of recalcitrant seed material to withstand lower water contents following rapid drying is ephemeral: flash-drying does not confer the property of desiccation-tolerance. Thus, flash-dried material would survive under ambient conditions for, at most, a few days only (Walters, *et al.*, 2001). Suitable storage methods must therefore be established for the germplasm of species producing recalcitrant seeds, whether in the form of the seeds themselves, or of excised axes or other explants.

2.2.8 Storage of recalcitrant seeds

Conventional seed storage, applied widely to orthodox seeds, relies on their maintenance at low moisture contents (i.e. under low RH conditions) and low temperatures. Under these conditions, the longevity of the seeds increases logarithmically with reducing water content and the seeds can be stored for long periods that are predictable in terms of the constants determined (per seed batch) before storage (Ellis and Roberts, 1980). For the viability equation to hold, however, the water content of the seeds must be lower than the equivalent of a water potential of about -14 MPa (Roberts and Ellis, 1989). This threshold water potential is similar to the -11 MPa identified by Vertucci (1993) to be the point at which all water in seeds is non-freezable. Since recalcitrant seeds cannot survive dehydration to such low water contents, storage in the dry state is not feasible. On the other hand, even if such seeds are stored in the fully hydrated state, they still lose viability within days to two or three weeks (in some tropical species) to 2 to 3 years in some temperate species (King and

Roberts, 1980; Farrant *et al.*, 1989; Fu *et al.*, 1990; Tompsett, 1992; Pammenter *et al.*, 1997; Motete *et al.*, 1997). However, in the case of temperate species such as *Quercus robur*, if fungi are present within the tissues then, although the seeds may retain viability, they become very debilitated, and are unlikely to be able to support seedling establishment (Berjak[1], pers. comm.).

The relatively longer storage lifespan of temperate recalcitrant seeds is achieved by their maintenance at low temperatures, a treatment which is inapplicable to the seeds of those tropical species which are chilling-sensitive below about 10 °C (Corbineau and Côme, 1988; Pammenter and Berjak, 1999). However, storage at higher temperatures encourages microbial proliferation, particularly since all recalcitrant seeds have been shown to harbour a spectrum of surface and internal fungal contaminants at harvest (Mycock and Berjak 1990; Berjak, 1996; Calistru *et al.*, 2000).

In an effort to control fungal contamination of recalcitrant seeds in storage, fungicides have been used. Coating seeds of *Hevea brasiliensis* with 0.3% (w/w) Benlate® a benomyl fungicide (Dupont, Wilmington, Delaware, USA) has been found to extend the longevity from 3 to 12 months (Chin, 1988). Other studies by Motete *et al.* (1997) and Calistru *et al.* (2000) demonstrate that by controlling fungal contamination, the storage lifespan of the highly recalcitrant seeds of *Avicennia marina* can be increased significantly. It is important to note, however, that surface application of fungicides as powders or slurries, will be effective in extending seed storage lifespan only if there is no internally located mycelium.

A further cause of deterioration during the storage of recalcitrant seeds is suggested to be the metabolic status of the seeds prior to, and during, storage. The seeds are shed while still metabolically active, and this metabolism continues towards germinative events during storage. This generates a requirement for additional water which is not provided during storage. As a consequence, the seeds have been suggested to suffer effectively from a relatively mild, but prolonged, and increasingly severe, water stress (Pammenter *et al.*, 1994). Those authors proposed that, just as in the case of slow drying, this water stress could

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bring about unregulated metabolism, a reduction in the efficiency of anti-oxidant mechanisms and consequent free-radical-mediated degradative processes. However, this water stress hypothesis, although not disproved, has not yet been substantiated (Motete *et al.*, 1997; Greggains *et al.* 2000).

Because of the visible manifestation of germination initiation during storage, the reduction of metabolic rate has been the focus of attempts to increase the storage lifespan of recalcitrant seeds. In chilling-tolerant, temperate species, this has been achieved by lowering the storage temperature (Suszka and Tylkowski, 1980; Pritchard *et al.*, 1995). In many instances, where the seeds are chilling-sensitive, other techniques have been used, including the application of the germination-inhibitor, ABA, prior to storage (Pammenter *et al.* 1997; Motete *et al.*, 1997).

Another approach used to curtail metabolism in storage is the manipulation of the storage atmosphere. Sowa *et al.* (1991)) showed that treatment of *Litchi chinensis* and *Dimocarpus longan* seeds with nitrous oxide, an anaesthetic which reduces respiration rates in seeds, increased storage lifespan; while Edwards and Mumford (1985) suggested an inverse relationship between cumulative respiratory activity and longevity of imbibed seeds of *Citrus aurantium*. However, a storage atmosphere rich in oxygen is still required to maintain viability in storage, particularly for recalcitrant seeds (Willan, 1985). This would preclude the use of air-tight containers, conventionally used for the storage of orthodox seeds, from use in the storage recalcitrant seeds.

Besides oxygen, the quality of light during seed storage may play a rôle in longevity, as it may affect precocious germination. Darkness particularly, has been shown to inhibit the germination of the seeds of some forest species (Teketay, 1998), which may be an adaptation to suppress germination if the seeds are buried deep into the soil or dispersed under vegetation canopy. However, despite the rôle of phytochrome and ultraviolet radiation in germination being well-documented (e.g. Carpenter and Boucher, 1992; Singh and Amritphale, 1992; Kim *et al.*, 1998; Leinonen and Dechantal, 1998; Toyomasu *et al.*, 1998), the actual effect differs from species to species, and there is heterogeneity

even between seedlots of the same species (Singh and Amritphale, 1992). Therefore the effect of light/dark on stored seeds must be determined empirically.

As water is required for germinative development, which contributes to the loss of viability in storage, it has been suggested that one way to extend the storage lifespan of recalcitrant seeds is to lower the water contents to levels that curtail germination (King and Roberts, 1980). According to those authors, such 'sub-imbibed' storage has been used to facilitate transport of recalcitrant seeds which, in effect, constitutes short-term storage. Another advantage cited for sub-imbibed storage is that it may reduce the proliferation of micro-organisms in storage (Hong and Ellis, 1996).

However, if, as suggested by Pammenter *et al.* (1994), germinative metabolism imposes a water stress, then any 'limited' drying before storage is likely to exacerbate the situation and lead to faster loss of viability in storage. In a study that tested this hypothesis, Drew *et al.* (2000) showed that sub-imbibed storage of the seeds *of Trichilia dregeana* induced ultrastructural damage accompanied by the reduction of storage lifespan to one third of that of the seeds stored in the fully hydrated state. A shortening of longevity following partial drying was also observed by Corbineau and Côme (1986, 1988) in the recalcitrant seeds of four tropical species.

A significant aspect of short-term drying is that it results in an initial increase in the totality and rate of germination (e.g Fu *et al.*, 1994; Tompsett and Pritchard, 1998; Pammenter *et al.*, 1998). While the basis of this phenomenon is not well understood, it implies that if seeds are mildly dehydrated before storage, they progress more rapidly towards germination and are therefore more vulnerable to the deleterious effects of hydrated storage than seeds stored at the shedding water content.

A consideration of the various factors that deleteriously affect the viability of recalcitrant seeds in storage illustrates that they can be maintained for only short periods, ranging from a few days or weeks, to several months at most, although the period may be longer in the case of certain temperate species. Effective longer-term storage, however, is achievable only via

cryopreservation, the storage of living biological material at ultra-low temperatures, normally at or near -196 °C, the temperature of liquid nitrogen (Engelmann and Dussert, 2000). This is approach is explored and discussed in Chapter 3. However, the development of any storage regime, whether short- or long-term, requires an understanding and prudent exploitation of the post-shedding physiology of the seeds.

2.3 Objective of this phase of the study

The species under investigation in this study typify many tropical species producing recalcitrant seeds: a high exploitation and extinction rate (discussed in Chapter 1), intensified by the absence of any, or useful, information on the physiology and / or storability of the seeds. This lack of information thwarts many efforts aimed at conservation. It is the objective of this part of the study, therefore, to characterize the behaviour of the seeds of *Trichilia dregeana*, *T. emetica* and *W. salutaris,* with the aim of establishing methods for their short- to medium-term storage of these seeds. Investigations on the storage lifespan and the response to dehydration of the seeds are reported, utilising the changes in viability and vigour, water content, and ultrastructural integrity, to draw conclusions.

2.4 Materials and Methods

2.4.1 Seed procurement and handling

Trichilia dregeana and *T. emetica* seeds were harvested over consecutive years, and more than once per season. Seed availability and quality was highly variable from year to year. For example, in certain seasons (e.g. 1996), seeding was prolific and continued for several months. Generally, such seeds seemed to harbour relatively little internal microflora and could be stored successfully in the medium term (up to five months, section 2.5.5.1). For other seasons (e.g. 2000) seed production was sparse, and seed-associated microflora posed a significant problem in the experiments. In yet other years (e.g. 2002) no seeds were available. Unless otherwise stated, the seeds used the experiments reported here (and in the following chapters) were conducted on high quality seeds which manifested no signs of associated micro-organisms.

Seeds of *T. dregeana* and *T. emetica* were collected from trees planted around Durban and Mtunzini (28° 56'S; 31° 45'E) municipalities on the sub-tropical east coast of South Africa. Freshly-matured, newly-opened fruits were harvested using telescopic lopping shears, and seeds extracted immediately. Whenever mature, unopened fruits were inadvertently harvested along with newly-opened fruits, the former were placed at 16 °C for about 3 days (by which time they had opened) and the seeds extracted.

Seeds meant for storage without the testas were de-coated manually, using a pair of forceps, within 48 h of collection. Since the seed-coat is fused with the aril, the only practical method of removing the aril while leaving the seed-coat intact (for seeds stored with the seed-coat) was to soak the seeds in water and wash off, with gentle rubbing, the consequently softened aril. Seeds were thus soaked for 3 hours and the aril removed to expose the black (*T. dregeana*) or brown (*T. emetica*) testa.

Cleaned seeds were surface-sterilised by soaking in 1% sodium hypochlorite solution for 20 min (*T. dregeana* and *T. emetica* only) followed by drying with paper-towel. The seeds were air-dried back to the original fresh weight, and dusted with Benlate.

In 2000, seeds of *T. dregeana* were found to be heavily contaminated with seed-borne fungi, and an endeavour was made to control the micro-organisms by soaking the seeds in a systemic anti-fungal cocktail of 0.2 ml l^{-1} Early Impact® (active ingredients: Flutriafol [triazole] and carbendazim [benzamidazole] Agrochemicals, South Africa), and 2.5 ml l^{-1} Previcur® (active ingredient: propamocarb-HC, AgrEvo, South Africa) for 4 h. A sub-sample of these seeds was coated with a customised alginate gel (Kelp Products, Simon's Town, Western Cape Province, South Africa) previously shown to have strong fungistatic properties (Motete *et al.*, 1997; Calistru *et al.*, 2000). Coating was achieved by dipping the seeds into the alginate gel solution (in the ratio of 4:1, alginate:distilled water), after which they were transferred to a 10 m*M* calcium chloride solution for 5 minutes to facilitate polymerisation of the gel. Another batch which had not been treated with systemic fungicides was similarly coated. All seeds were dried in a laminar airflow for about 2 h to attain the original fresh weight.

In the case of *W. salutaris*, fruits were either obtained from Silverglen Medicinal Plants Nursery at Chatsworth, Durban, and processed on the same day; or harvested from natural populations in Ngong, Kenya (1° 19' S; 38° 55'E), or Lushoto, Tanzania (4° 43'S; 38°, 17'E), and dispatched by courier to the laboratory in Durban South Africa. Where the fruits were too hard for the seeds to be extracted without injury, they were stored at 16 °C for up to 4 – 6 weeks to soften, and the seeds extracted carefully by hand. As *Warburgia* seeds were lethally harmed by sodium hypochlorite, they were not surface-sterilised, but were soaked in the fungicidal mixture described for *T. dregeana*, above.

2.4.2 Seed Storage

Following cleaning and sterilization, the seeds were stored in a monolayer on plastic mesh pre-sterilised by soaking in 1% sodium hypochlorite. This mesh was supported about 200 mm over water-saturated paper towel enclosed in translucent white plastic buckets (20-litre buckets for *T. dregeana* or 5-litre buckets for *T. emetica* and *W. salutaris*). Seed-containing buckets were placed at 6 °C, 16 °C and 25 °C.

Seeds of *W. salutaris* stored in the dehydrated state (see below) were enclosed in batches of 30 (comprising individual weekly test samples), in heat-sealed air-tight polythene bags, thus obviating the exposure of seeds for ongoing storage to ambient humidity. Bags were maintained at 6, 16 or 25°C, as were the containers used for hydrated storage.

2.4.3 Seed sampling

Hand sampling (ISTA, 1999) was used to obtain random samples for all tests.

2.4.4 Water content determination

The water content was determined gravimetrically, separately for embryonic axes and cotyledons. The material was heated in an oven at 80 °C for 48 h (this time having been pre-determined to achieve constant weight of the material), and the difference between fresh and dry weight (total water) was expressed as g water per g dry matter ($g\ g^{-1}$).

2.4.5 Drying

Two modes of drying were employed: slow and fast. Slow drying of *T. dregeana* and *T. emetica* seeds was done by maintaining whole seeds on a plastic mesh placed 200 mm above activated silica gel in a closed 20-litre plastic bucket. The 'slow' dehydration of *W. salutaris* seeds was designed to be faster than would be achieved by the method described above, because previous personal observations indicate that very slow dehydration of these seeds is generally lethal. Thus, the seeds were buried in activated silica gel held at 25 °C and sampled every 6 h.

Fast drying was performed only on embryonic axes, as the seeds of all three species under investigation are too large to dry sufficiently rapidly this way. Thus, excised embryonic axes were either placed on dry filter paper in a laminar air flow, or flash-dried as follows: axes were placed on a nylon gauze mounted over a computer cooling fan (12V, 1W) which was then placed in a 350-ml jar containing 100 g of activated silica gel. Electrical connections to the fan were made through a perforation in the screw-cap of the jar (Wesley-Smith, 2002). When switched on, the fan effected the flow of a fast, steady stream of dry air over the embryonic axes.

Slowly-dried seeds were sampled for water content determination and viability assessment daily, while flash-dried axes were sampled every 15 min.

2.4.6 Viability assessment

Whole seeds of *Trichilia dregeana* and *T. emetica* were sown in moist vermiculite, and maintained in a germination room (16 h light: 8 h dark photoperiod) at 25 °C (day) and 18 °C (night), germination being scored daily (for *T. emetica*), every two days (*T. dregeana*). For *W. salutaris*, vermiculite was found to be unsuitable, especially for cryopreserved seeds, and seeds of this species were sown in sand maintained at 25 °C, an assessed weekly for germination. A seed was scored as having germinated on the basis of radicle protrusion, and establishment was scored after the appearance of the first pair of leaves. For each sample, percentage germination, the germination index (the maximum percentage germination

attained divided by the number of days taken to attain that percentage), and percentage establishment were determined.

The viability of excised embryonic axes was determined by *in vitro* germination (see Chapter 3). Axes were surface-sterilized by soaking in 1% sodium hypochlorite, containing a few drops of Tween-20, for 20 min (for *T. dregeana*). In the case of *T. emetica*, surface sterilization was performed by soaking in 0.2% (w/v) mercuric chloride for 1 min. Sterilized axes were washed with sterile distilled water (three changes) and then cultured aseptically on an MS (Murashige and Skoog, 1962) nutrient medium, at a light intensity of 66 $\mu mol\ m^{-2}\ s^{-1}$ and a 16:8 light:dark photoperiod. Axes were scored as having germinated on the basis of radicle elongation beyond 5 mm, while shoot development was determined by the appearance of the first pair of leaves. Embryonic axes which produced callus instead of either roots or shoots were scored as viable but not germinated.

2.4.7 Ultrastructural studies

Small pieces of root and/or shoot apices, not exceeding 0.5 mm^3 in size, were cut from five replicate embryonic axes which, if dehydrated, had been maintained for at least 1 h on moist filter paper rehydrate, and processed for transmission electron microscopy as follows:

Samples were placed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C (primary fixation), and then transferred back to room temperature. After several rinses with phosphate buffer, the material was post-fixed in 0.5% aqueous osmium tetroxide (OsO_4) solution for 1 h, washed several times with phosphate buffer, and dehydrated in a graded acetone series. The tissue blocks then were infiltrated in a 50:50 acetone:epoxy resin (Spurr, 1969) mixture overnight, followed by infiltration in pure resin at 70 °C for 8 h. The blocks were then sectioned using a Reichert Ultracut E microtome. The ultra-thin sections (60 - 100 nm), collected on copper grids, were post-stained with a saturated aqueous uranyl acetate solution (10 min) followed by alkaline lead citrate for 10 min. The material was viewed, and images recorded photographically,

with a Jeol 1010 transmission electron microscope on Kodak® EM Film (Electron Microscopy Sciences, Washington, PA, USA).

2.4.8 Electrolyte conductivity

The leakage of electrolytes from seeds was measured using a CM100 multi-cell conductivity meter (Reid and Associates, Durban, South Africa). The conductivities of the leachate from ten replicate axes from each treatment (or axes excised from seeds following treatment), each soaked, immediately after dehydration, in 3 ml deionised water, were measured every 30 min for 18 h. Leakage was expressed in $\mu S\ cm^{-1}\ g^{-1}$ dry matter min^{-1}.

2.4.8 Measurement of respiration rate

The rate of respiration of excised axes/cotyledons was determined by their rate of evolution of CO_2, measured using an infrared gas analyzer, IRGA (ADC-255 MK3, Analytical Development Corporation, Hoddesdon, Herts, UK), that was first calibrated with known concentrations of CO_2. Axes or cotyledons were enclosed in an air-tight vial sealed with a plastic plug. Using hypodermic needles connected to the outlet and inlet ports of IRGA analytical ports via latex rubber tubes, the air in the vial was rapidly flushed out with CO_2-free air (ambient air that had been passed through a column of soda lime before introduction into the IRGA). The absolute concentration of any CO_2 in the vial was then measured, and the vial left for 30 min in a water-bath thermostatically controlled at 20 °C, to allow for respiration of the axes/cotyledons. Absolute CO_2 concentration was measured again, and the difference between first measurement and second gave the amount of CO_2 evolved. Respiration rate was expressed as $\mu mol\ CO_2\ h^{-1}\ g^{-1}$ dry weight of material. Three replicates of three axes/sets cotyledon were used per measurement.

2.5 Results and Discussion

2.5.1 Seed germination

For *T. dregeana* and *T. emetica*, seed germination was assessed by the protrusion of the radicle from between the cotyledons, while the emergence of the first pair of leaves was regarded as seedling establishment. Seeds of *W. salutaris*, which were sown in bottom-heated sand beds, were scored as germinated only when the shoot emerged. Hence, there was no distinction between germination and seedling establishment in this species.

Seeds of *W. salutaris* germinated readily without any pre-sowing treatment, but those of *T. dregeana* and *T. emetica* required the removal of both the aril and testa before successful germination occurred. In this study, the testa of *T. dregeana* seeds reduced not only the totality, but also the rate, of germination, with the inhibiting influence of the seed coat being intensified by the aril (Figure 2.1). With regard to *T. emetica,* Maghembe and Msanga (1988) showed that the removal of the aril and seed-coat was more effective than other pre-sowing treatments tested, such as application of gibberellic acid, in promoting germination. Therefore seeds of this species were sown, in this study, after the removal of the aril and testa.

The influence of the seed coverings on germination has been demonstrated in other recalcitrant seeds, including those of *Avicennia marina* (Farrant *et al.*, 1993). Those authors showed that the pericarp of *A. marina* is a reservoir of germination-inhibiting abscisic acid, which may serve to check germination before the seeds encounter suitable conditions for seedling establishment. In the case of *T. dregeana* and *T. emetica*, the bright-red aril serves the additional rôle of attracting animals, which aid in seed-dispersal (e.g. Wilson and Thompson, 1982; Wilson and Melampy, 1983). On animal consumption of the aril, which is closely associated with the testa, germination would be facilitated. In some species, such as *Chamaecytisus proliferus* (Leguminosae), the aril has been shown to contain starch, proteins and lipid – nutrients suggested to aid in myrmecochory (Tiano *et al.*, 1998), and the lipid-rich aril of seeds of *Trichilia* spp. rapidly attracts ants once the seeds are shed (personal observations).

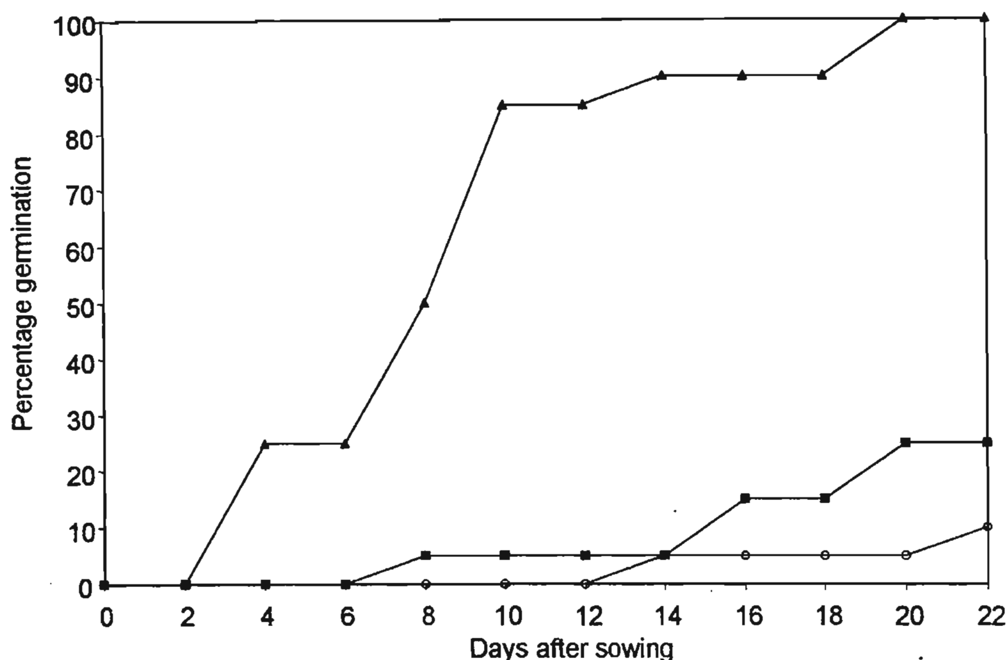


Figure 2.1 The germination time-course of seeds of *T. dregeana* sown intact with the aril (○), with the seed coat but aril removed (■), or without the aril and seedcoat (▲). n=20.

2.5.2 The effect of dehydration on the viability of seeds and embryonic axes.

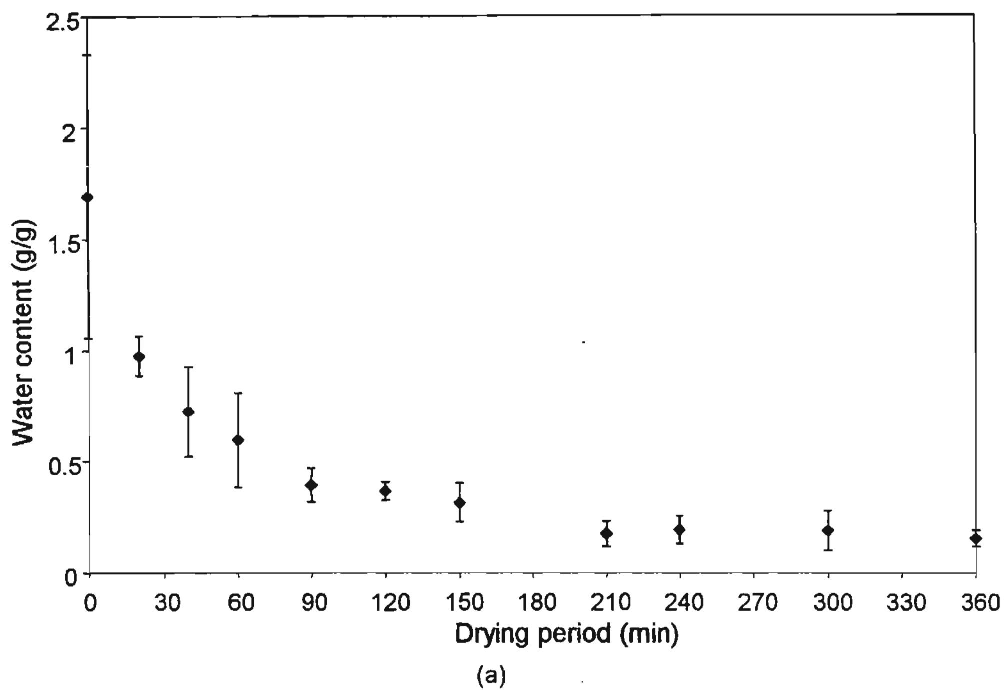
The significance of the rate of dehydration in determining the response of recalcitrant seed tissue to dehydration has been discussed in section 2.2.7. For large seeds, rapid rates of drying are generally unattainable simply because of their size. This necessitates the use of excised embryonic axes in operations that require rapid decrease in water content. The relative sizes of whole seeds and excised embryonic axes of the species used in this study are shown in Table 2.1.

Table 2.1. Comparison of the fresh mass of whole seeds, and embryonic axes, of *T. dregeana*, *T. emetica* and *W. salutaris*. Mass represents the mean ± SD of 10 seeds and excised axes weighed individually.

Species	Whole seed fresh mass (g)	Embryonic axis fresh mass (g)	Embryonic axis as fraction of whole seed
T. dregeana	0.76 ± 0.2	0.0030 ± 0.0008	0.004
T. emetica	0.36 ± 0.1	0.0013 ± 0.0004	0.004
W. salutaris	0.33 ± 0.04	0.0012 ± 0.0002	0.004

Although there were distinct differences among the species, the flash-drying time taken for the water content of excised axes to reach a constant value, in all cases occurred in 3.5 to < 1 h, with drying time for *T. dregeana* > *T. emetica* > *W. salutaris* (Figs 2.2a, 2.3a, 2.4a). However, when whole seeds were dried, it took a matter of days for axes to approach similar water contents to those attained rapidly on flash drying (Figs 2.2b, 2.3b, 2.4b). The drying rate of axes within intact seeds was again slowest for *T. dregeana* (which had not reached a constant value even after eight days), and *W. salutaris* dried most rapidly. Drying rates were related to the fresh mass of both whole seeds and excised axes for *T. dregeana* and *T. emetica*. In the case of *W. salutaris*, the more rapid axis drying rates relative to those attained for *T. emetica* must reflect the differences in chemical and physical factors, as both seed and axis fresh weights were practically the same for these two species (Table 2.1).

The pattern of water content loss, in which the rate is highest during the early stages of dehydration and subsequently declines, has been observed in most studies dealing with desiccation of non-orthodox seeds or excised embryonic axes, this effect being particularly marked in the latter (e.g. Farrant *et al.*, 1989; Pammenter *et al.*, 1991; Ntuli *et al.*, 1997). Among the species under investigation in this study, the embryonic axes of *W. salutaris* dehydrated most rapidly, reaching water contents approaching 0.1 g g^{-1} within 45 min.



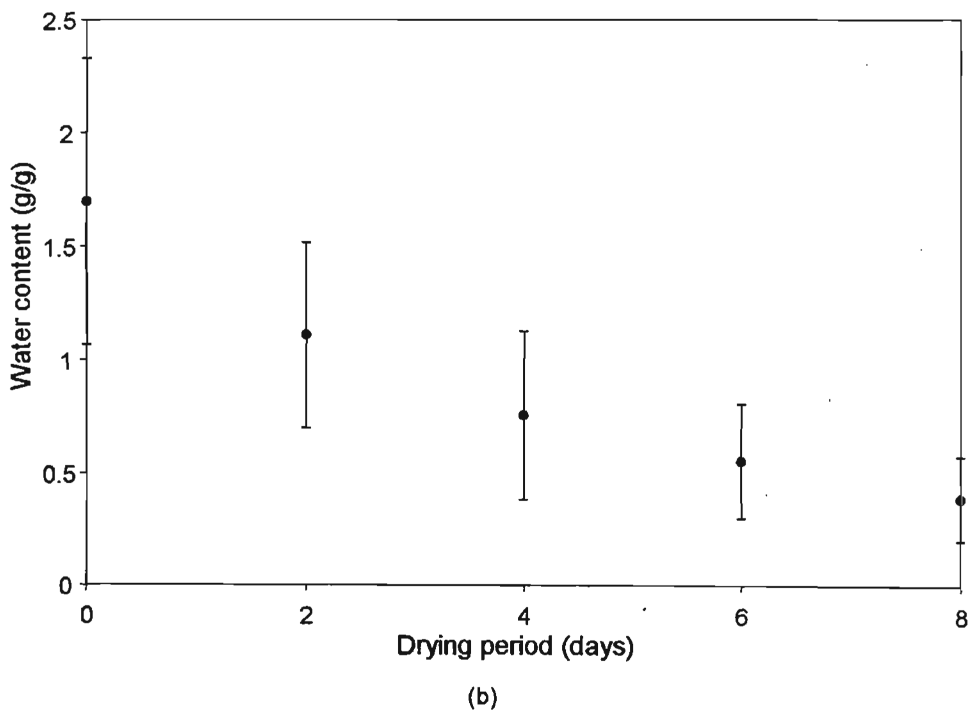
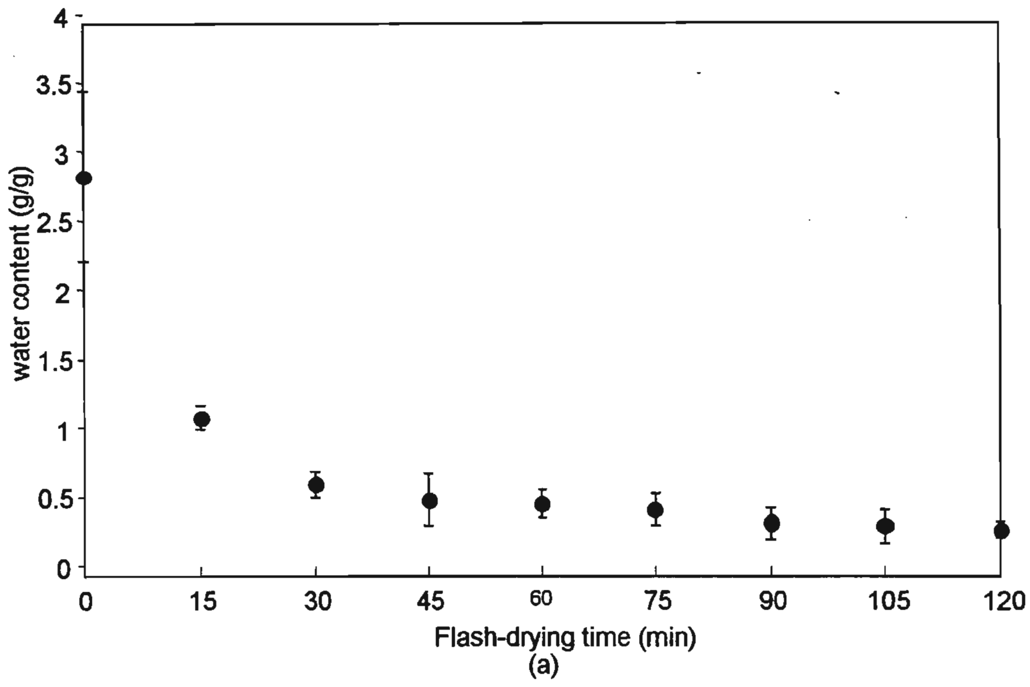
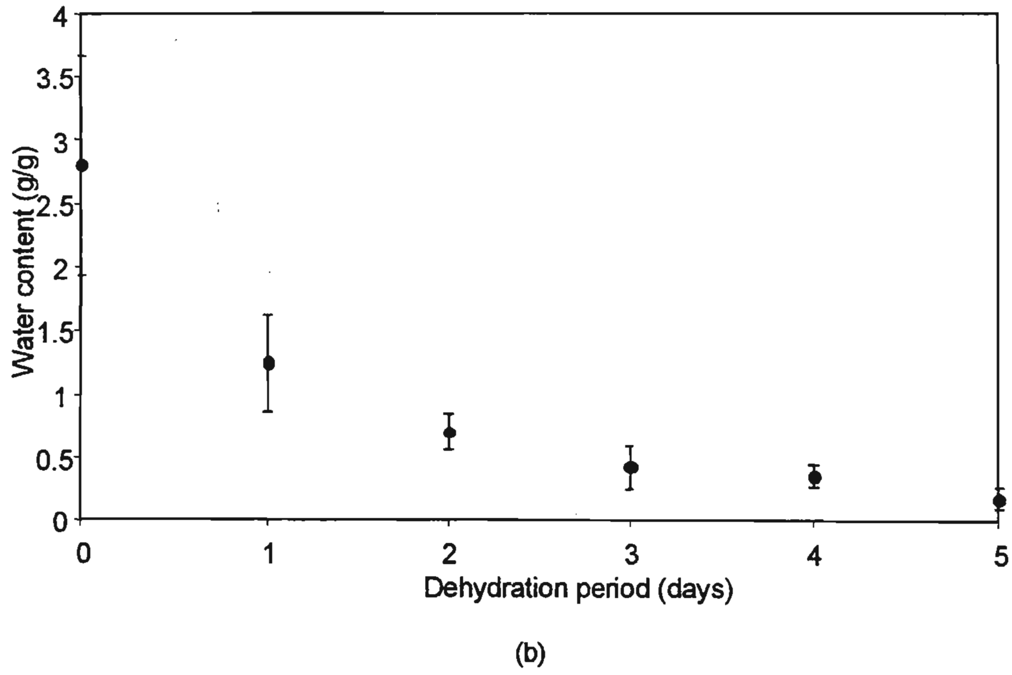


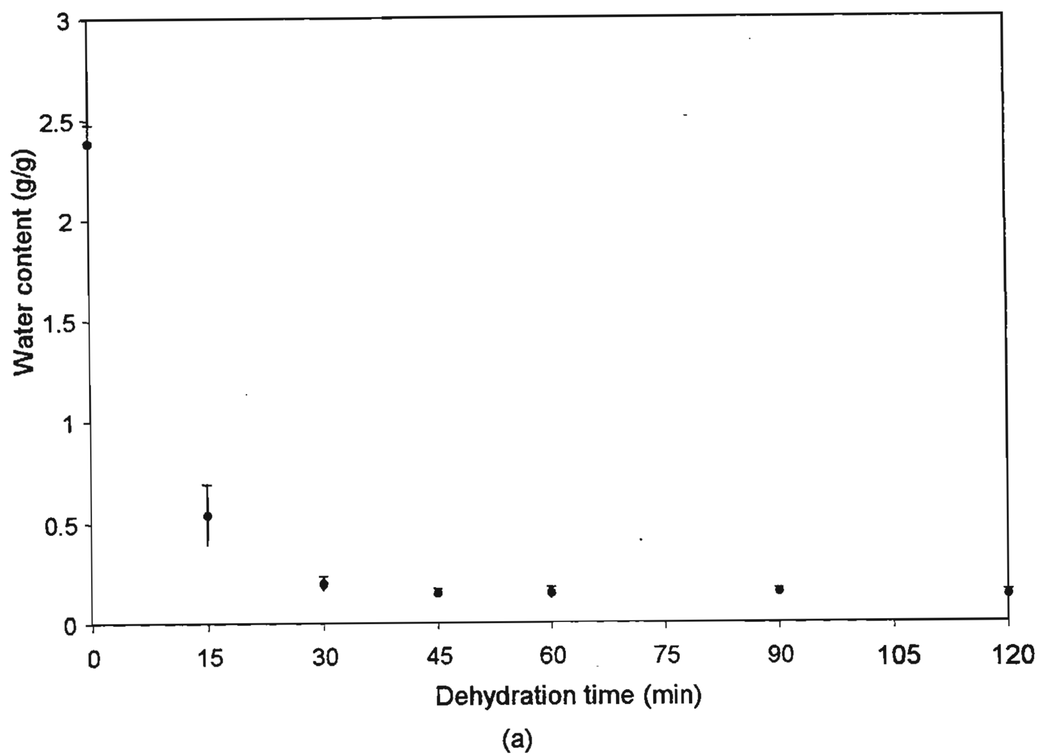
Figure 2.2 Drying time course for *T. dregeana* axes dehydrated after excision (a) or within the seed (b). Markers represent mean ± standard deviation of five individual axes.





(b)

Figure 2.3 Drying time course for *T. emetica* axes dehydrated after excision (a) or within the seed (b). Markers represent mean ± standard deviation of five individual axes.



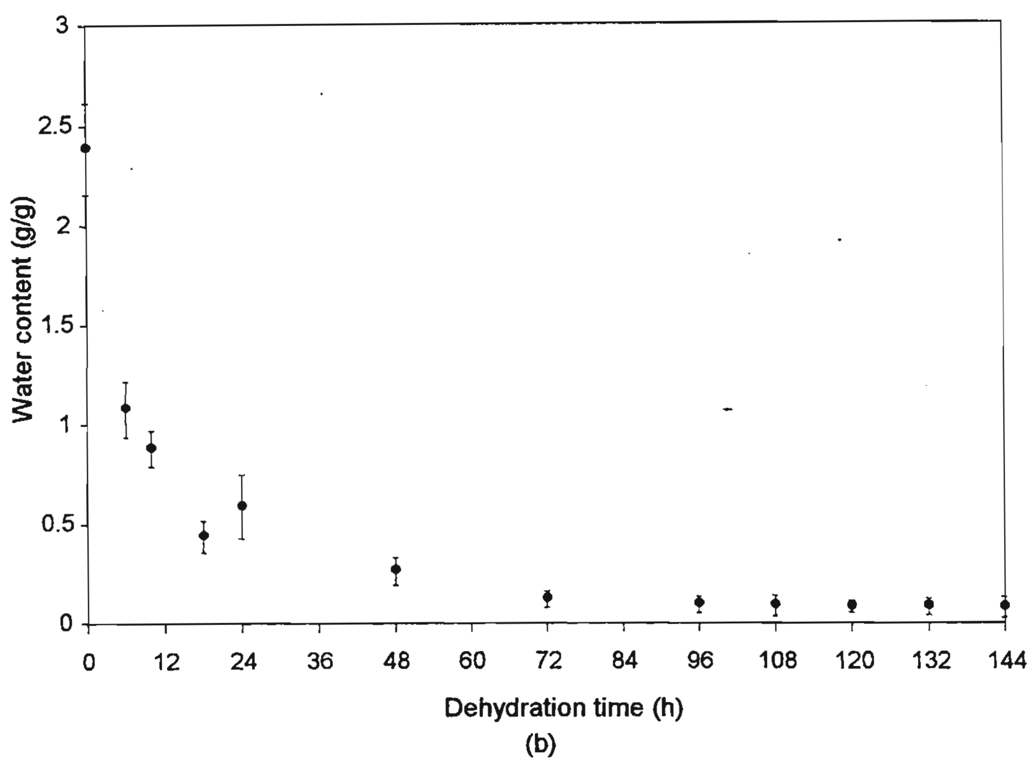


Figure 2.4 Drying time course for *W. salutaris* axes dehydrated after excision (a) or within the seed (b). Markers represent mean ± standard deviation of five individual axes.

In contrast, embryonic axes from seeds of *Trichilia dregeana* did not dehydrate to a similar extent, even when flash-dried for several hours, a situation markedly different from that for axes of comparable size, of many species. For example, flash-dried embryonic axes of tea were found to decline in water content from 3.5 g g^{-1} to 0.07 g g^{-1} within 2 h (Berjak *et al.*, 1993), while the water content of jackfruit embryonic axes reduced from 3.54 g g^{-1} to less than 0.2 g g^{-1} on flash-drying for only 90 min (Wesley-Smith *et al.*, 2001a). The relative resistance of the axes of *T. dregeana* to desiccation, also reported by Drew *et al.* (2000), may primarily be a function of the thick cuticle, probably combined with some intrinsic tissue characteristic. Such mechanisms would be advantageous in countering excess water loss in the relatively open environments in which this species often occurs naturally (Albrecht, 1993), particularly as the seeds lack a hard pericarp, as found in some recalcitrant seeds, which has been shown to lower the rate of water loss (e.g. Connor *et al.*, 1996; Pammenter *et al.*, 1998).

However, it must be noted that water in these axes may not be evenly distributed among the cells. In *T. dregeana* axes, very large cells in which vacuoles appear to be filled with amorphous masses occur regularly (Fig. 2.11a). If these masses are not hydrophilic, then the water content of those cells not containing them would be higher than the 'overall' values obtained for axes. If, in contrast, the amorphous masses are hydrophilic, then the water content of the other cells would be lower than reflected by the value for whole axes. Whether or not these cells occur with sufficient frequency to make a significant difference either way, is presently not known. However, similar cells are not encountered in *T. emetica* axes.

The embryonic axes of *T. emetica* are much smaller than those of *T. dregeana* (Table 2.1), and dry more quickly, probably primarily as a consequence of the greater surface area:volume ratio. Additionally, if the amorphous mass-filled cells have an influence on water retention in *T. dregeana* axes, their absence in *T. emetica* may influence the kinetics of drying.

2.5.3 The effect of dehydration on the viability and ultrastructure of seeds and embryonic axes

Slowly-dried seeds of *T. dregeana* and *T. emetica* were ungerminable at axis water contents 0.55 g g^{-1} (attained after six days) and 0.42 g g^{-1} (attained after 3 days) and lower, resp., while some excised embryonic axes remained viable to considerably lower water contents following flash-drying: 0.16 g g^{-1} for *T. dregeana* and 0.26 g g^{-1} for *T. emetica* (Figures 2.5 and 2.6). However, the axis water contents, attained by flash-drying, below which viability declined abruptly, were somewhat higher, around 0.2 and 0.4 g g^{-1} for *T. dregeana* and *T. emetica*, respectively. These damaging water contents are higher than those attainable by fully viable, mature orthodox seeds. However, the responses of *W. salutaris* differed from those of *T. dregeana* and *T. emetica*: whole seeds dehydrated in silica gel over six days retained viability to axis water contents as low as 0.1 g g^{-1}, although 60% and 100% of the excised embryonic axes had lost viability when flash-dried, to similar water contents over a 60- and 90-minute period, respectively (Figure 2.7).

A significant phenomenon observed in this study was that flash-dried embryonic axes of *Trichilia* spp. lost the capacity for shoot development at water contents at which germination (as defined by radicle growth) still took place (Figures 2.5 and 2.6). This is a common observation in studies dealing with dehydration of embryonic axes of recalcitrant seeds, and many workers report survival only in terms of root growth (e.g. *Theobroma cacao* [Chandel *et al.*, 1995] and *Araucaria hustenii* [Pritchard and Prendergast, 1986]). In more extreme cases, shoot formation has been observed only in control, undried axes, with all dehydration treatments precluding their ability for shoot formation (e.g. *Artocarpus heterophyllus* [Wesley-Smith *et al.*, 2001a]).

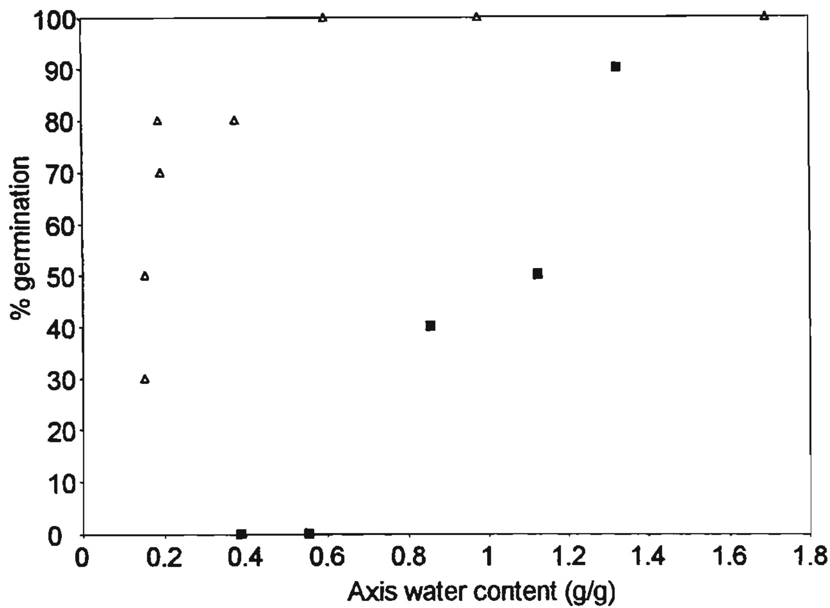


Figure 2.5 The relationship between axis water content, and the percentage germination of slowly-dried *T. dregeana* seeds (■), and flash-dried embryonic axes (Δ)

The differential response of shoot- and root-tips to dehydration is likely to be a consequence of different drying rates at the shoot- and root-poles of individual embryonic axes, resulting in different shoot and root water contents in each axis at the end of each flash-drying period. As shown in Figure 2.8, the water content of the shoot-pole was considerably higher than that of the root-pole in freshly-harvested material. Upon dehydration, however, the shoots of individual axes initially dehydrated more rapidly than the roots, after which the root and shoot water contents declined in parallel, with that of the shoots being lower than of the roots. The result of this drying pattern is suggested to have imposed a more severe stress upon the shoot meristem for prolonged periods.

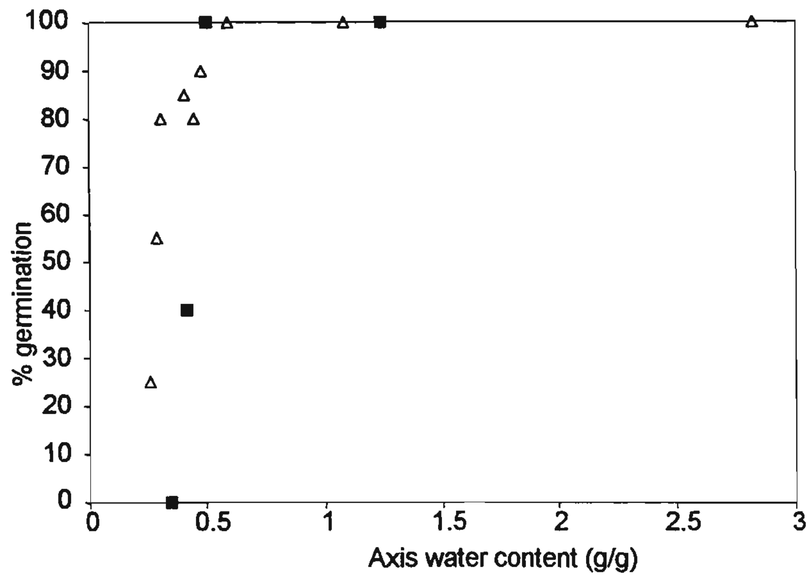


Figure 2.6 The relationship between axis water content, and the percentage germination of slowly-dried *T. emetica* seeds (■), and flash-dried embryonic axes (Δ).

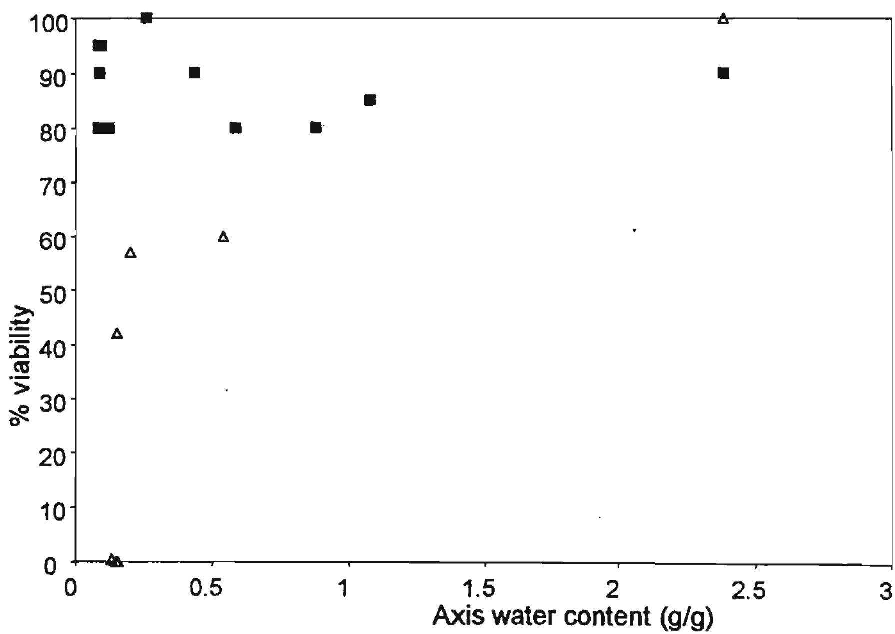


Figure 2.7 The relationship between axis water content, and the percentage germination of *W. salutaris* seeds dried in silica gel (■), and viability of embryonic axes dehydrated in a laminar air flow (Δ).

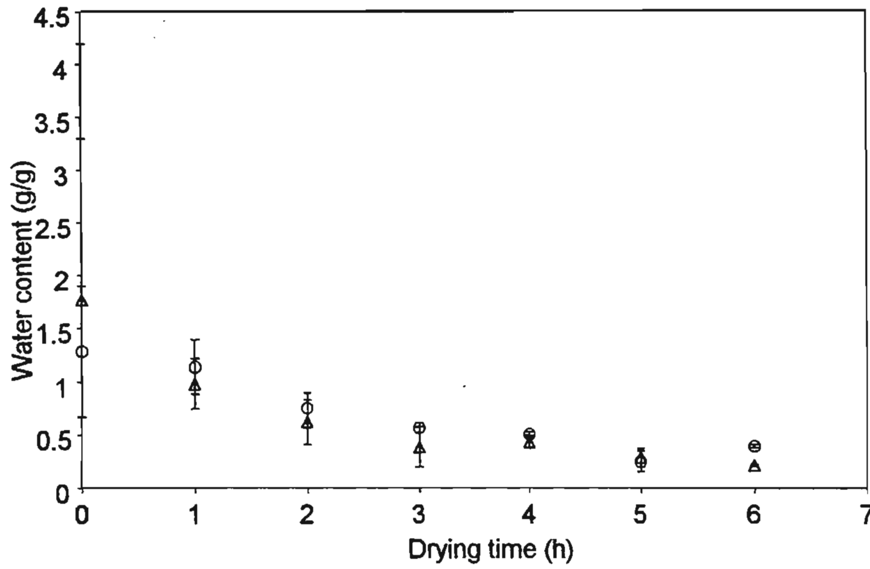


Figure 2.8 Changes in the water contents of shoot- (Δ) and root-tips (o) of the embryonic axes *of T. dregeana*. Each embryonic axis was dehydrated as a unit and, after each dehydration period, the shoot- and root-tip was separated for individual water-content determination. Markers represent the average ± standard deviation of five individual embryonic axes.

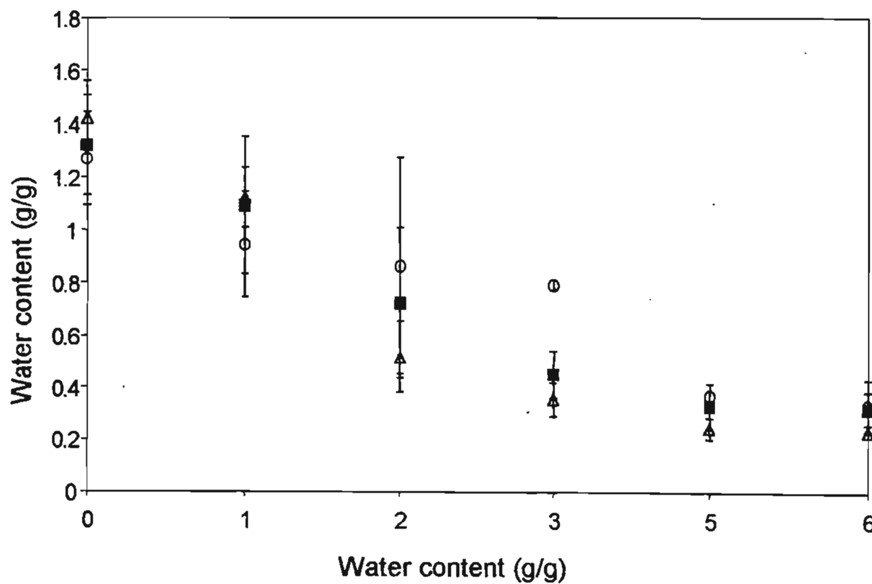


Figure 2.9 Water contents root-tips (o), hypocotyls (•), and shoot-tips (Δ) of axes of *T. dregeana* encapsulated with sodium alginate and flash-dried as entire units. Markers represent the average ± standard deviation of five individual embryonic axes.

The slower drying rate of the root pole is probably a consequence of the protection of the root apex afforded by several ranks of cells constituting the root cap Differential drying within embryonic axes affects the possibility of desiccating these explants to water contents low enough to facilitate successful cryopreservation (Chapter 3). Efforts were therefore made to achieve a more uniform drying rate throughout individual embryonic axes. This was done by initially encapsulating the axes with an alginate gel before desiccation. Two types of alginate gel were used: a fungicidal alginate gel (custom-manufactured from *Ecklonia maxima* by Kelp Products, in Simon's Town, Western Cape Province, South Africa), and a commercially prepared sodium alginate. Such encapsulation could have an added advantage in terms of reduction of freezing injury during cryopreservation (Dereuddre *et al.*, 1990).

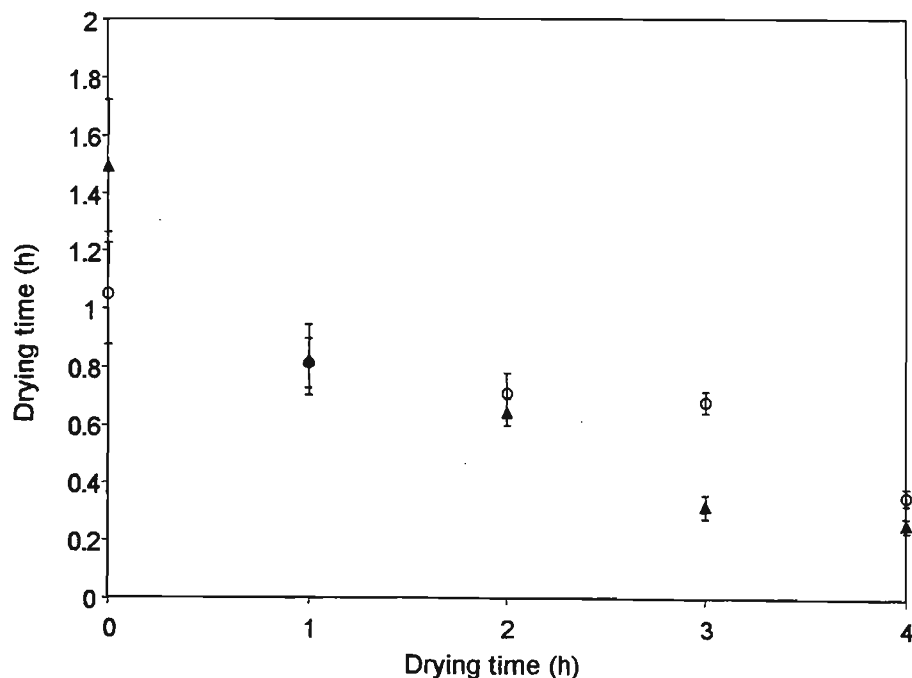


Figure 2.10 Water contents of shoot-tips (▲) and root-tips (○) of axes of *T. emetica* encapsulated with sodium alginate and flash-dried as entire units. Markers represent the average ± standard deviation of five individual embryonic axes

Encapsulation, with either type of alginate did not alter the pattern of drying in the embryonic axes, and the shoot tip still dried faster than the root apex, in both *T. dregeana* and *T. emetica* (Figures 2.9 – 2.10). The encapsulation was therefore adjusted such that only the shoot pole was encapsulated: however, the pattern of drying (not illustrated) remained unaffected. It therefore appears that the putative protection offered by the root-cap cells during dehydration cannot be mimicked by an alginate layer, the hydrodynamics of which would be different from that found in a living tissue.

Both flash-dried embryonic axes and embryonic axes dried slowly within whole seeds were processed for electron microscopy after rehydration for 1 h to avoid fixation artifacts (Wesley-Smith, 2001). Root and/or shoot meristematic and immediately adjacent cells were examined ultrastructurally, and the following general observations made (Figs 2.11 and 2.13): cells of undried embryonic axes of both *Trichilia* spp. were typical of freshly-harvested recalcitrant seeds, with features indicative of ongoing active metabolism such as many cristate mitochondria, numerous profiles of rough endoplasmic reticulum and Golgi bodies, small vacuoles probably formed from cytolysomes (as indicated by intravacuolar membranous inclusions (Fig 2.11b; 3.34a). Chloroplasts showing considerable inner membrane development were abundant in the shoot meristems (Figs 2.11d and 2.13 d), and the cytomatrical organisation in the non-dried condition was such that organelles were normally distributed throughout the cell (Figs 2.11b and 2.11a, c), an indication of the underlying cytoskeletal organisation.

On dehydration, the two *Trichilia* species suffered differing degrees of ultrastructural damage as water content declined:

2.5.3.1 *Trichilia dregeana*

In root meristematic cells of axes flash-dried to a water content of about 0.16 g g^{-1}, there was a general maintenance of cellular organisation, in which nuclear morphology appeared normal and organelles could easily be discerned (Fig 2.12a, b). As illustrated, however, the organelles were frequently seen to assume an orientation around the nucleus, which was probably as a consequence of the dismantling of the cytoskeleton as

water was lost. As the viability of *T. dregeana* declined sharply after flash-drying to water contents below 0.2 g g^{-1} (Fig. 2.5), this indirect evidence of cytoskeletal disorganisation could indicate its inability to re-constitute. Contrary to the general retention of ultrastructure of flash-dried material, cells from axes of seeds that had been slowly dried to the much higher water content (0.55 g g^{-1}) were extensively damaged, with hardly any subcellular organisation remaining (Fig. 2.12c).

2.5.3.2 *Trichilia emetica*

Flash-drying for 30 minutes achieved a similar axis water content, about 0.5 g g^{-1}, as slow drying for three days (Fig. 2.2). The ultrastructural situation in the axis cells of the material dried at the two different rates could not have been more different: cells from the flash-dried axes had a well-organised cytomatrix (Fig. 2.14a), with well-developed mitochondria and many relatively short profiles of rough endoplasmic reticulum, polysomes, and Golgi bodies (Fig. 2.14b), indicating the metabolic potential of the axes. In stark contrast, cells from axes of seeds dehydrated to similar water contents over three days displayed total subcellular destruction (Fig. 2.14c). The plasmalemma was completely detached from the cell wall and the entire cell contents had coalesced to such an extent that it was impossible to discern individual organelles.

In axes flash-dried for 90 minutes to a water content of about 0.3 g g^{-1} (80% germination and 25% shoot formation), cells from root-meristems presented an ultrastructure consistent with normal functioning including cristate mitochondria, discrete Golgi bodies, endoplasmic reticulum profiles, and well-constituted nuclear and vacuolar membranes (Fig 2.15a, b). However, many cells from shoot meristems exhibited distorted organelles with occasional blebbing (Fig 2.15c, d).

As shown above for both species of *Trichilia*, axis water contents of 0.3 g g^{-1} is not representative of the water content of the shoot pole, which is considerable lower. Hence, in the shoot meristem (which occupies the most superficial, hence least protected, cell layers), water contents would have been at damaging levels for most of the 90-min

dehydration period, thus accounting for the marked ultrastructural abnormality seen (e.g. Fig. 2.15c).

Further flash-drying the embryonic axes for 120 minutes reduced the water content to 0.26 g g^{-1}, and resulted in extensive subcellular damage to both root and shoot meristems (Fig 2.16)

It is noteworthy that cells from the embryonic axes of *T. dregeana* maintained relative ultrastructural integrity following rapid dehydration to 0.16 g g^{-1}, while those of *T. emetica* were completely destroyed at an axis water content of around 0.26 g g^{-1}, despite the fact that *T. emetica* axes are smaller than those of *T. dregeana* (Table 2.1). This implies *T. emetica* axes are more sensitive to extremes of dehydration than those of *T. dregeana*, as suggested by the results of germination following axis dehydration by flash-drying (Figs 2.5-2.6). If, however, conclusions were drawn from responses of whole seeds after dehydration, this would have led to the conclusion that *T. dregeana* is more sensitive to desiccation than is *T. emetica* (Figs 2.5-2.6).

2.5.3.3 *Warburgia salutaris*

In contrast to the situation in *Trichilia*, in *Warburgia salutaris*, cells from seeds extracted from freshly-harvested fruits showed a relatively less active state, with electron-translucent mitochondria and no evidence of endomembrane activity. Lipid bodies were prevalent, (Fig 2.17a), a feature not observed in axis cells of the *Trichilia* spp., but which has been reported in oil-rich *Azadirachta indica* (Meliaceae [Berjak *et al.*, 1995]).

After relatively slow dehydration of the seeds (over three days) to an axis water content of ~ 0.1 g g^{-1}, the subcellular integrity was unaffected (Figure 2.17b). However, rapid dehydration of excised axes (over 90 minutes in an airstream) to the same water content resulted in complete subcellular derangement, with no organellar organisation remaining (Figure 2.17c).

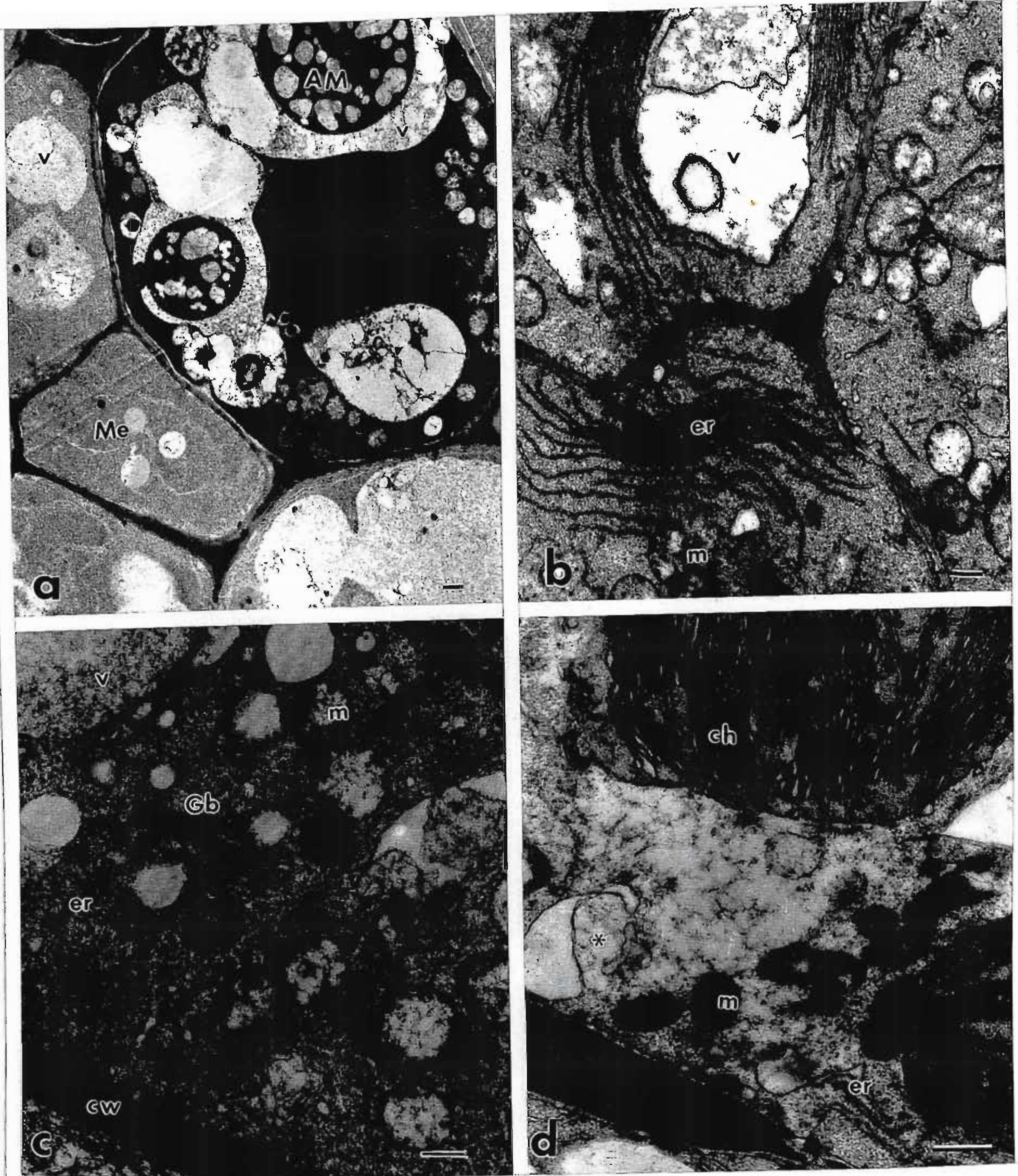


Fig. 2.11 Ultrastructure of meristematic cells (a, b root; c, d shoot) from embryonic axes of *T. dregeana*. (a), darkly-staining cells containing intravacuolar amorphous masses (AM) are scattered among meristematic cells (Me). (b), the cells exhibited a marked degree of endomembrane development, with abundant profiles of rough endoplasmic reticulum, and vacuoles often containing membranous material (asterisk). (c), the cytomatrix was dense with non-membrane-bound polysomes, and Golgi bodies and numerous mitochondria were present. (d), cells from the shoot meristem also had well-differentiated chloroplasts and mitochondria. The 'cleared' cytomatrical area (asterisk) is probably the site of nascent vacuolar formation (Lamb and Berjak, 1981). m, mitochondrion; ch, chloroplast; er, endoplasmic reticulum; Gb, Golgi body; v, vacuole; N, nucleus; Nu, nucleolus. Bar = 500 nm

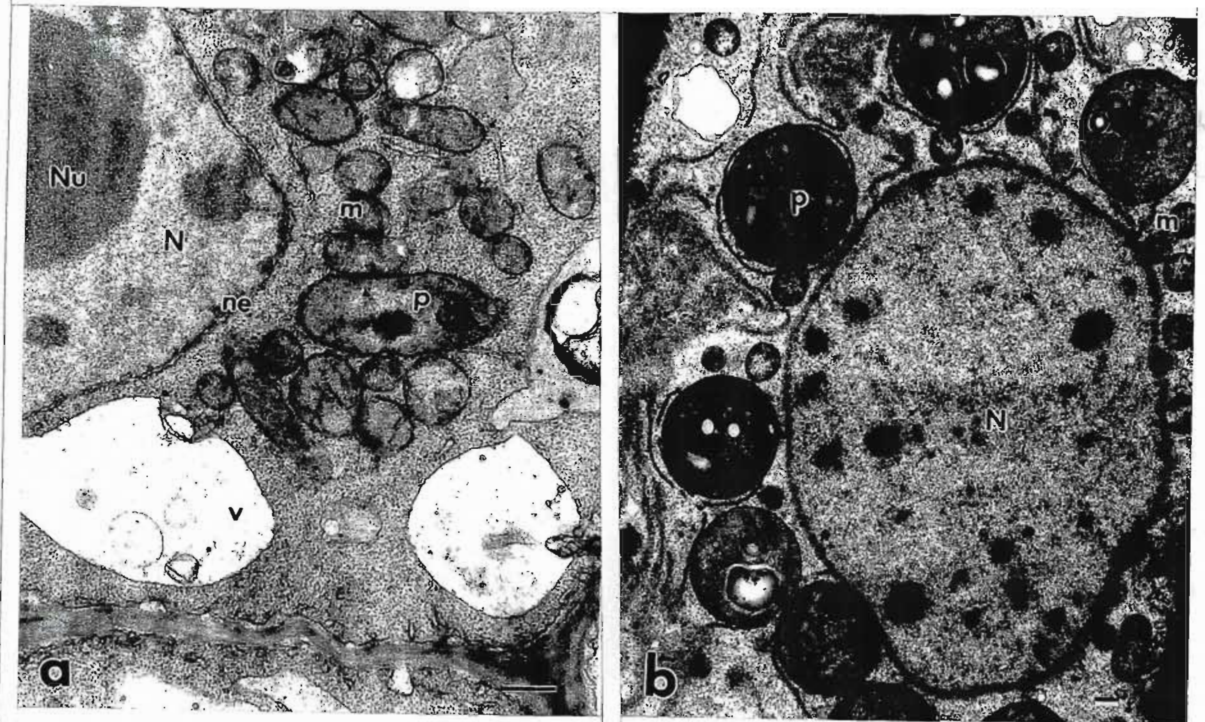
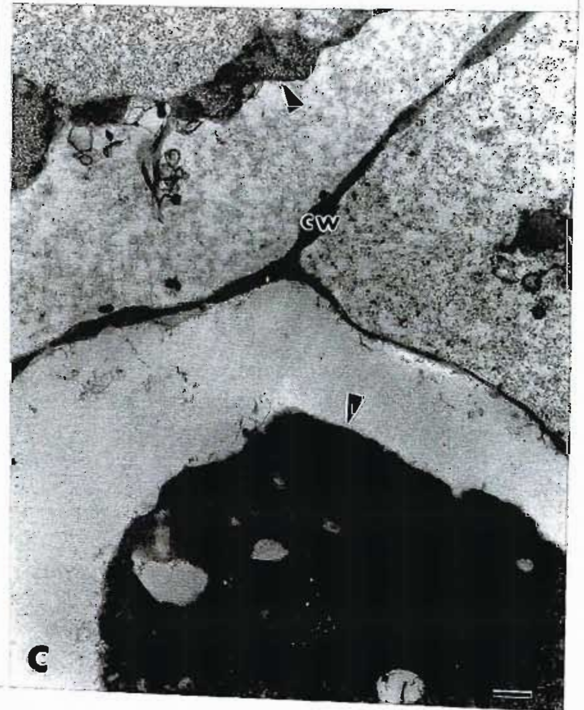


Fig 2.12 Ultrastructure of root meristematic cells of axes of *T. dregeana* dehydrated either to 0.16 g g^{-1} over 4 h (a) and (b) or to 0.55 g g^{-1} over six days (c). In all cases, axes were rehydrated for 1 h prior to processing. (a), cells of flash-dried axes maintained a functional ultrastructural appearance, although there was indirect evidence of deficient cytoskeletal re-assembly as evidenced by perinuclear clustering of organelles. This was particularly apparent in meristem derivatives, where plastids were located around the nucleus (b). In the axes dehydrated slowly, the cells lost all ultrustructural integrity at a considerably higher water content, Arrow-heads in (c) indicate the coalesced cytomatrix.
m, mitochondrion; v, vacuole; N, nucleus; Nu, nucleolus; ne nuclear envelope; p, plastid; cw, cell wall. Bar = 500 nm



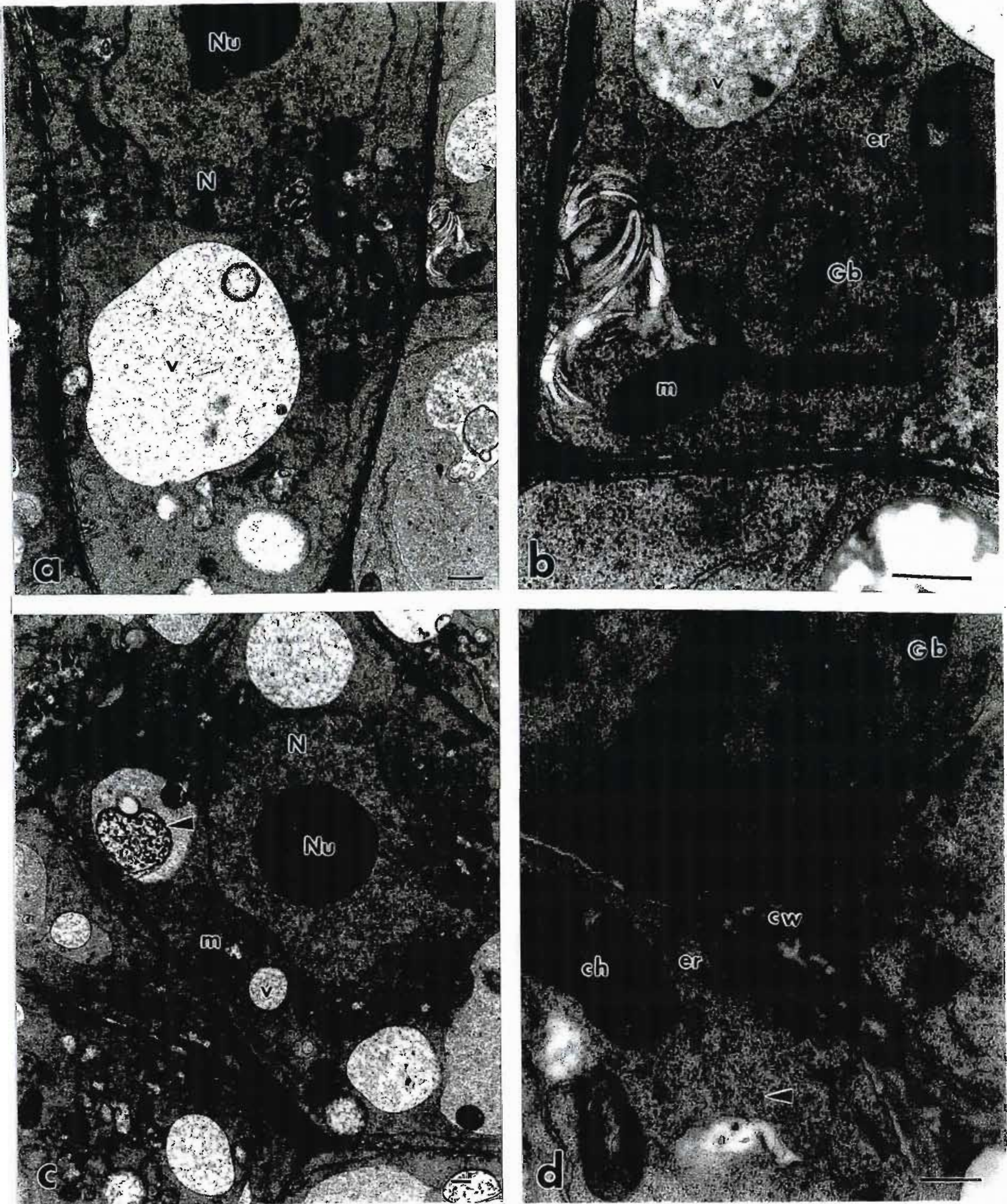
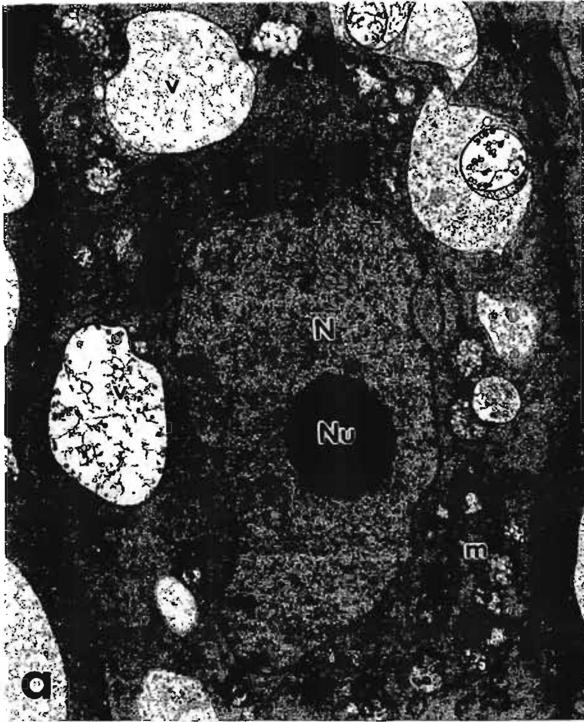


Fig. 2.13. The ultrastructural situation meristematic cells of *T. emetica*. (a) In roots, the cells were typified by slightly irregular nuclear profiles and a few well-developed vacuoles, with organelles distributed throughout the cytomatrix. (b) Well-developed mitochondria, Golgi bodies, rough endoplasmic reticulum, and polysomes, were indicative of active metabolism. (c) Cells from the shoot meristem contained a number of smallish vacuoles, which contained evidence of autophagic activity (arrow), nuclei of slightly irregular profile, dominated by prominent nucleoli, and a generally even distribution of (d) plastids/chloroplasts, mitochondria, Golgi bodies, rough endoplasmic reticulum and cytomatrical polysomes (arrow-head).
m, mitochondrion; ch, chloroplast; er, endoplasmic reticulum; Gb, Golgi body; v, vacuole; N, nucleus; Nu, nucleolus. Bar = 500 nm



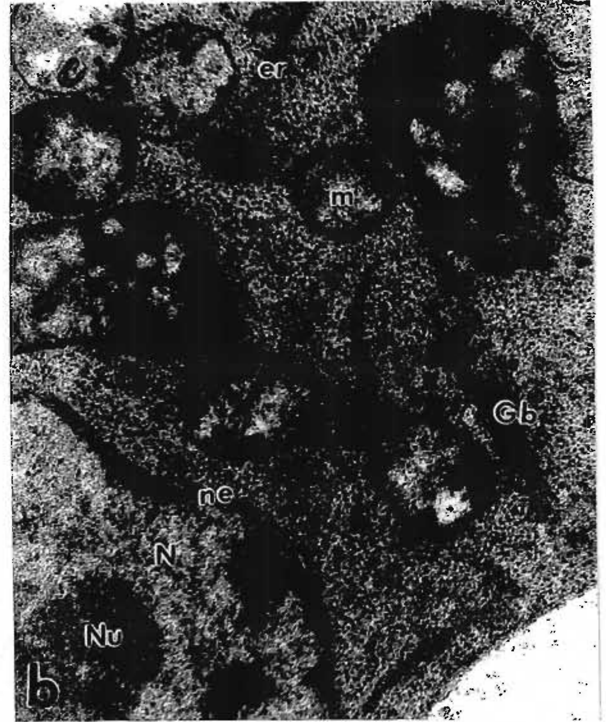
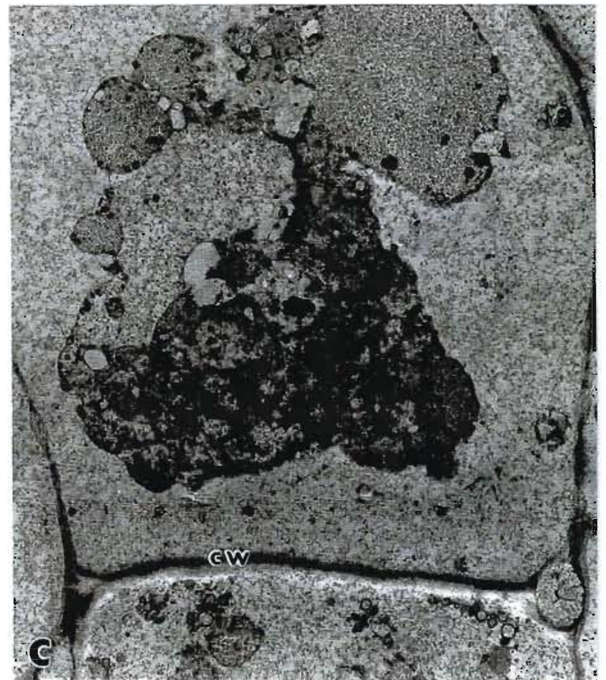


Fig 2.14. Root meristematic cells of embryonic axes of *T. emetica* flash-dried to *c.* 0.5 g g^{-1} over 30 min. (a) The ultrastructure was generally maintained, with an apparent normal distribution of organelles. (b) The occurrence of many cristate mitochondria, Golgi bodies, and endoplasmic reticulum was indicative that the potential for metabolic activity in the axes was not curtailed. (c) When the axes were dehydrated over 3 days to the same water content as those illustrated in (a) and (b), the ultrastructure was completely deranged, and no organelles could be discerned.
m, mitochondrion; er, endoplasmic reticulum; Gb, Golgi body; v, vacuole; N, nucleus; Nu, nucleolus; cw, cell wall. Bar = 500 nm



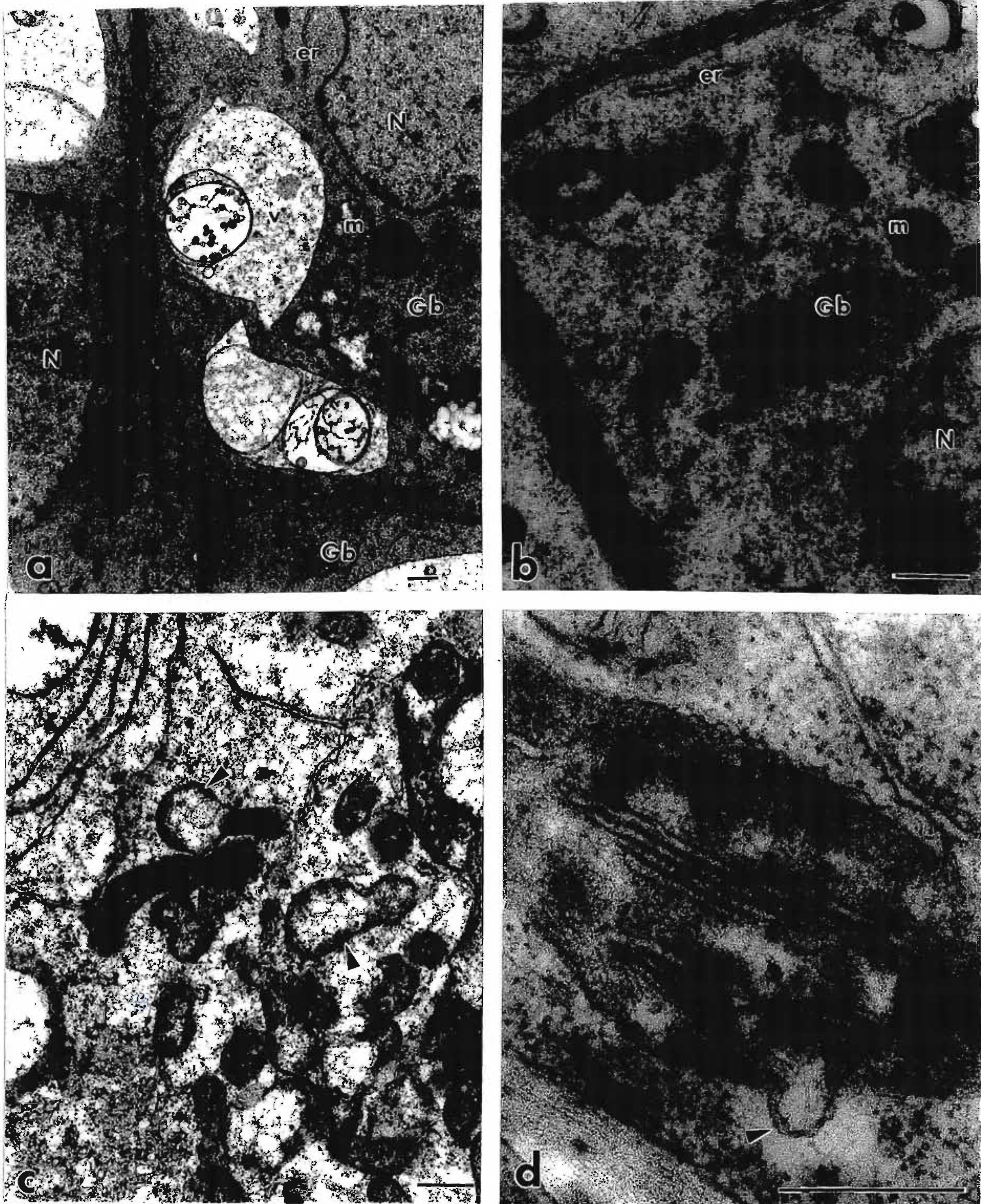


Fig 2. 15 The ultrastructure of embryonic axes of *T. emetica* dehydrated to *c.* 0.3 g g^{-1}, over 90 min. (a) and (b) Cells from the root meristem maintained the structure consistent with normal functional cells; while cells from the shoot meristems had abnormally-shaped organelles (e.g. arrow-heads in c), with irregularities such as the blistered membrane boundary of the plastid illustrated in (d), arrowed.
m, mitochondrion; er, endoplasmic reticulum; Gb, Golgi body; N, nucleus; Nu, nucleolus. Bar = 500 nm.

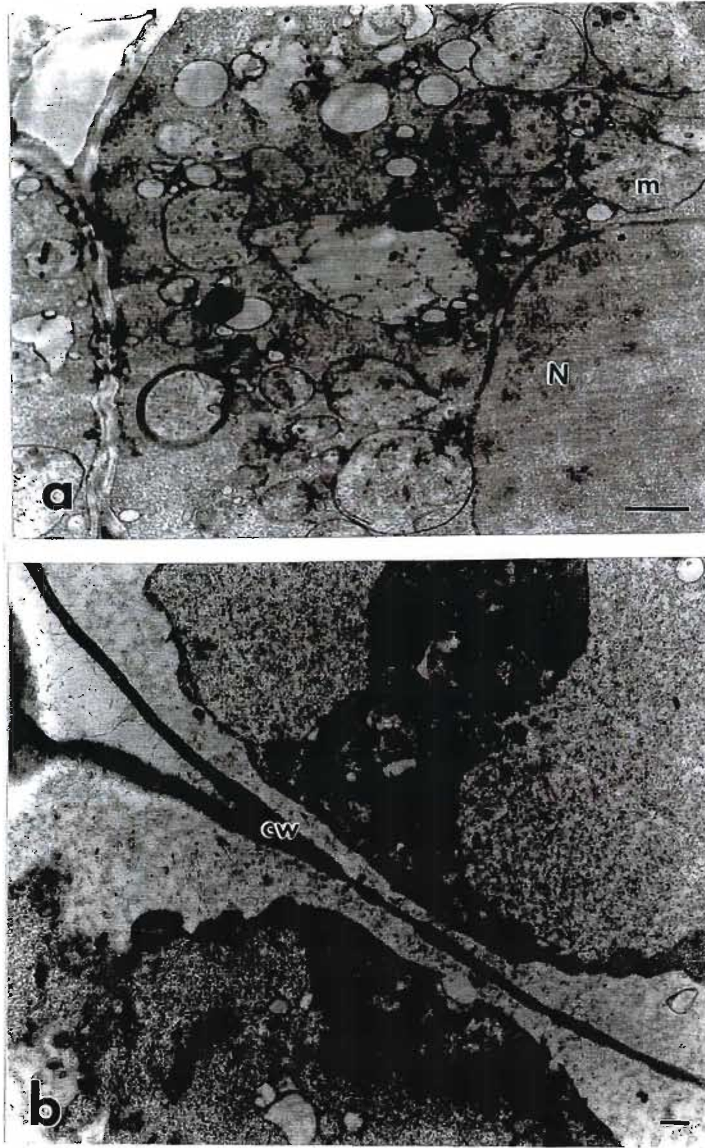


Fig 2.16. The ultrastructural situation in *T. emetica* axes flash-dried for 120 min to a water content of *c.* 0.26 g g^{-1} showed extensive damage to the clustered organelles in the root apex cells (a), while no organelles could be discerned in the cells of the shoot meristem (b).
m, mitochondrion; N, nucleus; cw, cell wall. Bar = 500 nm.

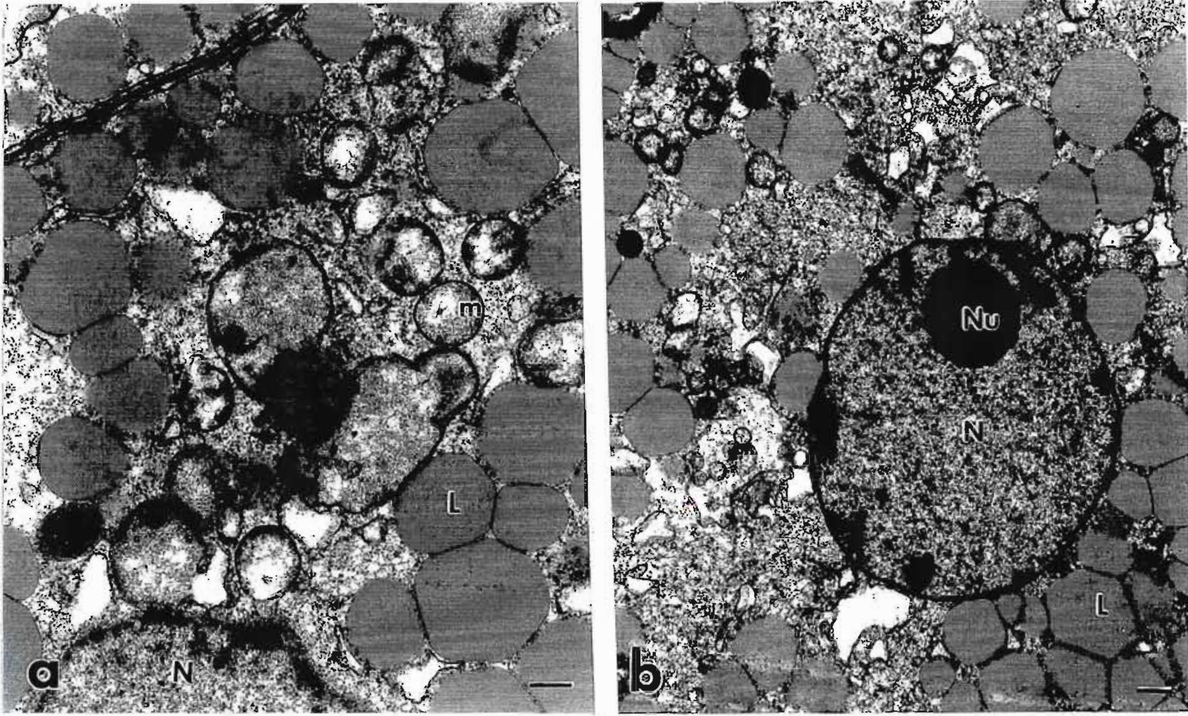


Fig 2.17 The ultrastructure of root meristematic cells of *W. salutaris* embryonic axes. (a) Axes from freshly-extracted seed, with the ultrastructure dominated by lipid bodies (L) and contained clearly resolvable, but de-differentiated organelles. (b) Cells from axes excised from seeds that had been dehydrated in silica gel, for 72 h, to *c.* 0.1 g g^{-1}, in which the ultrastructural integrity was maintained. (c) Cells from axes dehydrated in a laminar air flow for 90 min to *c.* 0.1 g g^{-1}. The lipid bodies had coalesced, and all ultrastructural organisation was lost.
m, mitochondrion; N, nucleus; Nu, nucleolus; cw, cell wall. Bar = 500 nm.

Besides ultrastructural and viability characteristics, another measure of desiccation damage in seeds is the loss of cell membrane integrity, which can be expressed in terms of the rate and extent of electrolyte leakage from the tissues. This has been used successfully to assess desiccation damage for a number of desiccation-sensitive seed tissues (e.g. Vertucci and Leopold, 1987; Pammenter *et al.*, 1991; Berjak *et al.*, 1992; Poulsen and Eriksen, 1992; Li and Sun, 1999; Wesley-Smith *et al.*, 2001a). In this study, the rate of electrolyte leakage was observed to increase with decreasing water content in embryonic axes of *T. dregeana* and *T. emetica.* The rate of increase was greater in axes dried slowly within whole seeds, reaching two and four times of that in flash-dried excised axes of *T. emetica* and *T. dregeana*, resp., at the lower water contents (Figures 2.18-2.19). A similar relationship between drying rate and electrolyte leakage was reported for embryonic axes of jackfruit (*Artocarpus heterophyllus*) by Wesley-Smith *et al.* (2001a), and indicates that flash-drying helps maintain the integrity of cellular membranes during dehydration and subsequent rehydration. Irrespective of the drying rate, however, far more solutes were leaked, and at a greater rate, from axes of *T. emetica* than from those of *T. dregeana* (compare Figs 2.18 and 2.19). This observation agrees with survival and ultrastructural observations, that axes of *T. emetica* are considerably more desiccation-sensitive than those of *T. dregeana*.

Rapid dehydration of desiccation-sensitive seed tissues facilitates viability retention to relatively low water contents. This is presumed to be the outcome of the very limited time afforded (during drying), in which deleterious metabolism-linked damage can occur (Pammenter, *et al.*, 1998; Pammenter and Berjak, 1999). Damage to subcellular membranes – which is curtailed during rapid drying – is prominent among the deleterious consequences of slow dehydration (Kioko *et al.*, 1998; Pammenter *et al.*, 1998). According to Berjak *et al.* (1989), slow-drying - certainly over a period of days, as was done in this study - allows the seeds to progress along the germination pathway which is accompanied by their increasing desiccation-sensitivity, while keeping the tissues at 'intermediate' water contents at which active, regulated metabolism gives way to de-regulated, 'out-of-phase' metabolism (Pammenter *et al.*, 1998) and generation of free radicals, which, in turn, cause peroxidative membrane damage (Senaratna and McKersie, 1986; Leprince *et al.*, 1990b; Hendry, 1993; Pammenter *et al.*, 1994; Smith and Berjak, 1995).

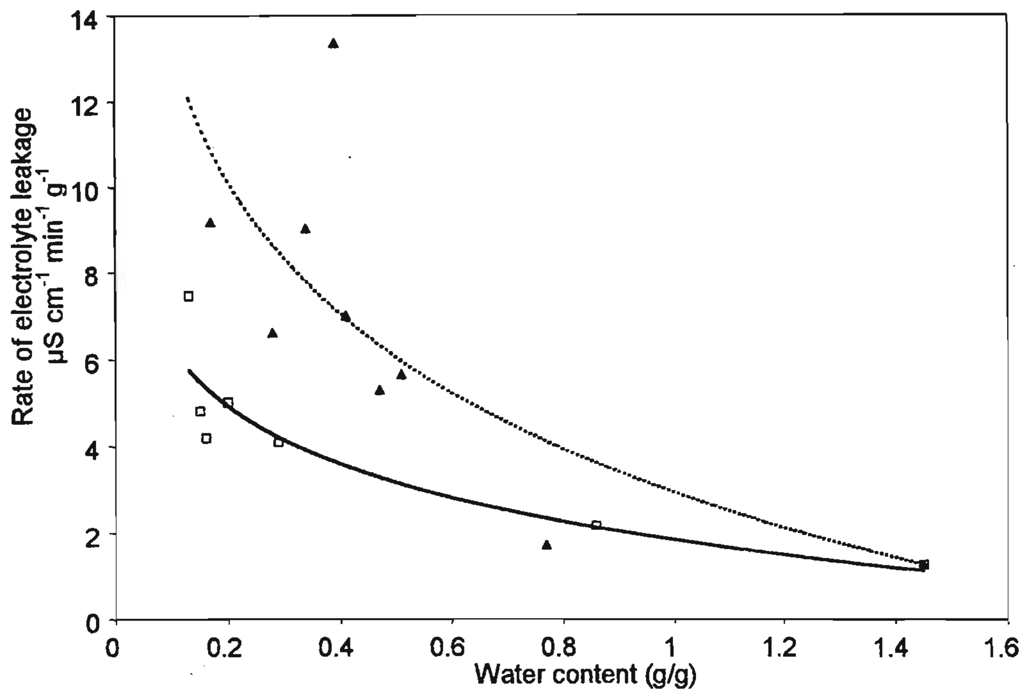


Figure 2.18 Log of the rate of electrolyte leakage from embryonic axes of *T. dregeana* dehydrated either slowly (dotted line, ▲) or fast (solid line, □).

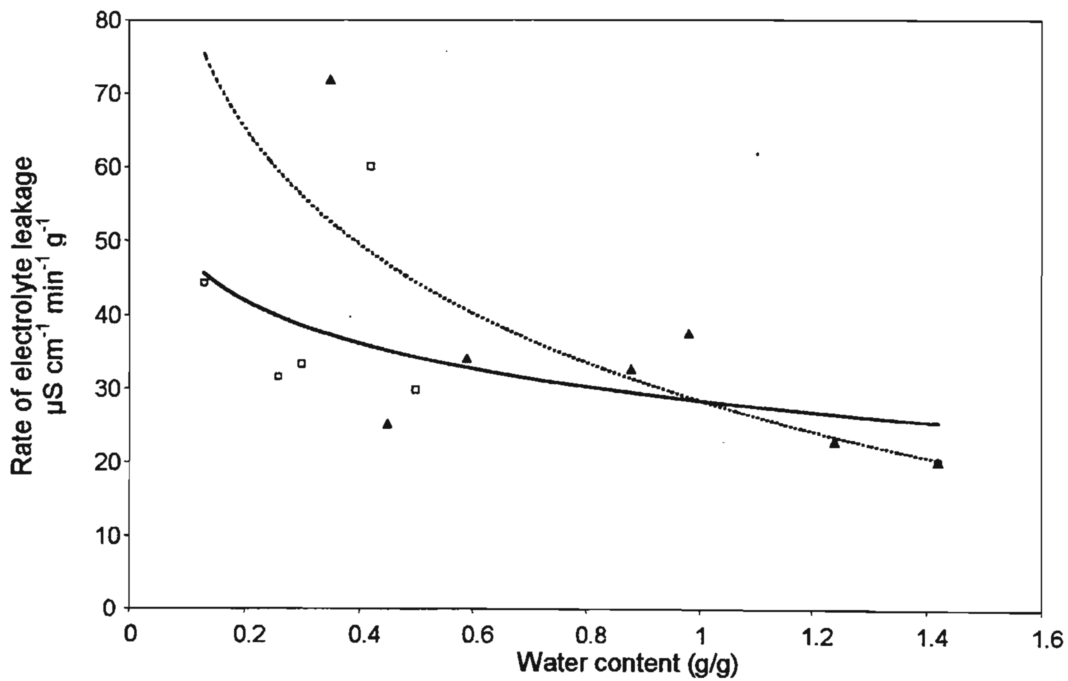


Figure 2.19 Log of the rate of electrolyte leakage from embryonic axes of *T. emetica* dehydrated either slowly (dotted line, ▲) or fast (solid line, □).

Nevertheless, regardless of the rate at which the embryonic axes are desiccated, there is a lower water content limit below which viability will be rapidly lost, and this limit is higher than that tolerated for extended periods by orthodox seeds, which can be desiccated to water contents ≤ 0.05 g g^{-1} without losing viability (Hong and Ellis, 1996). A spectrum of factors is implicated in the loss of viability as a result of desiccation in recalcitrant seeds, among which is their inability (unlike orthodox seeds) to tolerate the loss of non-freezeable water (Pammenter *et al.*, 1991). Those authors, working on the embryonic axes of *Landolphia kirkii*, suggested that the amount of non-freezable water required to maintain intracellular integrity represents the 'minimum tolerance level'. Finch-Savage (1992) also identified 'critical moisture contents' in *Quercus robur*, equal to the amount of 'matric-bound water', below which those seeds could not withstand dehydration. However, subsequent studies by Pammenter *et al.* (1993) demonstrated that sensitivity to very rapid desiccation in recalcitrant seeds is not necessarily accountable in terms of structure-associated water alone, as (those authors showed) some species lose viability at water contents in excess of the bound water levels in their tissues.

In contrast to the situation in *Trichilia* spp., axes slowly-dried seeds of *W. salutaris* maintained viability to water contents much lower than those tolerated by flash-dried embryonic axes. Whole seeds tolerated dehydration to axis water contents as low as 0.1 g g^{-1}, while the viability of excised flash-dried embryonic axes rapidly declined below a water content of 0.2 g g^{-1} (Fig. 2.7). This increased tolerance to slower, rather than faster, dehydration, is atypical of recalcitrant seeds (Berjak *et al.*, 1993; Pammenter *et al.*, 1998), but has been observed for embryos of developing orthodox seeds (Bochicchio *et al.*, 1994). This, coupled with the ability of the seeds to withstand dehydration to water contents as low as 0.1 g g^{-1}, indicates that these seeds may have certain protective mechanisms against dehydration, such as some of those outlined in section 2.2.3. However, very slow dehydration of *W. salutaris* seeds, by equilibration with ambient relative humidity, results in loss of viability at substantially higher water contents (Albrecht, 1993) than those tolerated after following the 'slow' dehydration used in this study. This implies that whatever protective mechanisms these seeds might have, they are considerably less efficient than those typical of orthodox types. Furthermore, unlike

typical orthodox seeds, *W. salutaris* seeds are shed at high water contents, a situation invariably encountered in recalcitrant and other non-orthodox (e.g. intermediate) seeds.

Even though the seeds of *W. salutaris* could withstand a relatively high degree of dehydration without losing viability, storage at the low water contents resulted in a loss of viability within a matter of weeks (Fig 2.37), reinforcing the placement of *W. salutaris* seed in the 'intermediate' category identified by Ellis *et al.* (1990). This is in contrast to reports by Albrecht (1993), who classified the seeds as highly recalcitrant. However, that author utilised a very slow rate of dehydration to test the desiccation response of the seeds. That dehydration technique, described by Kioko *et al.* (1993) involved drying the seeds in the shade, or in a heated enclosed room (in inclement weather) for several weeks. The likely consequence of this technique, certainly at elevated temperatures, is accelerated ageing (*sensu* orthodox seeds) during which loss of viability is occasioned by such processes as free radical activity and lipid peroxidation (Smith and Berjak, 1995), probably coupled with the effects of the seemingly ubiquitous seed-associated micro-organisms (see Chapter 3, section 3.3.1.3).

Other results that conflict with previously reported findings are those regarding the desiccation response of *T. dregeana* seeds. The present work demonstrates that rapid dehydration enables the axes of these seeds to withstand much lower water contents than if slow-dried. In contrast, Choinsky (1990) asserted that the rate of drying did not have any effect on the desiccation response. However, in that study, whole seeds were used for both rates of drying, and the rates attained with 'fast' drying could therefore not remotely approach the rates achieved by flash-drying of excised embryonic axes. Furthermore, slow and fast drying were carried out at different temperatures: fast drying at 25°C, and slow drying at 15 °C. At these temperatures, there are differences in the quantitative, and perhaps also qualitative, metabolism, which may be critical to the response of recalcitrant seeds to desiccation over relatively protracted periods (Berjak *et al.*, 1989).

When dehydrated at similar rates and temperatures, excised *T. dregeana* axes survived to water contents lower than those of *T. emetica*. Furthermore, a comparison of the

respiration rates of freshly-harvested seeds, measured in this study by the evolution of carbon dioxide, showed that embryonic axes of *T. dregeana* evolved 0.962 ± 0.37 µmol CO_2 h^{-1} g^{-1} dry weight while those of *T. emetica* evolved 4.00 ± 0.08 µmol CO_2 h^{-1} g^{-1} dry weight. These values indicate that the axes of *T. emetica* were respiring at around four times the rate of those of *T. dregeana*. As the degree of metabolic activity of seeds at shedding may be associated with desiccation sensitivity (Pammenter and Berjak, 2000) this, and the desiccation response, imply that *T. emetica* may be more recalcitrant than *T. dregeana*, despite the morphological similarity and phylogenetic closeness between the two species.

The dichotomy in the seed physiology of the two species may be related to the natural habitat. In Kenya, where natural populations of these species abound, personal observations are that the two species do not co-occur, with *T. emetica* being confined to the warm coastal and riparian forests in southern Kenya, and in the pockets of tropical rain forests found in western Kenya; while the range of *T. dregeana* extends to altitudes of about 3 000 m above sea level in the central Kenyan highlands on the slopes of Mt Kenya. Thus, *T. dregeana* would be adapted to periods of cool dry weather, whereas *T. emetica* naturally thrives in a warm, wet tropical habitat, conducive to a highly recalcitrant seed habit.

The variation in the degree of recalcitrance between the species mentioned above agrees with the notion of a continuum of seed behaviour, as proposed by Pammenter and Berjak (1994). According to those authors, seed behaviour fits along a sliding scale (ranging from highly recalcitrant on one hand, to minimally recalcitrant on the other), rather than into clearly-defined, discrete (i.e. all-or-nothing) divisions.

2. 5.4 The effect of dehydration on the rate of germination

In this study, limited dehydration increased the rate of germination in *T. dregeana* and *W. salutaris* seeds, as illustrated in Figures 2.20 and 2.22, in which the slope of the germination curve for seeds sown after limited dehydration is steeper that that of seeds sown fresh. The effect was not apparent for *T. emetica* (Fig. 2.21) under the conditions of

the present experiments. In *T. dregeana*, the germination rate was marginally higher in seeds dehydrated for 2 d, (to ~ 1.0 g g^{-1}), and dropped with further dehydration (Fig 2. 20). This trend was far more marked for *W. salutaris*, with seeds dehydrated from 2.4 g g-1 for 48 h (to 0.26 g g^{-1}) having the highest rate of germination (Fig 2.22). Additionally, the initiation of germination was substantially sooner for these *W. salutaris* seeds compared with those not dried, or dehydrated for 6 h (water content ~ 1 g g^{-1}).

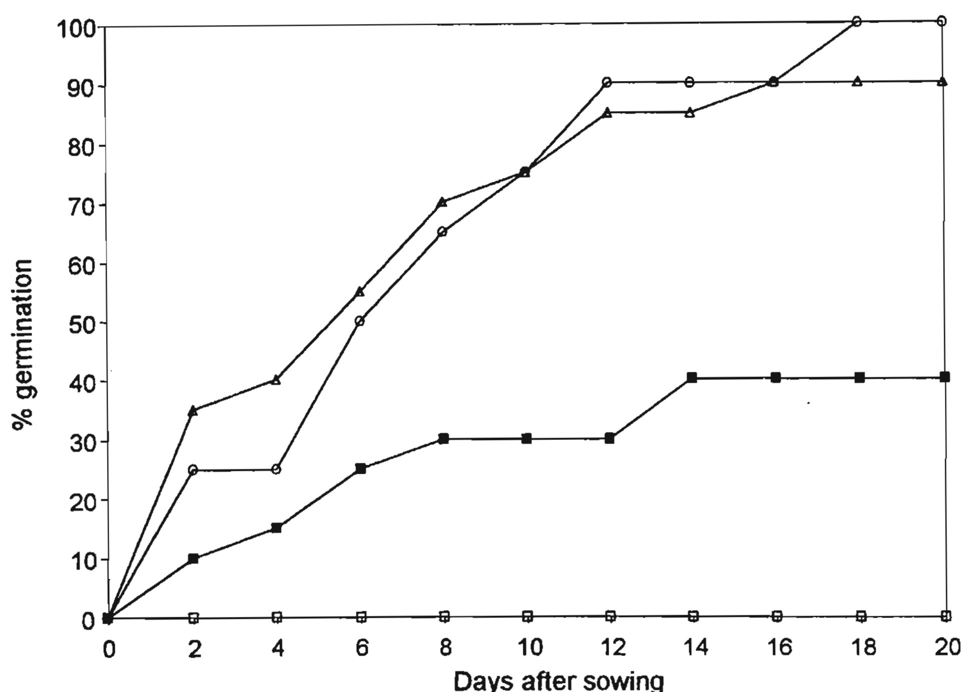


Figure 2.20 Germination time course of *T. dregeana* seeds dehydrated over silica gel to different axis water contents: o, not dried (1.3 g g^{-1}); Δ, dried to 1 g g^{-1} over 2 d; ■, dried to 0.75 g g^{-1} over 4 d; □, dried to 0.55 g g^{-1} over 6 d. The slope of each germination curve represents the germination rate.

It is noteworthy, however, that dehydration of *W. salutaris* seeds (which are tolerant to relatively low water contents) for longer periods was associated with increasingly adverse effects. This implies that, wherever in the continuum of seed behaviour (Pammenter and Berjak (1994) the seeds fit, they are not orthodox; it is possible that they are intermediate *sensu* Ellis *et al.* (1990).

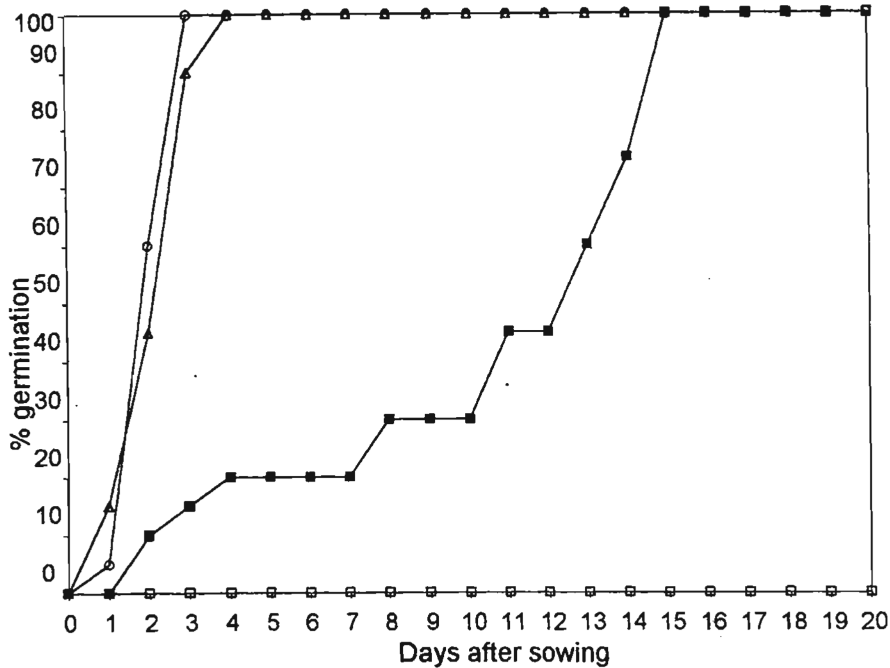


Figure 2.21 Germination time course of *T. emetica* seeds dehydrated over silica gel to different axis water contents: ○ not dried (2.8 g g^{-1}); Δ, dried to 1.2 g g^{-1} over 1 d; ■, dried to 0.7 g g^{-1} over 2 d; □, dried to 0.42 g g^{-1} over 3 d. The slope of each germination curve represents the germination rate

Stimulation of germination by short-term non-injurious dehydration is not restricted to whole seeds, as mildly flash-dried embryonic axes of *Trichilia* spp. evinced a similar response (results not shown). This phenomenon may be characteristic of recalcitrant seeds (Pammenter and Berjak, 1999), as it has been observed in several other species studied, e.g. *Ekebergia capensis* (Pammenter *et al.*, 1998) and *Camellia sinensis* (Diegel, 1991). The basis for such stimulation is only now being investigated. However, it is well-documented that dehydrins and low-molecular weight heat-shock proteins play a rôle in the response of plant embryos to mild stress and have been linked to seed vigour (Coca *et al.*, 1994; Derocher and Vierling, 1994; Bettey and Finch-Savage, 1998), and these may be involved in the stimulatory effect of mild dehydration stress on recalcitrant seeds. However, no dehydrins could be found in axes or cotyledons of mature *T. dregeana* seeds, even after dehydration- or ABA-induced stress (Kermode, 1997), this species may have different mechanisms underlying the stimulatory response.

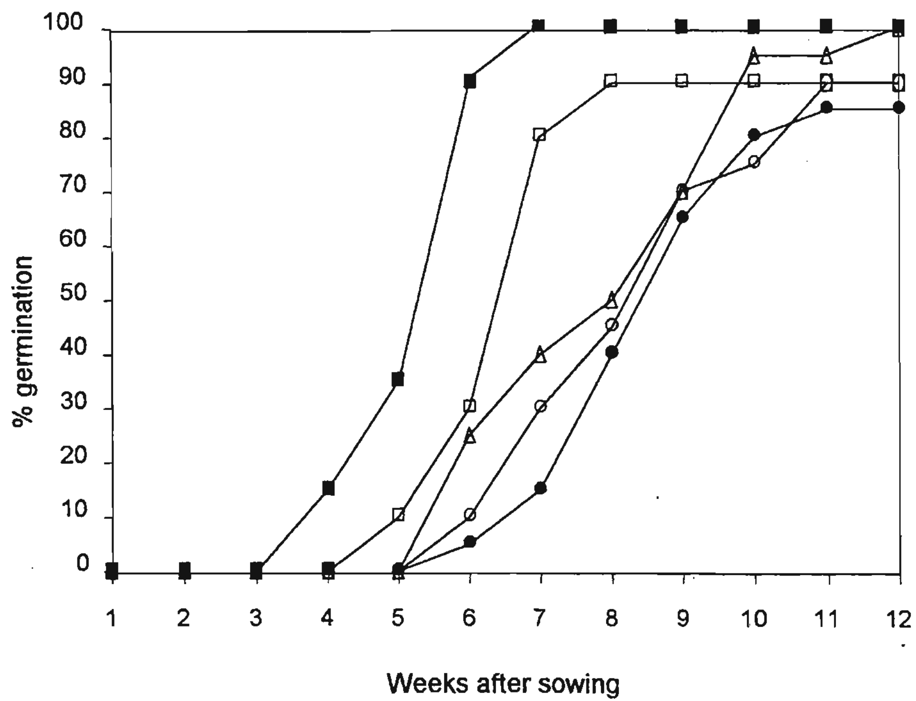


Figure 2.22 Germination time course of *W. salutaris* seeds sown without drying (○), or dehydrated in silica gel for 6 h to *c*. 1 g g^{-1} water content (Δ), or for 48 h to *c*. 0.26 g g^{-1} (■), or for 72 h to *c*. 0.12 g g^{-1} (□), or for 144 h to *c*. 0.08 g g^{-1}

In *T. emetica*, dehydration from 2.82 g g^{-1} to the water contents tested did not appear to effect a pronounced increase in germination rate. Dehydration over 1 d (to about 1.2 g g^{-1}) appeared to accelerate only the start of germination, while a 2-d drying period had a markedly adverse effect (Figure 2.21). It is, however, possible that seeds of *T. emetica* may be stimulated to germinate by dehydration interval(s) shorter than 1 d, as water content was presently more than halved during this period.

Despite the slight indication of stimulation of germination at the initial stages of dehydration, drying below a relatively high water content led to a steady reduction in the rate and totality of germination, underscoring the essentially desiccation-sensitive nature of the seeds.

2.5.5 Storage longevity of the seeds under investigation.

The seeds of all three species showed high germination capacity prior to storage, but the rate of germination varied widely among the species. The fastest-germinating seeds were those of *T. emetica*, which attained 100% germination within four days, while *T. dregeana* seeds, sown under the same conditions, reached full germination only after at least two weeks. It was five weeks before germination was first recorded seeds for seeds of *W. salutaris*, with an additional four weeks required for all the seeds to germinate.

It has been suggested that metabolic activity, which leads to germination, is a measure of recalcitrance in terms of desiccation-sensitivity (Pammenter and Berjak, 2000), and this implies that *T. emetica* is more recalcitrant than the other species studied, while *W. salutaris* is relatively the least recalcitrant of the species tested. This is in agreement with the implications that may be drawn from the results of desiccation-tolerance trials described and discussed in section 2.3.

2.5.5.1 *Trichilia dregeana*

In terms of longevity, previous information indicated that the seeds of *T. dregeana* would last in storage at ambient temperatures for only three to four weeks (Pammenter[2], pers. comm.), and would not germinate if exposed to temperatures of 15 °C or below (Choinsky, 1990). In the present investigation, the seeds were therefore initially stored at 25°C, and the influence of the seed-coat (which would presumably inhibit germination in storage) and light investigated

The total germinability of *T. dregeana* seeds in this study remained largely unchanged during a 5-month (150 d) storage period, regardless of whether the seeds were stored with or without coats, or in the dark or light (Figure 2.23). At the end of this period, most of the seeds had protruded radicles, with seeds stored without testas having all germinated after 91 d, whether stored in the dark or in the light. This is a manifestation of the ongoing germinative processes in storage, which was also illustrated by the steady rise in the germination index, calculated as the highest percentage germination divided by the number of days taken to achieve it (Figure 2.24), and the decrease in the establishment lag (the period it took for all germinants to develop shoots after radicle protrusion) as shown in figure 2.25. The phenomenon of germination in storage, initially recorded for its nuisance value (e.g. Chin and Roberts, 1980) was originally described in *Avicennia marina* by Pammenter *et al.* (1984), who emphasised that this major manifestation of metabolic activity was, in fact, the underlying cause of seed recalcitrance (Berjak *et al.*, 1989).

Whether the seeds were stored enclosed by the testa or not, in the dark or light, the axis ultrastructure was basically similar. Figure 2.26 shows the situation in seeds stored in the dark, with testas. The ultrastructure was largely undamaged, generally well-organised, and typical of highly metabolic plant cells, with active endomembrane systems (Fig 2.26). However, the accompanying autophagy and occurrence of occasional damaged organelles could be indicative of compensatory and deteriorative events caused by mild, but prolonged, water stress and perhaps the onset of uncoordinated, deleterious oxidative metabolism

[2] N.W. Pammenter, School of Life and Environmental Sciences, University of Natal, Durban, 4041, South Africa.

(Pammenter *et al.*, 1994; Smith and Berjak, 1995). Hence, even under non-dehydrating, conditions, these seeds of *T. dregeana*, which showed no signs of fungal contamination, may not have a post-harvest longevity much longer than five months.

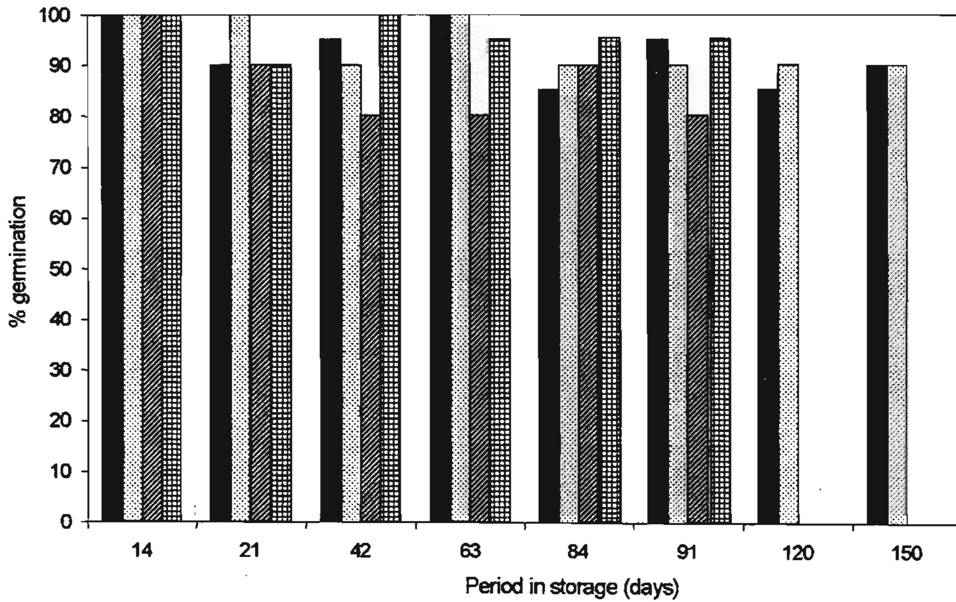


Figure 2.23. Final percentage germination of *T. dregeana* seeds stored either with testas in the dark (solid bars) or in the light (dotted bars); or without testas in the dark (hatched bars) or in the light (checked bars).

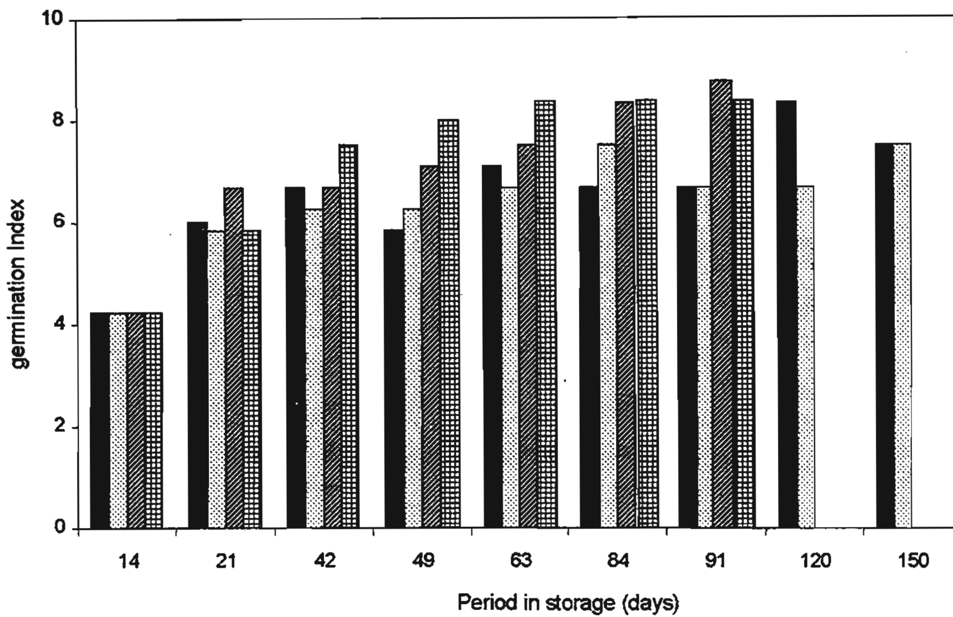


Figure 2.24 Germination Index, calculated as the maximum percentage germination divided by the number of days taken to achieve that percentage, of seeds of *T. dregeana* stored either with testas in the dark (solid bars) or in the light (dotted bars); or without testas in the dark (hatched bars) or in the light (checked bars).

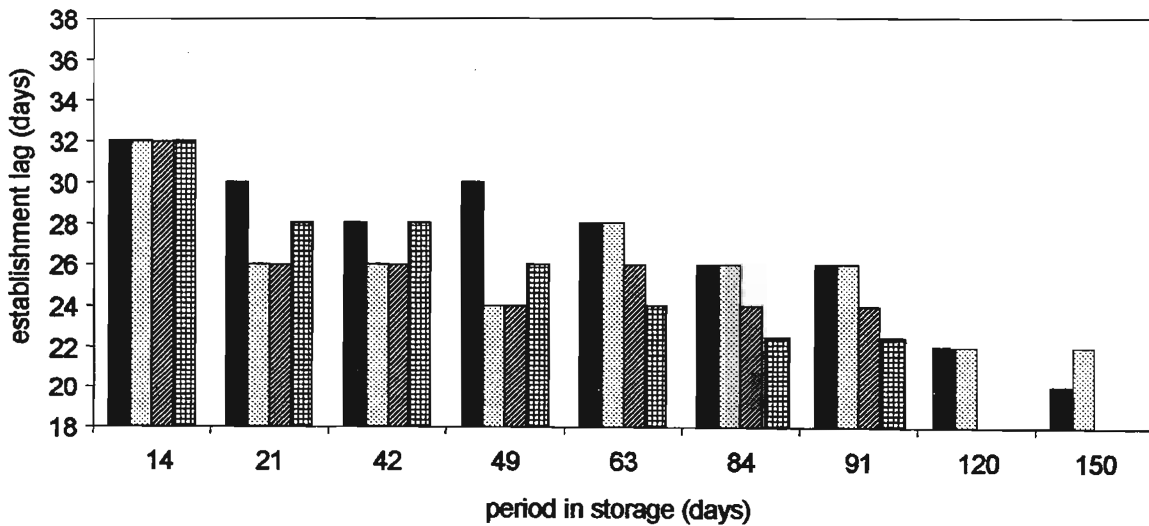


Figure 2.25 Establishment lag, calculated as the period taken by all germinated seeds to develop shoots, of *T. dregeana* seeds stored either in the dark with testas (solid bars) or without (dotted bars); or in the light with testas (hatched bars) or without (clear bars).

However, the 5-month storability of *T. dregeana* seeds described in this study is contrary to the earlier observations by Choinsky (1990) and those casually made by Pammenter (above). The widely divergent survival periods could be attributed to the variability among recalcitrant seeds of the same species in characteristics that influence longevity (such as maturity at harvest and relative desiccation sensitivity), time elapsed between shedding and collection, and importantly, whether or not infecting micro-organisms persist and/or are active, during the storage period. This variability occurs not only among species, but also among different harvests within a species, and from seed to seed within the same harvest (Berjak *et al.*, 1989; Finch-Savage and Blake, 1994).

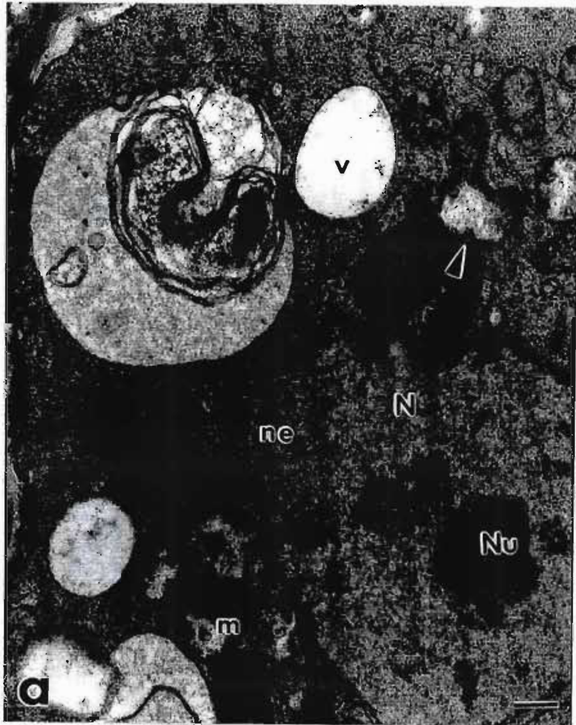
In previous studies on the germination of *T. dregeana* the variation in germination was higher than 40% between seeds from two different sites in Harare, Zimbabwe (Choinsky, 1990). In a recent storage trial using seeds from generally the same source as those used in this study, Drew *et al.* (2000) found that the seeds retained viability for only 16 to 21 days (during storage at 25°C and 16°C, resp.), much shorter periods than those presently recorded.

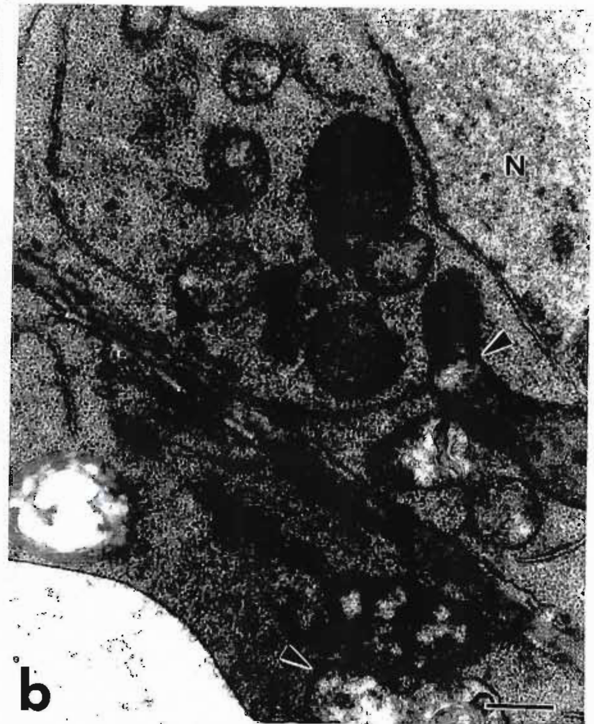
One of the major causes of seed deterioration in storage is microbial contamination, especially because the relatively warm and humid environment used for the storage of recalcitrant seeds provides an excellent *milieu* for fungal proliferation (Mycock and Berjak, 1990; Berjak, 1996). The application of surface anti-fungal dressings serves only to curtail the spread of infection but does not destroy the mycoflora within the seed tissues. In fact Drew *et al.* (2000) found that, despite the surface sterilization and application of a benomyl fungicide to *T. dregeana* seeds prior to storage, there was rapid decline of germinability in storage accompanied by fungal proliferation.

In 2000, only sparse production of *T. dregeana* seeds occurred, and these harboured fungal inoculum internally. In an effort to control seed-associated fungi in the interests of extending the storage lifespan of the seeds of *T. dregeana*, they were stored at 16 °C after the following treatments: surface-sterilisation with NaOCl; surface-sterilisation followed by treatment the systemic anti-fungal cocktail (detailed in section 2.4.1); surface-sterilisation treatment with

the anti-fingal cocktail and encapsulation in the fungicidal alginate gel mentioned previously and which has been successfully used to extend the storage longevity of *A. marina* seeds four-fold (Motete *et al.*, 1997; Pammenter *et al.*, 1997); or surface-sterilisation followed by coating with the alginate gel, without treatment with anti-fungal cocktail.

All the untreated (surface-sterilised only) seeds lost viability within four weeks, and storage longevity was increased by all treatments. However, irrespective of the treatment, all seeds lost viability within 10 weeks (Fig. 2.27), by which time marked fungal and bacterial proliferation was apparent. The rate of germination, represented by the germination index, rose within the first four weeks of storage, and then declined rapidly (Figure 2.28). It is significant that the highest rate of germination coincides with a slight reduction in the respiration rate (Fig 2.30). This may imply that the continuing development of the seeds of *T. dregeana* reached a plateau four weeks after harvest, indicating full physiological maturity and highest vigour (Jalink *et al.*, 1999). This is not akin to the metabolic quiescence associated with maturation drying in orthodox seeds (Kermode, 1990), but may be an indication of the indeterminate development associated with recalcitrant seeds (Finch-Savage and Blake, 1994), in which seeds are shed at different maturity stages, even from the same parent tree. As the degree of development has been shown to influence the response/vulnerability of recalcitrant seeds to fungal infection (Calistru *et al.*, 2000), the extraordinarily high level of fungal proliferation in this storage experiment may have been a factor of the stage at which the seeds were shed. From four weeks in storage onwards, the rate of respiration in storage increased (Fig 2.30), which was coincident with fungal proliferation (Fig. 2.29). It therefore appears that the activity of the anti-fungal cocktail is fungistatic, losing efficacy after four weeks. It is possible, however, that re-application of the fungicide mixture, might have further curtailed the proliferation of seed-associated microflora. Additionally, treatment trials using other fungicidal compounds require to be undertaken.





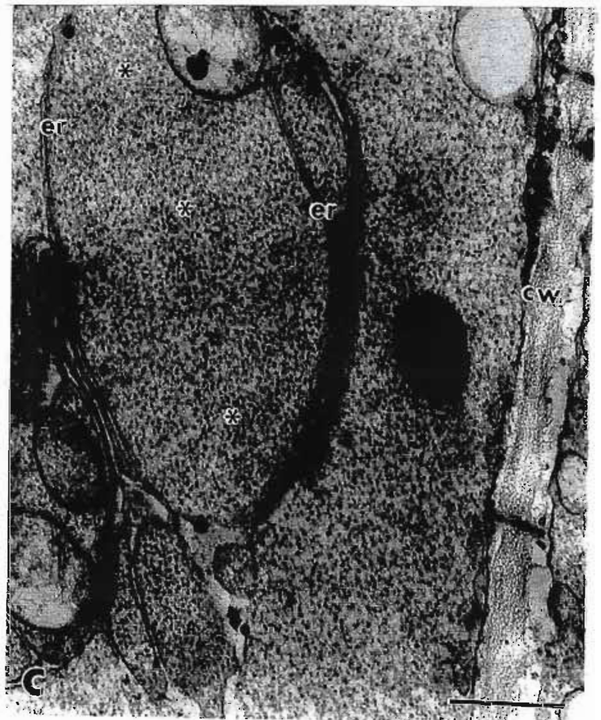


Fig 2.26 The subcellular situation in root meristematic cells of *T. dregeana* seeds stored at 25 °C for 5 months. The cells appeared to have maintained general ultrastructural organisation, with intact nuclear, vacuolar and plasmalemmal membranes (a); and metabolic activity could be inferred from numerous cristate mitochondria and profiles of endoplasmic reticulum (b). However, occasional aberrant mitochondria (arrowed), and evidence of autophagic activity (a); and the enclosure of large volumes of cytomatrix by ER-derived membranes (asterisk in c), indicative of autophagy of the sequestered material, suggested the effects of mild, but long-term stress.
m, mitochondrion; er, endoplasmic reticulum; v, vacuole; N, nucleus; Nu, nucleolus; ne, nuclear envelope; cw, cell wall. Bar = 500 nm.

In the present experiment, a single application of systemic fungicides did not extend the storage longevity of *T. dregeana* seeds usefully, as they were overrun by fungi and had lost viability within 10 weeks of storage at 16 °C. Even though this storage lifespan is longer than that observed in some previous studies on seeds of this species (e.g. Drew *et al.*, 2000), it is effectively less than half that observed in earlier stages of this study with high quality seeds which appeared not to harbour internal inoculum, and highlights the central rôle of fungi in viability loss of stored recalcitrant seeds, as suggested by Calistru *et al.* (2000).

Reducing the storage temperature, which curtails fungal proliferation, has been successfully applied in the storage of chilling-tolerant, temperate recalcitrant seeds (Suszka and Tylkowski, 1980), but was found to be inapplicable to seeds of *T. dregeana*. When fully hydrated seeds were stored at 6 °C following surface-sterilisation and treatment with systemic fungicides, they lost viability after only six weeks, demonstrating their chilling sensitivity. Ultrastructural examination of meristematic cells revealed that the cytomatrix was completely degraded, the cell walls were broken, and the cells were heavily colonised by bacteria (Fig 2.31). Bacteria are known to follow fungal successions in orthodox seeds (e.g. McLean and Berjak, 1987). Structural barriers such as the cell wall are degraded by fungi, which render the tissues and cells suitable for subsequent bacterial proliferation. Fungal degradation (here followed by bacterial proliferation) has been found to be accelerated by extrinsic or intrinsic factors favouring debilitation of recalcitrant seeds stored at the shedding water content (Calistru *et al.*, 2000) or partially dehydrated (Drew *et al.*, 2000).

Thus, neither hydrated storage at ambient or reduced temperatures, nor partially-hydrated storage (*sensu* Drew *et al.*, 2000), are suitable for the storage of the seeds of *T. dregeana*.

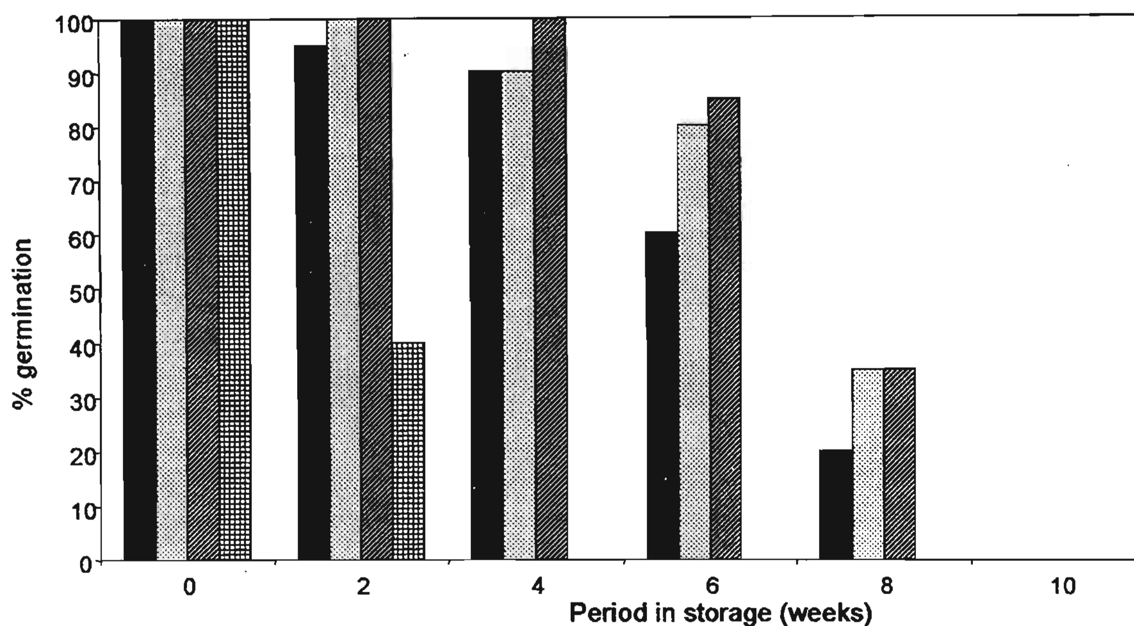


Figure 2.27. The final percentage germination of *T. dregeana* seeds stored at 16 °C, after drying back to the original fresh weight, following coating with a fungicidal gel (solid bars), soaking in systemic fungicides (hatched bars), soaking in systemic fungicides and coated with fungicidal gel (dotted bars), or untreated (checked bars).

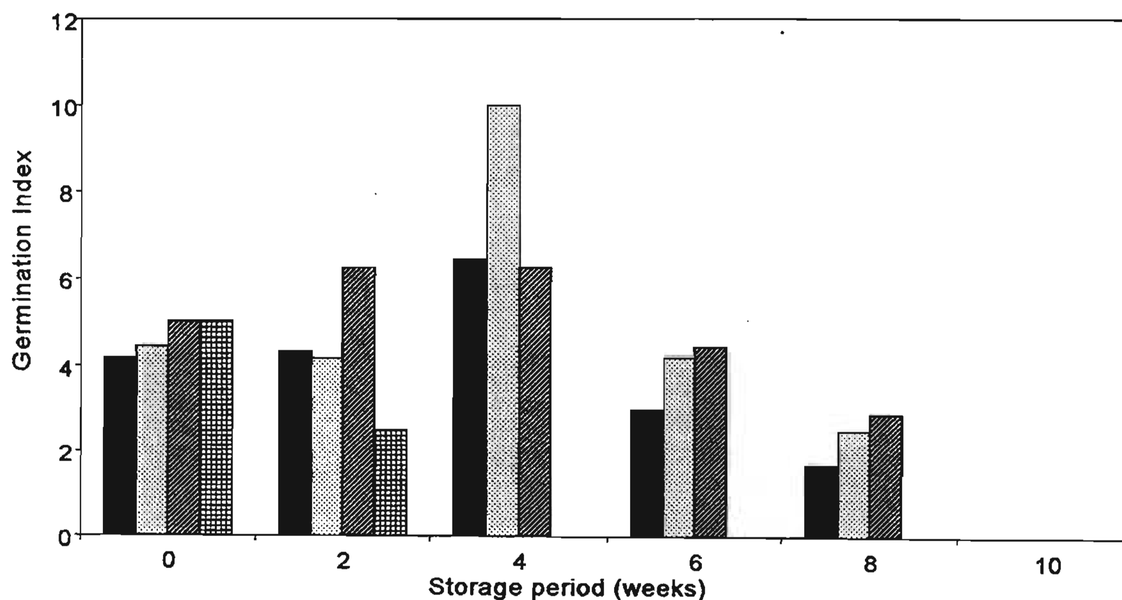


Figure 2.28. The germination index, calculated as the maximum germination attained divided by the number of days taken to reach it, of *T. dregeana* seeds stored at 16 °C, after drying back to the original fresh weight, coating with a fungicidal gel (solid bars), soaking in systemic fungicides (hatched bars), soaking in systemic fungicides and coated with fungicidal gel (dotted bars), or untreated (checked bars).

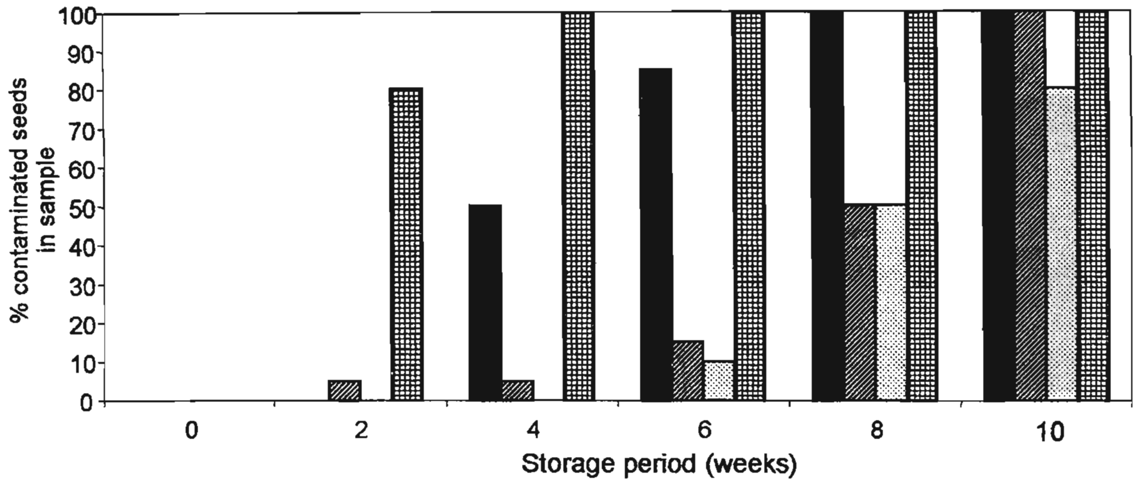


Figure 2.29. The percentage of seeds contaminated in the samples seeds of *T. dregeana* stored at 16 °C, after drying back to the original fresh weight, coating with a fungicidal gel (solid bars), soaking in systemic fungicides (hatched bars), soaking in systemic fungicides and coated with fungicidal gel (dotted bars), or untreated (checked bars).

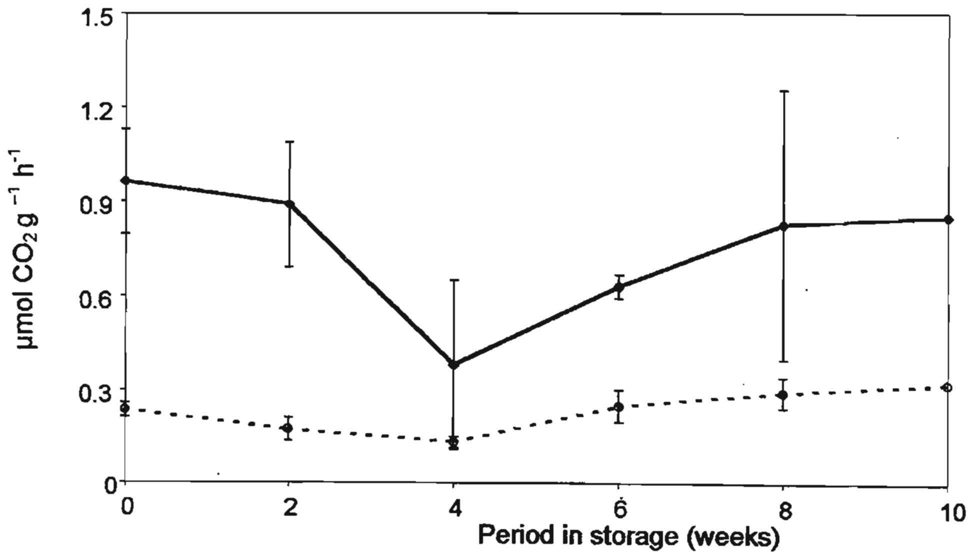


Figure 2.30. The rate of respiration of embryonic axes (solid line) and cotyledons (dashed line) of *T. dregeana* seeds, after soaking in systemic fungicides and being dried back to the original fresh weight, before storage at 16 °C.

2.5.5.2 *Trichilia emetica*

The situation of *T. emetica* seeds in storage resembled that of *T. dregeana*, but the former were much shorter-lived, and germinated considerably more rapidly in storage. In an attempt to curtail metabolic rate and reduce germination in storage, the *T. emetica* seeds were stored at 6 °C, in addition to 16 °C and 25 °C.

Those seeds stored at 6 °C all lost viability after 20 and 25 days (when stored without or with testas, respectively), as illustrated in Fig 2.32. Reference to that figure also shows that at 16 °C, the seeds stored with or without testas maintained almost total germinability for 60 days, although all had germinated in storage by then. Seeds stored at 25 °C attained total germination in storage within 35 and 40 days (with testas removed and intact, respectively).

It was notable that there was little discernible contamination of seeds stored either at 16 or 25°C, while those stored at 6°C were overrun by fungi and bacteria within 20 days. As all the seeds were from a common batch, this strongly suggests that vigorous, metabolically-active individuals under favourable conditions, have mechanisms to counteract micro-organism proliferation, as proposed for recalcitrant seeds by Berjak (1996). The germination index declined continually in all seeds stored at 6 °C, and increased steadily in all seeds stored at either 16 or 25 °C (Figures 2.33).

Ultrastructural studies showed that freshly-harvested seeds were actively metabolic state (Fig. 2.13; 2.34a), as would be expected of mature recalcitrant seeds, especially those which entrain germination rapidly. Examination of seeds stored at 6 °C showed signs of deterioration by day 10: for example, the cells had electron-translucent mitochondria and plastids with no discernible internal structure, even though cellular membranes such as the plasmalemma and nuclear envelope (not illustrated) appeared undamaged (Fig 2.34b, c). By day 20 of storage at 6 °C, the cells exhibited extensive ultrastructural derangement. The plasmalemma had receded from the cell wall and was discontinuous in many places, and organelles were extensively degraded (Fig 2.34c). All seeds stored 6 °C had lost viability within 20 d (Fig 2.32).

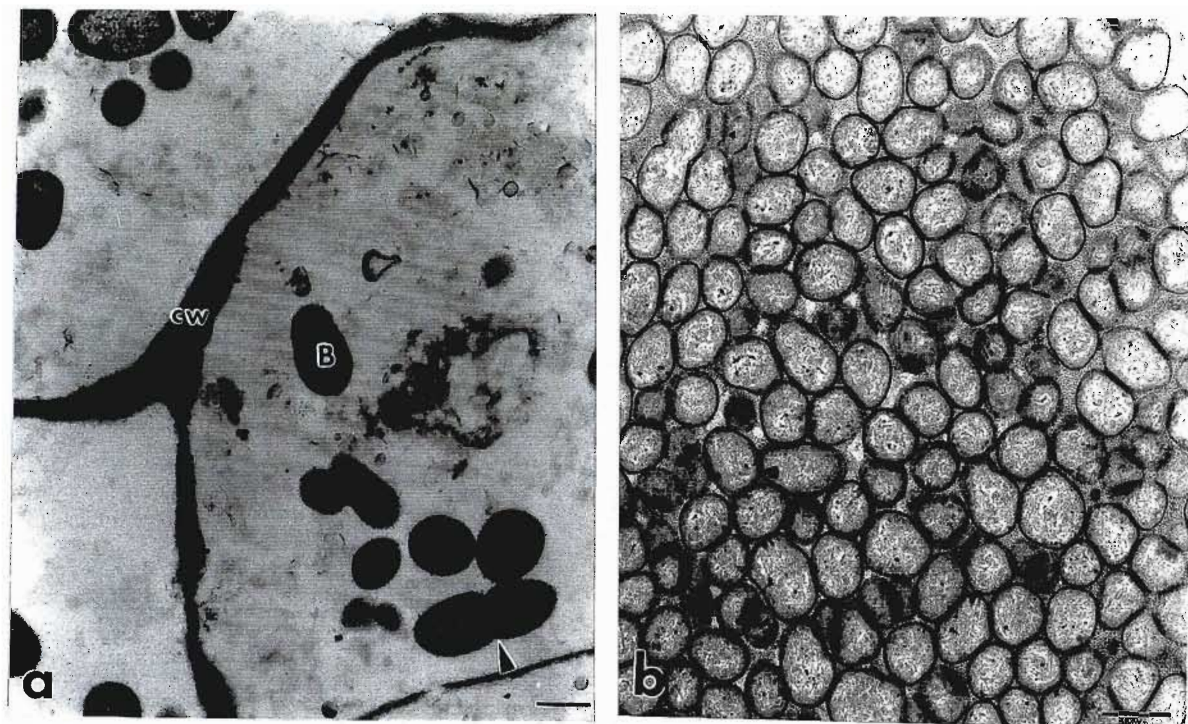
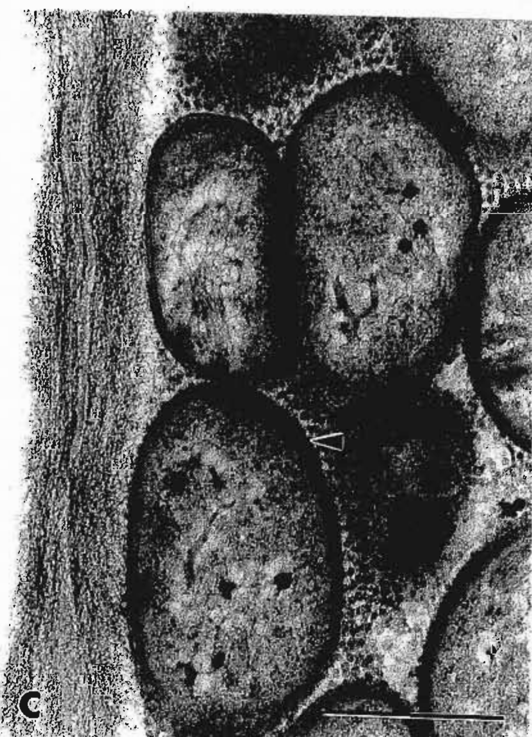
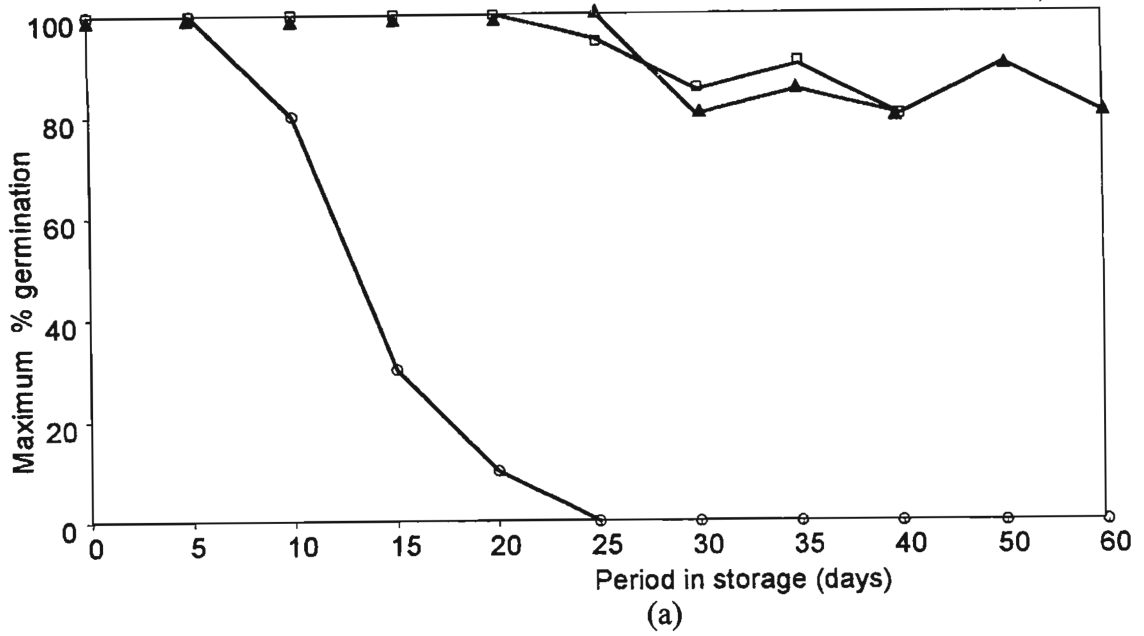


Fig 2.31 The ultrastructural situation in root meristematic cells of *T. dregeana* seeds stored at 6 °C for six weeks. Bacteria (B) are illustrated undergoing binary fission (arrow-head) within an axis cell devoid of recognisable subcellular structures (a); while in other cells, the interior was completely filled with bacteria (b). The robust cell wall (arrowed) of the bacteria indicates they are gram-positive (c).
Bar = 500 nm



In contrast to the ultrastructural situation in seeds stored at 6 °C for 20 days, cells of seeds stored at 16 and 25 °C, which were still fully germinable, showed an apparent absence of ultrastructural damage. Ongoing metabolism at these temperatures was indicated by numerous cristate mitochondria, Golgi bodies, profiles of endoplasmic reticulum, and a cytomatrix with abundant non-membrane bound polysomes (Fig 2.35), and many plastids, some containing sizeable starch grains.

By 60 days in storage, all the seeds had visibly germinated, radicles at least visible in all of them. However, ultrastructural damage was also evident in many cells of seeds stored at both 16 and 25°C. These cells had undulating cell walls with a peculiar wavy appearance (Fig 2.36). This may a further indication of prolonged mild water stress, which the seeds are suggested to have suffered as germination proceeded in storage. Other indications of stress were the poorly-formed (or lack of) cristae in mitochondria (Fig 2.36a), and damaged plasmalemma (Fig 2.36b). Significantly, fungal contamination was evident in only one or two seeds out of 30 at each sampling period, even at 60 days of storage. However, the deteriorating ultrastructural state, coupled with total germination of all seeds in storage, indicates that seeds of *T. emetica* have a useful storage lifespan of not much longer than two months.



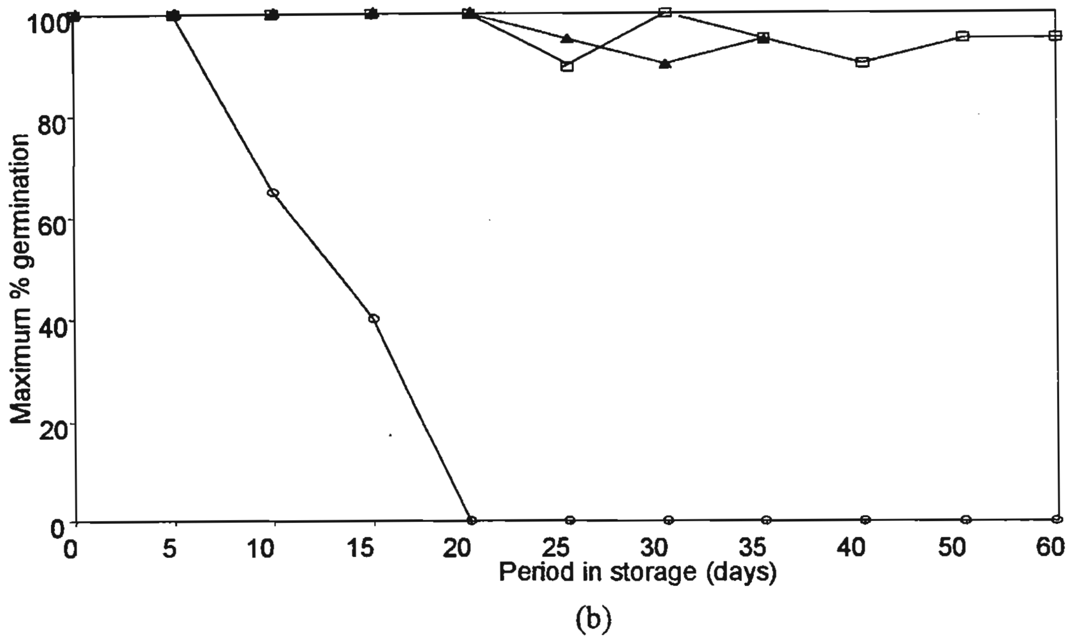
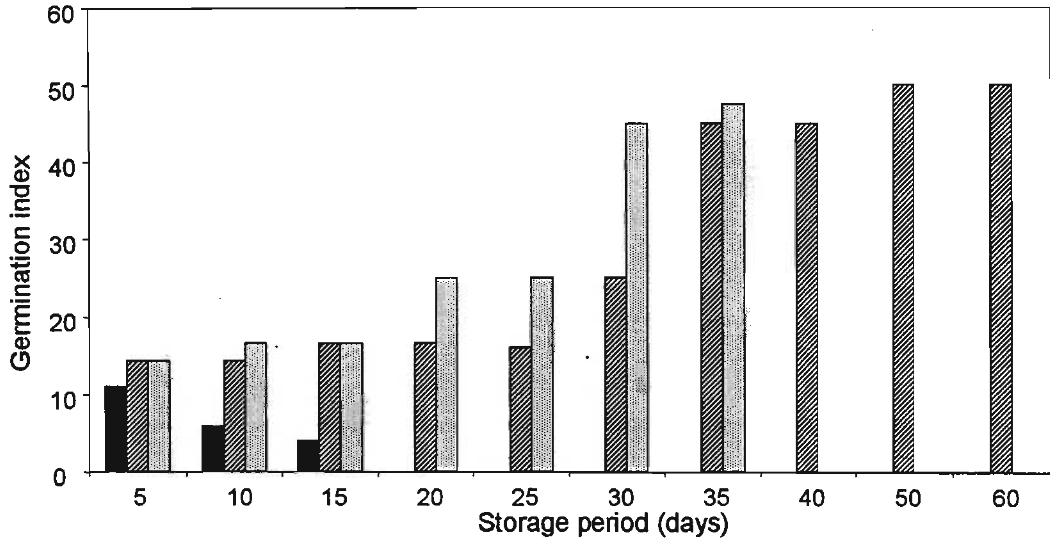
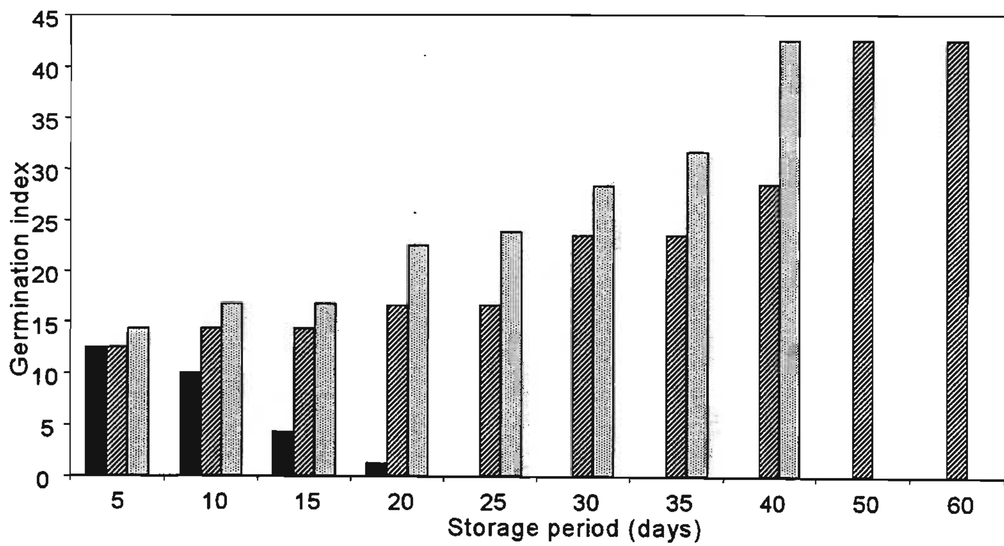


Figure 2.32. Final percentage germination of *T. emetica* seeds stored with testas (a) or without testas (b), at 6 °C (○), 16 °C (▲) and 25 °C (□). Note that all seeds stored at 16 and 25 °C had germinated in storage by day 60.

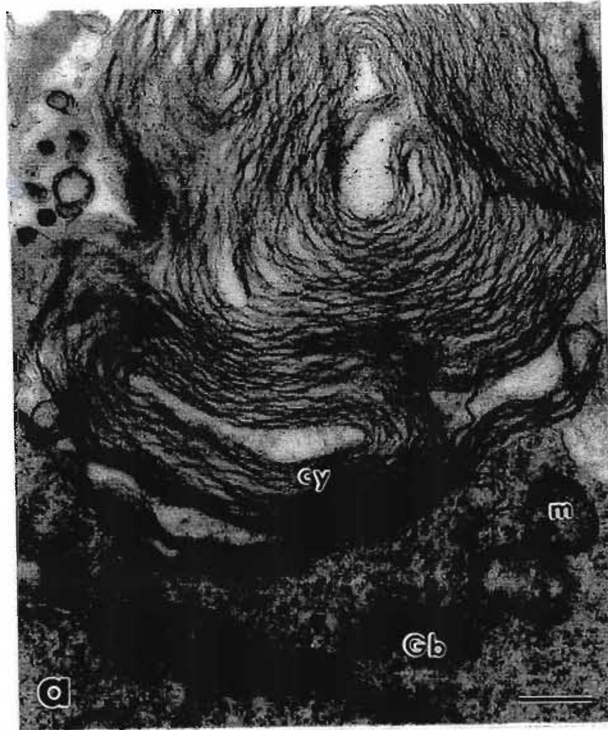


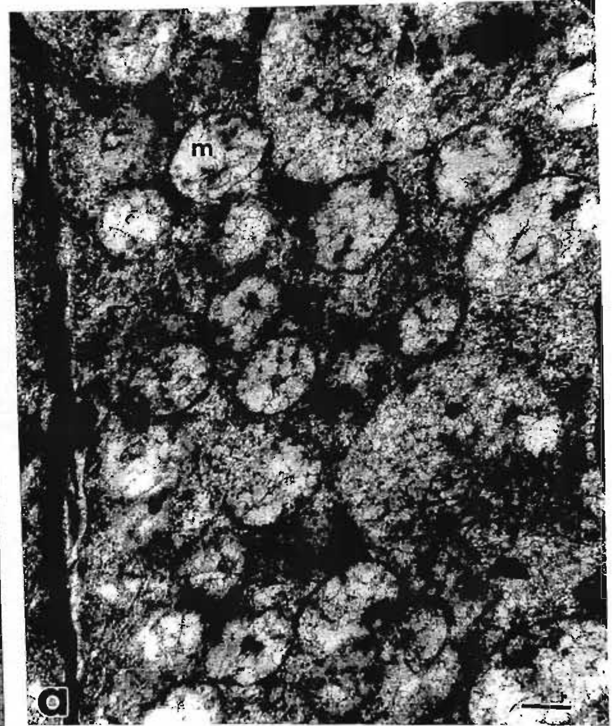
(a)



(b)

Figure 2.33. The germination index, calculated as the maximum germination attained divided by the number of days taken to reach it, of *T. emetica* seeds stored with testas (a) or without (b), at 6 (solid bars), 16 (hatched bars) and 25 °C (dotted bars).





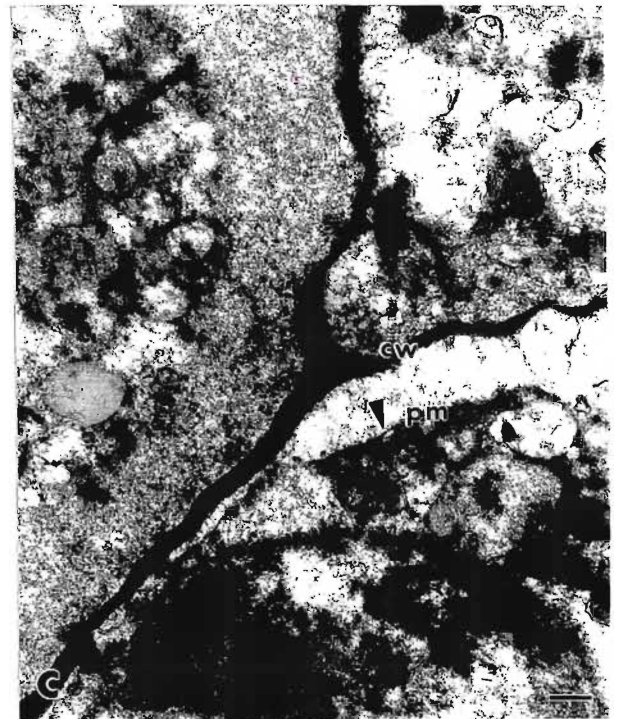


Fig. 2.34 The ultrastructure of root meristematic cells of seeds of *T. emetica* stored at 6°C. (a) The situation in axes of non-stored seeds provided evidence of their metabolically-active status, with cytolysome formation (cy), abundant polysomes and well-developed Golgi bodies and mitochondria illustrated here. (b) In seeds stored for 10 days, chilling damage was evidenced by electron-translucent, de-differentiated mitochondria. (c) Cells of seeds stored for 20 days suffered extensive ultrastructural damage, including conspicuous withdrawal of the plasmalemma (pm) from the cell wall (arrow-heads).
m, mitochondrion; Gb, Golgi body. Bar = 500 nm.

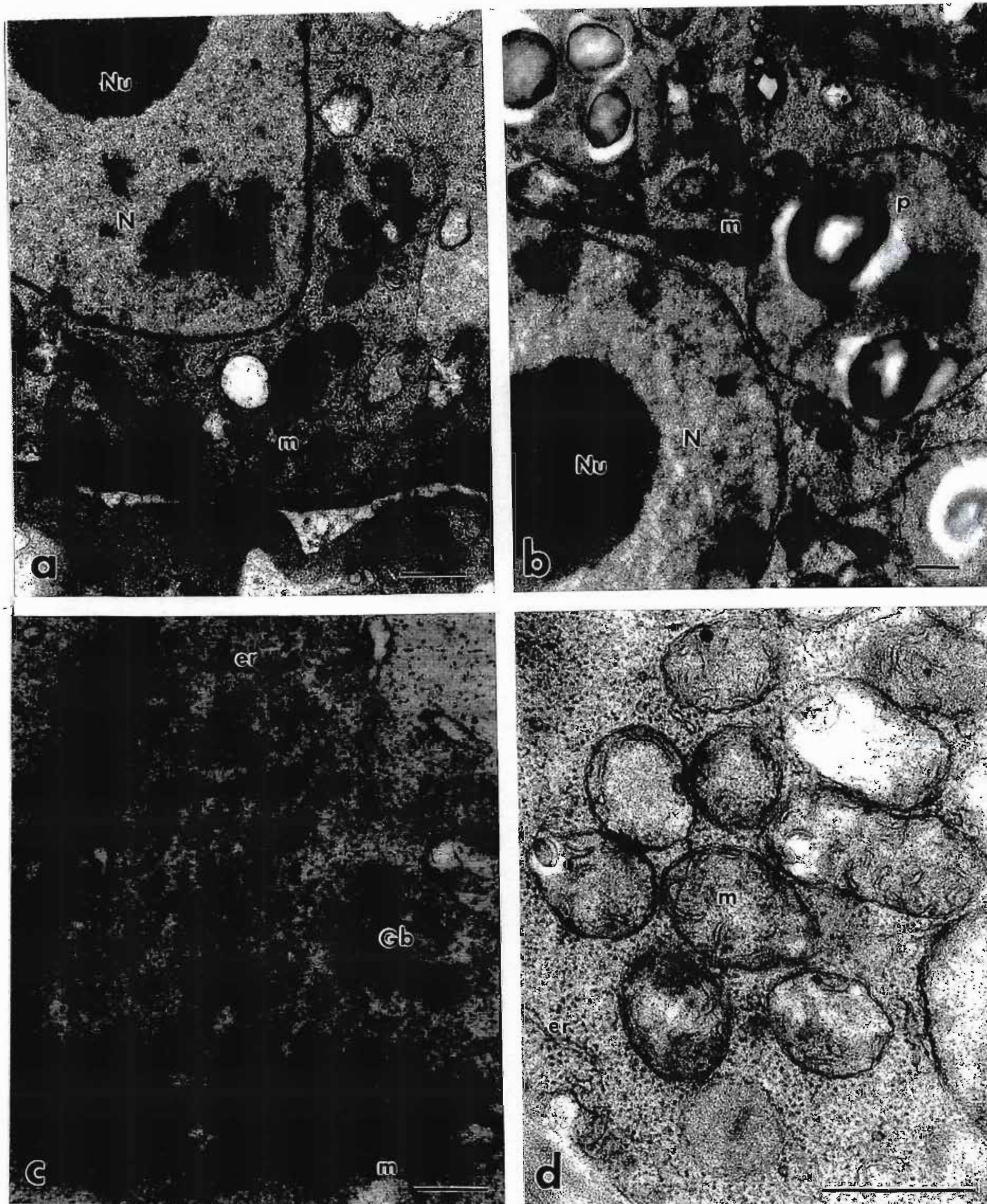


Fig 2.35 Ultrastructure of root meristematic cells of *T. emetica* seeds stored for 20 days at 16 °C (a and b) and 25 °C (c and d). The cells presented evidence of being actively metabolic, with mitochondria in which the cristae were well-developed, plastids, some of which contained substantial starch grains, rough endoplasmic reticulum and non membrane-bound polysomes, as well as Golgi bodies. m, mitochondrion; er, endoplasmic reticulum; Gb, Golgi body; N, nucleus; Nu, nucleolus; P, plastid. Bar = 500 nm.

2.5.5.3 *Warburgia salutaris*

Seeds in hydrated storage became overrun by proliferating fungi within the first week, despite the fact that they were newly extracted from the compact pulp of undamaged, closed fruits, soaked in systemic fungicides, and then stored under sterile conditions. Thus, hydrated storage was considered not feasible. This is in keeping with findings that fungal propagules generally are associated peripherally and often internally with non-orthodox seeds of tropical/sub-tropical provenance (Mycock and Berjak, 1990; Sutherland *et al.*, 2003), although there are exceptions, e.g. in the case of *Landolphia kirkii* (Apocyanaceae), seeds newly extracted from the fruit pulp were free of mycoflora (Mycock and Berjak, 1990).

For dehydrated storage, seeds were desiccated to a water content of ~ 0.1 g g^{-1} and hermetically stored at either 6, 16 or 25 °C. Viability of seeds stored at 16°C was 88% after 28 d, but there was a rapid decline to zero by day 49 (Fig. 2.37). Seeds stored at 25°C remained 100% germinable to day 28, but also lost viability rapidly thereafter. The viability of the seed batch at 6 °C declined to 10% after 28 d storage: full germinability was retained for only 7 d (Fig. 2.37). The seeds lost not only germinability during storage, but the rate of germination in viable seeds declined sharply after two weeks in storage in all three temperatures (Fig. 2.38).

In terms of their tolerance of relatively rapid dehydration to water contents ≤0.1 g g^{-1}, seeds of *W. salutaris* cannot be described as being typically recalcitrant: however, considering the limited period (~ 40 d and 49 d) within which viability declined to 50% and zero, respectively, during storage at either 16 or 25°C, they are certainly non-orthodox. Chilling sensitivity of the seeds in the dehydrated condition further suggests their categorisation as an intermediate type, but their curtailed lifespan once dehydrated implies that desiccation damage *sensu stricto* (Pammenter *et al.*, 1998; Walters *et al.*, 2001) occurs, albeit considerably more slowly than would take place in highly recalcitrant seeds.

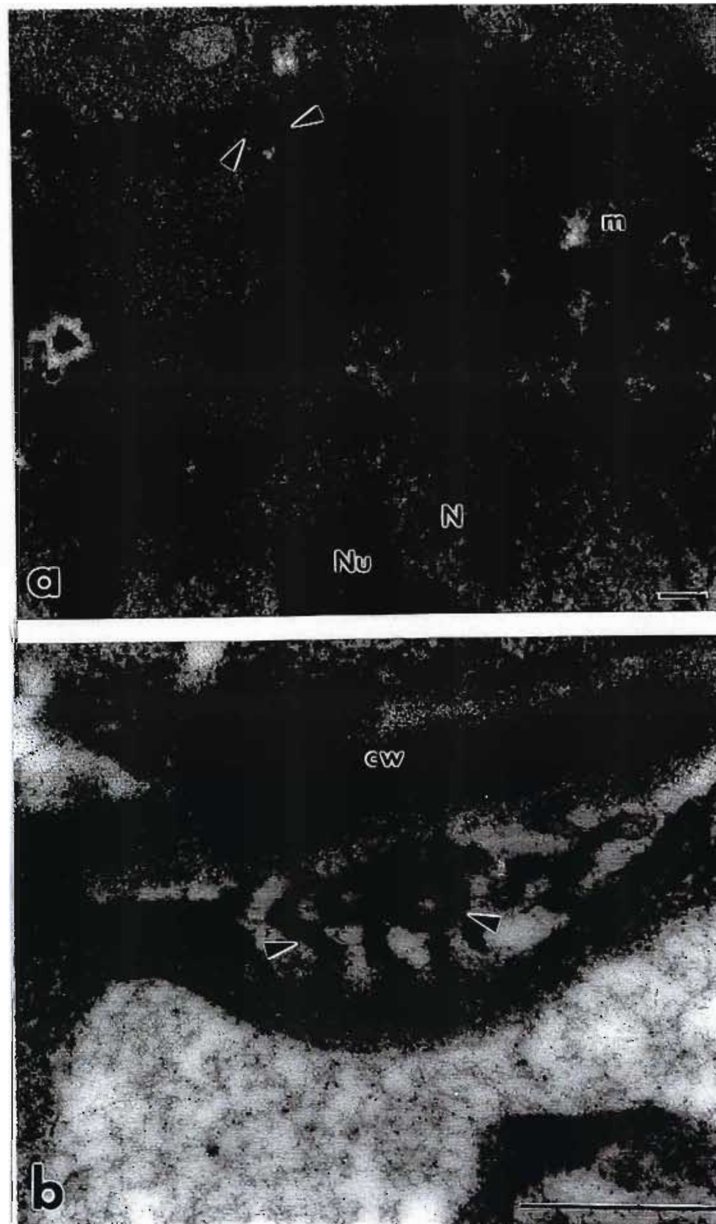


Fig 2.36 Axis ultrastructure of *T. emetica* seeds stored at 16 °C (a) and 25 °C (b) for 60 days. Cells from seeds stored at both temperatures had characteristically undulating cell walls (arrow-heads) and electron-translucent mitochondria with poorly-defined cistae (a). In b, vesicles and tubular structures formed by degraded plasmalemma, are evident (arrow-heads).
m, mitochondrion; N, nucleus; Nu, nucleolus; cw, cell wall. Bar = 500 nm

Such damage was evident in the ultrastructure of seeds stored at all three temperatures (Fig 2.39); storage brought about coalescence of lipid bodies, which has been described as a consequence of desiccation damage, manifest on rehydration, in lipid-rich desiccation-sensitive seeds (Leprince *et al.*, 1998).

Electron microscopy revealed that the axis cells of *W. salutaris* seeds contain a prominent component of lipid bodies (Fig 2.17), the consequence of which is an underestimation of the water activity by the measured water content (Secandé and Hoekstra, 1999). Therefore, a measured water content in the range of 0.1 g g^{-1} may have water activity equivalent to that of 'intermediate water contents' (Vertucci and Farrant, 1995), in which injurious, out-of-phase oxidative processes occur. Such processes may be temperature-dependent, as shown by Ntuli *et al.* (1997) and may come into play sooner at 25 than at 16 °C. While seeds stored at both temperatures had deteriorated after 42 d, the degree of degradation was more extreme in those maintained at 25 °C (Figs 2.39c and d). At 6 °C the seeds are suggested to have suffered extensive chilling damage during storage, as do non-orthodox seeds of certain other species of tropical origin (Corbineau and Côme, 1986; Chin *et al.*, 1989; Tompsett 1994; Hong and Ellis, 1998).

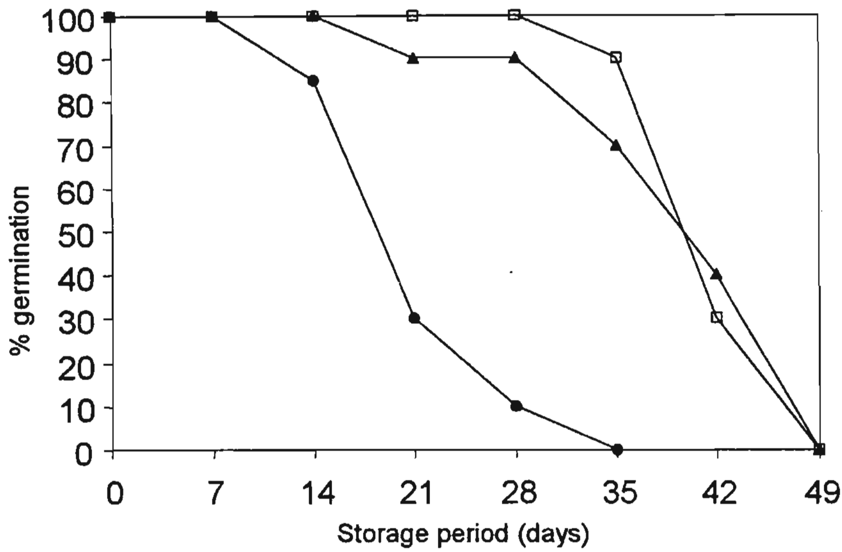


Figure 2.37. The change in final percentage germination over time, in seeds of *W. salutaris* stored at 6 °C (•), 16 °C (▲) or 25 °C (□).

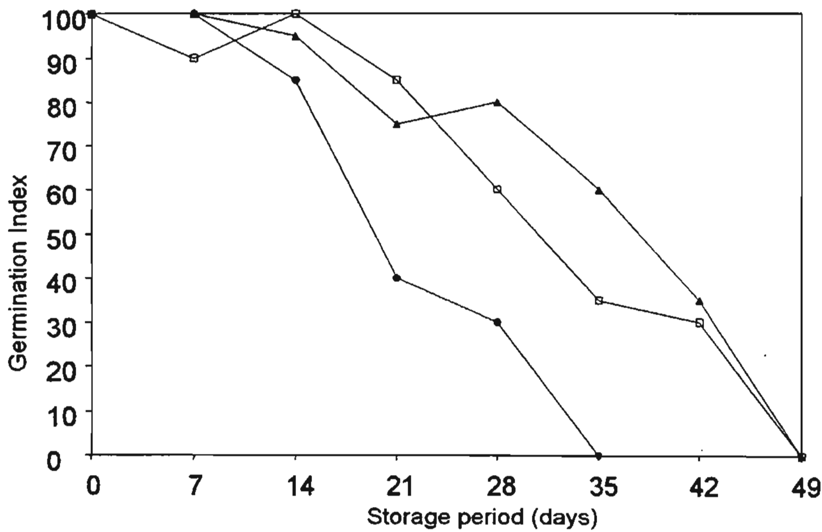


Figure 2.38. Germination Index, calculated as maximum percentage germination, divided by the number of days taken to attain that germination, of seeds of *W. salutaris* stored at 6 °C (•), 16 °C (▲) or 25 °C (□).

Chilling sensitivity in plants has been attributed to conformational transition in cell membranes from the liquid crystalline to the gel phase, often followed by lateral phase separation of the components of the membrane (Platt-Aloia and Thompson, 1987). This is associated with dysfunction of membrane proteins and leakage of cytoplasmic solutes from the cells and (Yoshida *et al.*, 1986). The transition temperature (T_m) of membranes in chilling-sensitive tropical plants has been estimated to be approximately 10 °C (Crowe *et al.*, 1989), therefore storage of the seeds of such plants at temperatures as low as 6 °C will result in fatal injury.

Alternatively or additionally, the seeds, which had been dried to about 0.1 g g^{-1}, may have suffered imbibition injury during rehydration. In order to minimise such damage in stored non-orthodox seeds, Secandé and Hoekstra (1999) suggested rehydrating the seeds at 30 – 40 °C. Those authors found that soaking dry *Azadirachta indica* seeds at 30 – 40 °C improved germination while soaking at 22 °C reduced the percentage of seeds that germinated. In this study, stored, dehydrated seeds were sown directly (and hence, rehydrated) at 25 °C.

2.5.6 Post-shedding behaviour: concluding comments

When stored fully hydrated, and at non-chilling temperatures (16 and 25 °C), seeds of *T. dregeana* and *T. emetica* germinated increasingly rapidly as the storage period progressed, a characteristic first described for *Avicennia marina* (Pammenter *et al.*, 1984), and later for three other, unrelated recalcitrant species by Farrant *et al.* (1989). Those authors showed that during storage, the recalcitrant seeds exhibited ultrastructural changes characteristic of germinating seeds, such as increased mitochondrial organisation, increased endomembrane activity, and reserve mobilisation. As a consequence of subcellular events indicating the progress of germination in hydrated storage, apparently enhanced rates of germination are observed when seeds are removed from storage and planted out.

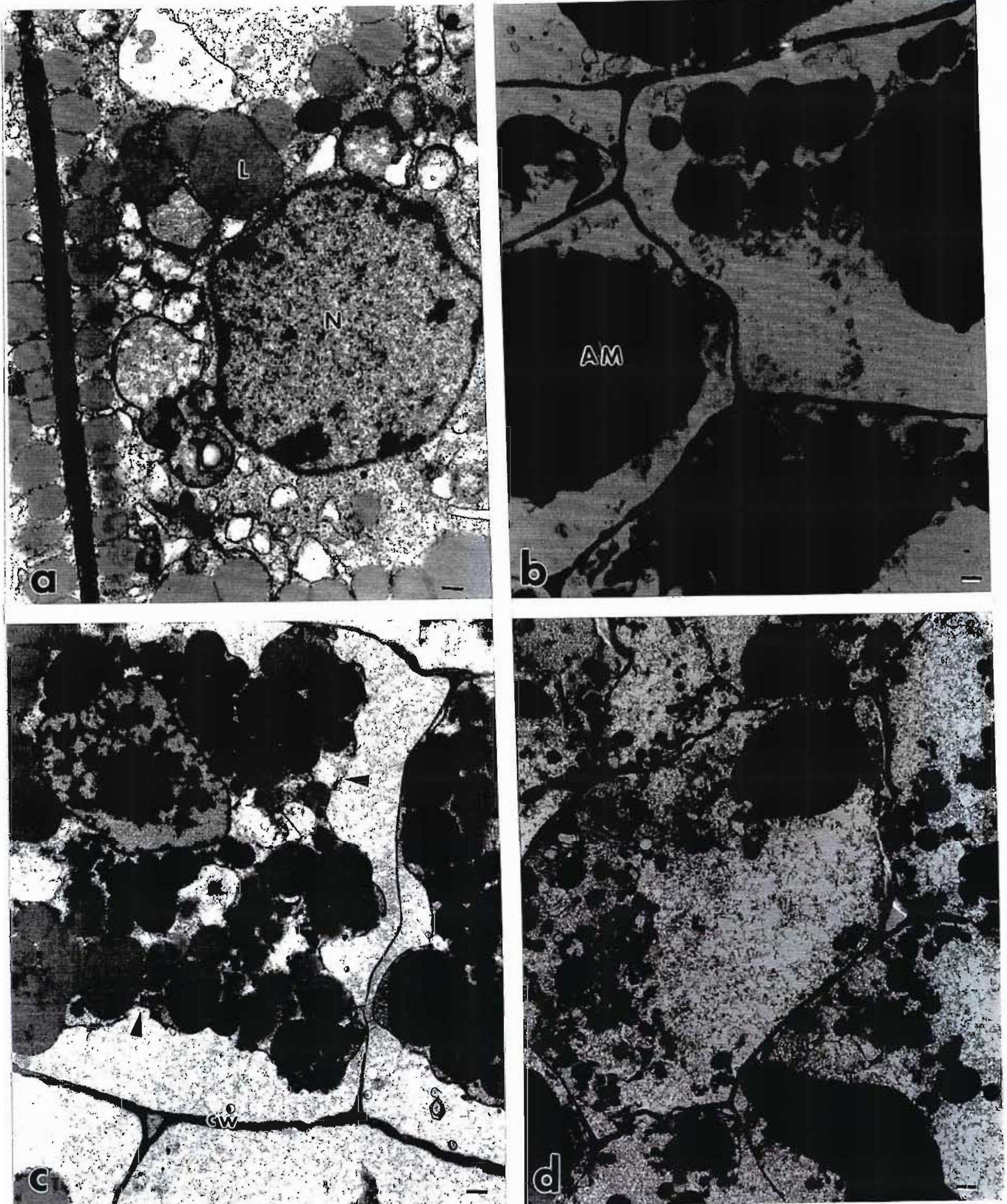


Fig. 2.39. The ultrastructural situation in embryonic axes of *W. salutaris* seeds immediately after dehydration to a water content of ~ 0.1 g g^{-1} (a), and then stored for 35 days at 6 °C (b), and for 42 days at 16 °C (c) and 25 °C (d). While the cells in non-stored seeds dehydrated to a water content 0.1 g g^{-1} contained many discrete lipid bodies (a), these became fused into an amorphous mass (AM), particularly after storage at 6 °C (b). Axis cells of seeds stored at 16 and 25 °C generally had far less lipid coalescence(c and d), but a collapse of entire cells was evident (arrowed).
N, nucleus; L, lipid body; cw, cell wall. Bar = 500 nm.

Even though hydrated storage of recalcitrant seeds encourages the continuation of germinative development which, in turn, may accelerate the loss of viability as a consequence of mild water stress, storage under desiccating conditions has been shown to result in far more rapid loss of viability. In studies by Farrant *et al.* (1989) the longevity of *Araucaria angustifolia*, *Landolphia kirkii* and *Scandoxus membranaceus* was halved when the seeds were stored under desiccating conditions rather than at their initial water contents, while Drew *et al.* (2000) showed that storing seeds of *T. dregeana* in a partially-dehydrated state reduced the storage longevity from 21 d (when stored under fully hydrated conditions) to eight days. The latter authors suggested that storing recalcitrant seeds at water contents lower than the shedding water content accelerates the onset and progress of deteriorative reactions as a consequence of water stress.

The present results also contribute to the generally observed characteristic of recalcitrant seeds, that, despite high initial germination capacities and optimum storage conditions, the seeds generally lose viability within relatively short periods (e.g. less than 11 weeks in *A. marina* seeds encapsulated in fungistatic alginate gel [Motete *et al.*, 1997], 10 weeks in *Pometia pinnata* [Soestina *et al.*, 1999] and three weeks in *Boscia senegalensis* [Danthu *et al.*, 1999]). Temperate species may last relatively longer in storage, especially if they can be cold-stored (Chin and Roberts, 1980).

The basis for the loss of viability of non-orthodox seeds in hydrated storage has been the subject of various studies (reviewed by Pammenter *et al.*, 1994 and by Berjak and Pammenter, 2001). As mentioned above, it has been suggested that, as these seeds proceed with germinative metabolism during storage, water becomes limiting and they die as a result of the deleterious effects of mild, but prolonged, water stress (Pammenter *et al.*, 1994). In this study, seeds of *Trichilia* spp. were stored fully hydrated, with little observable change in the water content during storage (e.g. Fig. 2.40). However, it was not ascertained if there were any changes in the seed or axis water potential during storage, which could indicate a measure of water stress. It is noteworthy, however, that such water stress was not detected (although not unequivocally precluded) during hydrated storage of the highly-recalcitrant seeds of *A. marina* (Motete *et al.*, 1997). Those authors suggested that fungal contamination,

which had been curtailed in that study, normally would constitute a major cause of deterioration in stored hydrated recalcitrant seeds. Calistru *et al.* (2000) clearly demonstrated that controlling fungal proliferation significantly extended the storage lifespan of stored recalcitrant *A. marina* seeds. This is also manifested by the results of the two storage experiments with *T. dregeana* seeds in the present study: in the first experiment where fungal contamination was observed to be minimal, the seeds survived in storage for at least five months, while they lost viability after only 10 weeks when fungal contamination was apparent.

It is also noteworthy that the associated microflora was far more prevalent in the seeds of some harvests, than of others, a situation also reported by Mycock and Berjak (1990), who also reported that seeds of particular species may be more resistant to infection than others. In one study, Mycock and Berjak (1990) found no bacterial or fungal infection in the seeds of *Castanospermun australe*, no bacterial infection in seeds of *Camellia sinensis*, and no trace of the usually ubiquitous fungus species, *Fusarium*, in the seeds of *Landolphia kirkii*. Those authors suggested that the thick-walled fruits of *C. australe* and *C. sinensis* served as mechanical barriers to microbial infection, and that the fruit pulp of the apocynaceous species, *L. kirki*, possessed some form of specific fungistatic activity. It possible that the seeds of *Trichilia* spp. used in this study have at least a degree of anti-fungal activity: chemical studies have yielded five limonoids from the seeds of *T. dregeana* (Mulholland and Taylor, 1980), and aqueous leaf extracts showed some antimicrobial activity (Desta, 1993).

Furthermore, the cotyledonary extract of *T. dregeana* appeared to be phytotoxic and fungitoxic (Chapter 3). Such anti-microbial activity would contribute to high-quality *Trichilia* seeds being relatively free of contamination during storage, and enhance their storage lifespan.

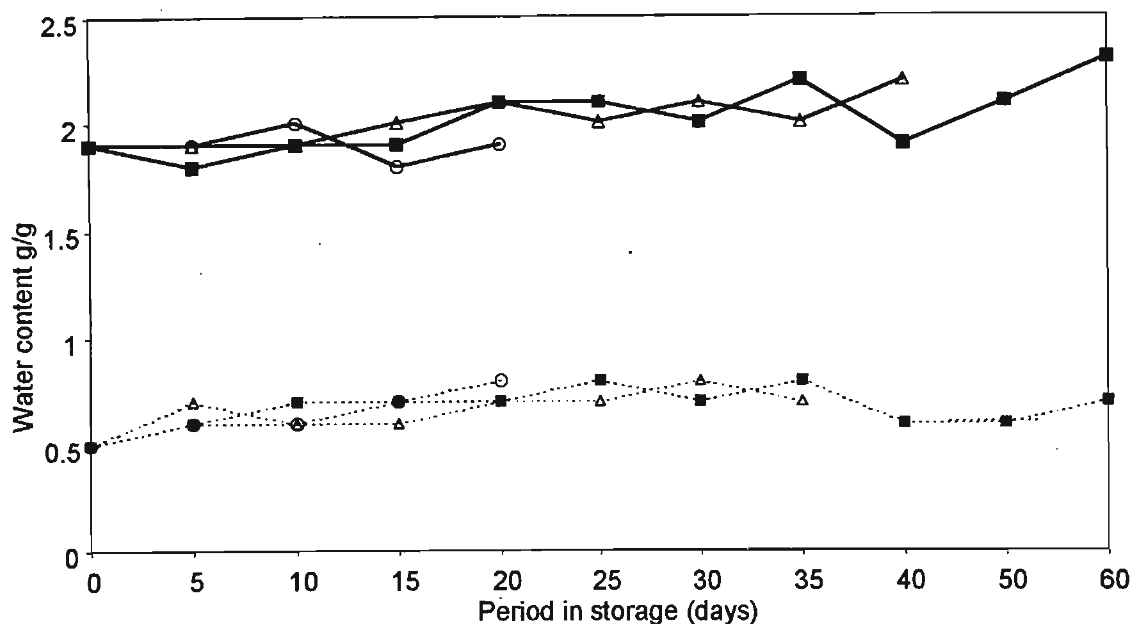


Figure 2.40 Changes in the water contents of the axes (solid lines) and cotyledons (dashed lines), with storage time, of stored *T. emetica* seeds stored with testas, at 4 °C (o), 16 °C (•) and 25 °C (Δ).

However, any innate anti-microbial ability may be jeopardised if the seeds are of inferior quality. For example, current observations indicate that seeds of most recalcitrant species collected at the end of the season, are of lower quality than early- or mid-season seeds (Berjak[1] pers. comm.), and generally harbour particularly heavy inocula of seed-borne fungi and/or bacteria, which appear to be impossible to eliminate. Thus, the quality of seeds prior to storage may play a pivotal rôle in determining their storage longevity.

From the evidence currently presented, it is apparent that the seeds of *T. dregeana* can be kept in storage for at least five months, depending on the initial seed quality, and provided that fungal contamination is excluded. However, there is significant inherent variability in the quality and contamination status of seeds from season to season, and frequently also

from seed batch to seed batch, which makes it impossible for the seeds of this species to be stored for predictable periods. *Trichilia emetica* seeds are also unstorable for useful periods, for example between seasons of production, because they rapidly germinate in storage. As they are sensitive to chilling, cold storage, which could reduce germination rate in storage, is impossible. However, some optimal temperature between 6 and 16 °C still requires to be identified. Even though seeds of *Warburgia salutaris* were able to tolerate considerable desiccation, they were unstorable for practical periods at the reduced water contents.

Thus, as is generally the case with non-orthodox seeds, neither hydrated storage, nor conventional means (low temperature and low water contents) can be used to preserve the germplasm of the three important plant species investigated in this study – one of which is virtually extinct in the wild, and the others of restricted wild populations, at least in South Africa. The only feasible means for the long-term storage of this germplasm is suggested to be cryopreservation. This aspect is explored in Chapter 3.

CHAPTER 3

Development of *in vitro* embryo germination media and cryopreservation of the germplasm of *Trichilia dregeana*, *T. emetica* and *Warburgia salutaris*.#

3.1 Introduction

The seeds of the species under investigation in this study are non-orthodox and unstorable for reasonable periods or for conservation purposes (Chapter 2), and demand for these species in the herbal medicine trade and for other ethnobotanical purposes remains high (Mander, 1998). Consequently, *Warburgia salutaris* is endangered and virtually extinct in the wild in South Africa (Scott-Shaw, 1999), while *Trichilia dregeana* and *T. emetica*, for which inventories are not available, may be in decline, as natural populations could not be located during the course of this study. Therefore the development of methods for the long-term preservation of the germplasm of these species is crucial.

For species producing non-orthodox seeds, the only feasible means for long-term germplasm preservation is presently envisaged to be cryopreservation (Stanwood, 1985; Berjak *et al.*, 1999). At the extremely low temperature of liquid nitrogen (-196 °C), at which tissues are usually cryopreserved, all metabolic activities and deteriorative processes are presumed to be immobilised, suspending ageing and genetic changes (Kartha, 1985). Therefore true-to-type genotypes should be conserved for theoretically unlimited periods, without the risk of the genetic drift, which accompanies conservation by other techniques such as conventional seed storage (Grout, 1991; Blakesley *et al.*, 1996; Engelmann, 1997, 2000). Furthermore, variations in moisture content which occur during storage at relatively 'high' low temperatures (e.g. -18 °C), and which may accelerate seed deterioration, do not occur at the ultra-low temperature of liquid nitrogen (Stanwood, 1985).

#Parts of this chapter were incorporated in, and published, in *Cryo-Letters* **19**, 5-13 (1998);

and reported in M. Marzalina, K.C. Khoo, N. Jayanthi, F.Y. Tsan and B. Krishanpillay (eds) *Recalcitrant Seeds: IUFRO Seed Symposium 1998*. FRIM, Kuala Lumpur, Malaysia. pp 96-109 and pp 265- 371 (1999);

However, few biological specimens can be frozen to sub-zero temperatures without adversely affecting cell viability. The fatal injury that accompanies freezing appears to be the consequence of either or both biochemical and/or physical processes. Biochemical injury occurs when, during freezing, ice is formed in the extra-cellular spaces and, as the ice crystals grow, water is drawn out of the cells. This may result in deleterious increases in solute concentrations and shifts in pH, bringing about an entirely abnormal intracellular milieu (Ring and Danks, 1994; Fleck *et al.*, 1999). Physical injury could result from physical shrinkage (which occurs as water leaves the cell), and the destruction of membrane systems within the cell by ice crystals (Singh and Miller, 1985; Wesley-Smith *et al.*, 1992; Wesley-Smith, 2002). Fleck *et al.* (1999) reported that oxidative stress-induced injuries may constitute a third category of freezing injury to living cells, as they found evidence of increased lipid peroxidation associated with the freezing of the alga *Vaucheria sessilis*.

In order to minimise freezing injury and attain survival of explants during and following cryopreservation, a number species-, tissue-, or even cell-type-specific parameters must be optimised. These parameters are listed below:

3.1.1 Choice of explant

The need to avoid freezing injury and to conserve true-to-type identity of the cryopreserved material limits the range of materials considered suitable for cryopreservation (Withers, 1986; George, 1993; Engelmann, 1997). To avoid freezing injury, small explants are favoured (Bailey and Zasadzinski, 1991), with cell suspension cultures being some of the smallest types used. However, the procedures used in obtaining cell suspension, or any other via a callus-mediated pathway, are likely to induce genetic aberrations (e.g. Moukadiri *et al.,* 1999). Furthermore, many cell suspension cultures may not be morphogenic (Grout, 1995; George, 1996), and therefore the use of these explants depends on the availability of genetically stable and morphogenically competent cell lines. Such morphogenic cell lines have been developed for a number of tree species, such as *Eucalyptus grandis* and *E. grandis* x *camaldulensis* (Blakeway *et al.*, 1993) *Lolium* sp. (Wang *et al.*, 1993), *Panax ginseng* and *Polyscias ficifolia* (Krivokharchenko *et al.*, 1999), and *Digitalis thapsi* (Moran *et al.* 1999).

The attributes potentially lacking in callus or callus-derived cultures, such as genetic stability and morphogenic competence, can be provided by apical meristems, meristems within larger buds, and possibly by somatic embryos obtained by direct somatic embyogenesis. These explants are therefore considered the most suitable for the cryopreservation of 'true' clonal progeny (Withers and Engelmann, 1997; Isaacs and Mycock, 1999; Chang *et al.*, 2000). Other attributes that favour meristems as explants for cryopreservation include their high nucleo-cytoplasmic ratio and cytoplasmic density, and relatively low water contents (Towill, 1985; Grout, 1995).

Another explant suitable for clonal cryopreservation is pollen, which has been cryopreserved for breeding experiments since the 1920s (Knowlton, 1922), and is routinely frozen for a number of species such as maize (Barnabas and Rajki, 1976; Georgieva and Kruleva, 1993), rapeseed (Chen and Beversdorf, 1992), tomato (Sacks and Stclair, 1996), pear (Reed *et al.*, 1998), and dogwood (*Cornus florida* [Craddock *et al.*, 2000]).

An important consideration in the conservation of endangered species is the maintenance of a wide genetic representativity in the cryopreserved germplasm. For this purpose, seeds provide the most suitable explants (Pita *et al.*, 1997). However, whole-seed cryopreservation is generally restricted to desiccation tolerant (orthodox) seeds (e.g. Touchell and Dixon, 1993; Berjak and Dumet, 1996; Pita *et al.*, 1997; Wang *et al.*, 1997). Recalcitrant seeds are unsuitable explants for cryopreservation because they are generally too large and dry slowly, consequently losing viability prior to attaining the low water contents needed to avoid lethal ice crystal formation during freezing. Removal of freezable water is important for orthodox seeds: as an example, prior to the cryogenic storage of the seeds of 13 threatened taxa in the Western Australian endemic genus *Dryandra* (Proteaceae), Cochrane *et al.* (2002) dried the seeds to a water content of 0.05 ± 0.01 g g^{-1}, an extent of dehydration lethal to recalcitrant seeds. Because of their size also, even if suitable water contents could be achieved without compromising viability, recalcitrant seeds cool too slowly, further facilitating ice crystal formation during freezing. For such seeds generally, zygotic germplasm can be cryopreserved only in the form of excised embryonic axes (Stanwood, 1985), as originally demonstrated for rubber (Normah *et al.*, 1986) and for Klinki pine (*Araucaria hunsteinii*) by Pritchard and Prendergast (1986).

Embryonic axes have been successfully used in the cryopreservation of the germplasm of several recalcitrant-seeded species (e.g coconut [Assy-Bah and Engelmann, 1992], *Prunus* sp. [Deboucaud *et al.* 1996], jackfruit [Thammasiri, 1999], *Quercus robur* [Berjak *et al.*, 1999] and *Aesculus hippocastanum* [Wesley-Smith *et al.*, 2001b]). However, presently, a practical difficulty with the use of embryonic axes is that 'survival' after cryopreservation is often in the form of unorganised callus or abnormal root or shoot growth only (e.g. Pritchard and Prendergast, 1986 [*Araucaria hunsteinii*]; Chandel *et al.*, 1995 [*Artocarpus heterophyllus*]; Normah and Marzalina, 1996 [*Baccaurea polyneura*] and it is unclear whether the surviving explants are able to develop into normal plants. Furthermore, post-thaw survival rates are often low (Pritchard and Prendergast, 1986; Chandel *et al.*, 1995; Chaudhury, 2000; Marzalina and Nashatul, 2000), implying possible genetic shifts as particular genotypes may be eliminated by cryopreservation, which would have unfavourable implications in the conservation of genetic diversity. However, where high post-thaw survival and normal growth are achievable, excised embryonic axes present the ideal explants for the cryopreservation of recalcitrant-seed zygotic germplasm.

3.1.2 Water content of explants prior to freezing

One approach to avoid the damage caused by ice crystal formation during cooling, is to reduce the amount of water in the explant prior to freezing. Drying increases cytoplasmic viscosity, reducing the intracellular mobility of water (Buitink *et al.*, 1998; Buitink *et al.*, 2000) and the readiness with which ice crystals are formed during cryogenic cooling (Luyet *et al.*, 1962; Rall and Fahy, 1985; Rall, 1987). The amount of drying possible is, however, limited in recalcitrant seeds, as these suffer desiccation damage below 0.45 g g^{-1} (hydration level 4, Vertucci and Farrant, 1995). On the other hand, if freezing is carried out at water contents above this level, lethal ice-crystal formation may be expected to occur (Becwar *et al.*, 1983). There is, however, a window of optimum water content at which recalcitrant embryonic axes can be cryopreserved, subtended by desiccation damage at the lower end, and by freezing damage at the other. This window can be extended at the lower end by flash-drying (Berjak *et al.*, 1990), which enables excised axes to tolerate relatively low water contents transiently (Chapter 2). Following flash-drying, the embryonic axes of recalcitrant seeds of several species have been successfully cryopreserved e.g. *Quercus robur* (Berjak *et al.*, 1999) *Ekebergia capensis* (Walker, 2000) and *Acer saccharum* (Wesley-Smith, 2002).

At the higher end, the water content window optimum for freezing can be extended by the utilisation of appropriate cooling rates, described below.

3.1.3 Rate of cooling

Fully hydrated cells freeze at *c.* -2 °C and ice crystal growth is arrested at *c.* -80 °C (Moor, 1973). The freezing injury suffered by explants is influenced by the rate at which they are cooled through this temperature range. Slow cooling (<10 °C min^{-1} [Wesley-Smith, 2002]) encourages extracellular freezing (Mazur, 1990) and, as water moves out of the cell forming ice externally, the cytoplasm becomes increasingly concentrated. At a particular optimum cytoplasmic concentration, the cells may be exposed to cryogenic temperatures without injury. However, cells that fail to achieve the necessary intracellular concentration and are supercooled, are likely to suffer lethal intracellular freezing if the temperature is decreased further (Mazur, 1990).

Several groups of workers have reported successful cryopreservation of non-orthodox seed germplasm using a slow-cooling procedure (e.g. Runthala *et al.*, 1993; González-Arnao *et al.*, 1998; Dussert *et al.*, 2000). However, this is often achieved by cooling in combination with cryoprotection treatment (discussed below) or dehydration to near-lethal water contents. In some cases, the dehydration is so severe that the explants develop abnormally even in the absence of cooling (e.g. Pence, 1992; Wesley-Smith *et al.*, 2001a). A more suitable approach has been suggested to be rapid cooling, by which the explants are taken through the ice-forming temperature range so quickly that ice crystals either do not form, or do not grow to injurious sizes (Wesley-Smith *et al.*, 1992). The intracellular solution may also become vitrified, forming a non-crystalline glass (James, 1983), and this obviates the damage that can be caused by intracellular ice. The rate of cooling necessary to attain vitrification can be lowered by partial of drying the tissue prior to freezing, and by the use of cryoprotectants, discussed below.

Owing to the limited drying that recalcitrant material can tolerate, very high rates of cooling, perhaps in combination with cryoprotection, must be employed for successful cryopreservation. Rapid cooling rates, of the order of 1 200 °C min^{-1}, have been used for the cryopreservation of hydrated pea axes (Mycock *et al.*, 1991), while excised embryonic axes of *Camellia sinensis* in the water content range of 0.6 - 1.1 g g^{-1} were successfully frozen at *c.* 550 °C sec^{-1} (Wesley-Smith *et*

al., 1992). This approach has also been used in the successful cryopreservation of a range of other specimens, for example erythrocytes (Fujikawa, 1981), protoplasts (Langis and Steponkus, 1990), mouse embryos (Rall and Fahy, 1985; Rall, 1987) and *Drosophila* embryos (Steponkus *et al.*, 1990; Mazur *et al.*, 1993). More recently, ultra-rapid cooling rates combined with partial dehydration have been used in the cryopreservation of excised embryonic axes from the recalcitrant seeds of *Quercus robur* (Berjak *et al.*, 1999), *Acer saccharinum* and *Poncirus trifoliata* (Wesley-Smith, 2002).

According to Wesley-Smith (2002), the faster the cooling and warming rates, the wider the window of optimum water content at which excised embryonic axes of recalcitrant seeds can be successfully cryopreserved.

3.1.4 Cryoprotection before freezing

Cryoprotectants are a heterogeneous group of compounds that reduce freezing injury to cells during cooling (Meryman and Williams, 1985). Since the original demonstration of cryoprotective action of glycerol (Lovelock, 1953), many compounds have been shown to have similar characteristics. These include natural cryoprotectants such as sorbitol and trehalose (Morrisey and Baust, 1976), and artificial ones such as ethanol, methanol, dimethylsulphoxide (DMSO), dextran, and polyvinylpyrrolidine (Meryman and Williams, 1985). The latter authors grouped cryoprotectants into penetrating (small molecules such as DMSO), and non-penetrating (larger molecules such as dextran).

Penetrating cryoprotectants are suggested to work by lowering the freezing point of intracellular solution through colligative action (Storey and Storey, 1996; Popova, 1997; Santarius and Franks, 1998). Thus, in proportion to the amount of water maintained in the liquid state below 0 °C, the concentration of toxic electrolytes in the nonfrozen cellular solution is minimised (Meryman and Williams, 1985). The larger, nonpenetrating cryoprotectants are thought to have a dehydrating action, withdrawing water from the cells by osmosis and reducing the amount of free water available for ice crystal formation (Storey and Storey, 1996).

Besides the colligative and dehydrating effects, other suggested modes of cryoprotection include interactions of small cryoprotective compounds, such as proline and threonine, with cell membranes at particular loci (Heber *et al.*, 1971); the coating of otherwise sensitive membranes by polymeric compounds so that the former are not denatured by increased concentration of salts resulting from freezing (Meryman, 1966); alteration of membrane permeability characteristics to avoid excessive dehydration (Phelps *et al.*, 1999); and the delay of ice nucleation (Skaer *et al.*, 1977).

Cryoprotection can be carried out either by soaking the explants in the cryoprotectant for appropriate periods (e.g. Meryman and Williams, 1985; Mycock *et al.*, 1995; Aronen *et al.*, 1999) or by preculturing the explants in a growth medium enriched with osmotica or cryoprotectants such as sucrose (e.g. Dumet *et al.*, 1994; Decruse *et al.*, 1999; Moran *et al.*, 1999).

A different approach to cryoprotection is the use of vitrification solutions (reviewed by Sakai, 2000). Vitrification is a physical process in which an aqueous solution undergoes a phase transition from a liquid to an amorphous state at the glass transition temperature, T_g, while avoiding ice crystallization (Fahy *et al.*, 1984). Glass transitions during cooling and rewarming in various materials have been recorded using thermal analyses (e.g. Dereuddre *et al.*, 1991; Tannouri *et al.*, 1991). A glass causes less dehydration of the tissue than ice, and results in lesser alterations in pH and solute concentrations. This may partly account for the correlation of increased survival rates of cryopreserved explants with the progressive disappearance of ice crystallization peaks and their replacement by glass transitions in cooling/warming differential scanning calorimetry (DSC) thermograms (Dumet *et al.*, 1993). It has been even been suggested that vitrification may be the only freeze-avoidance mechanism that enables hydrated cells, tissues and organs to survive the temperature of liquid nitrogen (Sakai, 1965, 1995, 2000).

The most commonly applied vitrification solution is a low-toxicity, glycerol-based solution designated PVS2, constituted by 30% (w/v) glycerol, 15% (w/v) PEG, 15% (v/v) DMSO, and 0.4 *M* sucrose (Sakai *et al.*, 1990, 1991), which supercools below -100°C and solidifies at -115 °C (Sakai, 2000). PVS2 has been used to successfully cryopreserve the meristems of woody plant species, such as apple and pear (Niino *et al.*, 1992), grape (Matsumoto *et al.*, 1998), *Grevillea scapigera* and *G. cirsiifolia* (Touchel and Dixon, 1996), and tea (Karanuki and Sakai, 1995).

The effectiveness of cryoprotection depends on a number of characteristics of the cryoprotectant, such as its chemical nature, the relative lack of toxicity, the molecular size and penetrating ability, interaction of mixtures of compounds, and the rate and temperature of application to the explant (Finkle *et al.*, 1985). These factors have to be established empirically, on a species-, tissue-, or even cell-type basis.

3.1.5 Thawing and rehydration

Following cryopreservation, the frozen tissues must be thawed in such a manner as to avert ice crystallization during warming. Even in vitrified conditions, as temperature rises and devitrification occurs, ice crystals start forming at the crystallization point and, depending on how long the tissues remain at that temperature, the crystals increase in size and can damage the cells (Reinhound *et al.*, 2000). Thus, the faster the intracellular solution passes the crystallization point, the less damage is envisaged to occur. Sufficiently rapid warming rates can be achieved by thawing in a microwave oven (Meryman and Williams, 1985), or by immersing the tissue in a thawing medium in a thermostatically controlled water bath at 35-40 °C (e.g. Berjak *et al.*, 1999; Wesley-Smith, 2002).

While water and liquid MS have been commonly utilised as thawing solutions for 'naked' specimens (e.g. Wesley-Smith *et al.*, 1992, 2001b), recent studies have shown that the normal development of explants after cryopreservation may be dependent on the inclusion, in the thawing medium, of cations appropriate to the assembly of cyto- and nucleo-skeletal elements, and perhaps to normalisation of other intranuclear processes. Berjak *et al.* (1999) used a 1:1 solution of 1 μM $CaCl_2.2H_2O$ and 1 mM $MgCl_2.6H_2O$ as the thawing medium, which improved not only the rate of post-thaw survival, but the proportion of normal plantlets attained after the cryopreservation of embryonic axes of *Quercus robur*. A similar response was obtained from embryonic axes of pea and somatic embryos of date-palm when the calcium and magnesium (in the ratio mentioned above) were included in the cryoprotection medium prior to freezing (Mycock, 1999).

3.1.6 *in vitro* regeneration systems

As cryopreservation of the zygotic germplasm of recalcitrant-seeded species is almost always necessarily restricted to embryonic axes, it is imperative that plant tissue culture protocols for

disinfection, inoculation and germination *in vitro*, and plantlet development, are developed *before* any cryopreservation experiments are carried out. This involves growing the excised axes on sterile nutrient media which contain minerals, a carbon source, and perhaps plant growth regulators - which would have been supplied mainly by the seed storage tissues. Successful *in vitro* germination depends on a number of factors, outlined below.

3.1.6.1 Aseptic conditions

Embryonic axes are cultured aseptically because micro-organisms not only compete for nutrients owing to their rapid growth, but may also produce enzymes and toxic metabolites that can kill the axes. Some fungi such as *Penicillium* and *Streptomyces* may also produce antibiotics against some phytopathogens or saprophytes, interfering with the natural microbial ecosystem, and boosting the predominance of parasitic fungi (Misaghi, 1982; Morrisey and Osbourn, 1999). To achieve asepsis, the axes (which, in the case of recalcitrant seeds, are often heavily contaminated by micro-organisms) must be surface-sterilised, and the culture medium must have been sterilised to inactivate any bacterial and / or fungal propagules that may be present.

Surface sterilisation may be achieved by soaking the axes, for a brief period, in any of a number of sterilising solutions, e.g. mercuric chloride (Reddish, 1957; Hartman *et al.*, 1997), ethanol (Reddish 1957; Bhojwani and Razdan, 1983), hydrogen peroxide (Pelczar *et al.*, 1986), or hypochlorites (Bhojwani and Razdan, 1983; Pelczar *et al.*, 1986). In order to improve the wettability of the explant and permeation of the sterilant into its crevices during sterilisation, a surfactant (e.g. Tween 20) is added in small amounts (0.1 – 0.01%) to the sterilant (Hartman *et al.*, 1997). Since sterilants are also toxic to the explant, the latter must be well-rinsed after surface-sterilisation, usually 3-4 times in sterile distilled water (Bhojwani and Razdan, 1983).

Surface-sterilzation alone is, however, ineffective if the microbial contamination is internal. In such cases, sterilisation may be achieved by treatment with hot water (e.g. Langensgerrits *et al.*, 1998; microwaves (e.g. Tisserat *et al.*, 1992) or systemic antimicrobial agents (e.g. Wilson *et al.*, 1993; Chanprame *et al.*, 1996). However, the treatment must inactivate the contaminant without damaging the explant, which, with few exceptions, is difficult to achieve by the application of heat to recalcitrant embryonic axes.

In order to sterilise the culture medium, moist heat in the form saturated steam under pressure is used. The medium is autoclaved at a temperature of 121 °C under a pressure of 106 kPa for 15 – 40 min, effectively destroying all forms of microbial life (Pierik, 1989). Certain thermolabile components of the medium cannot be autoclaved, and therefore are added to the cooled, autoclaved medium through bacteria-proof filter membranes of pore-size 0.45 μm or less. Such components include antibiotics, zeatin, abscisic acid, and certain vitamins (Bhojwani and Razdan, 1983).

Besides the explant and culture medium, other sources of microbial infection include the culture environment and instruments used. To prevent the entry of contaminants into culture, all transfers and other operations necessitating the opening of the culture vessels are carried out under strict aseptic conditions in a laminar flow hood. The instruments are sterilised by dipping in ethanol (96-100%), and flaming.

3.1.6.2 The effect of culture medium on *in vitro* morphogenesis

Achieving the objectives of plant tissue culture is dependent on the induction of a desired morphogenic response. The 'direction' in which an explant develops is critically influenced by the culture medium: the composition, particularly the balance between the organic, inorganic, and growth-regulator constituents of the medium must be appropriate for the desired morphogenesis (George and Sherrington, 1984).

The medium must also contain a utilisable carbon source, since the explants are usually not photoautotrophic. The most common carbon sources used are sucrose and glucose, with lactose, maltose, galactose, and starch being inferior alternatives (Gamborg and Shyluk, 1981). These carbon compounds are usually autoclaved in the culture medium for sterilisation, and this also hydrolyses sucrose into more effectively utilisable sugars such as fructose (Ball, 1955). That author also demonstrated that autoclaved sucrose, thus hydrolysed, was better than filter-sterilised sucrose for the growth of *Sequoia* callus.

The mineral nutrients required for *in vitro* growth of plant organs are generally those that are transported in the vascular system (Sharp *et al.*, 1980), and include the macronutrients (required in millimolar concentrations) nitrogen, phosphorus, potassium, calcium, sulphur and magnesium; and the micronutrients (required in micromolar concentrations) iron, manganese, zinc, boron, and molybdenum (Bhojwani and Razdan, 1983). Nitrogen may be additionally needed in organic forms (e.g. Gamborg and Shyluk, 1981), or in the form of adenine or the amino acids, glutamine or asparagine (Strauss, 1960; Risser and White, 1964).

The optimum constitution of the medium depends on the requirements of different individual species, cultivars, or clones (Pierik, 1989). Standard media have been formulated, and those after Murashige and Skoog (1962) and Gamborg *et al.* (1968) have proved to be well-balanced for most cultures and have found world-wide acceptance (Kartha, 1985). However, because many woody species are sensitive to the relatively high salt concentrations in these media, Lloyd and McCown (1981) formulated a 'woody plant medium'.

Growing plants synthesise the necessary vitamins, but under *in vitro* cultures, vitamins often become limiting (Gamborg and Shyluk, 1981). Thus, some vitamins such as thiamine (vitamin B_1), calcium pantothenate (vitamin B_5), pyridoxine (vitamin B_8), nicotinic acid (vitamin B_3), and myoinositol may need to be added to the medium to improve growth (Bhojwani and Razdan, 1983).

Even in nutritionally-balanced culture media, morphogenesis *in vitro* is still highly dependent on the interaction between naturally-occurring (endogenous) growth substances and exogenously-supplied analogues (George and Sherrington 1984). The main groups of plant growth regulators affecting morphogenesis are auxins and cytokinins. Auxins induce cell division, de-differentiation and root growth, while high levels strongly suppress organogenesis (Sharp *et al.*, 1980). On the other hand, high levels of cytokinins relative to auxins favour shoot growth for a large number of plant cultures (e.g. Sarul *et al.*, 1995).

Commonly-used auxins include indole-3-acetic acid (IAA) and its naturally-occurring stable analogues, indole-3-butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Various

cytokinins are used, including benzylamino purine (BAP), furfurylamino purine (FAP), kinetin, and zeatin. Although the growth regulators often influence each other synergistically in inducing the respective morphogenic effects, they have been shown to act independently (Gyulai *et al.*, 1994).

Besides phytohormones such as auxins and cytokinins, other growth promoters used to enhance *in vitro* morphogenesis include organic complexes such as coconut milk (e.g. Greer *et al.*, 1991), casein hydrolysate (e.g. Harry and Thorpe, 1991) and adenine sulphate (e.g. Philip *et al.*, 1992).

3.1.6.3 Environmental factors

Apart from the medium itself, environmental factors such as light, temperature and environmental gaseous composition also influence *in vitro* development and morphogenesis (Thorpe, 1980; Hughes, 1981; Amirato, 1989). Light is significant in terms of not only its presence or absence, but also in its physical characteristics such as wavelength and photoperiod (Kozai, 1991). Since *in vitro* cultures are often not photoautotrophic, the light requirements are different from those required for photosynthesis, but may be critical for photomorphogenic and phototropic responses (Thorpe, 1980). For example, high light intensities have been shown to increase free-radical damage during *in vitro* regeneration of cryopreserved embryonic axes of *Zizania palustris* (Touchell and Walters, 2000).

Temperature affects plant growth *in vivo* (e.g. Wayne *et al.*, 1998), and similar effects may be encountered *in vitro*. Generally, cultures are maintained at a constant temperature of about 25 °C (e.g. Thorpe, 1980; Berjak *et al.*, 1999; Wesley-Smith, 2002)

In spite of a consideration of all the factors that determine an optimal *in vitro* medium, this is frequently species-, genotype-, and cell-type specific, and has to be established empirically.

3.1.7 Common cryopreservation techniques

The techniques employed in the cryopreservation of plant germplasm can be grouped into two: classical and new (Withers and Engelmann, 1997). Those authors consider classical techniques to be those that involve freeze-induced dehydration, and new ones to be based on vitrification.

3.1.7.1 Classical cryopreservation techniques

Classical cryopreservation techniques generally involve slow cooling, in which extra-cellular ice is formed and the cells are dehydrated as intracellular water is lost to the exterior. In optimal conditions most, or all, freezable water leaves the cell, avoiding intracellular ice formation (Engelmann, 2000). Upon thawing, however, crystallization may occur, in which the melting ice re-forms into thermodynamically more stable, larger, and more damaging crystals (Mazur, 1984). This is usually avoided by very rapid thawing (e.g. Wesley-Smith, 2002).

Freezing by classical cryopreservation procedures is often done in two steps, with the first step cooling the specimen to a defined freezing temperature, and the second comprising immersion into liquid nitrogen (e.g. Brison *et al.*, 1995, 1997). For maximum efficiency and reproducibility, the techniques require expensive programmable freezers, although domestic freezers are used in some laboratories (Kartha and Engelmann, 1994).

Classical techniques have been particularly useful in the cryopreservation of undifferentiated systems such as cell suspension cultures (e.g. Withers and Engelmann, 1997; Swan *et al.*, 1999), and the shoot apices of cold-tolerant species (e.g. Brison *et al.*, 1995, 1997; Reed and Chang, 1997) and some tropical species (e.g. Escobar *et al.*, 1997).

3.1.7.2 New cryopreservation techniques

Vitrification-based techniques are operationally less complex than classical techniques, as the critical step in avoiding ice crystal formation is dehydration, and not freezing. Thus, if explants are amenable to dehydration to sufficiently low water contents with little or no decrease in survival compared with non-dehydrated controls, cryopreservation can generally be achieved with little or no further drop in survival (Engelmann, 1997). Seven different vitrification-based procedures can be identified (Engelmann, 2000), as listed below:

3.1.7.2.1 Encapsulation-dehydration

This technique is based on the alginate-gel encapsulation used in the production of artificial seeds. Explants are encapsulated in alginate beads, pre-grown on a medium enriched with 0.5 – 1.0 *M* sucrose, partially desiccated to a water content of about 20% (fmb), and then plunged into liquid nitrogen (Benson, 1995). The technique has been widely applied in the cryopreservation of somatic explants (e.g González-Arnao *et al.*, 1998) and of zygotic materials such as orchid seeds (Wood *et al.*, 2000), with generally high recovery rates.

3.1.7.2.2 Vitrification

The procedure termed 'vitrification' involves the treatment of explants with highly concentrated, cryoprotective, and dehydrative 'vitrification' solutions before being plunged into liquid nitrogen. The explants are then rapidly thawed, washed to remove cryoprotectants, and grown *in vitro* for recovery. It is a procedure that has been applied for the successful cryopreservation of cell suspension cultures, somatic embryos, and shoot apices of many species (e.g. Sakai, 1995, 1997; Shatnawi *et al.*, 1999).

3.1.7.2.3 Encapsulation-vitrification

This is a combination of encapsulation-dehydration and vitrification procedures, in which the samples are encapsulated in alginate beads and cryopreserved using the vitrification technique. It has been successfully applied in the cryopreservation of shoot meristems of species such as carnation (Tannouri *et al.*, 1991) and lily, wasabi (*Wasabia japonica*) and *Armoracia* (Sakai, 1997)

3.1.7.2.4 Desiccation

This technique is relatively simple in that it involves only the dehydration of the explants followed by direct immersion into liquid nitrogen. It is routinely applied in our laboratory in the cryopreservation of excised embryonic axes of recalcitrant seeds (e.g. Wesley-Smith *et al.*, 1992; Berjak and Dumet, 1996; Berjak *et al.*, 1999; Walker, 2000; Wesley-Smith, 2002; Wesley-Smith *et al.*, 2001b). Desiccation is usually performed rapidly by flash-drying (Berjak *et al.*, 1989; Wesley-Smith *et al.*, 2001a; Wesley-Smith, 2002), which facilitates the axes tolerating relatively low water contents prior to freezing. However, the degree of dehydration required to avoid freezing injury

may be detrimental to the axes, leading to unorganised callus or abnormal growth in the thawed explants (e.g.. Chandel *et al.*, 1995; Wesley-Smith *et al.*, 2001a).

3.1.7.2.5 Pregrowth

This techniques involves culturing the explants on a medium enriched with cryoprotectants, such as sucrose, followed by freezing by direct immersion in liquid nitrogen. It has been successfully applied in the cryopreservation of apices of many species such as *Musa* (Panis, 1995), *Holostemma annulare* (Decruse *et al.*, 1999), and cassava (Charoensub *et al.*, 1999).

3.1.7.2.6 Pregrowth-desiccation

This is a combination of pregrowth and desiccation, in which explants are pregrown on media containing cryoprotectants, dehydrated (usually in the laminar air flow cabinet or over silica gel), and then frozen rapidly. It has been applied to the cryopreservation of the explants of various species, for example axillary buds of asparagus (Uragami *et al.*, 1990), zygotic embryos of coconut (Assy-Bah and Engelmann, 1992), somatic embryos of oil-palm (Dumet *et al.*, 1993), and the embryonic axes of several woody species (Dumet *et al.*, 1996) and nodal explants of *Antirrhinum microphyllum* (Gonzalez-Benito *et al.*, 1998).

3.1.7.2.7 Droplet freezing

In this technique, explants are pretreated with cryoprotectants, placed on aluminium foil in small droplets of the cryoprotectant, and then frozen directly by rapid immersion into liquid nitrogen. The technique is apparently not widely applied, but has been used for the successful cryopreservation of potato meristems (Schäfer-Menuhr *et al*, 1997) and apple shoot-tips (Zhao *et al.*, 1999).

3.1.8 Objective

This aim of this phase of the study was to establish methods for the cryopreservation of the zygotic germplasm of the species under investigation. In order to achieve this, several essential initial objectives were pursued:

(i) development of an *in vitro* germination protocol(s) for excised embryonic axes of *Trichilia dregeana* and *T. emetica*

(ii) investigation of the effects of cryoprotection on *in vitro* germination of the axes of *T. dregeana* and *T. emetica*

(iii) determination of appropriate freezing and thawing techniques for the embryonic axes of *T. dregeana* and *T. emetica*

(iv) attempts to develop *in vitro* methodology for excised axes of *Warbugia salutaris*

(v) development of a suitable method for the cryopreservation of seeds of *Waburgia salutaris*.

3.2 Materials and methods

3.2.1 Establishment of *in vitro* axis germination medium

3.2.1.1 Surface sterilisation of axes

To ascertain the efficacy of different sterilants, surface-sterilisation of embryonic axes was done by immersing the axes, for various periods, as shown in Table 3.1, after which the axes were rinsed three times with sterile distilled water.

As surface-sterilisation is ineffective for internal microbial contamination, embryonic axes were also treated by incorporating anti-microbial agents in the germination medium. For *W. salutaris*, the following anti-microbial agents were tested, both singly and in combination: the fungicide, Benomyl WP® (Sanachem, South Africa) 0.1 mg l^{-1}; the antibiotic, Penstrep® (500 units of penicillin G and 5 mg streptomycin ml^{-1}, Sigma-Aldrich, St. Louis, MO, USA) 50 µg ml^{-1}; and an antibiotic cocktail constituting Rifampicin® (3-[4-Methylpiperazinyl-iminomethyl]rifampicin, Sigma-Aldrich, St. Louis, MO, USA) 20 µg ml^{-1} and Trimethoprim® (2,4-Diamino-5-[3,4,5-Trimethoxybenzyl]pyrimidine, Sigma-Aldrich, St. Louis, MO, USA) 30 µg ml^{-1}. Additionally, the efficacy of a customised fungicidal alginate gel (Chapter 2, section 2.5.3) was tested by

incorporating it at 400 ml l^{-1} of germination medium, and by encapsulating the axes prior to *in vitro* germination. Encapsulation was carried out as described in Chapter 2, section 2.4.2. Fungicides were co-autoclaved with the nutrient media, while antibiotics were added to the cooled, autoclaved medium through sterile bacteria-proof acetate filters (Cameo 25AS®, Micron Separations Inc., USA), pore-size 0.22 µm.

Table 3.1 Type, concentration and duration of application of the surface sterilants used disinfect embryonic axes of *Trichilia dregeana*, *T. emetica* and *Warburgia salutaris*.

Surface sterilant	Concentration	Duration of application	Species applied to
Sodium hypochlorite	0.5% (v/v)	5, 10, 20 min	*Warburgia salutaris*
Sodium hypochlorite	1% (v/v)	5, 10, 20 min	*Trichilia dregeana, T. emetica, W. salutaris*
Mercuric chloride	0.2% (w/v)	30 s, 1 min, 2 min	*W. salutaris*
Silver nitrate	1% (w/v)	5 min, 10 min	*W. salutaris*
Calcium hypochlorite	5% (w/v)	5, 10, 20 min	*T. emetica, W. salutaris.*

Trichilia dregeana embryonic axes from seeds of some harvests were particularly contaminated internally, and therefore the following anti-microbial agents were included in the *in vitro* germination medium: antibiotics, kanamycin (50 µg μml^{-1}) and Penstrep (50 µg ml^{-1}); and the fungicides, Benomyl (0.1 mg l^{-1}) and an anti-fungal cocktail of 0.2 ml l^{-1} Early Impact® (active ingredients: triazole [94 g l^{-1}] and benzamidazole [150 g l^{-1}], Agrochemicals Pty Ltd., South Africa), and 2.5 ml l^{-1} Previcur N® (active ingredient: propamocarb-HCl [722 g l^{-1}], AgrEvo S.A. Pty Ltd., South Africa). As in the case with *W. salutaris*, the anti-microbial agents were tested both singly, and in factorial combination.

3.2.1.2 *in vitro* germination medium

In order to develop an optimal *in vitro* axis germination medium, the following formulations of macro- and micro-nutrients were tested (Appendix A): MS (Murashige and Skoog, 1962) medium (full-strength, half-strength, and quarter-strength), Woody Plant Medium (WPM, Lloyd and McCown, 1981), and Gupta and Durzan's (1985) DCR medium. Zeatin and IAA were included in the media as the cytokinin and auxin respectively, in ratios indicated in Table 3.2. All the media tested contained 3% sucrose as a carbon source, at pH 5.6 – 5.8, and were solidified with 0.8% (w/v) agar.

Besides auxin and cytokinin analogues, organic complexes were also assessed for the ability to promote *in vitro* axis germination for *T. dregeana*. These were casein hydrolysate, coconut milk, and cotyledon extract from the seeds of *T. dregeana*.

Table 3.2 Concentrations and ratios of IAA:zeatin tested for the enhancement of *in vitro* germination of embryonic axes of *Trichila dregeana*.

[Zeatin] mg l^{-1}	[IAA] mg l^{-1}	Zeatin / IAA ratio
0	0	0
0.04	0.02	2.0
0.08	0.02	4.0
0.08	0.01	8.0
0.20	0.02	10
0.20	0.01	20
0.40	0.01	40
1.0	0.01	100

The cotyledonary extract was prepared as follows: cotyledons were ground in distilled water in the ratio 1 g fresh weight to 10 g water and the mixture boiled for 20 min in a water bath controlled at 100 °C. After cooling, the boiled mixture was strained through double muslin cloth and vacuum-filtered through a double layer of filter paper (Whatman® No. 1, Whatman International Ltd., Maidstone, England) and centrifuged at 10 000 g for 30 min. The supernatant was decanted and filtered through sterile filters: firstly through a 0.8 mm filter, and then through a 0.2 mm filter into a sterile culture bottle. The filtrate was then filter-sterilised through a 0.22 µm filter and introduced in the following concentrations (v/v) to cooled nutrient medium: 5%, 10%, 20%, and 50%.

Besides the finely-homogenised cotyledon extract, a variant was prepared, which was not filtered, but mixed with 10 g l^{-1} agar (with no other nutrients added) and autoclaved. Embryonic axes were then plated onto the medium.

Coconut milk (purchased tinned from a supermarket) was filter-sterilised into cooled medium at concentrations of 5, 10, 20 and 30 ml l^{-1} of medium, while adenine sulphate (40, 60 and 80 mg l^{-1} of medium) was co-autoclaved with the germination medium.

3.2.2 Cryoprotection

The following cryoprotectants were tested: DMSO and glycerol (5 and 10% [v/v] each); PVP and dextran (10 and 20% [w/v] each); and sucrose (0.5 M and 1M). Each cryoprotectant was used both singly and in factorial combination with each of the others. Explants were cryoprotected by soaking them in the lower concentration of the cryoprotectant solution for 30 min, followed by a further 30 min in the higher concentration.

Another cryoprotection method used was that of Dumet *et al.* (1994) and Dumet and Berjak (1996), in which the axes were pregrown on a germination medium containing 0.75 *M* sucrose for 16 h before being desiccated and frozen. Additionally, the method of Fu *et al.* (2000) was used for cryoprotection. In this method, the axes were asceptically cultured on Woody Plant Medium (WPM, Lloyd and McCown, 1981) containing 3% sucrose for three weeks, after which they were transferred to a fresh medium containing 27% sucrose and incubated for 7 d. Axes were then successively transferred to fresh media containing sucrose at concentrations of 40% (for 5 d), 55%

(for 5 d), and 65% (for 2 d). At the end of each stage, survival and water content of the axes were determined.

3.2.3 Flash-drying / rapid drying

Flash-drying was carried out to previously-established, non-injurious water contents (Chapter 2) – by passing a stream of dry air over the axes in a flash-dryer (*Trichilia dregeana*), or by placing them on the bench of a laminar air flow cabinet (*T. emetica*). In some instances for *T. dregeana*, the shoot apex was encapsulated with an alginate gel (described in chapter 2, section 2.4.2) in an attempt to prevent its excessive dehydration. For axes that were cryoprotectected for freezing, they were treated with the cryoprotectants before dehydration.

3.2.4 Cooling

Whole seeds of *Warburgia salutaris* were enclosed in 1.0 ml cryotubes (NUNC®, Daigger, USA) and immersed into liquid nitrogen, in which they were kept for at least 1 h. Five seeds were frozen in each cryotube.

Embryonic axes of *Trichilia dregeana* and *T. emetica* were frozen by one of six different means:

(i) Axes were enclosed in cryotubes and plunged into liquid nitrogen. Five axes were contained in each cryotube.

(ii) Axes were individually mounted onto a wooden sliver using a thin layer of glycerol, and then plunged rapidly into melting isopentane (-140 °C) held in a liquid nitrogen reservoir, before being transferred into the liquid nitrogen.

(iii) Axes were enclosed in 10 mm x 10 mm envelopes (five axes per envelope) made from a single layer of commercial light-weight aluminium foil, and plunged, using a pair of fine forceps, into liquid nitrogen.

(iv) Axes were cooled at 0.5 °C min^{-1}, on the specimen stage of a differential scanning calorimeter (DSC-7, Perkin Elmer, USA), to either -20 °C, -40 °C or -80 °C and then rapidly transferred to liquid nitrogen.

(v) Axes were cooled ultra-rapidly by being plunged into nitrogen slush (liquid nitrogen sub-cooled to -210 °C [Echlin, 1992]). For this purpose, axes were first mounted on cryo-ultramicrotomy specimen supports (Leica, Austria) using a thin layer of low-temperature

adhesive (Tissue-Tek®, Miles Scientific, USA), and then plunged at 1.2 m s^{-1} into a 1 600 mm-deep container of nitrogen slush, using a spring-loaded device and set-up developed by Wesley-Smith (2002). Alternatively, ultra-rapid cooling was achieved by making nitrogen slush in a 100 mm x 50 mm polystyrene container and tumble-mixing the slush with the embryonic axes in a second container. Ten embryonic axes were frozen simultaneously using this approach. In order to make the nitrogen slush for this method, the polystyrene container was filled with liquid nitrogen and placed in a desiccator, which was then evacuated with a vacuum pump until the liquid nitrogen had visibly solidified.

(vi) Axes were frozen using the vitrification technique as follows: the axes were soaked in the PVS2 vitrification solution (Sakai *et al.*, 1991), for 30 or 60 min at 25 °C, then suspended in 1.0 ml cryotubes with 0.5 ml fresh PVS2, and the tubes directly immersed in liquid nitrogen.

3.2.5 Thawing/Rehydration

Explants were thawed, and simultaneously re-hydrated, by immersing for 1 min in one of two thawing media in a water bath thermostatically controlled at 40 °C: distilled water, or a 1:1 solution of 1μ*M* $CaCl_2.2H_2O$ and 1m*M* $MgCl_2.6H_2O$. Axes that were frozen by direct plunging into liquid nitrogen or slush were directly immersed into the thawing medium, while those explants frozen in cryotubes or aluminium envelopes were thawed in the vials and then re-hydrated in either water or calcium / magnesium solution.

3.2.6 Assessment of survival

Survival of thawed embryonic axes was assessed by plating them, immediately after thawing and rehydration, onto the optimal *in vitro* axis germination media previously established (Chapter 2). They were placed in the dark for 48 h and then transferred to a 16h:8h light:dark photoperiod at a light intensity of 200 μE m^{-2} s^{-1}), at the same temperature. Survival was then assessed in terms of normal plantlet formation, root elongation or callus proliferation.

Whole seeds of *W. salutaris* were sown either in vermiculite in germination trays placed at 25 °C, or in bottom-heated sand-beds maintained at 25 °C, and watered twice daily. Survival was scored in terms of shoot emergence.

3.2.7 Transmission electron microscopy

Material for examination by transmission electron microscopy was processed as described in Chapter 2, section 2.5.7.

3.2.8 Water content determination

Water contents were determined gravimetrically as described in Chapter 2, section 2.4.4.

3.3 Results and discussion

3.3.1 Establishment of an *in vitro* axis germination medium

3.3.1.1 Sterilisation of explants

The seeds of the species used in this study, viz. *Trichilia dregeana*, *T. emetica*, and *Warburgia salutaris*, were expected to have high levels of microbial contamination, as recalcitrant seeds usually harbour a wide spectrum of fungi and bacteria (Mycock and Berjak, 1990; Berjak, 1996). Some of the micro-organisms isolated from such seeds include more than 20 fungal species (Singh and Singh, 1990), with species of *Aspergillus*, *Fusarium*, *Penicillium*, *Curvularia*, *Pseudomonas* and *Trichoderma* being associated with the recalcitrant seeds of just one tropical species (Calistru *et al.*, 2000) growing in the same geographical area as the species used in this study. The majority of these fungi were found on the surface of seed tissues (testa, cotyledons, embryonic axes) at harvest, with progressive fungal invasion of the internal seed tissue (e.g. Calistru *et al.*, 2000). Hence, surface-sterilisation of embryonic axes was considered imperative before any attempt was made to culture these *in vitro* (as is necessary after cryopreservation) in anticipation that this procedure would be sufficient to eliminate contaminants.

3.3.1.2 *Trichilia dregeana*

Embryonic axes of *Trichilia dregeana* were routinely surface-sterilised by soaking in 1% NaOCl for 20 min, which generally excluded all contamination, while the axes generally achieved 100%

germination. However, this procedure was not able to eliminate fungal/bacterial contamination from embryonic axes of seeds from all harvests or every season. For example, seeds harvested in July 2000 remained 100% contaminated despite surface-sterilisation. Hence, the following anti-microbial agents were included in the germination media and evaluated for sterilisation: the fungicides, Benomyl and a mixture of Previcur N and Early Impact. These were tested either singly or in factorial combination with the antibiotics, Penstrep and kanamycin. A mixture of Previcur N - Early Impact and kanamycin proved to be the most effective in eliminating the embryo-associated contamination (Table 3.3).

Table 3.3 Percentage contamination and germination (radicle growth) of *T. dregeana* embryonic axes cultured on WPM-based media containing different anti-microbial agents. The axes were assessed after 14 d.

Anti-microbial agent	**% contamination**	**% germination**
Benlate	84	8
Previcur N-Early Impact	52	15
Kanamycin	100	0
Penstrep	100	0
Benlate +kanamycin	40	8
Benlate +Penstrep	60	35
Previcur N-Early Impact+kanamycin	5	88
Previcur N-Early Impact+Penstrep	36	45

Particular fungal contamination may not be invariably associated with a particular species (e.g. Kioko *et al.,* 2003; Sutherland *et al.*, 2003), and the extent and type of microbial contamination depends on environmental factors such as conditions during flowering and fruit set, prevailing humidity, the geographic location of the parent plant, and even the stage in the fruiting season, at

which the seeds are harvested. Hence, while surface sterilisation with 1% NaOCl was sufficient to exclude contamination in *T. dregeana* axes from seeds harvested early in some seasons, this treatment was ineffectual in sterilising embryonic axes of seeds harvested late in the 2000 season. For the latter axes, co-culturing with a mixture of the fungicidal cocktail of Previcur N-Early Impact and the antibiotic kanamycin was the most effective means of eliminating contamination in culture (Table 3.3). This mixture of antimicrobial agents was also found to be the most effective of the compounds tested for sterilisation, in the field, of embryonic axes extracted on site (Makhathini, 2001) from the same locality from which seeds were harvested in 2000.

While all the three fungicides tested are systemic, Previcur N and Early Impact are, collectively, effective against a wider spectrum of fungal species (including *Aphanomyces, Bremia, Plasmopara, Pseudopenorospora, Pythium, Rhynchosporium, Puccinia, Erysiphe* and *Septoria*), than Benomyl®, which is targeted to *Botrytis, Fusarium, Rhizoctonia, Verticillium* and *Sclerotinia* (Makhathini, 2001). It is noteworthy that in some studies (e.g. Hannweg, 1995) the use of Benomyl and Previcur N in combination was found to be less effective than either of the two fungicides alone. However, as the microbial contamination may be unique to each explant, species or provenance, it is necessary to determine the appropriate surface-sterilisation/disinfection protocol empirically.

3.3.1.2 *Trichilia emetica*

Embryonic axes of *T. emetica* showed different reponses to the various surface-sterilants. Sodium hypochlorite, one of the most commonly used surface-sterilants in plant tissue culture, was injurious to the axes, which attained an average germination of only 60% when contamination was eliminated. On the other hand, calcium hypochlorite (which is often less injurious) was not effective in eradicating fungal infection. Mercuric chloride, applied for either one or two minutes, effectively eliminated contamination, while enabling the axes to retain full germinability (Table 3.4). As a consequence of these responses, 0.2% mercuric chloride (for 1 min) was the method of choice.

Table 3.4. The effects of the various surface sterilants tested, on contamination and axis germination (root and shoot development) for axes of *T. emetica*. Twenty axes were used per treatment, and observations recorded after 14 days in culture.

Surface sterilant	Duration of application	% contamination	% axis germination
1% NaOCl	5 min	70	15
	10 min	10	50
	20 min	0	60
0.2% $HgCl_2$	30 s	30	30
	1 min	0	100
	2 min	0	100
$Ca(OCl)_2$	5 min	100	0
	10 min	85	50
	20 min	55	30

3.3.1.3 *Warburgia salutaris*

The following surface-sterilants were found to be lethal to embryonic axes of *W. salutaris*: NaOCl (0.5 to 1%, applied for 5, 10 and 20 min), and $AgNO_3$ (1%, applied for 5 and 10 min). Dipping the axes for 1 min in 100% ethanol did not kill them, but was not effective in eliminating contaminants.

Plating the axes on media containing the customised anti-microbial alginate gel described in chapter 2 (section 2.5.3) produced similar results to those of surface-sterilisation with NaOCl: contamination was curtailed, but so also was axis germination (Table 3.5).

Table 3.5 The effect of plating embryonic axes of *W. salutaris* (n=20) on a medium containing a customised organic alginate gel. This gel has been shown to have anti-microbial properties, and was either autoclaved before incorporation into the medium, or used without autoclaving. Embryonic axes were excised asceptically from seeds sterilised by soaking in 1% $AgNO_3$ for 5 min. (+ or - indicate the application, or not, of a particular procedure).

Seeds sterilised	**alginate autoclaved**	**% contamination**	**% germination**
-	-	100	0
+	-	25	0
-	+	100	0
+	+	0	0

As all the axes remained contaminated despite surface-sterilisation, or died if the surface-sterilant was effective in combating the micro-organisms, the sterilisation technique was modified, so that anti-microbial agents were included (singly or in combination) in the culture medium (Table 3.6). Thus, the fungicide Benomyl was included into the germination medium (at a concentration of 0. 1 mg l^{-1}), along with antibiotics Penstrep (50 µg ml^{-1}), and an anti-biotic cocktail consisting of Rifampicin (20 µg ml^{-1}) and Trimethoprin (30 µg ml^{-1}).

The inclusion of anti-microbial agents in the medium reduced the microbial contamination that was otherwise unchecked by surface sterilisation, but only for a period shorter than two weeks, after which the axes were all overrun by fungi and bacteria (Table 3.6). Similar anti-microbial agents, particularly the penicillins, have been shown to not only check contamination in many plant cultures (e.g. Kneifel and Leonhardt, 1992), but also to stimulate *in vitro* growth (Holford and Newbury, 1992). Moreover, one of the constituents of the antibiotic cocktail, Rifampicin, has been found to be the most effective inhibitor of common plant-infecting bacteria, such as the pink-pigmented facultative methylotropic bacteria (Chanprame *et al.*, 1997).

Table 3.6. The effect of the inclusion of anti-microbial agents in the germination medium, on *in vitro* germination (root and shoot production) and contamination in embryonic axes of *W. salutaris*. The basic medium used was MS salts and vitamins, containing 30 g l^{-1} sucrose and 8 g l^{-1} agar. Germination and contamination were assessed on 20 axes per treatment, after 14 days in culture.

Anti-microbial agent	% germination	% contamination
Penstrep	60	100
Antbiotic cocktail	55	100
Benomyl	55	100
Penstrep + Benomyl	70	100
Antibiotic cocktail + Benomyl	75	100

However, the effectiveness of antibiotics in plant tissue culture is affected by culture conditions such as pH and the concentration of cations (e.g. calcium and magnesium) which change with time (Falkiner, 1988). In some instances, this may be overcome by the frequent sub-culturing of explants onto fresh antibiotic-containing media in order to maintain their efficacy.

In this study, owing to the inadequacy of the elimination of contaminants from the excised axes of *Warburgia salutaris*, subsequent cryopreservation techniques were carried out on whole seeds (see below).

3.3.2 Optimisation of axis germination medium

In order for excised embryonic axes to germinate, the embryonic plant must be supplied with appropriate amounts of macro- and micro-nutrients and other growth factors, which are normally derived from the seed storage tissues. In intact seeds of dicotyledonous species such as *Trichilia dregeana*, *T. emetica* and *Warburgia salutaris*, the cotyledons serve this purpose (Robbins *et al.*, 1966), and continue to supply the juvenile seedling with nutrients until leaves develop and attain the ability to carry out photosynthesis.

Thus, when the cotyledons are severed from the embryonic axes, the rôle of the former must be substituted by the germination medium. The optimal concentrations and ratios of various growth regulators depend on the species and purpose of culture (George, 1993), with cytokinins and auxins being particularly important for *in vitro* germination (Khan, 1975).

In this study, it was not possible for embryonic axes of *Warburgia salutaris* to be effectively sterilised, and therefore *in vitro* germination was investigated only with the axes of *Trichilia dregeana* and *T. emetica*. Germination was considered as the development of the embryonic axis to a stage where its structures indicated that it was capable of onward growth into a satisfactory plant, and a crucial criterion was the development of both roots and shoots.

Trichilia dregeana was the first species of which seeds were available, thus the initial investigations were done on this species. Due to the close taxonomical relationship, and morphological, and possibly physiological similarity between the seeds of the two *Trichilia* species, it was intended that the findings obtained from studying *T. dregeana* be applied to *T. emetica* probably with minor modifications.

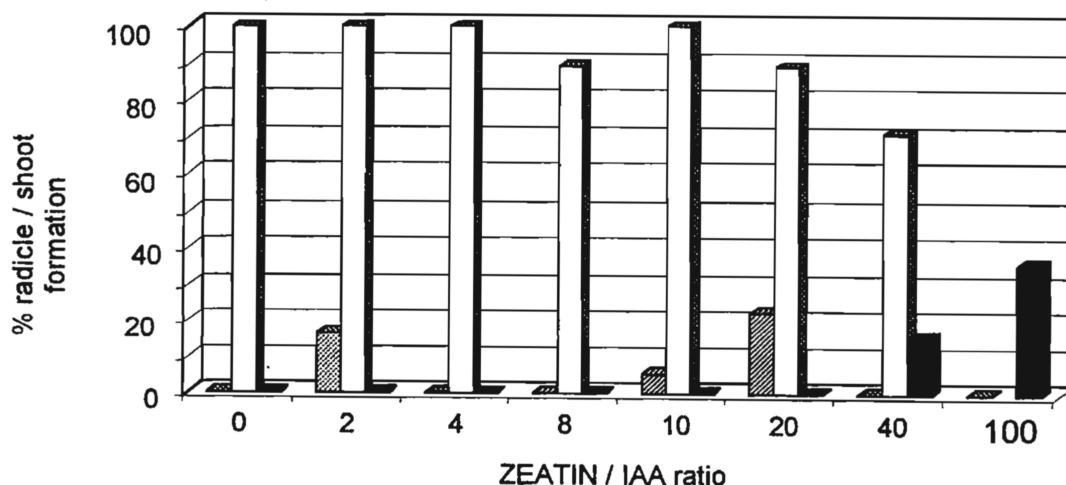


Fig 3.1 Percentage of embryonic axes of *Trichilia dregeana* attaining root lengths of 5 mm or more (clear bars), developing roots and shoots (hatched bars), or forming callus (solid bars) following *in vitro* culture in MS medium containing different cytokinin:auxin ratios.

When embryonic axes of *T. dregeana* were cultured on media containing different ratios of plant growth regulators (Table 3.2), the axes developed long radicles (except at the heightened zeatin:IAA ratios), some up to 100 mm, but no shoots developed (Figs 3.1 – 3.2). In media with higher cytokinin:auxin ratios, the radicles showed much less linear elongation, became bulbous, and tended to form callus. Even though radicle elongation occurred reproducibly in practically 100% of axes cultured on most media, subsequent shoot formation was not observed in more than 20% of the axes on any of the media tested. Thus, despite clear indications that the axes could germinate *in vitro*, there was need for the optimisation of other factors besides the ratio of auxins to cytokinins in the germination media. One such factor was the total concentration and form of specific nutrients in the media. To this end, nitrogen was supplied to the medium in either the reduced (NH_4^+) or oxidised ($N0_3^-$) form, without altering the total amount of nitrogen in the medium, and in the form of casein hydrolysate.

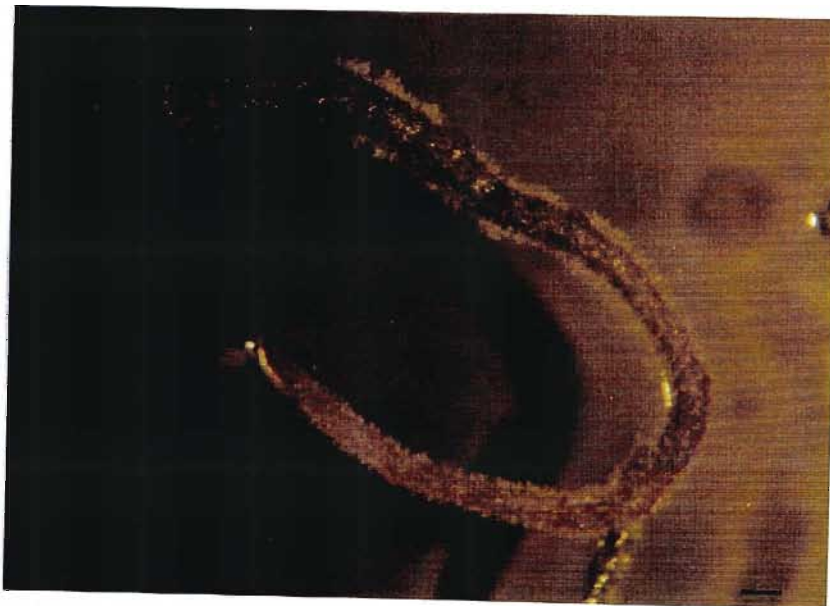


Fig. 3.2. Excised embryonic axis of *Trichilia dregeana* plated on MS medium, without any plant growth regulators. The axes developed long radicles, but there was no shoot development. Bar = 2 mm

Axis germination was also attempted in media with less total nitrogen (Gupta and Durzan's [1985] DCR medium); without any nitrogen (i.e. without stock 1 of the MS medium formulation); and without iron (i.e. MS medium without $FeSO_4.7H_20$ in stock 5). These were tested without any plant growth regulators, and the results are summarised in Fig. 3.3.

Significant differences in shoot development and germination were expected as a result of this manipulation of nitrogen and iron. The nitrogen content of a medium is the most important determinant of plant growth or morphogenesis (George, 1993), and media containing only NO_3^- have been used successfully for the shoot culture of some species (Boxus, 1974). However, the presence of some form of reduced nitrogen is required for the continued development of zygotic embryos in culture (Mauney *et al.*, 1967; Norstog, 1967, 1973). The proportions of reduced to oxidized forms of nitrogen also has an effect on the action of plant growth regulators, with higher proportions of reduced nitrogen enhancing the effects of cytokinins (Meyer and Abel, 1975), which would possibly stimulate germination and shoot formation. Furthermore, addition of nitrogen in the organic form of casein hydrolysate has been shown to promote *in vitro* morphogenesis (Chang *et al.*, 2000), including shoot formation in woody species (Harry and Thorpe, 1991), and to be necessary for plantlet formation from somatic embryos of sandalwood, *Santalum album* (Rugkhla and Jones, 1998).

However, in this study, there was no improvement in germination following the above-mentioned alterations in the form and concentration of nitrogen and iron (Fig. 3.3). Radicle elongation was observed in a high proportion of the axes, but shoot development was erratic, and the axes eventually died.

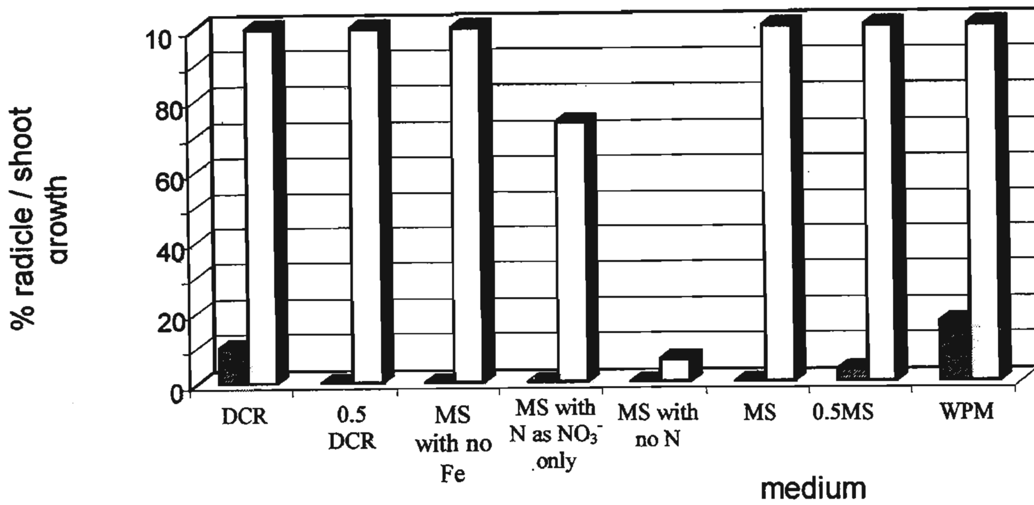


Fig 3.3 Percentage of embryonic axes of *Trichilia dregeana* attaining root lengths of 5 mm or more (clear bars) and subsequently developing shoots (hatched bars) following *in vitro* culture in different media formulations.

Another compound, adenine sulphate, was therefore tested to promote shoot development and germination, with concentrations of 40, 60 and 80 mg l^{-1} being investigated. The growth regulatory effects of adenine have been noted for over 60 years, since Bonner and Haagen-Smit (1939) found that the compound promoted an expansion in the area of leaf discs floated on sugar solutions. It has subsequently been found that adenine has cytokinin activity and can improve growth (Nwankwo and Krikorian, 1983) and stimulate shoot formation (Philip *et al.*, 1992). It is often added to media in amounts ranging from 40 - 80 mg l^{-1} (George, 1993).

In this investigation, the inclusion of adenine sulphate in the media failed to induce shoot formation. Axes grown on the medium containing 40 and 60 mg l^{-1} adenine sulphate all developed roots with none developing shoots. Axes on the medium containing 80 mg l^{-1} responded in a manner similar to that of axes plated in media with very high cytokinin: auxin ratios – only 30% developed roots (with much reduced elongation relative to axes on the other adenine-containing media), and all the axes became bulbous. High concentrations of adenine sulphate have the same effects as an excess of cytokinin, and suppress apical dominance. Nickerson (1978) found that 80 mg l^{-1} adenine was too high a concentration to promote shoot formation.

Stimulation of growth by adenine can be unpredictable and may depend on other characteristics of the medium or explant (Chiariotti and Antonelli, 1988), such as the type and concentration of cytokinin used, as adenine is thought to act as a synergist to cytokinins (Beach and Smith, 1979). Start and Cumming (1976) also found that when adenine sulphate was added to a cytokinin-containing shoot initiation medium, it increased shoot formation from African violet leaf sections, whereas on the basal medium with only adenine sulphate, shoot formation was delayed and roots alone were formed. Adenine sulphate has also been shown to have inhibitory effects on shoot formation in a number of species (Jarriet *et al.*, 1985; Philip *et al.*, 1992).

It was therefore not clear whether the lack of shoot formation in adenine-containing media used in this study was due to inhibitory effects of adenine, or because the compound simply could not overcome the factor (s) causing lack of their development. As no satisfactory shoot development had been obtained on any of the other media tested, the latter explanation was considered more feasible.

Manipulation of the inorganic constituents of nutrient media performed in this study failed to stimulate reasonable *in vitro* germination of *T. dregeana* embryonic axes. However, plant culture media may also include various organic additives, one of the most commonly-used of which is coconut milk, the liquid endosperm of *Cocos nucifera* (George, 1993). It is a highly heterogeneous mixture of many amino acids and nitrogenous compounds, sugars and sugar alcohols, organic acids, growth substances, and inorganic elements (George, 1993). In many plant tissue cultures, coconut milk has been shown to have promotive effects on growth and morphogenesis (e.g. Vasil and Vasil, 1981; Jana *et al.*, 1994; Rugkhla and Jones, 1998; Thomas, 1999).

However, in this study, no shoot growth was recorded in media supplemented with coconut milk at concentrations of 5, 10, 20 and 30 ml l^{-1}. The axes showed considerable elongation of radicles, but no shoot formation, and the axes later died.

Although coconut milk does promote growth *in vitro*, this additive is not specifically defined and its composition can vary considerably (Swedlund and Locy, 1988), thus results may not necessarily be reproducible. Furthermore, coconut milk has been shown to be particularly inhibitory to the growth of embryos of some species, including the embryo shoot apices of wheat (Smith, 1967; Noh *et al.*, 1988), although it is not known which ingredients cause the inhibition (George, 1993).

It was apparent that *in vitro* germination of *T. dregeana* axes could be dependent on some factor(s) other than the inorganic or organic constituents of the germination media. Such factors may be present in the cotyledons, from where they are normally supplied to the germinating axes. In an attempt to include any such factors, the cotyledons were incorporated into the germination media. They (cotyledons) were either ground and used as a germination medium (solidified with agar) or used to prepare a liquid cotyledon extract (section 3.2.1.2) and added to the germination media.

The media containing cotyledon extracts (autoclaved / filter-sterilised) were clearly unsuitable for axis germination. Radicle elongation occurred in less than 20% of the axes, with axes cultured in undiluted cotyledon extract all dying without developing radicles (Fig 3.4). This was observed for media containing both the filter-sterilised and autoclaved extracts. It may be, therefore, that homogenisation of the cotyledons releases compounds that are toxic to the embryonic axes. This was corroborated by subsequent results obtained in this laboratory (unpublished) which demonstrated the anti-microbial and phytotoxic properties of extracts from cotyledons of *T. dregeana* seeds. In those later studies, the growth of *T. dregeana* axes was completely inhibited by the inclusion of the cotyledon extract at levels higher than 4% (v/v). The concentrations of extract used in the present study ranged from 5% upwards, and were therefore above the toxic limit. In the *in vivo* situation, there may exist mechanisms which physically partition, or keep the concentration of the inhibitory compounds lower than the toxic levels. However, during preparation of the extracts, the processes of autoclaving or homogenisation and centrifugation prior to filter-sterilisation, may either disrupt cytomatrical organisation and compartmentalization, or denature the proteins involved, thus liberating substances that are inhibitory, or even toxic, to the axes.

There is little published information regarding the putatively phytotoxic compounds in *T. dregeana* cotyledons, but these have been shown to contain at least four different limonoids (Mulholland and

Taylor, 1990). Limonoids, commonly produced by members of the Meliaceae, are recognised for their toxic effects against a wide range of life forms, and used in insecticide formulations (Dua *et al.*, 1995; Nagpal *et al.*, 1996).

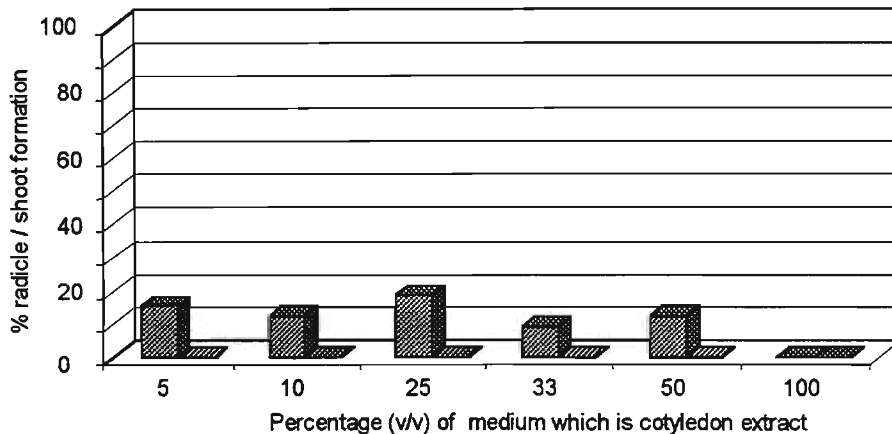


Fig 3.4 Percentage of embryonic axes of *Trichilia dregeana* attaining root lengths of 5 mm or more (solid bars) and developing shoots (hatched bars) following *in vitro* culture on MS medium constituting different strengths of cotyledon extract.

When all the 30 media formulations so far tested for axis germination in this study were considered, there was ubiquitous root development without full germination, and in all cases, the axes subsequently became necrotic. This led to the speculation that the lack of shoot development may have been related to problems on the shoot apex itself rather than with the germination media. As the zygotic axis of *T. dregeana* is very small (*c.* 0.003 g, Chapter 2), it is possible that the extent of the lesions after severing the cotytledonary attachment might have precluded shoot development. To test this, axes were excised in such a manner as to leave the shoot uninjured, by leaving the cotyledonary attachments intact, and including a segment of both cotyledons with the excised axis (Figure 3.5). Axes thus excised were then incubated on a germination medium that consisted of basal MS salts and vitamins, without any growth regulators.

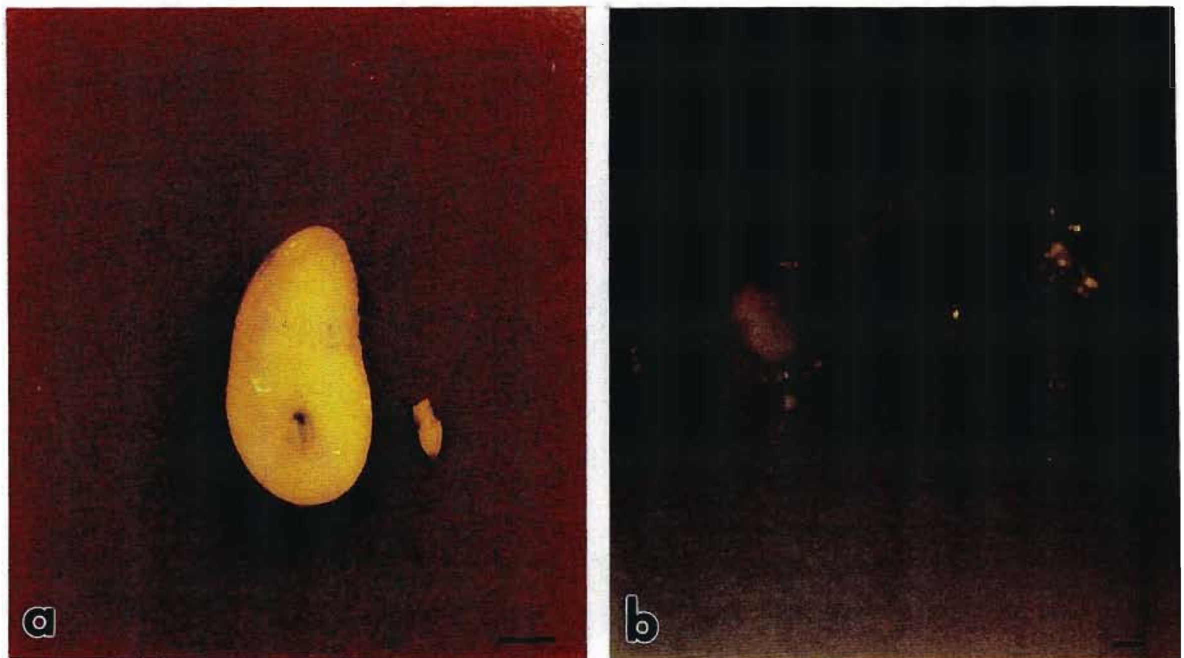


Fig 3.5 (a) *Trichilia dregeana* cotyledon (left) and embryonic axis appropriately excised to include some attached cotyledon (right), showing the relative sizes of the two organs. (b) Comparison of embryonic axis excised without (left) and with (right) cotyledonary attachment. Bar = 10 mm

After about four to five weeks in culture, the axes had developed not only radicles, but shoots were visible in all cases (Figure 3.6). The effect of not severing the cotyledonary attachments, or of leaving a segment of each cotyledon attached to the axis was evidently enough to promote germination, including shoot development. It is considered likely that not severing the cotyledonary attachment obviated injury to the shoot apex. In contrast, the injury suffered by the apical tissues when the attachments were cut through flush with the axis surface, may have been so severe as to eliminate the shoot growth capacity. Both the apical meristem and area around the cotyledonary attachment have been shown to be necessary for shoot development from embryos. Histological studies in rice embryos showed that shoots developed from epidermal and sub-epidermal cell layers of two fixed sites on the abaxial side of the base of the first leaf, which is very close to the cotyledonary attachment (Nakamura and Hattori, 1995).

Fig 3.6 Shoot growth from an embryonic axis of *T. dregeana* excised appropriately, and cultured on MS medium without any plant growth regulators. Bar = 0.5 mm

Thus, when the cotyledonary attachments of *T. dregeana* were excised completely from the axes, such sites may have been severed and removed along with the cotyledons and this may therefore have removed the morphogenetic competence for shoot development. This is different from observations made for some species, that the shoot-regenerating meristem can be re-programmed and induced by cytokinin treatment after excision (Buising *et al.*, 1994). However, the degree of injury of the *T. dregeana* axes, coupled with the response to such a stress for this species (for which there is no available information), may have rendered them unable to recover or to regenerate shoots. It is noted that further studies, in which the size of the cotyledonary blocks is whittled down progressively to the situation where only the attachment sites remain, are necessary to clarify the issue of lack of injury vs an actual vital rôle of the cotyledonary tissue in promoting shoot development in *T. dregeana*.

Once it was possible to achieve full germination of axes following appropriate excision, trials were carried out to determine the optimum concentration and ratios of plant growth regulators for *in vitro* germination. Embryonic axes were therefore germinated on media containing the same concentrations of auxins and cytokinins as shown in Table 3.2, and the performance in various media assessed in terms of growth rate and the time taken for shoots to develop. The results of these trials are summarised in Table 3.7.

The hormones tested have been shown to accelerate the mobilisation of reserves and enzymes into embryonic axes from the attached cotyledons (Taneyama *et al.*, 1995). For example, IAA induces the production of lipoxygenases in zygotic embryos (Wang *et al.*, 1999), while NAA (another auxin) increases the efficiency of the desaturation-related processes in extra-plastidial compartments (Liu, *et al.*, 1995). Cytokinins have been shown to be responsible for directing shoot development in excised embryonic axes (Morre *et al.*, 1999), and to confer protection against oxidative stress during germination (Gidrol *et al.*, 1994).

In order to test the effect of macro- and micro-nutrient concentration on the performance of *in vitro* germinating axes, different strengths of MS medium (full-, half-, and quarter-strength), as well full-strength Woody Plant Medium (WPM), were tested.

The results (Table 3.7) showed that WPM, without any added phytohormones, stimulated *in vitro* growth and germination that was significantly superior to that in all the other media formulations tested. This may be because the nutrient concentrations in WPM, developed specifically for woody plants (Lloyd and McCown, 1981), are more suited to their morphogenesis of axis tissues of woody plants than are those in MS medium (which was originally formulated for a herbaceous species, tobacco [Murashige and Skoog, 1962]).

In contrast, however, similarly-excised axes of *Trichilia emetica* performed the best on full- and half-strength MS media, without any plant growth regulators (Table 3.8). This species is faster-growing than *T. dregeana* (personal observations during this study were that *T. emetica* axes germinated and developed shoots within two weeks in culture, while *T. dregeana* axes took at least

eight weeks to reach a similar stage of development) and may therefore require the higher levels of nutrients supplied by MS media.

The inability of the exogenously-supplied plant growth regulators to stimulate *in vitro* morphogenesis indicates that these compounds may be synthesized *de novo* in the embryonic axes, as demonstrated for yellow lupin, *Lupinus luteus* (Nandi *et al.*, 1995). Those authors found that reserve mobilisation during seed germination was regulated by a cytokinin emanating from the embryonic axis itself. Similar findings were reported by Villalobos and Martin (1992) for chickpea (*Cicer arietimun*) embryonic axes. Auxins, such as IAA, have also been shown to be produced *de novo* in the embryonic axes of several species, such as soybean (Bialek and Cohen, 1992) and pea (Hirasawa *et al.*, 1994).

Contrary to those findings, however, a study of the seeds of *Avicennia marina*, a tropical recalcitrant-seeded species, showed that the embryonic axis relied on the cotyledons for additional cytokinin supply to effect germination (Farrant, 1991). That author found that the cotyledons contained cytokinins at an extraordinarily high concentration of 2 100 ng g^{-1} dry mass of cotyledon. In an analysis of plant growth regulators in cotyledons of *Trichilia dregeana*, the following levels of plant growth regulators were found: zeatin, 5.15 ng g^{-1}; zeatin riboside, 21.3 ng g^{-1}; and IAA, 17.4 ng g^{-1} (Farrant[1], pers. comm.). Thus, the cotyledonary levels of these plant hormones are hundred-fold lower in *T. dregeana* than in *A. marina*, suggesting that it is unlikely that the cotyledons of the former species act as a source of the hormones for the germinating embryonic axis. It is therefore proposed, in this study, that embryonic axes of *T. dregeana* and *T. emetica* synthesised the necessary auxins / cytokinins *de novo* during *in vitro* germination, and that exogenous supply of these hormones was superfluous, and possibly even inhibitory.

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Table 3.7. Germination of embryonic axes of *T. dregeana* cultured on various medium formulations, containing different ratios of zeatin:IAA. Measurements represent the average (±sd) of 20 individual embryonic axes, assessed after eight weeks in culture.

Medium	Zeatin / IAA Ratio	Root length (mm)	% of explants with shoots
MS	0	21.2±2	20
	2	21.2±1.6	20
	10	12.4±0.8	15
	40	11.6±0.8	0
	100	9.2±1.6	0
½ MS	0	17.8±0.4	20
	2	9.6±0.0	35
	10	7.6±0.4	0
	40	5.6±0.0	0
	100	5.6±0.0	0
¼ MS	0	11.2±0.4	15
	2	7.6±0.4	5
	10	7.2±0.4	0
	40	10.4±0.4	0
	100	9.2±0.4	0
WPM	0	36.0±5.2	100
	2	20.8±2.8	75
	10	22±1.6	30
	40	22.8±5.6	5
	100	17.2±3.6	0

Table 3.8. Germination of *T. emetica* embryonic axes on media containing different ratios of zeatin:IAA. Measurements represent the average (±sd) length of roots of 20 individual embryonic axes, assessed after two weeks in culture.

Medium	**Zeatin / IAA ratio**	**Root length (mm)**	**% of explants with shoots**
MS	0	44.5 ±2.8	95
	2	35.2±2.4	95
	10	35.7±2.8	60
	40	23.5±2.2	55
	100	15.3±2.8	30
½ MS	0	46.5±1.7	90
	2	39.7±2.5	80
	10	24.9±1.4	65
	40	29.1±1.4	40
	100	10.4±2.2	45
¼ MS	0	22.6±1.4	55
	2	20.8±2.6	60
	10	11.4±1.5	45
	40	5.6±1.2	40
	100	5.7±0.9	30
WPM	0	22.6±2.2	55
	2	12.7±1.4	55
	10	6.6±3.4	35
	40	6.2±0.1	40
	100	15.7±3.6	30

3.3.3 Cryopreservation of excised embryonic axes of *Trichilia dregeana* and *T. emetica*

Dehydration of explants prior to cryopreservation reduces the amount of water in the tissues, leaving less for the formation of ice crystals. For zygotic axes of a number of species, dehydration has been successfully employed in curtailing intracellular ice formation and enhancing survival after cryopreservation (e.g. Wesley-Smith *et al.*, 1992; 2001b; Normah *et al.*, 1986; 1997; Berjak and Dumet, 1996; Berjak *et al.*, 1999; Normah *et al.*, 2000). However, the level of dehydration that embryonic axes of *T. dregeana* and *T. emetica* could tolerate while retaining the capability to resume normal growth and development, was in the order of 0.3 g g^{-1} (Chapter 2) – considered too high for conventional freezing of embryonic axes (Bajaj, 1985). Therefore, either alternative freezing techniques and/or cryoprotectants had to be employed in attempts to facilitate survival after cryopreservation.

3.3.3.1 The effect of freezing techniques on survival after cryopreservation

In this study, the following freezing techniques, achieving different cooling rates, were used: step-wise cooling, freezing in cryotubes, direct immersion of axes (naked or enclosed in aluminium foil envelopes or wire mesh baskets) into liquid nitrogen, immersion into melting isopentane, and plunging the axes into nitrogen slush.

For the axes of both *T. dregeana* and *T. emetica,* none of the cooling rates achievable by the spectrum of methods used, resulted in survival after cryopreservation, unless cryoprotectants were employed (below). Typical results of cryopreservation of *T. dregeana* by freezing in nitrogen slush, and those of freezing *T. emetica* embryonic axes in melting isopentane, are presented in Tables 3.9 and 3.10, respectively. In contrast to these results, rapid freezing in melting iso-pentane has been used for the highly successful cryopreservation of excised axes of *Quercus robur* (Berjak *et al.*, 1999), *Ekebergia capensis* (Walker, 2000), *Aesculus hippocastanum* (Wesley-Smith *et al.*, 2001b) and *Poncirus trifoliata* (Wesley-Smith, 2002); while freezing in nitrogen slush been shown to be effective in the cryopreservation of axes of *Acer saccharium* (Wesley-Smith, 2002) and *Aesculus hippocastanum* (Wesley-Smith *et al.*, 2001b). The success of these freezing techniques is attributed to the cooling rates achieved. Immersing axes into melting isopentane (-140 °C) can achieve cooling rates in the order of 40 000 °C min^{-1}, while plunging embryonic axes into nitrogen slush (-210 °C) attains cooling rates of about 10 000 °C min^{-1} (Wesley-Smith *et al.*, 2001b). At

such cooling rates, the embryo tissues go through the ice nucleation temperatures too rapidly for ice crystals to grow, and the intracellular solution is thought to either vitrify into a glass (James, 1983), or to form microcrystalline ice (Wesley-Smith *et al.*, 1992), both of which are considered to be non-injurious to cellular membranes.

The cooling rates attained depend not only on the cryogen used, but also on the thermal mass of the material (Meryman, 1956; Bald, 1987). Wesley-Smith (2002) suggests that cooling rates in excess of 6 000 °C min^{-1} are attainable in tissues with a dry mass of 1-2 mg, with rapid cooling becoming increasingly more difficult to achieve for larger specimens. After appropriate dehydration, the embryonic axes used in this study, which had part of the cotyledon attached, had a mass up to 7 mg (*T. dregeana*) and 5 mg for those of *T. emetica*. It is therefore unlikely that the extremely high cooling rates necessary for vitrification of partially-hydrated tissue could be attained.

Table 3.9 Percentage survival (shoot and/or root growth) of non-cryoprotected *T. dregeana* embryonic axes, flash-dried for up to 4 h, and frozen individually by rapid plunging into 1 600 mm of nitrogen slush (liquid nitrogen sub-cooled to -210 °C). Axes were then transferred to, and held in, liquid nitrogen for 1 h and thawed (and simultaneously rehydrated) in a 1:1 solution of 1μ*M* $CaCl_2.2H_2O$ and 1m*M* $MgCl_2.6H_2O$ at 40 °C for 1 min, and plated on WPM (Lloyd and McCown, 1981) medium. Assessments were recorded after 12 weeks in culture. n=20, and axes that were contaminated were discarded from culture and not included in assessment.

		% survival	
Drying period (min)	**Water content (g/g)**	**Not frozen**	**Frozen**
0	1.5 ± 0.31	100	0
15	1.2±0.33	100	0
30	0.88±0.17	95	0
45	0.85±0.11	100	0
60	0.86±0.08	90	0
90	0.64±0.12	95	0
120	0.68±0.09	90	0
150	0.58±0.13	94	0
180	0.24±0.10	88	0
240	0.16±0.07	50	0

Table 3.10 Percentage survival (shoot and/or root growth) of non-cryoprotected *T. emetica* embryonic axes, flash-dried for up to 2 h, and individually frozen by plunging them rapidly into melting isopentane (-140 °C) held in a liquid nitrogen reservoir. Axes were then transferred to, and held in, liquid nitrogen for 1 h and thawed (and simultaneously rehydrated) in a 1:1 solution of 1 μM $CaCl_2.2H_2O$ and 1 mM $MgCl_2.6H_2O$ at 40 °C for 1 min, and plated on MS (Murashige and Skoog, 1962) medium. Assessments were recorded after six weeks in culture. n=20, and axes that were contaminated were discarded from culture and not included in assessment.

Drying period (min)	Water content (g/g)	% survival	
		Not Frozen	Frozen
0	2.82±0.34	97	0
15	1.63±0.21	100	0
30	0.50±0.17	85	0
45	0.47±0.10	80	0
60	0.45±0.08	75	0
90	0.31±0.06	80	0
120	0.26±0.03	70	0

A further possible basis for the zygotic axes of *Trichilia* species being apparently less amenable to freezing than those of the species mentioned above, is that those species may be more acclimated to low temperatures. *Acer saccharium, Quercus robur, Aesculus hippocastanum* and *Poncirus trifoliata* are temperate species, and plants growing in these areas have been shown to have increased tolerance to freezing, following cold-acclimation by exposure to low non-freezing, non-injurious temperatures (Reed and Chang, 1997). Indeed, Walker (2000) reported that seeds of *Quercus robur* could be stored at chilling temperatures (as low as 6 °C) for at least three months without any loss in viability or vigour, and unpublished observations in our laboratory indicate a similar situation for *Ekebergia capensis*. Those species may therefore lie towards the less recalcitrant end, relative to *Trichilia* spp., of the continuum of seed behaviour proposed by Pammenter and Berjak (1994).

Alternatively, or additionally, the inability of the non-cryoprotected axes of *Trichilia* spp. to survive cryopreservation may be a function of the intracellular water. At water contents low enough for

cryopreservation, the major proportion of the intracellular water is likely to be non-freezable (Vertucci, 1990), and recalcitrant seeds do not survive the loss of non-freezable water (Pammenter *et al.*, 1991), which is in the region of 0.2 - 0.4 g g^{-1} (Vertucci and Leopold, 1987). The axes of *T. emetica* and *T. dregeana* tested for cryopreservation had water contents within this range, but it is not known what fraction might have been constituted by the freezable component. Even a small proportion of freezable water could have provided the basis for lethal ice crystallisation during the necessarily low cooling rates dictated by the relatively large thermal mass of the axis/cotyledonary block explants.

Although they survived dehydration, it seems that the explants could not survive the additional stress imposed by freezing. However, apparent viability of non-orthodox axes (or seeds, as in the case of *Warburgia salutaris*, reported later) immediately after dehydration has been found to differ substantially from the results obtained if the explants are stored even for short periods. Such observations have been made for several species in our laboratory (unpublished data) and their basis has been discussed by Walters *et al.* (2001). Thus, it may be concluded that in the dehydrated state, such specimens suffer desiccation damage *sensu stricto* (Pammenter *et al.*, 1998; Walters *et al.*, 2001), the onset of which might follow some hours or days later, or might occur almost immediately. The latter situation may well pertain in the dehydrated axes of *T. dregeana* and also *T. emetica*. If this is the case, then the successful cryopreservation may be achievable only if the axes are introduced into the cryogen in the shortest possible time after the requisite degree of dehydration has been attained. In the present study, the time elapsed was in the order of 20-30 min.

Furthermore, at reduced water contents, rapidly-cooled embryonic axes may suffer stresses other than those imposed by ice damage alone (e.g. Wesley-Smith *et al.*, 1992). Such stresses may emanate from phase transitions of molecules other than water during cooling or warming. Polar lipids in membranes of seeds have phase transition temperatures as low as –90 °C (Vertucci, 1989b, c), and these increase with decreasing water content (Bryant *et al.*, 2001). Thus, the embryonic axes of some species can survive cooling to temperatures above the lipid phase-transition temperature (-70 °C in the case of *Landolphia kirkii* [Vertucci *et al.*, 1991]), but lose viability if cooled further. Those observations may be extrapolated to suggest similar phase transitions could

have played a rôle in *Trichilia* embryonic axes, dried to levels close to their limit of desiccation tolerance, when exposed to very low temperatures.

Additionally, it is possible, in view of the slow cooling rates that must have been presently achieved for axis-cotyledon segments of *T. dregeana* and *T. emetica*, that vitrification of cell contents was not achieved on freezing, as this has been shown, particularly in slowly-cooled specimens, to depend on a variety of critical factors, such as cryoprotection (Sakai, 2000). This has been demonstrated for the shoot tips of a number of species (e.g. Kartha, 1985; Niino *et al.*, 2000; Takagi, 2000), cell suspension cultures of *Brassica* (Langis *et al.*, 1989), polyembryogenic cultures of oil-palm (Dumet *et al.*, 2000b), embryonic axes of *Pisum sativum* (Mycock *et al.*, 1991), peach, (Deboucaud *et al.*, 1996), coconut (Assy-Bah and Engelmann, 1992), and in seeds of many Western Australian native species (Touchel and Dixon, 1993).

3.3.3.2 Effect of cryoprotectants on survival of explants

A preliminary study was conducted on immature embryonic axes of *T. dregeana*, in which they were excised from cotyledons and cryoprotected with the following compounds, both singly and in factorial combination with each other: glycerol, sucrose, DMSO, dextran and polyvinylpyrrolidone (PVP). From the results (not shown here), explants survived only when treated with glycerol, or with the mixture of DMSO and dextran. However, not all survived: sixty percent of explants treated with glycerol and 80% of those treated with DMSO and dextran stained red, indicating viability retention, with the conventionally-used solution of 2,3,5-triphenlytetrazolium chloride (ISTA, 1999), while explants treated with the other cryoprotectants did not stain at all, but 100% of untreated explants gave the positive response.

In later studies, mature excised embryonic axes were found to be less damaged by cryoprotection. In *T. dregeana,* the percentage of embryonic axes germinating after cryoprotection alone ranged from an average of 69% (cryoprotected with sucrose) to 98% (cryoprotected with DMSO) while cryoprotected axes of *T. emetica* achieved germination percentages ranging from 80% to 98% (Tables 3.11 and 3.12).

Generally, the use of cryoprotectants has a major limitation imposed by their phytotoxicity (Finkle *et al.*, 1985; Bajaj, 1985; Canavate and Lubian, 1994), which may either irreversibly affect cell structure, or reversibly retard growth processes within the explant (Mycock *et al.*, 1991). The extent of toxicity varies with the type and concentration of cryoprotectant and the plant species used (Kartha, 1985; Mycock *et al.*, 1991). Glycerol on its own was found to be non-injurious to the axes, as has been reported in other investigations (e.g. Kartha, 1985; Molina *et al.*, 1994). Explant response to the more toxic cryoprotectants such as DMSO, may have been alleviated by combining them with other compounds, as the cytotoxicity of an individual cryoprotectant may be reduced if it is used in combination with another (Bajaj, 1985; Withers, 1985).

The adverse effects of any of the cryoprotectants may have been reversed had the explants been washed free of the cryoprotectants prior to viability testing. Mycock *et al.* (1991) showed that washing off a mixture of DMSO and glycerol from hydrated pea axes after cryopreservation counteracted the deleterious effects that this mixture had on their germination. However, Withers and King (1979, 1980) and Withers (1979) found that washing of freshly-thawed material was not beneficial and was often deleterious due to changes of osmotic pressure during the post-thaw washing phase. This damage is more serious in poorly-cryoprotectable cells (Watanabe *et al.*, 1999), a category into which axes of *Trichilia* species are suggested to fall, because of the lipid-rich cuticle encapsulating the axis. Successful cryopreservation has also been carried out, without cryoprotectant-washing, for different types of explants of several species, such as embryonic axes of peach (Deboucaud *et al.*, 1996) and coconut (Assy-Bah and Engelmann, 1992), meristems of black currant (Dumet *et al.*, 2000a), and shoot-tips of mango (Huang *et al.*, 2000).

While the embryonic axes generally maintained high levels of viability after cryoprotection alone (Tables 3.11 and 3.12), this study aimed at freezing axes that had not only been cryoprotected, but also subsequently dehydrated, a treatment which must gradually increase the concentration of penetrating cryoprotectants in the cells. In general, survival was decreased for cryoprotected, dehydrated axes, with this effect being most marked for axes of *T. dregeana* cryoprotected with DMSO (Table 3.11). This cryoprotectant has been shown to be phytotoxic at

concentrations higher than 20% when applied for 20 min or more (Canavate and Lubian, 1994). This may account for the drop in the viability of axes of *T. dregeana* cryoprotected with DMSO and dehydrated (Table 3.11). This cryoprotectant also binds to the monomers of actin and reduces the extent of actin filaments (Morriset *et al.*, 1994) visible in cells. Such disruption of the cytoskeleton may further reduce the percentage of axes developing into normal plantlets following cryoprotection and dehydration.

Cryoprotection also lowered the water content of embryonic axes of *T. dregeana* and *T. emetica*, with sucrose being the most dehydrating of the compounds tested (Figs. 3.7 and 3.8). On flash-drying of cryoprotected axes, cryoprotectant mixtures containing sucrose kept the water content of the axes consistently lower that of non-cryoprotected axes, while cryoprotection with glycerol or DMSO (singly or in combination) maintained the water content of axes higher than that of non-cryoprotected, dehydrated axes dried for similar periods.

Dumet and Berjak (1996) reported similar reduction in water content following sucrose cryoprotection of the embryonic axes of five tropical recalcitrant-seed species, including *T. dregeana*. Sucrose and other non-penetrating cryoprotectants act through dehydration which could promote vitrification during freezing (Vanoss *et al.*, 1991; Storey and Storey, 1996).

3.3.3.3 Effect of cryoprotection on survival after cryopreservation

In *T. dregeana*, survival after cryopreservation was attained only by axes cryoprotected with glycerol, or with the mixtures of glycerol and DMSO or glycerol and sucrose (Table 3.11). Surviving axes did not develop into normal plantlets, but showed callus proliferation from the root-pole of the embryonic axis (Fig 3.9). This type of growth persisted, even when the embryonic shoot-pole of the embryonic axis was protected against excessive dehydration by alginate gel encapsulation prior to dehydration and cryopreservation.

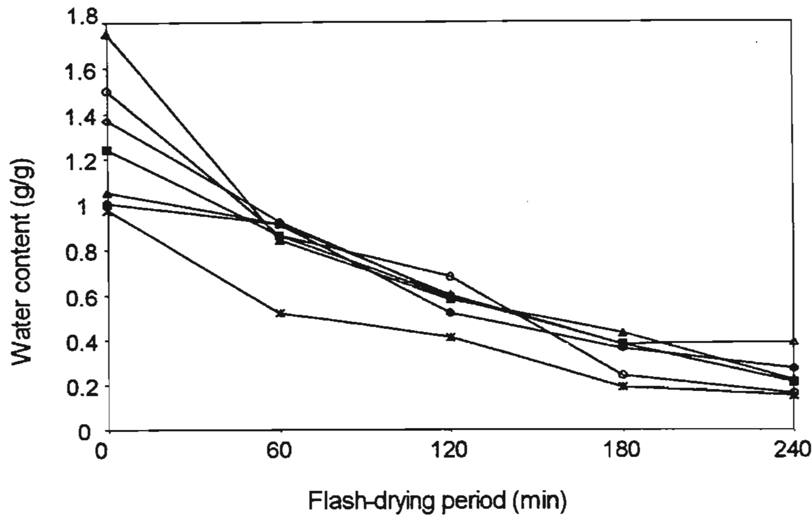

Fig. 3.7. Drying time course for *T. dregeana* axes dehydrated after cryopreservation with DMSO (▲), glycerol (●), sucrose (*), glycerol+DMSO (Δ), sucrose+DMSO (■), sucrose+glycerol (◇), or not cryoprotected (○). Markers represent mean of five individual axes.

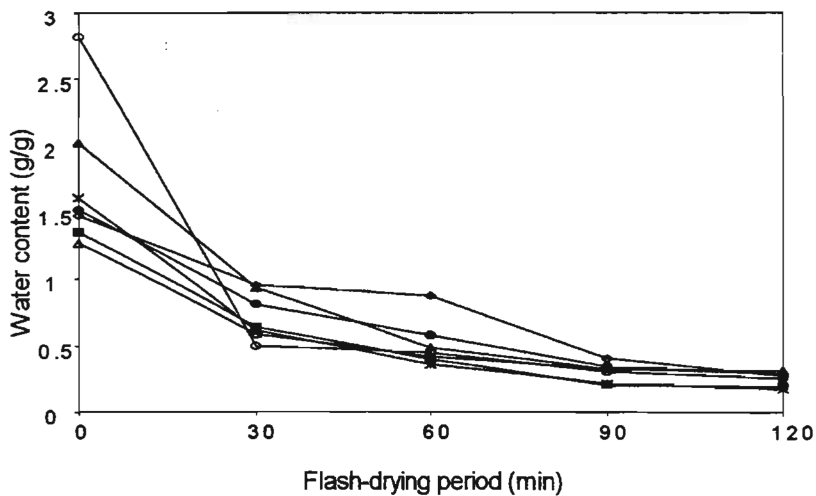

Fig. 3.8. Drying time course for *T. emetica* axes dehydrated after cryopreservation with DMSO (▲), glycerol (●), sucrose (*), glycerol+DMSO (Δ), sucrose+DMSO (■), sucrose+glycerol (◇), or not cryoprotected (○). Markers represent mean of five individual axes

Whereas no survival in any form was attained after cryopreservation of non-cryoprotected axes of *Trichilia* spp. regardless of the freezing rate/technique employed (section 3.3.3.1), the application of cryoprotectants had a dramatic effect. In *T. emetica*, all the cryoprotectants tested promoted survival after freezing, whether they were applied singly or in combination. When the axes were rehydrated in water during thawing, the surviving axes regenerated only in the form of apparently normal roots, and the highest survival was 43%, attained after cryoprotection with a mixture of DMSO and glycerol (Table 3.12). On rehydration in a 1:1 solution of solution of 1μ*M* $CaCl_2.2H_2O$ and 1m*M* $MgCl_2.6H_2O$ (below, section 3.3.1.3.5), 37% of the axes cryoprotected with DMSO and glycerol developed shoots (Table 3.12 and Fig 3.10). It is significant that embryonic axes in which shoot development was attained, were obtained from seeds that had been kept in storage (at 16 °C) for three weeks prior to any manipulation. The effect of axis development on their amenability to cryopreservation is discussed below.

It is apparent that DMSO, either singly or in combination with other cryoprotectants, was instrumental in facilitating the potential for cryopreservation of embryonic axes of both *T. dregeana* and *T. emetica*. The compound is a permeating cryoprotectant for which the efficacy for cryoprotection of hydrated tissues has been widely demonstrated (e.g. Mycock *et al.*, 1991; Muldrew *et al.*, 1996; Schäfer-Menuhr *et al.*, 1997; Ashmore *et al.*, 2000). It permeates the cells more readily than either sucrose or glycerol, and water removed from the cells during freezing can be replaced by DMSO, resulting in only a transient cell volume change and minimum intracellular damage (James, 1983; Kartha, 1985).

Combinations of cryoprotectants are generally more effective than single compounds (Finkle *et al.*, 1985), and, in the present study, a combination of DMSO with glycerol afforded improved cryoprotection. Glycerol itself is classed as one of the most effective cryoprotectants used, and is classified as solute that offers 'highest' protection against freezing injury (Sakai and Yoshida, 1968). It is therefore used in many cryopreservation protocols (reviewed by Pichugin, 1993), and is a constituent of common cryoprotection mixtures such as PVS1 (Uragami *et al.*, 1989), PVS2 (Sakai *et al.*, 1990), PVS3 (Nishizawa *et al.*, 1992) and PVS4 (Takagi, 2000).

Table 3.11 Percentage germination (roots and shoot development) of *T. dregeana* embryonic axes following cryoprotection, flash-drying for 3 h, and freezing in cryotubes. The axes were maintained in liquid nitrogen for 1 h and thawed (and simultaneously rehydrated) either in distilled water or / Ca^{2+}-Mg^{2+} solution. Axes were plated on WPM (Lloyd and McCown, 1981) medium and assessed after 12 weeks in culture. n=20 for non-frozen axes, and 40 for freezing experiments, with axes that were contaminated being discarded from culture and not included in the assessment. C, survival only in the form of callus proliferation from the root pole.

Cryoprotectant	**Treatment / survival**			
	Not dried, Not frozen	**Dried, Not frozen**	**Dried, Frozen, H_2O-rehydrated**	**Dried, Frozen, Ca/Mg-rehydrated**
No Cryoprotectant	100	88	0	0
DMSO	97.5	52.8	0	40 C
Glycerol	93.7	68	20C	45C
Sucrose	68.75	60	0	25C
Glycerol+ Sucrose	81.25	60	40C	55C
DMSO + glycerol	75	60	70C	62.5C
DMSO + sucrose	87.5	70	0	32.5C

Fig. 3.9 (a) Embryonic axes of *T. dregeana* following cryopreservation and growth on WPM medium for 12 weeks. The only form of growth attained by the axes was callus proliferation from the root-pole of the axes (arrow-heads). Bar = 1.3 mm.

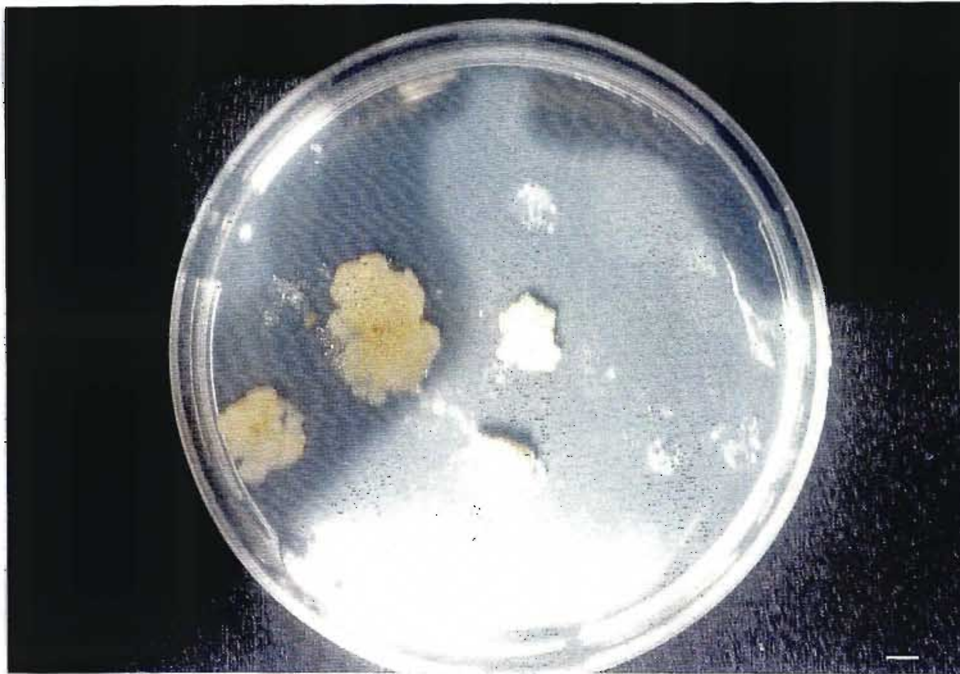

Fig 3.9 (b). Callus, regenerated by surviving *T. dregeana* embryonic axes, cultured in the dark on medium supplemented with 1 mg l^{-1} picloram. Even though this medium had been used to induce embryogenic callus from fruit-wall explants (results not presented here), the callus obtained from cryopreserved axes remained non-embryogenic. Bar = 4 mm.

Table 3.12 Percentage germination (root and shoot development) of *T. emetica* embryonic axes following cryoprotection, flash-drying for 90 min, and freezing in cryotubes (five axes per cryotube). The axes were maintained in liquid nitrogen for 1 h and thawed (and simultaneously rehydrated) in either distilled water or / Ca^{2+}-Mg^{2+} solution. Axes were plated on MS (Murashige and Skoog, 1962) medium and assessed after six weeks in culture. n=20, for non-frozen axes, and 40 for freezing experiments, with axes that were contaminated being discarded from culture and not included in the assessment Brackets indicate the percentage of cryopreserved axes that produced shoots.

Cryoprotectant	**Treatment / % survival**			
	Not dried, Not frozen	**Dried, Not frozen**	**Dried, Frozen, H_2O-rehydrated**	**Dried, Frozen, Ca/Mg-rehydrated**
No Cryoprotectant	96.9	80	0	0
DMSO	97.5	95	7.5	55 (0)
Glycerol	97.5	76.9	47.5	62.5 (17.5)
Sucrose	92.5	62.6	11.6	35 (0)
Glycerol+ Sucrose	80	70.9	36	44.4 (23.8)
DMSO + glycerol	97.5	62.5	43.1	69.4 (36.6)
DMSO + sucrose	86.9	72.5	32.3	45 (25)

Fig. 3.10 (a) Embryonic axes of *T. emetica* germinating on MS medium, after six weeks in culture. Axes that were excised appropriately with small blocks of cotyledonary tissue and plated on this medium developed both roots and shoots. Bar = 4 mm.

Fig. 3.10 (b) *Trichilia emetica* embryonic axes cultured on MS medium for six weeks after cryopreservation and rehydration in distilled water. Even though the axes survived cryopreservation, no shoot development occurred. Bar = 5 mm.

Fig. 3.10 (c) Embryonic axes of *T. emetica*, cultured on MS medium, following cryopreservation (after cryoprotection with DMSO and glycerol) and rehydration in a 1:1 solution of 1 μM $CaCl_2.2H_2O$ and 1 mM $MgCl_2.6H_2O$. Some (37%) of the axes thus rehydrated, developed shoots (arrow-heads) after about six weeks in culture. Bar = 4mm.

When used as a cryoprotectant, DMSO also stabilizes the liquid matrix of cell membranes (Yu and Quinn, 1994), and has also been shown to offer some protection against the cytoskeletal damage incurred by freezing in organisms as diverse as mammalian gametes (e.g. Gilmore *et al.*, 2000), yeast cells (e.g. Lewis *et al.*, 1994), and plant cell cultures (e.g. Morriset *et al.*, 1994). However, DMSO itself has been shown to have some disruptive properties at high temperatures of exposure (Johnson and Pickering, 1987; Morriset *et al.*, 1994).

However, neither DMSO nor glycerol, alone or in combination, although permitting callus formation, was sufficient to facilitate post-thaw onward growth of cryopreserved of embryonic axes of *T. dregeana*. An alternative method of applying cryoprotectants to explants is their pre-culture in a medium enriched with cryoprotectants for an appropriate period, with sugars such as

sucrose, fructose, maltose, ribose, and mannitol having been utilized in this manner to facilitate the cryopreservation of somatic explants (e.g. Assy-Bah and Engelmann, 1992; Dumet and Berjak, 1996; Dumet *et al.*, 2000a). The application of this technique for zygotic embryos is less common, and Dumet and Berjak (1996) found it to be unsuitable for the cryopreservation of zygotic embryos of *Camellia sinensis* and *Azadirachta indica*, and ineffective in facilitating the cryopreservation of embryonic axes of *Artocarpus heterophyllus* and *Landolphia kirkii*. The results of those authors suggested, however, that sucrose pre-treatment appeared to have the potential of increasing the survival to low water contents of subsequently dehydrated embryonic axes of *T. dregeana*.

In the present study, however, use of the sucrose pre-growth method of Dumet and Berjak (1996) with embryonic axes of *T. dregeana* resulted in further growth only in the form of unorganised callus, and did not facilitate survival after cryopreservation (Table 3.13). Ultrastructural examination of the root meristematic cells of axes pre-grown in a high-sucrose medium showed that the plastids were swollen and markedly distended, and had accumulated starch following the 7-d exposure to sucrose (Fig 3.11 b). There were also other abnormalities, such as a clearing of the nucleolar area (not shown) and the appearance of intranuclear bundles of fibrils (Fig 3.11 d), which suggested a derangement of the nuclear matrix (i.e. that region of the nucleoskeleton occupying the internal volume of the nucleus). Autophagy of organelles by intravacuolar engulfment was also apparent (Fig 3.11 c), implying that the former were damaged beyond functionality.

In all the cryopreservation trials described, only limited survival of embryonic axes of *T. dregeana* occurred, and, where post-thaw growth was evident, this was restricted to callus regeneration from the root-pole. No survival of any form was manifested in the embryonic shoot after freezing, which is suggested to be due to the higher degree of desiccation damage suffered by the shoot meristem (section 2.5.3). In an attempt to increase the desiccation tolerance of embryonic shoots before cryopreservation, Fu *et al.* (2000) developed a sucrose pre-treatment method, in which germinating embryonic axes were cultured in increasing concentrations of sucrose and tested for desiccation- and freezing-tolerance. Those authors, working on wampee (*Clausena lansium*), found that, even though the nascent roots withered with increasing sucrose concentration, the excised plumules were capable of regenerating adventitious roots.

The technique of Fu *et al.* (2000) was therefore applied to *T. dregeana* embryonic axes in an attempt to improve the desiccation tolerance of shoot-tips. However, the axes lost viability at water contents too high for cryopreservation, with the lowest tolerated water content becoming higher as the sucrose concentration increased (Fig 3.12).

Table 3.13 The effect of sucrose pre-growth on the water content, survival, and tolerance to cryopreservation, of embryonic axes of *T. dregeana*. Embryonic axes were pre-grown on a germination medium containing 0.75M sucrose for 7 days, some were dehydrated in a laminar air flow for 4 h only, while others were plunged in liquid nitrogen in cryotubes after being dried. n=20

	Water content		**% survival**	
	Shoot-tip	**Root-tip**	**Callus**	**Germination**
Not treated, not dried, not frozen	3.75 ± 0.53	1.5±0.2	0	100
Treated, not dried, not frozen	1.0±0.17	1.3±0.09	40	0
Treated, dried, not frozen	0.25 ± 0.05	0.35±0.19	0	0
Treated, dried, frozen	-	-	0	0

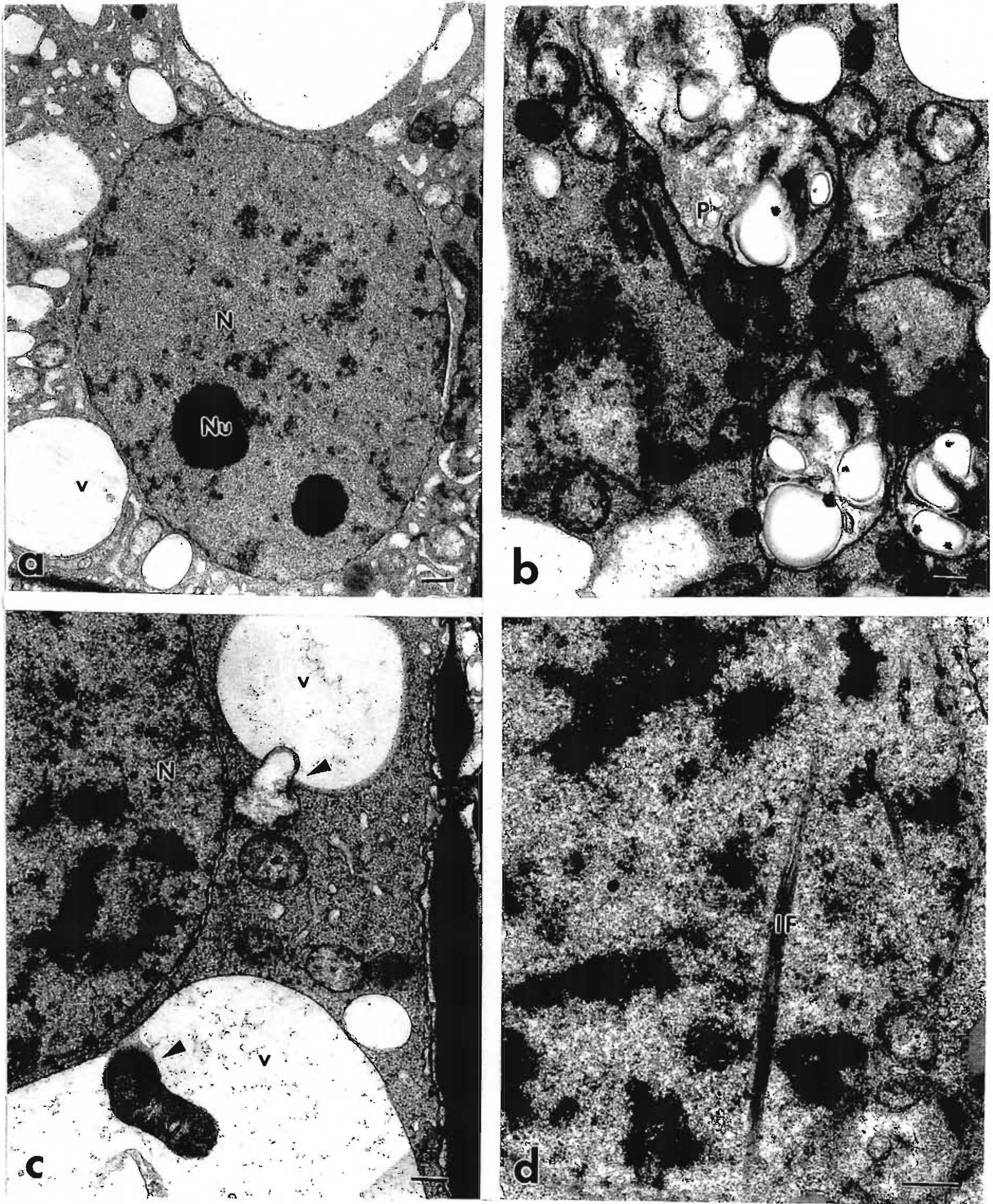

Fig. 3.11 Ultrastructure of cells from the root meristem of axes of *T. dregeana* treated with 0.75 *M* sucrose for 7 days, showing distended starch-containing plastids (P, in b) with disorganised internal membranes. Evidence of autophagy of organelles (arrows-heads in c) and bundles of intranuclear fibrils (IF in d) were observed. Fig. 3.11a represents the ultrastructure observed in meristems of axes not cultured on the high sucrose medium.
v, vacuole; N, nucleus; Nu, nucleolus. Bar = 500 nm.

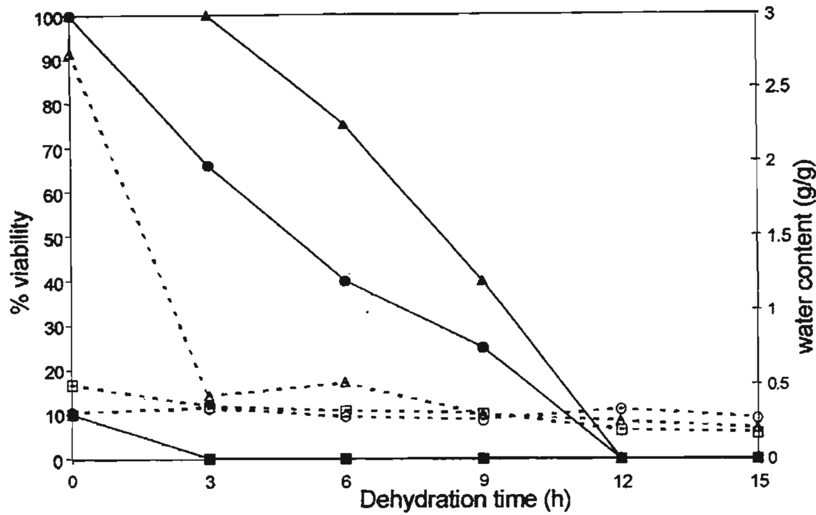

Fig 3.12. Changes in viability (solid line and solid symbols, primary y axis) and water content (dashed lines and clear symbols, secondary y axis) of embryonic axes of *T. dregeana* pregrown in increasing concentrations of sucrose and dehydrated over silica gel, according to the method of *Fu et al.* (2000). ▲, Δ, axes cultured on WPM for three weeks; ●, ○, axes cultured on WPM for three weeks, and then transferred to a medium containing 27% sucrose and cultured for 7 d, followed by 5-d culture on a medium containing 40% sucrose; ■, □, axes treated as above, then transferred onto medium containing 55% sucrose for 5 d, followed by culture on a medium containing 65% sucrose for 2 d.

The failure of the sucrose pre-growth approach (Fu *et al.,* 2000) to improve desiccation tolerance in *T. dregeana* axes was not altogether unexpected, as previous employment of this sugar has been shown to be injurious to the axes of this species, as described above. Alternative compounds that have been employed to improve the desiccation-tolerance of the axes of recalcitrant seeds are calcium chloride and ascorbic acid (Song[2], pers. comm.). However, these, too, did not improve the desiccation and / or freezing tolerance of *T. dregeana* axes (results not shown).

From the present results, it appears that the cryopreservation is far less feasible for embryonic axes of *T. dregeana* than those of *T. emetica,* as the techniques that were successful in the cryopreservation of the latter, and other species, were ineffective, or actually damaging, to *Trichilia dregeana.* An investigation was therefore performed to ascertain whether this apparent

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inability of the axes to survive cryopreservation could be explained in terms of ultrastructure and tissue organisation of the axes. The axes were cryoprotected with glycerol and DMSO, dehydrated and cryopreserved, and then plated onto germination medium for a recovery period of 1 week, and examined. The following observations were made:

- The root cap of freshly-excised axes was composed of about 12 ranks of highly-vacuolated cells (Fig 3.13a), as opposed to the compact root-cap cells which might have been expected to protect the axis during dehydration and freezing (Berjak *et al.*, 1999). Those authors attributed successful maintenance of root meristems integrity of *Quercus robur* axes, to the protection afforded by such root cap cells.
- The shoot meristem, on the other hand, was wholly superficial, and the apical tissue included scattered large cells of heterogenous appearance (Fig. 3.13b). Ultrastructurally, the shoot apical meristematic cells appeared highly active, with frequent profiles of rough endoplasmic reticulum, many polysomes, well-developed Golgi bodies, and abundant mitochondria (Fig. 3.13c). A distinctive feature of both the root- and shoot-tip cells was that they lacked any starch or lipid deposits, and were highly vacuolated (Fig. 3.13d).
- Upon cryoprotection and dehydration (in the laminar flow to the non-injurious water content of *c.* 0.35 g g^{-1}), but prior to freezing, the root cap presented a mosaic of variously deteriorated cells, with only 11% of the outer zone appearing active and undamaged (Fig. 3.14a). The root cap, a continuously regenerating structure, has been suggested to play an important rôle in enabling an embryonic axis to survive cryopreservation (Berjak *et al.,* 1999), In this case, even after the recovery period, root cap regeneration had not returned to normal.
- In the root meristem, while some cells appeared entirely normal and metabolic, with well-developed mitochondria, plastids and endoplasmic reticulum, as well as polysomes, others showed abnormalities including etiolated ER profiles and rounded plastids lacking internal membranes (Fig. 3. 14b). In some cases also, although presenting evidence of metabolic activity, cells exhibited derangement, in the appearance of abnormally-shaped organelles (Fig. 3.14c). There was also indirect evidence of cytoskeletal damage, in the clustering of organelles in the perinuclear area

(Fig. 3.14d). These are indications of the intracellular damage suffered by axes even before they were frozen.

- In the shoot meristems of surviving axes, many cells also presented similar localisation of organelles around the nucleus (Fig. 3.15a). In the nucleus, there was indirect evidence of nucleoskeletal abnormality in that the chromatin had lost its characteristic association with nuclear periphery (Fig. 3.15b cf 3.15c). Subsequent studies by Merhar *et al.* (2002) showed when the water content of embryonic axes of *T. dregeana* was reduced from 2.83 g g^{-1} to 0.21 g g^{-1} in 3 h, the shoot meristematic cells lost the ability for nucleoskeleton reassembly, compromising nuclear organisation and probably leading to the death of the cells.

Following cryopreservation;

- The surviving axes regenerated callus from the root-pole. Light micrographs showed a distinctive pattern of groups of surviving cells, probably the callus precursors, flanking the degraded root meristem region (Fig. 3.16a). It was apparent that many cells had not retained the typical polygonal shape, but were spherical (Fig 3.16b). This may be due to the loss of the constraint imposed by neighbouring cells, and/or a manifestation of the disorganisation, and possibly a random re-constitution, of the cytoskeleton. These cells showed evidence of a marked degree of metabolic activity – a high frequency of Golgi bodies, rough endoplasmic reticulum, abundant polysomes and well-developed mitochondria – and a difference in the spatial disposition normally encountered in root cells (Fig 3.16c).
- The cells of all the shoot-tips examined were in an advanced state of deterioration Fig 3.16d).

The abnormality of surviving root apex cells, coupled with extensive degradation of all those of the shoot apex, would preclude axis germination to produce a normal plant.

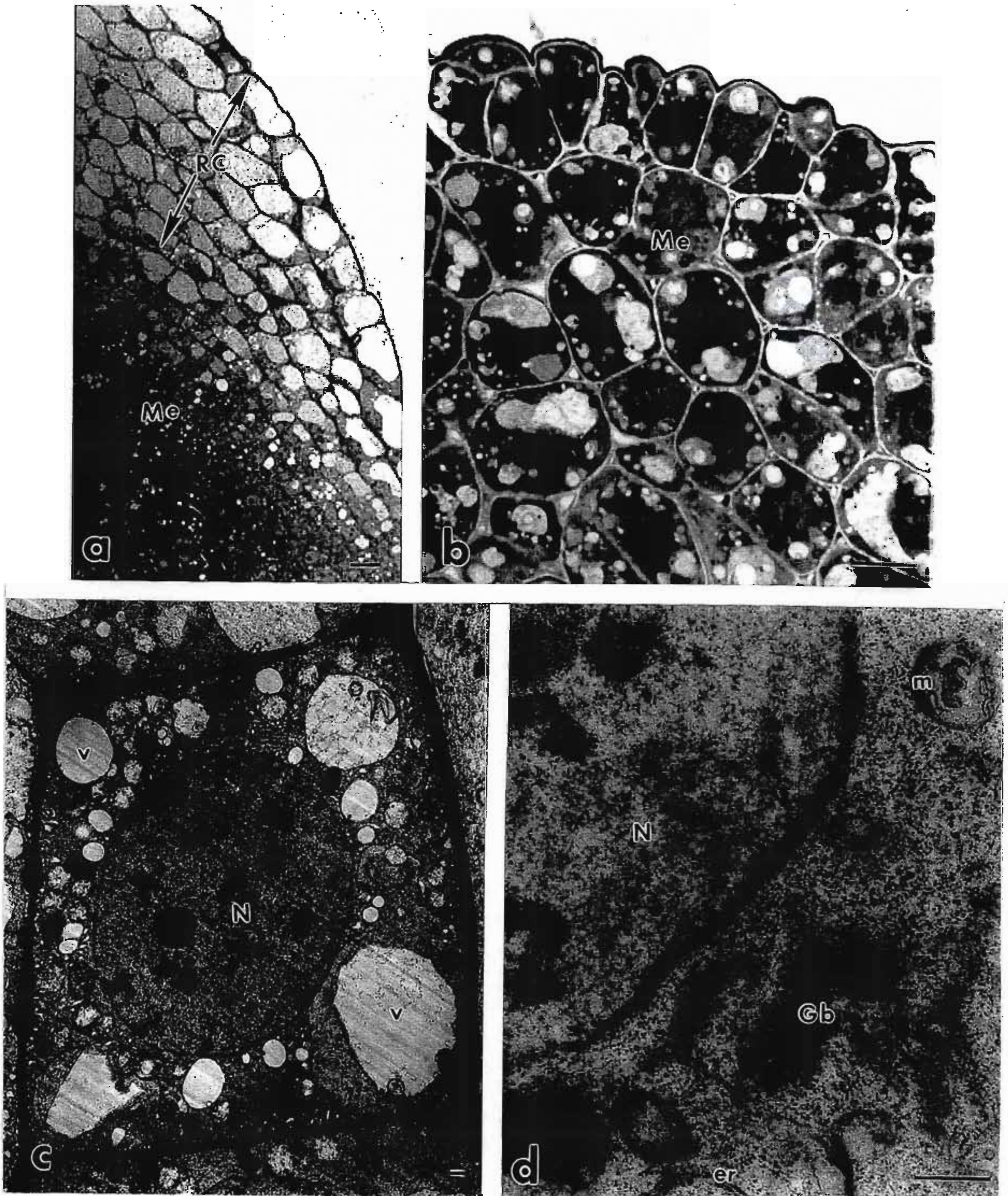


Fig 3.13. The structure of freshly-excised, non-treated axes of *T. dregeana*: light micrographs showing the root cap (RC) and meristem (Me) in a; the superficial shoot meristem (b); and electron micrographs of shoot apical meristem cells showing the degree of vacuolation (c), and presenting evidence of a high level of metabolic activity, in terms of the incidence of mitochondria, endoplasmic reticulum, Golgi bodies, and numerous polysomes (d).
m, mitochondrion; er, endoplasmic reticulum; Gb, Golgi body; v, vacuole; N, nucleus; Nu, nucleolus. Bar = 6.25 μm for (a) and (b), and 500 nm for (c) and (d).

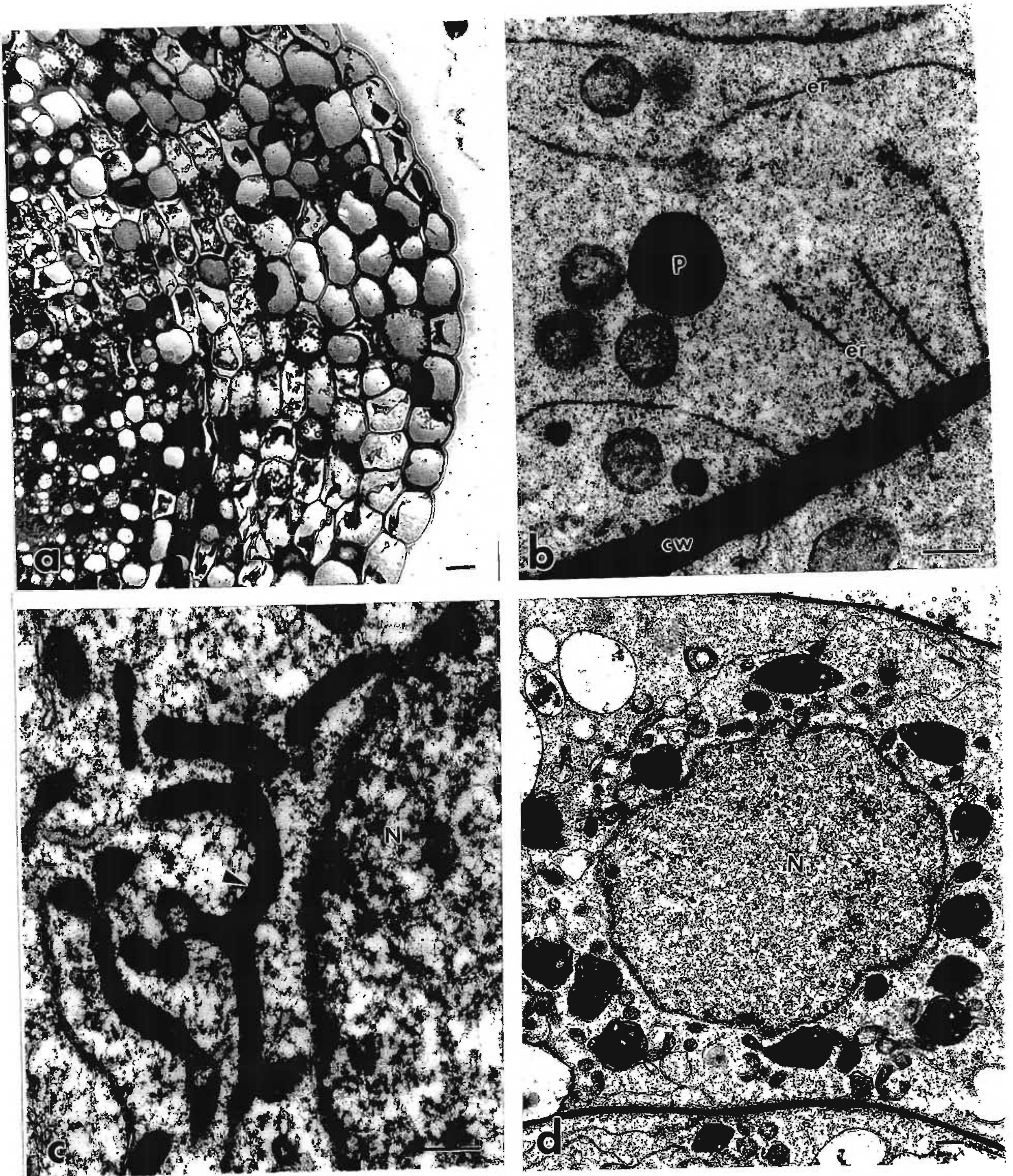


Fig 3.14. The structure of the root apex cells of axes of *T. dregeana* cryoprotected and dehydrated to 0.35 g g^{-1}. (a), light micrograph of the root cap; (b), the effects of dehydration evidenced by abnormally thin ER profiles, which seem to have been laterally compressed, and plastids that had become rounded and lost internal membranes; (c), aberrant, elongated organelles (arrow-heads); (d), organelles abnormally aggregated around the nucleus.
er, endoplasmic reticulum; N, nucleus; cw, cell wall. Bar = 6.25 µm for (a); and 500 nm for (b-d).

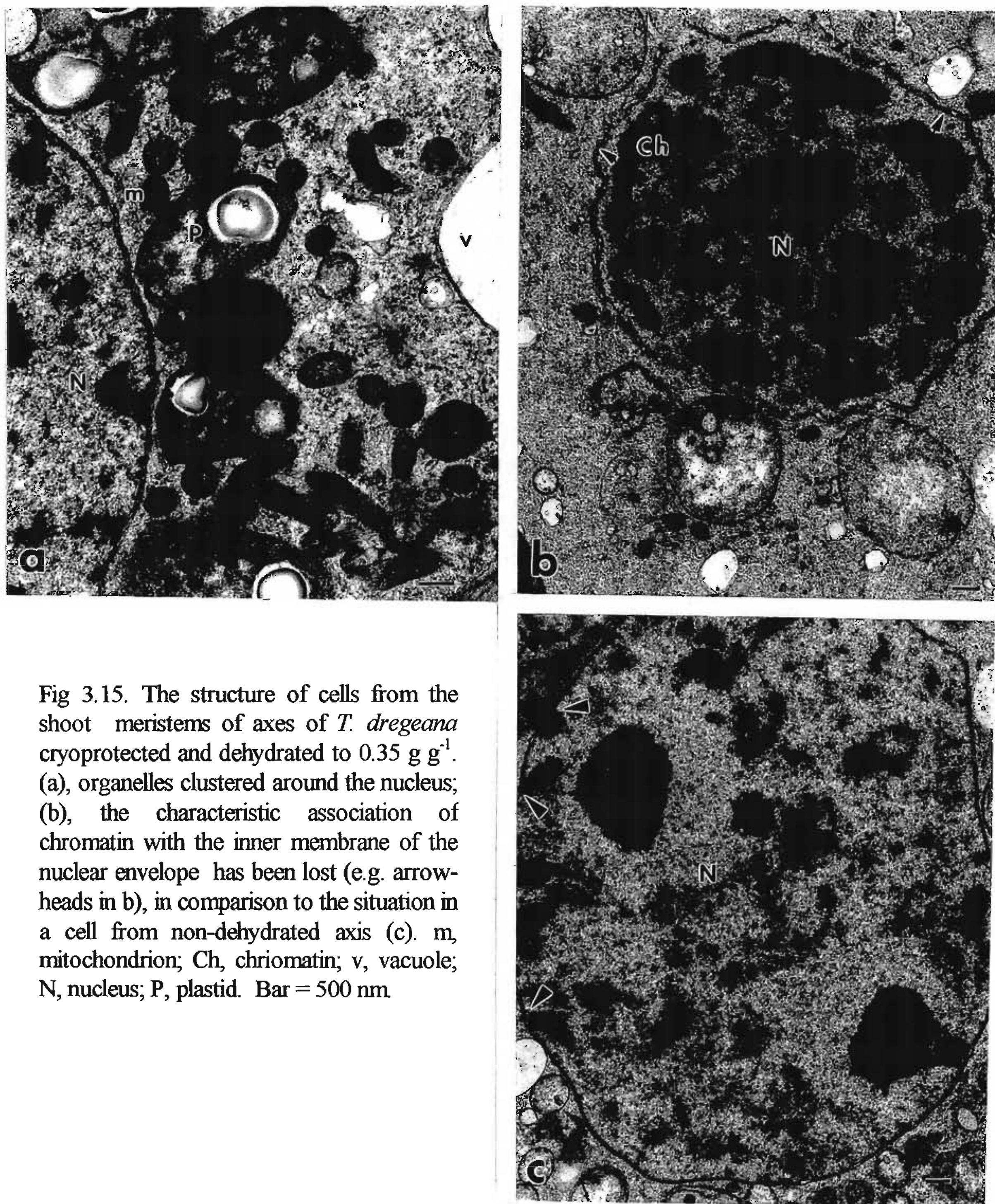


Fig 3.15. The structure of cells from the shoot meristems of axes of *T. dregeana* cryoprotected and dehydrated to 0.35 g g^{-1}. (a), organelles clustered around the nucleus; (b), the characteristic association of chromatin with the inner membrane of the nuclear envelope has been lost (e.g. arrowheads in b), in comparison to the situation in a cell from non-dehydrated axis (c). m, mitochondrion; Ch, chriomatin; v, vacuole; N, nucleus; P, plastid. Bar = 500 nm.

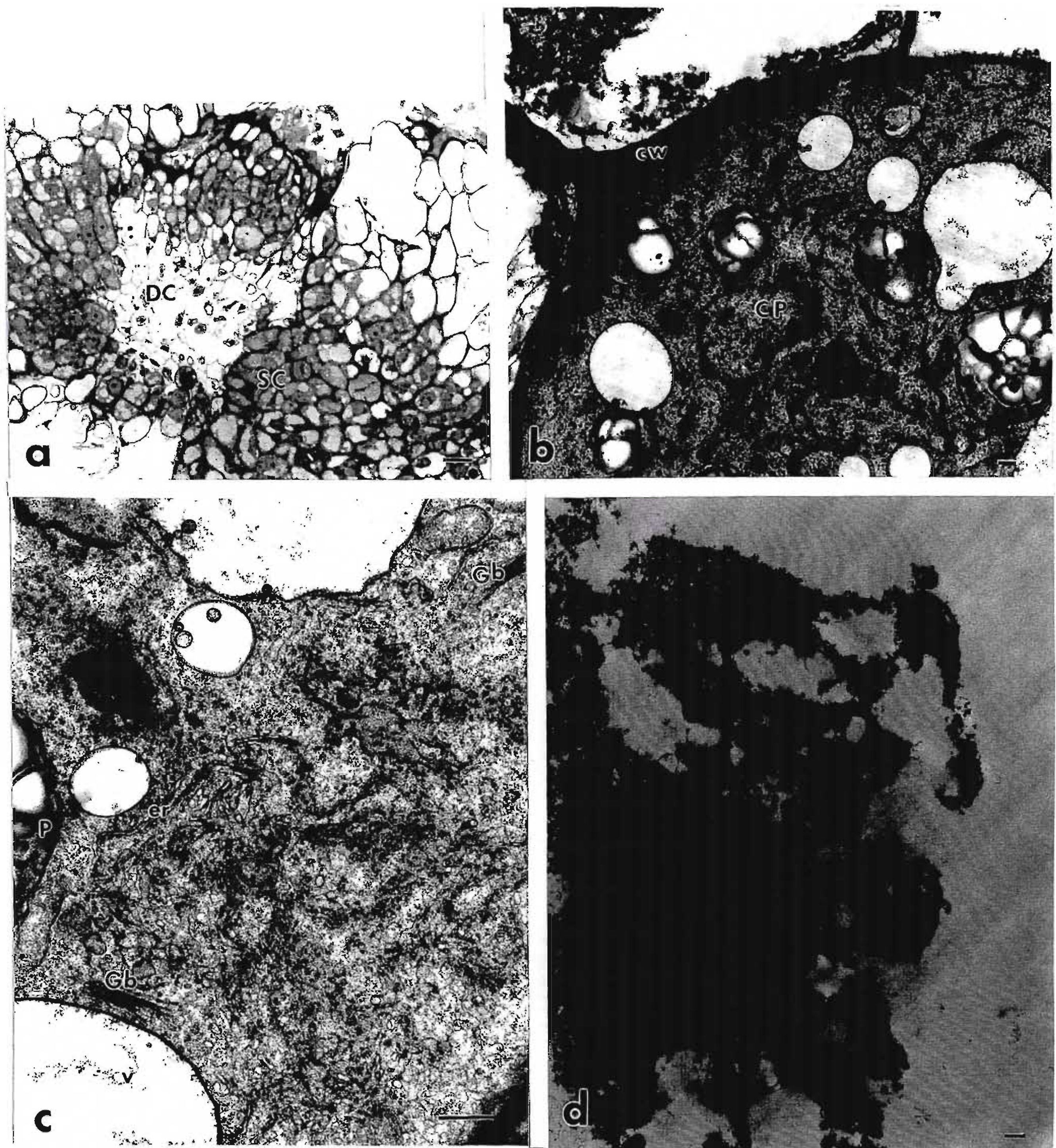


Fig 3.16. Surviving cells after cryopreservation of *T. dregeana* embryonic axes. (a), light micrograph showing groups of surviving cells (SC) flanking a region of dead cells (DC); (b), spherical-shaped callus precursor cell (CP) contiguous with the remains of extensively deteriorated cells; (c), the ultrastructure of the callus precursor cells indicated a highly degree of metabolic activity; d, cells from shoot meristems of axes following cryopreservation had a completely deranged ultrastructure.
er, endoplasmic reticulum; Gb, Golgi body; v, vacuole; P, plastid; cw, cell wall. Bar = 6.25 µm for (a), and 500 nm for (b-d).

The observations indicate that responses of embryonic axes of *T. dregeana* to dehydration and cryopreservation may have been compromised by a number of tissue, cell and intracellular features of this species:

- the highly-vacuolated, small root cap
- a high degree of tissue heterogeneity
- generally, the degree of vacuolation
- the absence of intracellular reserves, such as large starch grains or lipid bodies, which would provide some resilience when water is lost
- the relatively large size of the axes.

Together, these features are suggested to contribute to a lack of uniform response when axes were subjected to any particular treatment. For this species, and others with similar cellular and tissue characteristics, the only effective means of germplasm cryopreservation may be via somatic explants, such as embryogenic cell cultures, somatic embryos, or the meristems of apical or axillary buds. Additionally however, if the callus developing from the root pole of axes (after retrieval from cryopreservation) has embryogenic potential, then this could afford a means of capitalising on the genetic diversity of zygotic material.

3.3.3.4 Regeneration of embryonic axes after cryopreservation

Following cryopreservation and rehydration in a Ca^{2+}/Mg^{2+} solution (described below), 37% of *T. emetica* axes regenerated apparently normal roots and shoots, and developed into plantlets (Fig. 3.10). However, regardless of cryopreservation protocol used, embryonic axes of *T. dregeana* survived only in the form of unorganised callus developed from the root pole (Fig 3.9).

The lack of normal growth in surviving cryopreserved embryonic axes of tropical recalcitrant-seeded species is a widely-observed phenomenon (e.g. Pritchard and Prendergast, 1986; Pence, 1990; Chandel *et al.*, 1995; Normah and Marzalina, 1995; Berjak *et al.*, 2000; Wesley-Smith *et al.*, 2001a). This, according to Berjak *et al.* (2000), could be partly a consequence of the nucleoskeleton (Moreno Díaz de la Espina, 1995) and cytoskeleton (Shiboaka and Nagai, 1994) in the surviving cells, failing to reconstitute after cryopreservation. Such failure must result in a loss of control, perhaps precipitating chaotic intracellular and intranuclear events. It is relevant that

cytoskeletal abnormality has been linked to freezing injury in plant cells (Rikin *et al.*, 1980; Fosket, 1989).

In freeze-tolerant plant cells, depolymerisation of the cytoskeleton is necessary for the realisation of maximum freezing tolerance (Kerr and Carter, 1990), which has been suggested to help maintain a more fluid membrane system during the shifts to lower temperatures (Aszalos *et al.*, 1985). Upon thawing, however, the cytoskeleton must be re-constituted to facilitate the resumption of normal cellular activities (Bartolo and Carter, 1991). Such repolymerisation begins within 15 min (Hardham and Gunning, 1979; Hogetsu, 1986), to one hour (Bartolo and Carter, 1991) after thawing, and growth of the elements occurs at rates as high as 0.6 μm per min (Hardham and Gunning, 1979). Failure or inefficiency of repolymerisation, common in freeze-intolerant tissue, is correlated with freezing injury (Bartolo and Carter, 1991).

A novel approach has recently been applied in attempts to facilitate the repolymerisation of cytoskeletal elements after cryopreservation: the application of appropriate ratios of calcium and magnesium ions (Wolfe, 1995) that promote cyto- and nucleoskeletal assembly. According to Mycock (1999) a 1:1 solution of 1 μ*M* $CaCl_2.2H_2O$ and 1 m*M* $MgCl_2.6H_2O$ is optimal for plant cells. These are provided either in the solution in which cryoprotectants are dissolved (e.g. Mycock, 1999), or as a thawing medium (e.g. Berjak *et al.*, 1999, Walker, 2000), which simultaneously thaws and rehydrates explants after cryopreservation.

The Ca^{2+} / Mg^{2+} solution described above was tested in this study for thawing and rehydrating axes, and had a significant effect on post-thaw survival. The highest survival of cryopreserved *T. emetica* axes increased from 4 % to 69%, with 37% of the axes cryoprotected with a mixture of DMSO and glycerol, developing shoots after cryopreservation (Table 3.12). In contrast, none of the axes rehydrated in water showed any signs of shoot development. In *T. dregeana*, although no growth followed cryopreservation, survival was attained after use of the Ca^{2+}/Mg^{2+} solution, in treatments which had formerly not facilitated post-thaw viability retention, such as axis cryoprotection with DMSO only, sucrose only and DMSO mixed with sucrose (Table 3.11). Similar results were obtained by Berjak *et al.* (1999), who reported an increase of post-thaw shoot formation from 0 to 70% after thawing the axes of *Quercus robur* in the Ca^{2+} / Mg^{2+} solution. In the original application

of this thawing medium by Mycock (1999), there was an increase in the total number of pea embryonic axes recovering from cryostorage, and a concomitant reduction in the number of cryopreserved somatic embryos of date palm regenerating as callus.

Assembly, stability, or dis-assembly of the cytoskeleton is critically dependent on appropriate concentrations of Ca^{2+} and Mg^{2+} (Wolfe, 1995). Calcium has been shown to be a ubiquitous signal in plant cells, mediating a diverse range of responses such as specific gene expression in response to cold (Knight *et al.*, 1996), photomorphogenesis in response to red light (Shacklock *et al.*, 1992), free radical scavenger induction in response to oxidative stress (Price *et al.*, 1994), proline synthesis in response to salinity or drought (Knight *et al.*, 1997), and thermotolerance in response to heat shock (Gong *et al.*, 1998). It is therefore possible that calcium is involved in either the signalling or the gene expression necessary for the cytoskeletal re-assembly following freeze-induced de-polymerisation. Furthermore, calcium is stored, within the cell, in endoplasmic reticulum, constituted of membranous networks that are particularly vulnerable to freezing injury (Wesley-Smith *et al.*, 1992; Merymann and Williams, 1995; Singh and Miller, 1995; Wesley-Smith, 2002). Thus, following injury, calcium may leak out of the intracellular reservoirs and be leached out during water thawing/rehydration, hence needing to be supplied exogenously (Berridge *et al.*, 1998). This may partly explain the dramatic improvement in post-thaw survival of embryonic axes when appropriate concentrations of calcium ions are supplied during thawing and / or rehydration.

Another consequence of the damage to intracellular calcium reservoirs is an activation of stress signals which switch on the genes associated with apoptosis (Berridge *et al.*, 1998). During normal cellular functioning, there is a continuous shuttling of Ca^{2+} between the endoplasmic reticulum and mitochondria (which also sequester some Ca^{2+} ions during normal signalling); and if this shuttle is disrupted by damage to the endoplasmic reticulum, the resulting build-up of calcium in the mitochondria initiates a programme of events that lead to cell death (Berridge *et al.*, 1998). Thus, as the shoot meristems of individual axes were more damaged by freezing than root meristems, it is possible that the shoot meristem switched into apoptosis, and were not able to recover after cryopreservation. This may also apply to embryonic axes which are not able to regenerate shoots after drying alone (chapter 2, section 2.5.2; Wesley-Smith *et al.*, 2001a) as the shoot meristems suffered more membrane damage that root meristems after specific drying periods.

Magnesium ions are involved in the polymerisation of G-actin monomers to form the polymeric F-actin microfilaments (Wolfe, 1995). It is only after a monomer binds Mg^{2+}, that it becomes incorporated during microfilament assembly, with Mg^{2+} also needed to aggregate the filaments into bundles (Haaf *et al.*, 1998).

3.3.3.5 Cryopreservation of seeds of *Warburgia salutaris*

3.3.3.5.1 Effect of dehydration on survival after cryopreservation

While cryopreservation studies were done on embryonic axes of *Trichilia dregeana* and *T. emetica*, the excised axes of *Warburgia salutaris* could not be used because no *in vitro* regeneration method could be established (Section 3.3.1.1.). However, whole seeds of this species, unlike those of the *Trichilia* species, tolerated relatively rapid desiccation to very low water contents (Section 2.5.3.3). The potential for cryopreservation was therefore assessed for seeds dried to different axis water contents, down to 0.08 g g^{-1}.

Several cryopreservation studies were carried out, utilising seeds harvested in different years and from different provenances. In the first trial, fruits were obtained from Kenya in 1998 and, as they were still too hard for seeds to be extracted without injury, were stored for 4-6 weeks at 16 °C, until the pulp was soft enough for safe seed extraction. Upon dehydration to an axis water content of 0.1 g g^{-1}, some of these seeds could withstand cryopreservation in liquid nitrogen, with 30% germination after thawing (Fig. 3.17). The ultrastructure of axes that survived to germinate after cryostorage showed that their capacity for intracellular organisation and intensive metabolic activity had remained unimpared (Fig 3.18c).

However, the survival of only 30% of the seeds after freezing may be ascribed to two factors: the variability in the maturity stages of the seeds at harvest, and the germination medium. In these trials, the seeds were set to germinate in vermiculite at 25 °C and, while this was adequate for vigorous seeds, individuals that were less vigorous tended to be overrun by fungi proliferating on this water-retaining medium. Thus, in later experiments, bottom-heated sand beds, also at 25 °C, were used for germination instead of vermiculite in seedling trays. Besides the better drainage afforded by the

sandy medium, the wider-apart spacing of seeds helped curtail the spread of fungi from locations of infected/non-viable individuals.

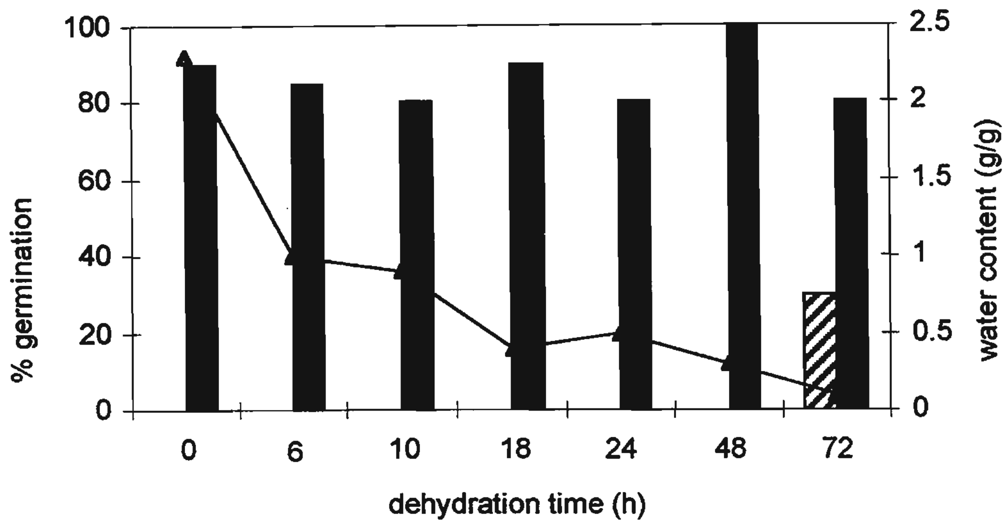
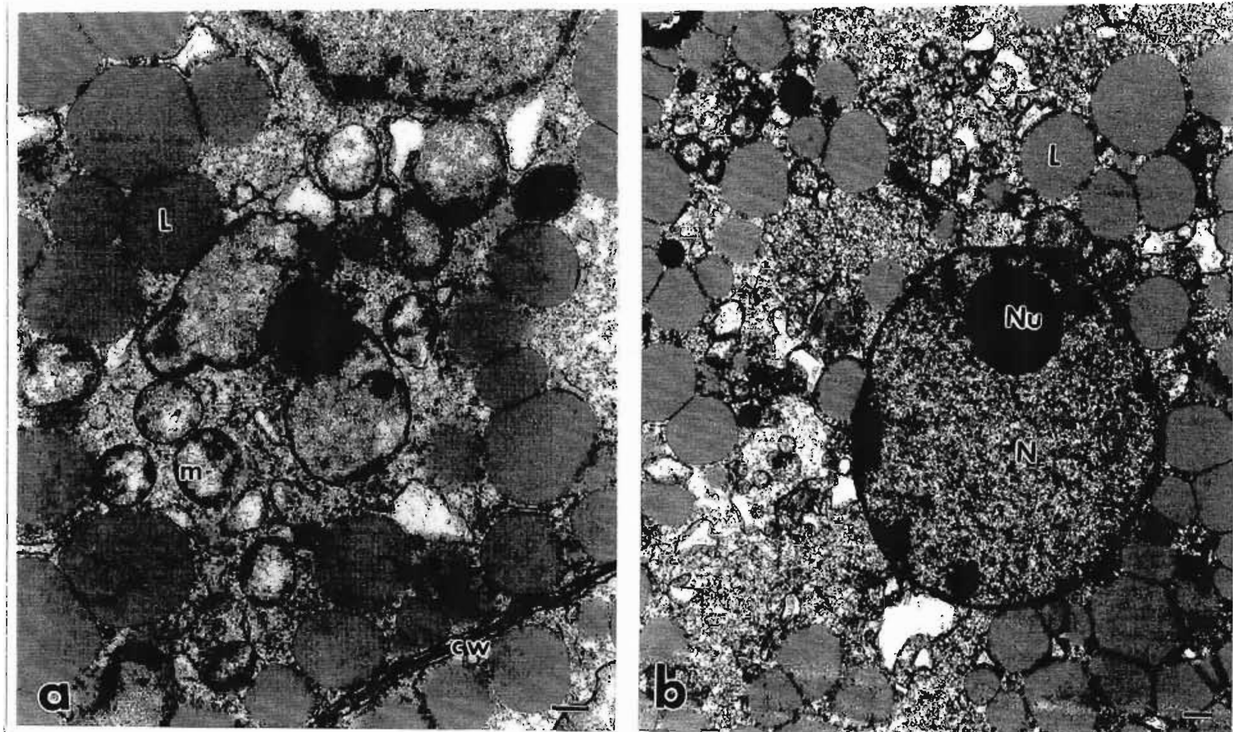


Fig. 3.17. Percentage germination (n=20) of seeds of *W. salutaris* following dehydration alone (shaded bars) or dehydration and cryopreservation (hatched bars). Seeds were buried in activated silica gel for up to 72 h. The curve shows the changes in axis water content (average of five individual axes per drying period) during the course of dehydration.

3.3.3.5.2 The effect of developmental stage of *Warburgia salutaris* seeds on survival after cryopreservation.

The seeds used in the initial cryopreservation trials are assumed to have been at different developmental stages as the fruits received and used were at different stages of ripeness. The stage of development of non-orthodox seeds influences not only the response to stresses such as desiccation (Chapter 2, section 2.2.1), but also the amenability to cryostorage (Chaudhury *et al.*, 1991; Pence, 1991; Chandel *et al.*, 1996; Finch-Savage, 1996). Thus, in the next harvest, from



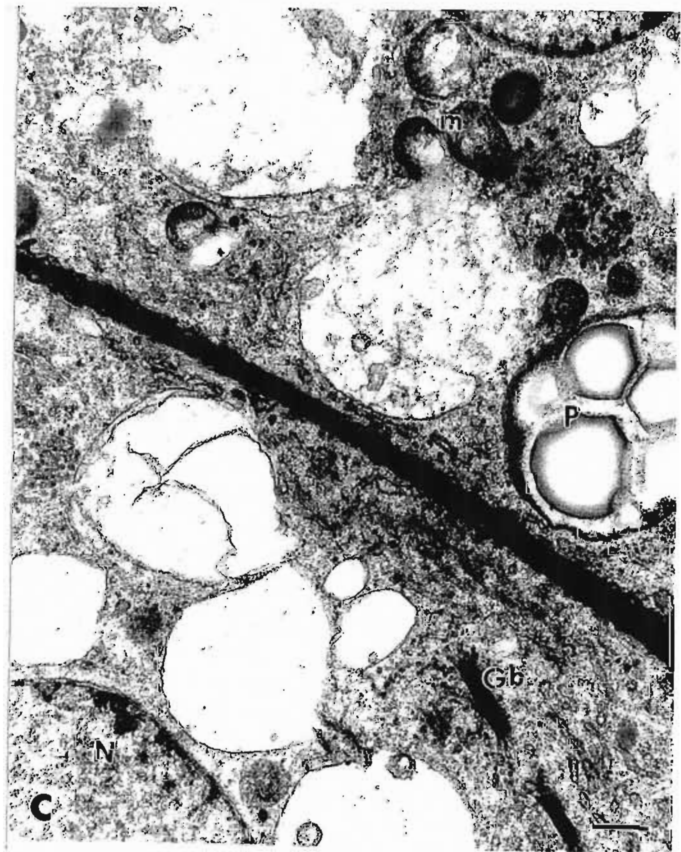


Fig 3.18 (a). The ultrastructure of embryonic axes of *W. salutaris* kept in storage to ripen, was dominated by lipid bodies, while organelles were relatively de-differentiated. (b), Following dehydration in silica gel to an axis water content of *c.* 0.1 g g^{-1}, the ultrastructure of the embryonic axes remained apparently unaffected. (c), Axes from seeds dehydrated to *c.* 0.1 g g^{-1}, cryopreserved in liquid nitrogen, and placed in germination medium for 10 days. The occurrence of many Golgi bodies (Gb), mitochondria with well-developed cristae (m), profiles of rough endoplasmic reticulum (er), polysomes, and starch-containing plastids (P) attest to the progress of active metabolism after retrieval from cryostorage. These axes later germinated into apparently normal seedlings.
N, nucleus; Nu, nucleolus; cw, cell wall.
Bar = 500 nm.

Lushoto in Tanzania, the fruits were categorised into three maturity stages, depending on fruit colour and hardness: green/hard; green-yellow/soft, and brown/soft. Seeds were extracted immediately from the green-yellow and brown fruits, while the green/hard fruits were kept at 16 °C until soft, before seed extraction was attempted. The seeds from fruits at the different maturity stages were dehydrated to *c.* 0.1g g^{-1} by burial in activated silica gel, and then frozen in batches of five per cryotube. While all the seeds survived dehydration with little loss of viability, those from green-yellow fruits had the highest post-thaw survival (Table 3.14), with a germination percentage of 65%. Seeds from brown fruits had the lowest survival after cryopreservation (40%), while those from green fruits that had been kept in storage to ripen showed 55% survival after freezing. The seedlings obtained from cryopreservation were similar to seedlings from non-frozen seeds, both morphologically and in terms of growth rates in the greenhouse and later, in the shade-house (Fig. 3.19).

Microscopical examination revealed that cells from embryonic axes of seeds from fruits at different maturity stages, differed considerably in ultrastructure. Embryonic axes of seeds from green-yellow fruits showed a relatively quiescent state, with the cytomatrix almost exclusively occupied by lipid bodies, and inconspicuous mitochondria appearing occasionally (Fig. 3.20b). The high lipid body:organelle ratio, organellar de-differentiation, and general cellular compaction is suggested contributed to resilience of the cells during freezing, and possibly played a major rôle in enabling seeds from green-yellow fruits to survive cryopreservation.

Seeds from fruits that were green, hard, and considered immature, were not extracted. For experimentation, however, the axes were examined electron microscopically. The ultrastructure of these cells was indicative of a high level of metabolism, with obvious indicators such as numerous cristate mitochondria, frequent profiles of endoplasmic reticulum, and Golgi bodies (Fig. 3.20a). Cells of these axes were also relatively highly vacuolated, and had a lower lipid body:organelle ratio than axis cells from seeds extracted from fruits that were harvested at a similar stage, but stored until soft (compare Fig. 3.18a with Fig. 3.20a).

Table 3.14. Percentage germination of seeds of *W. salutaris*, extracted from fruits at different maturity stages, after dehydration to an axis water content of *c.* 0.1 g g^{-1} and cryopreserved in liquid nitrogen. n=20.

Maturity stage of fruit	**% germination after:**	
	Dehydration only	**Dehydration and cryostorage**
Green, softened in storage	100	55
Yellow-green	100	65
Brown	100	40

In contrast to the axes from fruits harvested green, or yellow-green, those from brown fruits had an ultrastructure indicative of substantial damage. The cells had abundant lipid bodies, many of which were fused into substantial masses (Fig. 3.20c), a feature identified as a consequence of deterioration in non-orthodox seeds (Smith and Berjak, 1995; Leprince *et al.*, 1998), and which might be induced by fungal infection. Even though seeds from other maturity stages also harboured fungi (as evidenced by the heavy contamination of excised embryonic axes [section 3.3.1.3]), brown fruits had visible fungal inoculum in the pulp and fruit wall.

While the seeds may have been able to tolerate fungal contamination when fresh or dehydrated, indicated by survival (Table 3.14), many appeared unable to withstand the additional stress occasioned by freezing. Thus, it is possible that the activity of associated fungi may have contributed to the lower post-thaw survival of seeds from brown fruits, than those from yellow-green or from ripened green fruits (Table 3.14).

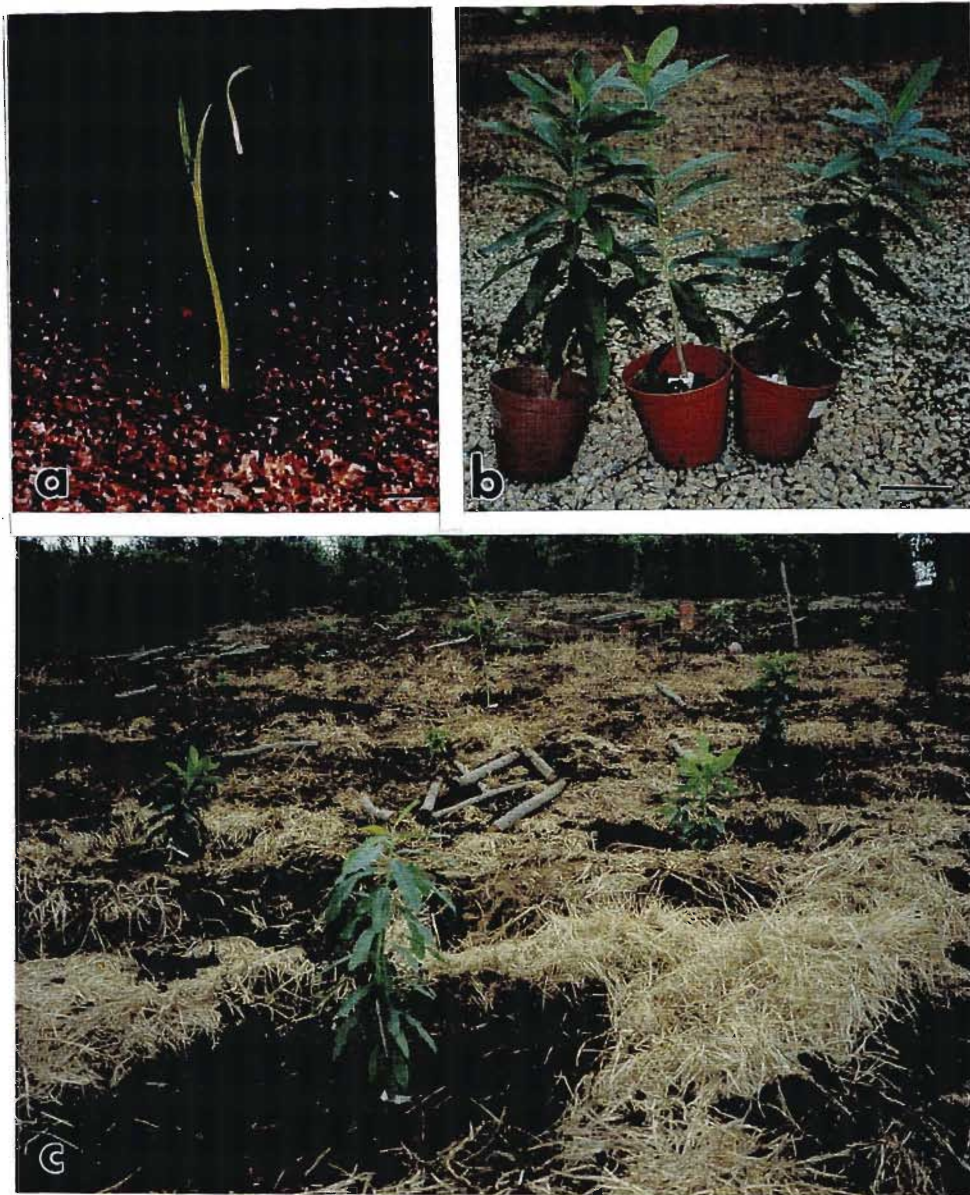


Fig 3.19 (a). Nascent seedlings from seeds sown in bottom-heated sand beds, following dehydration to an axis water content of *c.* 0.1 g g^{-1}, and cryopreservation in liquid nitrogen. (b) A comparison of typical seedlings obtained from a control (non-dehydrated, non-cryopreserved) seed (left), a dehydrated seed (middle) and a cryopreserved seed (right), after six months in a shade-house. There were no discernible differences in morphology among seedlings from the different experimental groups of seeds. (c), Seedlings from control, dehydrated, and cryopreserved seeds, immediately after planting in the field at Eston, KwaZulu-Natal, South Africa (29°52'S, 30°30'E). Bar = 5 mm in (a), and 0.1 m in (b).

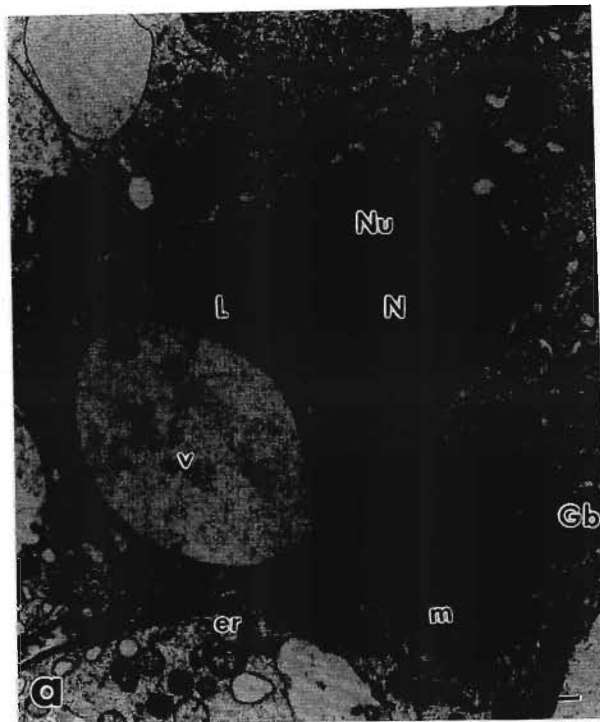
The higher post-thaw survival attained by seeds from freshly-harvested yellow-green fruits as compared to those from fruits that were ripened by storage for several weeks (Table 3.14) may be explained by considering the differences in ultrastructure between the two groups of seeds. The cells from force-ripened fruits showed a lesser degree of organelle de-differentiation and accumulated less lipid than fruits that ripened naturally on the parent tree, with a concomitantly lower degree of compaction both before, or after, dehydration (compare figures 3.18a with 3.20b). These intracellular variations reflect differences in physiological status, which could well underlie differences in responses to stresses such as dehydration and cryopreservation (Pammenter and Berjak, 1999).

The influence of seed maturity on cryopreservability has been observed in a number of species. For example, when tea seeds at three different maturity stages were dehydrated and cryopreserved, the most mature seeds had the highest post-thaw survival (Chaudhury *et al.*, 1991). In a parallel study on jackfruit, those authors found, in contrast, that axes from partly-mature seeds were the most suitable for cryopreservation even though there was no difference in the desiccation tolerance among the three stages tested, and Chandel *et al.* (1996) found a similar response from the seeds of neem (*Azadirachta indica*). For highly-recalcitrant cocoa seeds, Chandel *et al.* (1995) reported no survival after cryopreservation, but found that axes from immature seeds were more amenable to manipulation and desiccation than axes from seeds at other stages of maturity.

3.3.3.5.3 The influence of provenance of survival after cryopreservation

Besides the stage of maturity at testing, another factor that apparently influenced the amenability of *W. salutaris* seeds to cryopreservation was the provenance. When seeds from yellow-green fruits from two different provenances, Lushoto in Tanzania and Silverglen Nature Reserve in South Africa, were cryopreserved, those from Tanzania had consistently higher rates of post-thaw survival (Fig. 3.21).

The responses of non-orthodox seeds to many stresses such as desiccation, and probably cryopreservation, is influenced by habitat, with seeds from warmer, more moist habitats being more susceptible than those of cooler, drier, or temperate origins (Pammenter and Berjak, 1994).



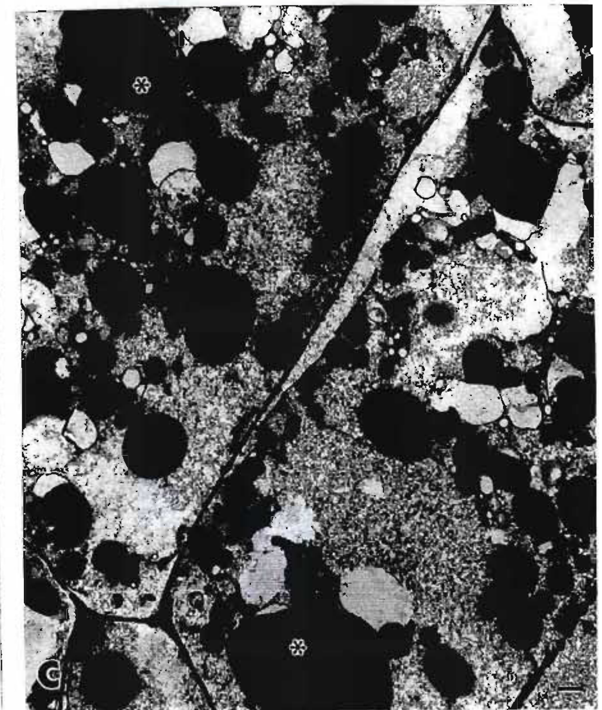


Fig 3.20 (a). Cells from axes of seeds from green / hard fruits. The cells had relatively large vacuoles devoid of obvious accumulations of insoluble material, a scattering of lipid bodies and clearly-defined organelles including Golgi bodies, mitochondria, plastids and profiles of rough endoplasmic reticulum, a situation indicative of a high rate of metabolism. (b), Cells from axes of seeds from fruits harvested at the green-yellow stage of maturity were dominated by masses of lipid bodies, among which cytomatrix containing dedifferentiated organelles and compressed vacuoles, is compacted. The cells had a higher lipid body:organelle ratio than those from seeds ripened in storage (Fig 3. 18a), facilitating higher tolerance to cryopreservation. (c), The ultrastructure of cells from axes of seeds extracted from brown / soft fruits had de-differentiated organelles, but showed deterioration such as fused lipid bodies (asterisk), which is suggested to be the consequence of fungal activity .
m, mitochondrion; er, endoplasmic reticulum; Gb, Golgi body; v, vacuole; N, nucleus; Nu, nucleolus; L, lipid body; cw, cell wall. Bar = 500 nm.

In this study, the seeds from Tanzania originated from a high-altitude (1 680 m above sea level) habitat with a mean temperature of 20 °C and annual rainfall of 600 – 800 mm; while those from South Africa were obtained from a humid habitat at sea-level, with an average temperature of 20.4 ± 8 °C and mean annual rainfall of 1000 mm.

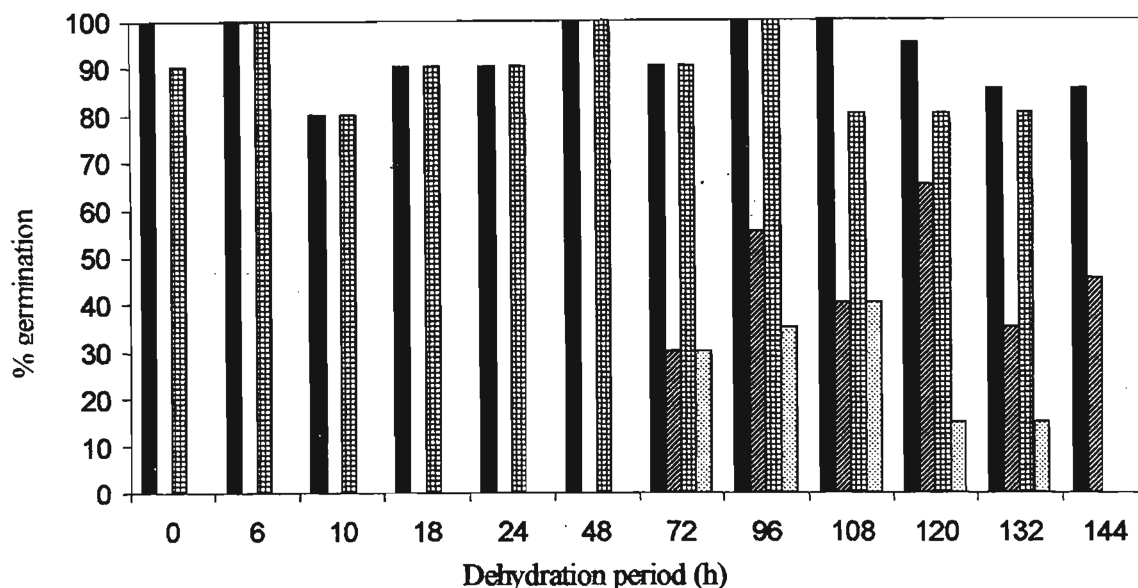


Fig. 3.21 Changes in germination totality, with dehydration period, of seeds from yellow-green fruits of *Warburgia salutaris* from Tanzania (solid bars, dehydrated only; hatched bars, dehydrated and cryopreserved) and South Africa (checked bars, dehydrated only; dotted bars, dehydrated and cryopreserved). Seeds from each provenance were dehydrated by burying in activated silica gel, and germinated in sand beds thermostatically controlled at 25 °C. n=20.

Aside from the differences in response to freezing that may be occasioned by the differences in provenance, recent studies using polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPDs) indicate that the trees from the two provenances may represent disparate species (Lamb[3], pers. comm.). Given that non-orthodox seeds have a wide variation in physiological properties within a single species (Berjak and Pammenter, 2001; 2002; 2003), differences in the response to freezing would not be unexpected if, indeed, the two populations of seed (Tanzania and South Africa) are separate species.

[3] J.M. Lamb, School of Life and Environmental Sciences, University of Natal, Durban, South Africa.

APPENDIX A:

Nutrient composition of standard *in vitro* culture basal media used in this study.

Compound	Medium / Concentration mg l^{-1}		
	MS	WPM	DCR
Macronutrients			
K_2SO_4		900	
KNO_3	1900		340
NH_4NO_3	1650	400	400
$Ca(NO_3)_2.4H_2O$		556	556
$CaCl_2.2H_2O$	440	96	85
$MgSO_4.7H_2O$	370	370	370
KH_2PO_4	170	170	170
Micronutrients			
$Na_2.EDTA$	37.3	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8	27.8
$MnSO_4.H_2O$	16.9	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2
KI	0.83		0.83
$CoCl_2.6H_2O$	0.025		0.025
$CuSO_4.5H_2O$	0.025	0.25	0.25
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
Vitamins			
Glycine	2.0	2.0	2.0
Thiamine.HCl	0.1	1.0	1.0
Pyridoxine.HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Myo-inositol	100	100	200

CHAPTER 4:

Is genetic integrity maintained following cryopreservation? A case study of *Warburgia salutaris*.[#]

4.1 Introduction

A primary goal of germplasm conservation is the preservation of genetic integrity and diversity in order to preserve the adaptability and the evolutionary potential in the populations subsequently raised from the conserved material (Avise, 1994). The lack of genetic diversity is associated with vulnerability to extinction (Sulaiman and Hasnain, 1996; Godt and Hamrick, 1998; Woodroffe and Ginsberg, 1998), and small populations are particularly susceptible. This is because of the associated changes in demography and loss of genetic diversity through genetic drift (the random change in allele frequencies in a population [Burgman *et al.*, 1993; Woodroffe and Ginsberg, 1998]) and inbreeding depression (the consequence of an increase in the homozygosity of unfavourable recessive alleles, which results in attributes of decreased fitness such as lowered fertility, susceptibility to disease and reduced growth rates [Charlesworth and Charlesworth, 1987; Falconer, 1989; Caro and Laurenson , 1994; Ballou, 1995; Kerrigan *et al.* 1998]).

Small, isolated populations have been shown to experience accelerating extinction due to the spiraling positive feedback between the population and extinction-driving factors. In

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[#] Parts of this chapter were incorporated in, and published as a chapter in: G. Nicolas, K.J. Bradford, D. Côme and H. Pritchard (eds) *The Biology of Seeds: Recent Research Advances*. CAB International, Wallingford, UK. (in press).

Small, isolated populations have been shown to experience accelerating extinction due to the spiraling positive feedback between the population and extinction-driving factors. In this feedback, demographic stochasticity and inbreeding reduce the ability of the small population to adjust to environmental changes. This leads to a further decline in the population, making it even more susceptible to demographic stochasticity, which accentuates fragmentation and inbreeding. This is termed 'the extinction vortex' (Gilpin and Soulé, 1986), and is illustrated in figure 4.1

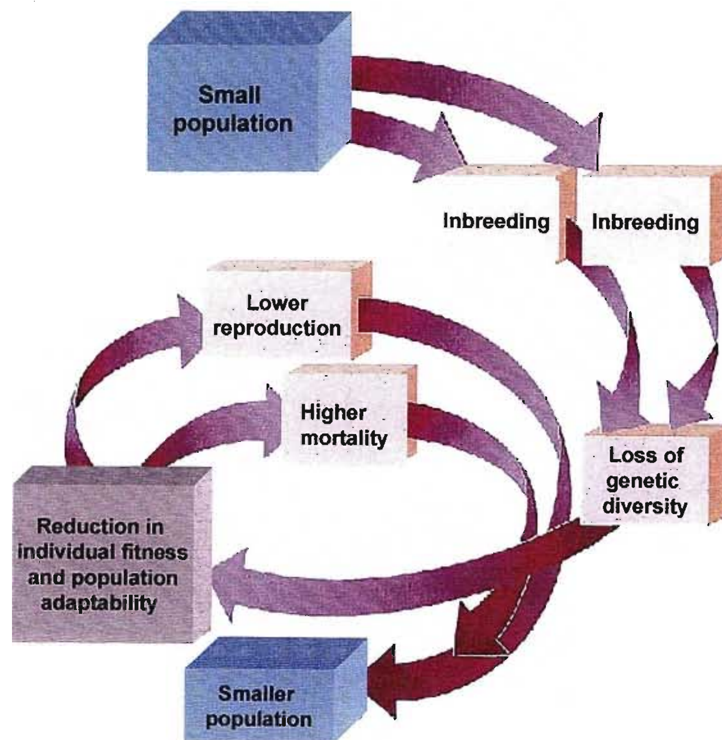

Fig 4.1 The 'extinction vortex', caused by positive feedback effects of small population size, inbreeding, and loss of genetic variability (adapted from Gilpin and Soulé, 1986).

The properties of small populations that accelerate extinction, characterise *Warburgia salutaris*, particularly in South Africa, where there are merely 11 sub-populations, of which only two exceed 20 individuals (Scott-Shaw, 1999). Furthermore, that author reports that each of the 11 subpopulations comprises ramets of only one clone. The low population and genetic diversity may have contributed to reduced fitness, and which leads to reduced vigour and fertility. This may partly account for the fact that no seedlings of this species have been observed in the wild (Scott-Shaw, 1999). This is in contrast to eastern Africa where large natural populations exist and, despite predation, copious seed production and seedling recruitment is observed (Mbuye *et al.*, 1994). Additionally, natural extinction rates of *W. salutaris* are exacerbated by increasing demand for bark and other plant parts in the herbal medicine trade which was recently estimated to be 4 000 000 kg y^{-1} in Durban, South Africa, alone (Mander, 1998); and in which *W. salutaris* produces the most favoured bark (Cunningham, 2002).

It is therefore imperative to conserve the germplasm of this species, which is now virtually extinct in the wild in South Africa (Scott-Shaw, 1999). Such conservation can be efficiently achieved via cryopreservation of the seeds (Chapter 3). However, the genetic fidelity of the plants obtained from the cryopreserved material must be maintained, not only to sustain the available genetic diversity, but also to retain the genetic information needed for biosynthesis of the pharmacological products for which the plant is valued; these include warburganal, muzugadial, polygodial, mukaadial and ugadensidial (Warthen *et al.*, 1983; Taniguchi and Kubo, 1993).

The maintenance of genetic stability following cryopreservation has been demonstrated for explants of a number of species, such as shoot-tips of ginseng (Yoshimatsu, *et al.*, 1996) and potato (Schäfer-Menuhr *et al.*, 1997); embryogenic cell suspension cultures of barley (Fretz and Lörz, 1994); and nucellar cells of navel orange (Kobayashi *et al.*, 1994).

On the other hand, some studies have indicated that cryopreservation may induce genetic aberrations. In an assessment of the genetic fidelity of cryopreserved potato shoot-tips, Perazzo *et al.* (2001) found that samples from one-year cryopreserved accessions differed from non-stored samples in multiple morphological characteristics and that they had significant DNA differences. Ohsako *et al.* (1997) found that freeze-thaw cycles reduced the amount of genomic DNA in somatic and gametic cells of some animal species. Furthermore, some common pre-cryopreservation measures may also cause genetic changes. For example, cryopreservation following cryoprotection with DMSO was shown to increase intraclonal variation in embryogenic cultures of *Abies cephalonica* from 1.6 % to 16.8 % (Aronen *et al.*, 1999).

Even though seeds may present a physiologically and perhaps genetically, more stable system for cryopreservation than do cell suspension cultures or somatic embryos, genetic changes have been reported in plants obtained from cryopreserved seeds. Harding *et al.* (2000) found changes in chromatin and DNA methylation to have occured, which may have significance in gene expression, in seedlings obtained from cryopreserved seeds of mahogany (*Swietenia macrophyla*).

Therefore the maintenance of genetic integrity or diversity after cryopreservation is not assured, and needs to be determined empirically for different explants / species. This is particularly important for endangered species, which may already have highly reduced genetic variability.

4.1.1 Techniques commonly used to study genetic diversity

Any genetic changes during cryopreservation have a significant effect on germplasm conservation programmes, and several techniques are available for detecting genetic differences / changes in plant populations. The techniques include isozyme analysis, restriction fragment length polymorphisms (RFLPs), and polymerase chain reaction-based genetic markers such as microsatellites (also known as simple sequence repeats, SSRs), amplified fragment length polymorphisms (AFLPs) and random amplified polymorphic DNA markers (RAPDs).

4.1.1.1 Isozyme analysis

Isozymes are different molecular forms in which a protein may exist while having the same enzymatic specificity (Buth, 1984). Genetic polymorphism for isozymes exists within the same population (Stebbins, 1989; Wendel and Weeden, 1989), and therefore individuals which vary in their genetic characteristics can be separated by variability in isozyme banding patterns on a gel plate. Isozyme analysis has thus been applied in genetic studies in diverse plant species such as garlic (*Allium sativum* [Maass and Klaas, 1995]), plantain (*Musa* spp. [Lebot *et al.*, 1993]), and *Brighania* (Campanulaceae), an endangered woody genus endemic to Hawaii (Gemmill *et al.*, 1998).

Although isozymes present a cheap and rapid means of probing taxonomic relationships and genetic diversity, they have the disadvantage of being developmentally regulated, i.e. they are expressed only at specific developmental stages and/or in certain tissues or organs (Beckmann and Soller, 1983). Isozymes are also largely uniform with respect to plant cultivars (Novo *et al.*, 1979), and therefore afford less precise data than techniques which assess DNA diversity directly.

4.1.1.2 Restriction fragment length polymorphisms (RFLPs)

RLFPs are based on restriction endonucleases which recognise and cleave DNA at, or adjacent to, short, specific sequences of nucleotides, producing DNA fragments that are between a few hundred and a few thousand base pairs in length (Zabeau and Roberts, 1979). The number and length of such fragments depends on the restriction endonuclease used, and is characteristic of the genomic DNA sequence tested (Young, 1993). RFLP differences between organisms are inherited in a Mendelian fashion, and have been used to produce genetic linkage maps (e.g. for *Brassica oleracea* [dos Santos *et al.*, 1994] and for cow pea, *Vigna unguiculata* [Menéndez *et al.*, 1997]). RFLP markers associated with specific traits can be identified and used in quantitative trait analysis, (e.g. for soybean [Maughan, 1994]). However, the technology is labour-intensive and takes several days to yield a result, and generally does not efficiently target tightly-linked markers (Haley *et al.*, 1994).

4.1.1.3 Microsatellites

Microsatellites, also known as 'simple sequence repeats' (SSRs), are composed of varying numbers of tandemly repeated 1 to 5 base-pair motifs flanked by conserved regions, and are widespread throughout eukaryote genomes (Lagercrantz *et al.*, 1993), in which the number of repeats can range from ten to several hundred thousand (Haymer, 1994). Because these repeats are flanked by conserved DNA sequences, primers complementary to these regions can be used to amplify the locus by polymerase chain reaction (PCR, described below) which employs a ^{32}P- labeled nucleotide to allow visualization of the amplified product by autoradiography after polyacrylamide gel electrophoresis (e.g. Maughan *et al.*, 1995). Microsatellites have important qualities that make them desirable as molecular markers: they are highly variable (Akkaya *et al.*, 1992; Lagercrantz *et al.*, 1993; Akagi *et al.*, 1996) and all alleles in an individual are perceptible, which is not the case for some dominant markers such as those of RAPDs, discussed below (Akagi *et al.*, 1996). Thus, microsatellites have been used to detect genetic variability and differentiation in, and among, populations (e.g. in winter wheat varieties [Donini *et al.*, 2000]); to identify hybridisation between species (e.g. for soybean plant introductions [Narvel *et al.*, 2000]); to characterise genetic relationships (e.g. for cultivated melon, *Cucumis melo* [Staub *et al.*, 2000]); to determine patterns of pollen and seed dispersal (Richardson *et al.*, 2002); as well as to facilitate paternity analysis and evaluate recent genetic history, such as population bottlenecks (Goldstein *et al.*, 1999). However, a disadvantage of microsatellites is the number of faint bands in the PCR profile that are caused by replication slippage, which occurs at a repetitive

sequence when the new strand mis-repairs with the template strand (Perry *et al.*, 1994) and which may lead to scoring errors.

4.1.1.4 Amplified fragment length polymorphisms (AFLPs)

The AFLP technique (Vos *et al.*, 1995) is a combination of restriction enzyme digestion and the polymerase chain reaction (described below). In this technique, DNA is digested with restriction enzymes, generating a pool of DNA fragments of varying sizes. Specific adaptor oligonucleotides with fixed sequences, are then ligated to the fragments generated by the restriction enzymes and amplified with the PCR technique using the adaptor sequences as primer sites. Thus, a selection of all restriction fragments in a reaction is amplified. The technique produces a dense, but reliable, banding pattern of typically 30-100 fragments (Vos *et al.*, 1995).

AFLPs are widely used in genetic studies in plants, e.g. for assessment of genetic diversity in cotton (Pillay and Myers, 1999), genetic analysis of *Metrosideros bartlettii*, a rare myrtaceous New Zealand tree, of which there are only 31 surviving individuals (Drummond *et al.*, 2000), as well as for the investigation of the level and pattern of DNA variation of *Arabidopsis thaliana* at the entire genome level, for 38 ecotypes distributed throughout the world (Miyashita *et al.*, 1999).

AFLPs give substantially more information than do RAPDs. For example, using AFLPs, Cervera *et al.* (1996) obtained approximately 10-fold the number of informative markers

obtained by RAPDs in *Populus* spp. However, AFLPs require large amounts of DNA relative to RAPDs, and can be more time-consuming.

4.1.1.5 Polymerase chain reaction-based random amplified polymorphic DNA (PCR-RAPDs).

The RAPD technique, also known as 'arbitrarily primed polymerase chain reaction' (AP-PCR) utilises PCR, which was first described by Saiki *et al.* (1985), and was developed for the purpose of revealing DNA polymorphisms (Welsh and McClelland, 1990; Williams *et al.*, 1990). In this technique, the genomic DNA is subjected to alternating high and low temperatures, during which the DNA double strand separates into single strands (high temperature), and primers anneal to the separated strands, allowing synthesis of new DNA strands (low temperature). By repeating the high and low temperature cycles, an exponential increase in the number of copies of the stretch of DNA complementary to the primer, is produced. The high product output enables direct visualisation by electrophoresis through an agarose or polyacrylamide gel, and the resulting banding pattern constitutes a DNA fingerprint for the particular genome and primer.

The DNA fingerprints produced by PCR-RAPDs allow for the estimation of the similarity between genomes to be made (e.g. Wade, 1999), and may be used in the identification of species and varieties (e.g. van Bockstaele, 2000), genetic linkage map construction (Lodhi *et al.*, 1995; Yang and Quiros, 1995; Lin *et al.*, 1996) and parentage analysis (Ragot and Hoisington, 1993)

Of all the methods mentioned above, the RAPDS technique represents the cheapest option (Ragot and Hoisington, 1993), with the added advantages that it does not require prior knowledge of the genomic DNA sequence being tested (Connolly *et al.*, 1994), and needs only small quantities of DNA (e.g. in sugarcane, nanogram amounts of genomic DNA are used [Al-Janabi *et al.*, 1993; Harvey *et al.*,1994]). It also gives superior results to those of karyotyping (Campos *et al.*, 1994), isozymes (Orozco-Castillo *et al.*, 1994) and RFLPs (Williams *et al.*, 1990). The use of RAPDs also enables the analysis of a large part of the genome. The presence of a single RAPD band is diagnostic for a sequence totalling 20 bp (using 10-base primers) in the target genome. Hence, an average of 10 amplified bands per primer means that each primer is diagnostic for 200 bp. This is in contrast to, for example, RLFP, which is diagnostic for only 12 bp per probe-enzyme combination.

Another advantage of the use of RAPDs is that it can be used to sample repetitive DNA (Williams *et al.*, 1990). Repetitive DNA comprises a significant part of the genome of many eukaryotes, and the changes in this type of DNA may occur in a different manner to, or to a different extent from, those occurring in single copy sequences or genes (Begun and Aquadro, 1993). Thus, RAPDs provides information in areas previously inaccessible with, for example, RLFPs, and this is particularly useful for germplasm of limited genetic diversity (Foolad *et al.*, 1993).

A drawback of RAPD markers is that they are inherited in a dominant fashion (Säll and Nilsson, 1994; Williams *et al.*, 1993), such that the RAPD reaction produces a fragment with template DNA from individuals that are homozygous dominant or heterozygous for an amplified allele, and no fragment is produced in a homozygous recessive locus. Even when homozygous dominant or heterozygous loci are present, certain crops lack detectable polymorphism, while a fraction of primers may be unable to amplify certain DNA fragments due competition among the fragments for amplification (Williams *et al.*, 1993; Halldén *et al.*, 1996).

RAPD amplification is based on specific physical and chemical parameters, and therefore non-optimal reaction mixtures or conditions can introduce DNA amplification artefacts or ambiguous products, obscuring genetic analysis (Reidy *et al.*, 1992; Hansen *et al.*, 1998). Results may also be obscured by the co-migration of bands, a phenomenon in which different fragments of similar size co-migrate during separation by gel electrophoresis.

The above factors may hamper the usefulness, and increase the cost, of RAPD markers in probing genetic diversity. However, with the attendant advantages, the PCR-RAPD technique has been widely and successfully applied in plant genetic studies, examples of which are outlined in Table 4.1. This technique was used for genetic assessment of *Warburgia salutaris* in the current study.

4.2 An overview of the RAPD methodology

The process of PCR-RAPD is illustrated in Fig 4.2. The genomic DNA is denatured at relatively high temperature in the presence of the four different nucleotides (dATP, dGTP, dCTP and dTTP), thermostable enzyme (*Taq* polymerase), and short oligonucleotide primers (5 – 20 bases in length [Caetano-Anollés *et al.*, 1991; Dinesh *et al.*, 1993). A buffer containing an optimum concentration of $MgCl_2$ is also included in the reaction mixture, as *Taq* polymerase requires the presence of Mg^{2+} for activity, and these cations also affect the melting and annealing temperatures of the primer:template hybids (Nagaraju *et al.*, 2001). The primers used in PCR-RAPDs are designed without prior knowledge of the genomic DNA sequence (Williams *et al.*, 1990), in contrast to specific PCR where primers are homologous to sequences flanking the area or gene of interest (Hoelzel and Green, 1992).

Table 4.1 A summary of some applications of PCR-RAPDs in the investigation of plant genetic diversity.

Species / common name	Investigation	Reference
Wheat	Genetic diversity among cultivars	Figliuolo and Spagnoletti-Zeuli, 2000.
Mahogany (*Swietenia macrophylla*)	Molecular stability following cryopreservation of seeds	Harding *et al.*, 2000
Lolium multiflorum and *L. perenne*	Genetic stability of cell suspension-derived plants	Wang *et al.*, 1993.
Picea mariana	Genetic stability of somatic embryogenesis-derived plants	Isabel et al., 1993.
Potato	Genetic stability following cryopreservation of shoot-tips	Schäfer-Menuhr *et al.*, 1997.
Maize	Genetic stability following cryopreservation of pollen	Shi *et al.*, 1996.
Cassava (*Manihot esculenta*)	Genetic stability following *in vitro* storage of slow-growth cultures	Angel *et al.*, 1996.
Panax ginseng	Stability of T-DNA after cryopreservation of hairy-roots	Yoshimatsu *et al.*, 1996.
Barley (*Hordeum vulgare* cultivars Gimpel and Igri)	Genetic stability following cryopreservation of embryogenic suspensions	Fretz and Lörz, 1994.
Tea (*Camellia sinensis*)	Genetic diversity and stability among excellent tea germplasms	Chen *et al.*, 1999
Christmas bush (*Ceratopetalum gummiferum*)	Genetic diversity among Australian cultivars	van Bockstaele, 2000.
Scirpus triqueter, S. tabernaemontani,	Taxonomy	Wade, 1999.
Alfalfa (*Medicago sativa*)	Detection of quantitative trait loci	Ivashuta *et al.*, 1999.
White spruce (*Picea glauca*)	Analysis of somaclonal variation	DeVerno *et al.*, 1999.
Rice	Genetic variation following cryopreservation of callus	Moukadiri *et al.*, 1999

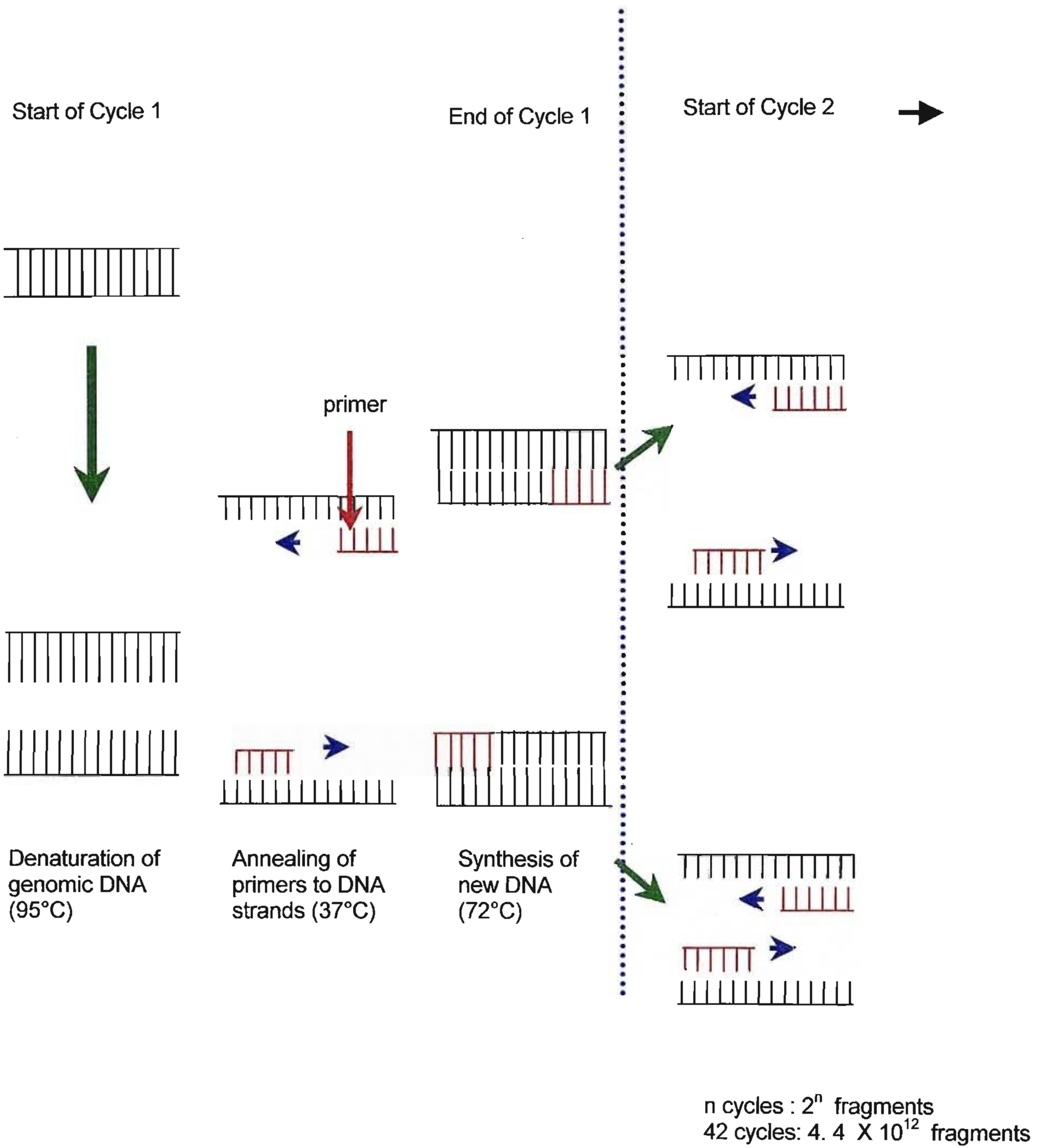

Fig 4.1 Diagrammatic representation of the polymerase chain reaction (PCR)

When the reaction mixture is exposed to the high temperature (92 - 95 °C), the double-stranded genomic DNA separates into single strands. On cooling the mixture (e.g. to 37 °C), the primers to anneal to complementary sequences on the single-stranded DNA. The temperature is then raised (to 72 °C, in this study) to allow DNA synthesis (primer extension) to occur. The temperature is then raised again to 92 -95 °C for the newly-synthesised DNA to separate, and the cycle of denaturation, annealing and elongation is repeated. Successive cycles should result in an exponential increase in the number of DNA copies (i.e. amplification). However, amplification is dependent on two main factors: the nature of the starting material (genomic DNA), and the efficiency of annealing and extension.

4.2.1 Nature of starting material

Only small quantities (as little as 5 ng – 0.12 pg) of DNA are required for PCR-RAPDs. The genomic DNA must, however, be free of compounds that may inhibit amplification, such as residual RNA or protein, which compete with the primer for binding sites (Castiglione *et al.*, 1993), or EDTA, phenol and other detergents that may be present from the extraction process (Taylor, 1991). Furthermore, the target DNA must be fully denatured at temperatures of 92 – 95 °C, and intact over the length that is to be amplified in order that amplification products are of appropriate length (Taylor, 1991).

4.2.2 Efficiency of annealing and extension

The first step in the formation of amplification products is the annealing of the denatured single-stranded DNA with a primer. For optimum amplification, the primers must be 8 – 20

nucleotides in length, as shorter or longer primers give products which are difficult to interpret (Welsh and McClelland, 1994). In order that primers bind only to the target DNA and not to one another, they should also not be self-complementary or complementary to each other, (Taylor, 1991). The number of amplification products is also inversely correlated with an increase in the temperature at which annealing occurs, and is affected by the concentration of magnesium ions in the buffer (Williams *et al.*, 1990).

Extension of DNA following priming, is dependent on the appropriate concentration of deoxynucleside triphosphates (dNTPs), which provide the nucleotides for DNA synthesis. This concentration should be high enough to be in excess after each cycle of reaction so that the synthesis steps may be repeated until the desired amount of amplification is achieved; but not so high as to bind to $MgCl_2$ and reduce the concentration of Mg^{2+} available (Taylor, 1991).

The enzyme employed in PCR-RAPDs is isolated from the thermostable bacterium *Thermus aquaticus (Taq)*, which can tolerate incubation temperatures of up to 95 °C (Saiki *et al.*, 1988). While this has the advantage that the enzyme is active at the temperatures optimal for primer annealing and DNA synthesis (Taylor, 1991), the commercially produced *Taq* polymerase lacks the exonuclease 'proof-reading' function of DNA polymerase 1, and therefore the fidelity of the polymerase chain reaction catalysed by this enzyme may be low. In order to minimise the mistakes that may arise during DNA synthesis, all four dNTPs are used at equal concentrations, and the total concentration of dNTPs is kept as low as possible, since high dNTP concentrations may increase the error rate by driving the reaction in the

direction of DNA synthesis, thereby creating more opportunity for error. Similarly, the PCR reaction periods should be kept as short as possible in order to minimise the time available for mis-replication (Eckert and Kunkel, 1993).

Another factor that affects the efficiency of annealing and extension is the pH of the reaction mixture (Cheng *et al.*, 1994), with a reported 9-fold increase in error rates if the pH changes by just 1 unit (Cline *et al.*, 1996)

4.2.3 Visualisation of amplification products

Once DNA has been amplified, the products are separated on an electrophoretic gel, which is typically composed of either polyacrylamide (for separation of fragments of lengths between 6 – 1000 bp) or agarose (for fragments 70 – 800 000 bp long) or both (Sambrook *et al.*, 1989). In order to minimise the heat generated by the current passing through the gel (which, in turn, causes a distortion of DNA bands), the concentration of the gel is kept low and the gel itself may be submerged in a buffer during electrophoresis (Sealy and Southern, 1990), or the voltage and/or running time is reduced. However, the optimal voltage and running time depend also on the size of the fragments and the level of resolution desired. Generally, at low voltage, the migration of linear DNA is proportional to the voltage applied, but as the voltage is increased, the mobility of the higher molecular mass fragments is increased differentially (i.e. the larger fragments tend to 'catch up' with the smaller fragments). Thus, the effective range of separation is decreased as the voltage is increased (Micklos and Freyer, 1990). For best resolution, according to those authors, 0.8% agarose

gels are run at no more than 0.5 V per mm (as determined by the distance between the electrodes).

In order to visualise the RAPD fragments on the gel, a tracking dye (the most commonly used ones being bromophenol blue and xylene cyanol) may be introduced in the sample wells along with the DNA (Sambrook *et al.*, 1989), or the gel may be stained with either ethidium bromide and viewed under ultraviolet light (agarose gels [Sealy and Southern, 1990]) or with silver nitrate (polyacrylamide gels [Blum *et al.*, 1987]).

Once conditions for polymerase chain reaction and gel electrophoresis are optimized, PCR-RAPDs provides a powerful, yet relatively inexpensive, tool for genetic analysis.

4.3 Objective.

The objective of this study was to assess, using PCR-RAPDs, the effect of the cryopreservation of seeds on the genetic fidelity of *Warburgia salutaris*.

4.4 Materials and Methods

4.4.1 Acquisition of leaf samples for DNA extraction

For this study, seedlings from seeds obtained from natural populations in Tanzania (Chapter 2, section 2.4.1) were used. As it was not feasible to extract DNA from the same seed before, and after, cryopreservation (discussed in section 4.5.3, below), ten individual seedlings were randomly identified from each of three populations: seedlings from seeds

that were sown without dehydration or cryopreservation; seedlings from seeds that were dehydrated only (not frozen); and seedlings from seeds that were dehydrated and cryopreserved in liquid nitrogen. From each of these seedlings, *c.* 2 g of young leaves were harvested and kept overnight in the dark at 6 °C, in order to reduce the accumulation of carbohydrate generated by photosynthesis.

4.4.2 DNA extraction and quantification.

DNA was extracted using the hot CTAB method developed by Doyle and Doyle (1987), with minor modifications, as follows:

a) Tissue samples were mixed with 2 mg polyvinylpyrrolidone (in order to prevent polysaccharides, secondary metabolites and polyphenolics from co-isolating with the DNA [Koonjul *et al.*, 1999]), and ground to a fine powder in liquid nitrogen using a mortar and pestle.
b) 2 X CTAB extraction buffer (Appendix B), that had 2-ß-mercaptoethanol (BME) added to a final concentration of 0.2%, was heated in a water bath at 60 °C for 5 min. Five hundred µl of this solution were then added to the ground leaf material in a 1.5 ml microcentrifuge tube, gently mixed, and the mixture incubated in the water bath at 60 °C for 45 min.
c) An equal volume of CI (Appendix B) was added to the mixture, and the tubes gently inverted for 10 min to extract the DNA.
d) The mixture was centrifuged at 10 000 g for 10 min.

e) The upper aqueous phase was transferred to a sterile Eppendorf tube using a wide-bore micropipette.

f) Steps c – e were repeated.

g) The DNA was precipitated by addition of 1000 µl of 95 % ethanol. This was left to precipitate overnight at 4 °C to increase the yield of DNA.

h) The DNA was pelleted by centrifuging for 3 min at 10 000 g

i) The ethanol was decanted and the DNA pellet dried for approximately 15 min (until the ethanol had just evaporated) in a vacuum desiccator.

j) The pellet was re-suspended in 100 µl TE buffer (Appendix B) for 1 h at 4 °C, after which 100 µl of 3 *M* sodium acetate and 250 µl of 95 % ethanol were added.

k) The solution was left to precipitate overnight at 4 °C.

l) The solution was centrifuged for 5 min at 10 000 g and the ethanol was decanted. 1 000 µl of 70 % ethanol were added to wash the pellet, which was then re-centrifuged for 2 min at 10 000 g, and the ethanol decanted.

m) The pellet was then dried in a vacuum desiccator until all visible traces of ethanol had evaporated.

n) The pellet was re-dissolved in 200 µl of 1 X TE buffer at 4 °C, and the purity and concentration of the DNA sample determined.

4.4.3 Estimation of the purity and concentration of DNA

The purity and concentration of the DNA were determined spectrophotometrically. TE buffer, made with sterile milliQ water and autoclaved, was used to dissolve the DNA and calibrate the spectrophotometer. The absorbance spectrum of the DNA samples was

checked to ensure that DNA was present: nucleic acids absorb UV light at 260 nm, with a characteristic peak. If an unusual spectrum was obtained, the sample was discarded, and DNA re-extracted from the sampled plant.

The purity was assessed by calculating the ratio of the absorbance at 260 nm to that at 280 nm (A_{260}:A_{280}), as a ratio of 1:1.8 corresponds to a pure preparation of DNA; and the DNA concentration determined based on the relationship that an optical density of 1 at 260 nm corresponds to a concentration of 50 μg μl^{-1} of double-stranded DNA (Sambrook *et al.*, 1989)

In order to check for high molecular weight DNA, 30 μl of each DNA sample, with 7 μl of loading dye (Appendix B), were electrophoresed on a 1 % agarose gel (described below). At a given voltage, high molecular weigh DNA migrates to a lesser extent than low molecular-weight DNA, and forms a sharp, discrete band (a smear indicates broken or degraded DNA). By comparing the fluorescence of the bands obtained with that of λ-DNA of known concentration, the concentration of the DNA in the sample was calculated and checked against that indicated by spectrophotometry.

4.4.4 Agarose gel electrophoresis

One perecent (w/v) agarose in 1X TBE buffer was prepared (mixture was boiled until agarose was fully dissolved), and 30 μl ethidium bromide added to the cooled solution when barely warm. This was then poured into the gel apparatus, combs inserted, and left to set at room temperature. The apparatus was then filled with 1X TBE buffer to submerge the gel

and wells. 30 μl of sample DNA was dispensed into sterile 0.5 ml Eppendorf tubes, and 7 μl of loading dye added and thoroughly (but gently) mixed. The total content of each PCR tube was then loaded into the gel wells, and electrophoresed at 100 V for 2 h at room temperature.

4.4.5 Amplification of DNA

Once it had been established that the DNA was of high molecular weight and the concentration and purity had been determined, PCR reactions were run following the protocol used by Bishop (1995). The reactants necessary for amplification were prepared as a master mix, in concentrations that had been previously determined empirically. The master mix consisted of: Stoffel® buffer, 25 m*M* $MgCl_2$, 10 U μl^{-1} *Taq* polymerase (all supplied in kit form [Applied Biosystems, Foster City, CA, USA]); a dNTP mix that consisted of equal amounts of dATP, dGTP, dCTP, and dTTP (100 m*M* sodium salt solutions, supplied by Roche Diagnostics GmbH, Mannheim, Germany); oligonucleotide primer (Appendix C, supplied by Operon Technologies, Alameda, CA, USA); and sterile milliQ water. The quantity of master mix made depended on the number of reactions (DNA samples) to be run, as illustrated in Table 4.2. The master mix was prepared using sterile containers, and in a preparation room into which no DNA samples were taken, in order to minimize cross-contamination of samples. All components were stored at -80 °C, thawed, kept on ice during the preparation of the master mix, and re-frozen on completion.

The total volume for each PCR reaction was 24 μl, of which 16 μl comprised the master mix, and 8 μl comprised DNA sample and water in varying proportions, depending on the

quantity of DNA needed for the reaction. In order to determine the optimum amount of DNA for PCR in this study, 20 ng μl^{-1} DNA stock solutions were prepared from the original DNA samples, and either 1 or 8 µl (requiring 7 or 0 µl of water, resp.) of representative samples used for the reaction.

Table 4.2 Composition of the PCR master mix.

No. of reactions	**1-3**	**4-5**	**6-8**	**10**	**12**	**16**	**20**	**40**
Water (µl)	23.3	38.78	64.2	77.75	96.3	124.4	155.5	311
Stoffel buffer (µl)	7.2	12	19.2	24	28.8	38.4	48	96
dNTPs (µl)	0.7	1.2	1.9	2.4	2.88	3.84	4.8	9.6
$MgCl_2$ (µl)	11.5	19.2	30.7	38.2	46.1	61.5	76.4	152.8
Primer (µl)	4.8	8.04	12.8	16.1	19.6	25.8	32.2	64.4
Taq polymerase (µl)	0.5	0.8	1.3	1.6	2	2.6	3.2	6.4
Total (µl)	**48**	**80.4**	**128.1**	**160.05**	**192.5**	**256.5**	**320.1**	**640.2**

The reaction mixture was put into thin-walled Eppendorf PCR tubes, overlaid with mineral oil (to avoid evaporation from the reaction mixture), and placed in an ESU University® thermal cycler for the amplification reaction. The cycler was programmed for 42 cycles, each consisting of a denaturation step at 94 °C (lasting for 180 seconds in cycle one, and 120 seconds in cycles 2-42), followed by an annealing step at 39 °C (for 120 seconds in all 42 cycles), and an elongation step at 72C (lasting 180 seconds in cycles 1 - 41, and 420

seconds in the 42nd and last cycle to ensure that primer extension reactions proceeded to completion).

4.4.6 Polyacrylamide gel electrophoresis (PAGE)

In order to separate the amplification products, non-denaturing polyacrylamide gel electrophoresis was used to separate amplification products into discrete bands according to molecular weight. The following method, adapted from Sambrook *et al.* (1989) was used:

a) A Hoefner Scientific Instruments vertical gel apparatus was used. The glass plates and spacers were thoroughly cleaned with detergent, and then wiped with 70% methanol and the glass plates set up in the casting stand with the spacers facing each other between the two glass plates.

b) Eight percent polyacrylamide gel containing TEMED (N,N,N',N'-tetramethylenediamine) (Appendix B) was poured between the glass plates and spacers. Gel combs were inserted, and the set-up left at room temperature for 1 h for the acrylamide to polymerise.

c) The wells of the gel were then flushed out with 1X TBE buffer (Appendix B)

d) Seven microlitres of gel-loading dye (Appendix B) was mixed with 15 μl of amplified DNA sample in 0.5 ml Eppendorf tubes and the total contents introduced into the sample wells using a microcapillary pipette.

e) The gel was placed in an electrophoresis tank which was then filled with 1XTBE buffer, and electrophoresis run at 120 V for 2 h, or until the loading dye could be seen within 20 mm of the bottom edge of the gel.

f) Once electrophoresis was complete, the gel was removed from between the glass plates and placed into 70% methanol as a fixative, before being stained using the non-ammoniacal silver staining procedure (described below).

4.4.7 Staining of polyacrylamide gels

Following electrophoresis, the gels were placed in a plastic staining trays on an orbital shaker, and taken through the following successive changes in the indicated solutions:

a) Fixative (70% methanol) for 10 min.

b) Oxidising solution (0.5 g potassium dichromate + 100 µl conc. HNO_3 + 500 ml sterile milliQ water) for 10 min.

c) Sterile milliQ water for 3 x 5 min

d) 100 ml sterile milliQ water + 50 µl conc. HNO_3 for 5 min. If the gel was still yellow, this step was repeated.

e) 0.2 % $AgNO_3$ solution for 25 min in the dark (container covered with aluminium foil)

f) Developer (Appendix B). When the developer turned dark, it was poured out and replenished. This was repeated until bands appeared clearly resolved.

g) 5% acetic acid for 3 – 5 min to stop reaction, followed by distilled water.

h) Blotted dry, sealed with clear plastic film, and photographed, after which the gels were stored at 4 °C.

4.4.8 Photography

Agarose or polyacrylamide gels were photographed digitally, immediately following electrophoresis, using a UVItec® camera and gel documentation system (Uvitec Limited, Cambridge, UK).

4.4.9 PCR-RAPD data analysis

Amplified fragments were manually scored: the presence of a specific amplified DNA band was scored as a '1', while the absence of such a band was scored as a '0'. Two scorers were used to introduce objectivity to the process. Bands neither clearly present nor absent, were scored as a '9', indicating that the data were not available. The data were then analysed with the computer programme, NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System [Rohlf, 1989]). The similarity between individuals and populations was measured using SIMQAL (Similarity for Quantitative data Algorithm), and the similarities clustered using the SAHN cluster analysis. The resulting relationships were portrayed in the form of a phenogram. Nucelotide diversity within populations and nucleotide divergence between populations were calculated using the RAPDIP computer programme (Clark and Lanigan, 1993).

4.5 Results and Discussion

4.5.1 DNA extraction and quantification.

The concentration of DNA extracted from different seedlings, determined by spectrophotometry, ranged from 20 to 1015 ng μl^{-1} (Table 4.3). This variation was also reflected by the varying brightness of the bands attained on agarose gels after electrophoresis (Fig 4.3). The brightness, determined by the UVIdoc® computer application (Uvitec Limited, Cambridge, UK), was directly correlated to the concentration as determined by spectrophotometry. However, by comparing the brightness of extracted DNA

samples with that of λ-DNA of known concentration, it was apparent that the results obtained by spectrophotometry over-estimated concentration of high molecular-weight DNA in the samples, probably as a result of the contribution of contaminants such as polysaccharides, polyphenolics, or secondary metabolites (Scott and Playford, 1996), to the sample absorbance.

Table 4. 3 Concentration of DNA in the extracts from leaves of seedlings raised from 'control' seeds (T1C – T20C), dehydrated seeds (T1D-T20D) and cryopreserved seeds (T1F – T20F). The concentrations were determined spectrophotometrically, on the basis that an optical absorbance of 1 at 260 nm corresponds to a DNA concentration of 50 ng μl^{-1}.

Control		**Dehydrated**		**Dehydrated and frozen**	
Sample	DNA concentration ng μl^{-1}	Sample	DNA concentration ng μl^{-1}	Sample	DNA concentration ng μl^{-1}
T1C	50	T1D	120	TIF	240
T2C	210	T4D	1015	T3F	215
T5C	135	T6D	365	T7F	170
T6C	405	T8D	120	T6F	795
T11C	475	T10D	380	T8F	60
T15C	350	T12D	475	T10F	650
T16C	150	T13D	435	T15F	495
T17C	60	T16d	160	T16F	410
T19C	390	T17D	850	T19F	555
T20C	430	T20D	285	T20F	260

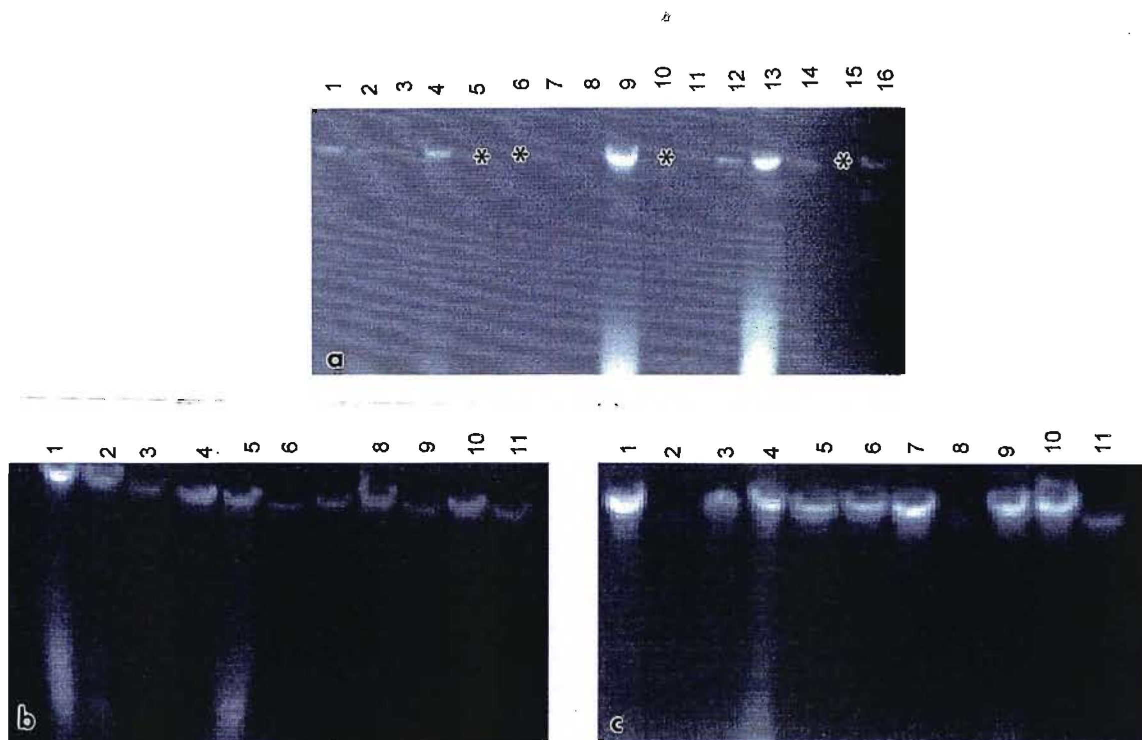

Fig. 4.3 Ethidium bromide-stained 1% (w/v) agarose gels illustrating the presence of high 1molecular weight DNA: (a) The samples extracted had different DNA concentrations, as illustrated by the varying brightness of the bands. Where no bands were present, (wells with asterisk), DNA was re-extracted from those samples. Lanes 1-5, control samples; lanes 6-10, dehydrated samples; lanes 11-15, cryopreserved samples; lane 16, molecular weight marker. (b) High molecular weight DNA from some seedlings from 'control' seeds (lanes 1-5), and from dehydrated seeds (lanes 6-10). Lane 11 contains λ-DNA. (c) High-molecular weight DNA from the 10 seedlings raised from cryopreserved seeds (lanes 1-10), with λ-DNA in lane 11. The smears downstream of the gels emanate from RNA.

4.5.2 Amplification of DNA

Preliminary results indicated that DNA amplification was attained with 20 – 80 ng template DNA in the PCR reaction mixture (Lamb[1], pers. comm.), and therefore all the extracted DNA samples were diluted to a working concentration of 20 ng μl^{-1}, from DNA solutions of this concentration 1 and 8 µl were used for PCR, corresponding to 20 and 160 ng template DNA, respectively, and amplification tested with Primer OPA 03.

The reaction produced consistent bands (Fig 4.4), indicating that the PCR worked equally well with amounts of genomic DNA ranging from 20 – 160 ng in the reaction mixture. As the use of 8 µl of genomic DNA solution did not need the addition of water (hence reducing handling), it was decided to use this volume in all subsequent PCR reactions. This volume of solution corresponded to 6.6 ng μl^{-1} DNA (160 ng of genomic DNA in 24 µl of reaction mixture). This is within the range of DNA concentration suggested to be optimum for PCR, which is 1-10 ng μl^{-1} for eukaryote genomes (Finnzymes, 2002).

Electrophoresis of amplification products resulted in a total of 104 scorable fragments produced by eight primers, all of which were utilised in the analysis. Some workers report scoring of only a subset of primers, particularly those which show the greatest polymorphism (e.g. Connolly *et al.*, 1994; Moukadiri *et al.*, 1999; Edwards, 2000), which may exaggerate the magnitude of the calculated DNA diversity. Examples of the bands obtained with some of the primers are illustrated in Figures 4.5 and 4.6.

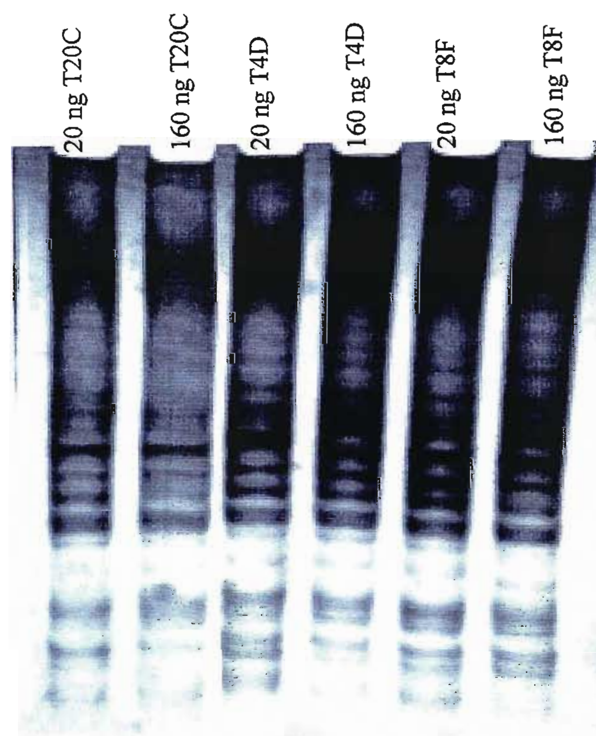

Figure 4.4.Amplification products electrophoresed on an 8% polyacrylamide gel, illustrating the effect of varying the amount of genomic DNA in the reaction mixture from 20 to 160 ng, i.e. from 0.8 to 6.6 ng DNA μl^{-1} of reaction mixture. The bands were consistent over this range of DNA concentration.

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[1] J.M. Lamb, School of Life and Environmental Sciences, University of Natal, Durban, 4041, South Africa.

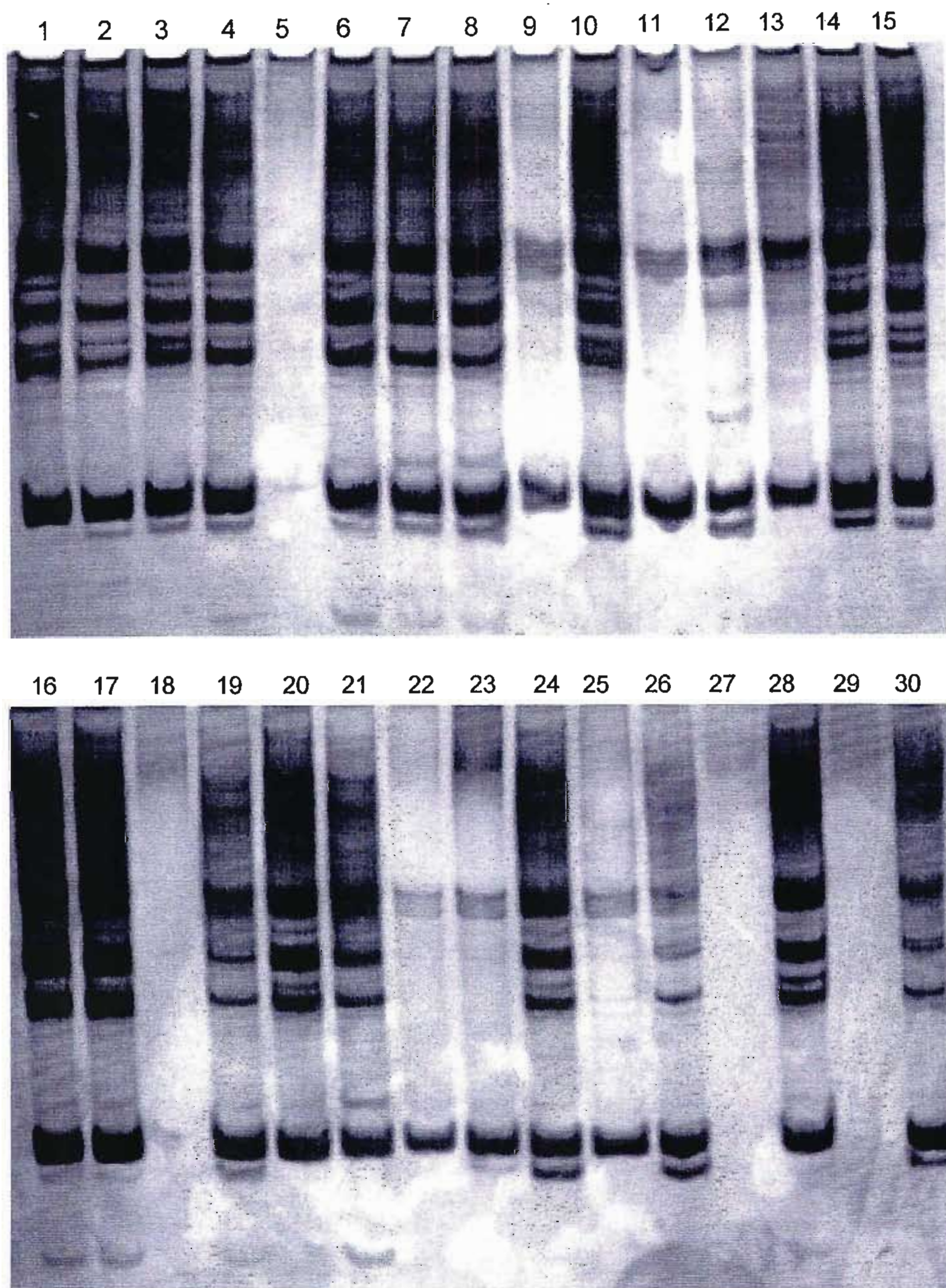

Fig 4.5 RAPD profiles obtained following amplification, with primer OPA 06, of DNA from seedlings raised from 'control' seeds (lanes 1-10), dehydrated seeds (lanes 11-20) and cryopreserved seeds (lanes 21-30). No amplification products could be visualized for samples in lanes 5, 18, 27 and 29. For such samples, the concentration of DNA needed for amplification was re-optimised.

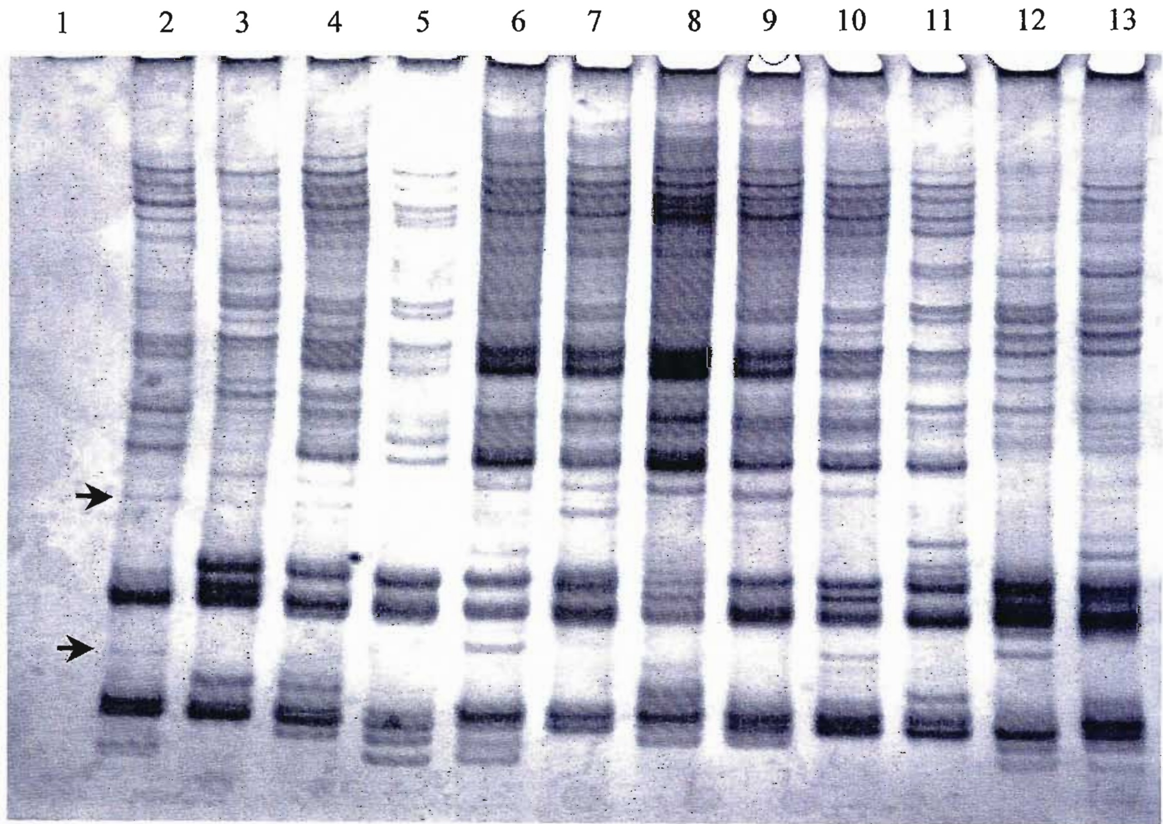

Figure 4.6. PCR-RAPD products generated using primer OPA09. Lane 1, distilled water (negative control); Lanes 2 – 5, seedlings from freshly-harvested, untreated seeds; lanes 6 - 9, seedlings from dehydrated seeds; Lanes 10 - 13, seedlings cryopreserved seeds. Arrows indicate polymorphic fragments.

Under optimum PCR conditions, the number of loci amplified per primer varies, with some workers reporting averages as low as two (Aronen *et al.*, 1999) to others observing an average of 90 (Borowsky and Vidthayanon, 2001). Lower levels of amplification require larger numbers of primers, as ideally, the number of markers needed for studies of aspects such as genetic integrity, should be equal to, or larger than, that required to obtain a saturated linkage map of the species (Isabel *et al.*, 1993). This number is currently unknown for many indigenous plant species such as *Warburgia salutaris*, but is estimated to be larger than 200 – 300 in clonal conifer material (Carlson *et al.*, 1991; Neale and Williams, 1991). Hence, the number of fragments obtained and used in this study was not ideal. However, it was within the range currently applied to study plant genetic diversity (e.g. Isabel *et al.*, 1991; Aronen *et al.*, 1999; Moukadiri *et al.*, 1999).

Of the 10 primers used in this study, two (OPA 02 and OPA 18) did not yield any amplification products. While some workers report amplification by all the primers tested (e.g. Nagaraju *et al.*, 2001), others have reported amplification by only a proportion of the tested primers (e.g. 81% [Menéndez *et al.*, 1997]; 85% [Wu *et al.*, 2002]). This implies that some of the tested primers may have no homology with the DNA of the species investigated.

4.5.3 Analysis of amplified polymorphic DNA

Among the bands scored, polymorphisms were detectable within and between the three treatment populations under study: seedlings from freshly-collected, non-dried seeds; seedlings from seeds that were dehydrated only; and seedlings from seeds that were

cryopreserved in liquid nitrogen. These polymorphsms were used to generate a phenogram of all individuals (from all three treatments), illustrated in Fig. 4.8. The percent similarities among individuals ranged from about 95% to 65%, and there was variation among individuals within all treatments. This variation was unrelated to either dehydration or cryopreservation, as there was no clustering in the phenogram according to treatments. Clustering has been used to discriminate genetically between closely related populations (e.g. Hébert *et al.*, 2000; Nagaraju *et al.*, 2001; Germain *et al.*, 2002).

The amplification products in this study were also utilised in calculating nucleotide diversity within treatments and nucleotide divergence between treatments. Nucleotide diversity represents the average number of nucleotide differences per site between two randomly-chosen DNA sequences in a population, and is an efficient assessment of natural populations (Nei and Li, 1979). RAPDs are particularly useful in estimating nucleotide diversity because they sample large numbers of unlinked or loosely-linked sites scattered throughout the genome. The three treatment groups exhibited varying nucleotide diversities: 0.45 ± 0.08% for seedlings from freshly-sown seeds; 0.55 ± 0.09% for seedlings from dehydrated seeds; and 0.32 ± 0.06 % for seedlings from cryopreserved seeds. These levels of diversity are generally higher than those in some animal species surveyed (aphids, grasshoppers and leeches), in which the average nucleotide diversity was 0.0135 ± 0.0032% (Borowsky, 2000). This may be because in plants, a large number of mating systems (ranging from pure self-pollination to pure out-cross pollination, with a high variation among genera and species [Armbruster *et al.*, 2002]) produces a richer variety of population genetic structures than in animals, with wind-pollinated plants

exhibiting the highest levels of heterozygosity followed by animal-pollinated plants (the category to which *W. salutaris* belongs, based on floral morphology [Dyer, 1975]), and then self-pollinated plants (Hamrick and Godt, 1990). In some cultivated plant populations, lower nucleotide diversity has been observed (e.g. 0.007% in *Arabidopsis thaliana* [Purugganan and Sudith, 1998], and 0.0049% in *Pinus sylvestris* [Dvornyk *et al.*, 2002]). The relatively high nucleotide diversity observed in the present study may be a consequence of sampling from natural populations (the seeds were collected from natural stands in Tanzania [Chapter 2, section 2.4.1). It may also imply that *W. salutaris* is outcrossing, with partial or complete self-incompatibility (no information is available on the mating system of this species), as such species have higher nucleotide diversity (Liu *et al.*, 1999).

In assessing the effect of cryopreservation on genetic fidelity, it would have been ideal to use tissue from individual seeds before, and after, cryopreservation. However, this was impossible as the excision of tissue samples was found to be lethal to the seeds. Hence, nucleotide divergence (a measure of the genetic distance between populations [e.g. Brown *et al.*, 1994]) among the seedlings obtained from the three treatments (control, dried, and cryopreserved) were compared with nucleotide divergence within the populations. It was significant that the nucleotide divergence between the populations of seedlings (ranging from 0.08% to 0.17%) was much lower than the nucleotide diversity within them (Fig. 4.9).

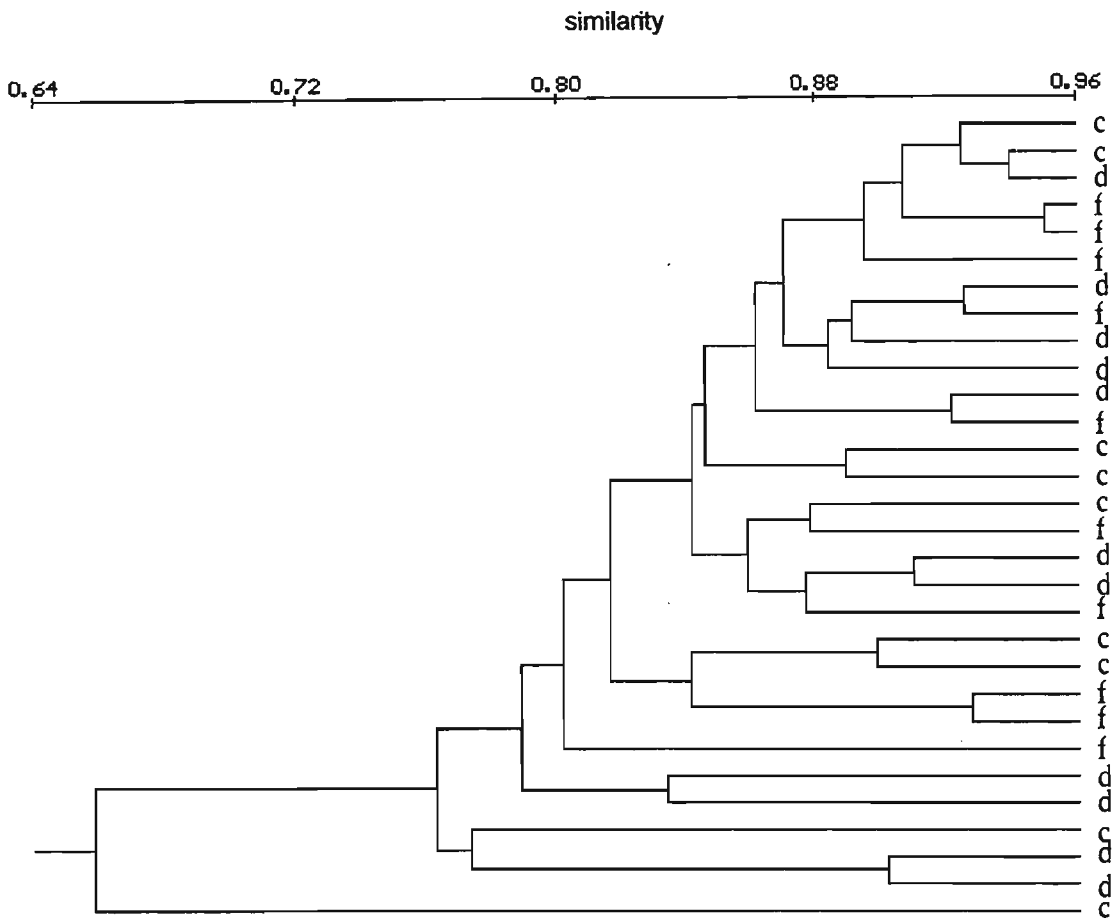

Figure 4.7 Phenogram showing the absence of clustering according to treatments, in the genetic relationships within the seedlings obtained from three seed-treatments: c, control; d, dehydrated; f, dehydrated and frozen.

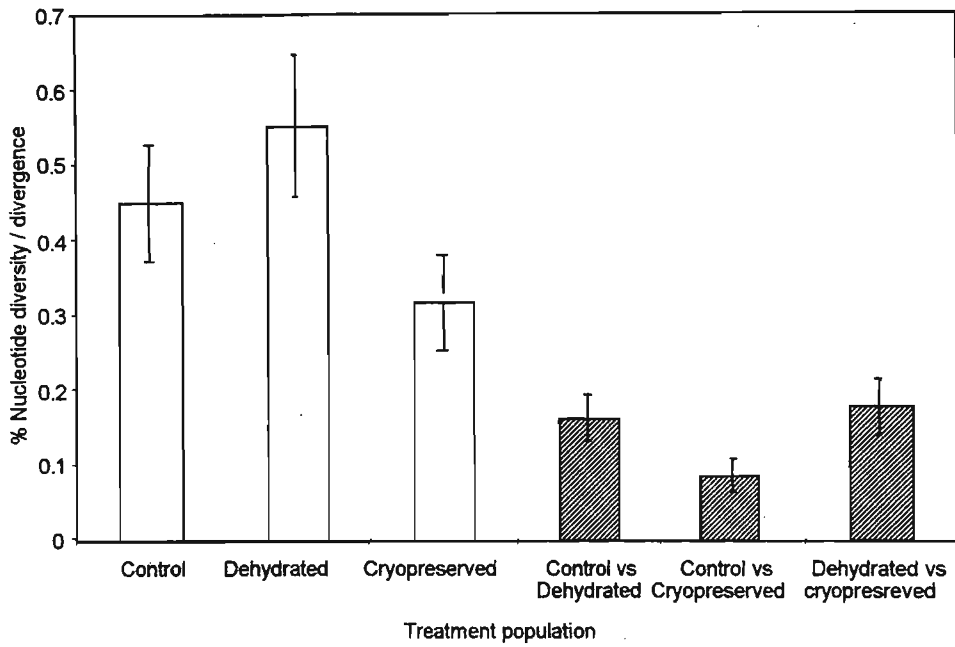

Figure 4.8. Nucleotide diversity within the populations of seedlings obtained from control (freshly-harvested, untreated), dehydrated, and cryopreserved seeds (clear bars); and nucleotide divergence between the populations (hatched bars).

Thus, there was higher variation within each treatment, than between the treatments, showing that neither dehydration nor cryopreservation introduced significant genetic variability within the seeds.

4.6 Conclusion

Warburgia salutaris, one of the most highly utilised medicinal plants in Africa, is virtually extinct in the wild in countries such as South Africa, with only depauperate, genetically-narrow, cultivated populations remaining. The resulting necessity for the conservation of the germplasm of this species can be met via cryopresrevation of the seeds, which are otherwise unstorable by the conventional means of low temperature and reduced water content. However, because cryopreservation could result in genetic aberrations in the plants established from the cryo-stored seeds, it was imperative to assess the effect of seed cryopreservation on the genetic integrity of this species. This study has shown that neither dehydration alone, nor dehydration and cryopreservation, have any effect on the genetic fidelity of seedlings obtained from seeds thus treated. Cryopreservation may therefore be used as an effective means of germplasm and genetic conservation of *Warburgia salutaris*, and possibly other endangered / over-utilised indigenous medicinal plants.

Similarity matrix for all samples used in this study.

The similarity ranged from 0.609 (between samples T12D and T13D) to 0.951 (between samples T1F and T8F)

	T11C	T15C	T16C	T17C	T19C	T1C	T20C	T2C	T5C	T6C	T10D	T12D	T13D	T16D	T17D	T1D	T20D	T4D	T6D	T8D	T10F	T15F	T16F	T19F	T20F	T3F	T6F	T7F	T8F	TIF
T11C	1.000																													
T15C	0.923	1.000																												
T16C	0.694	0.653	1.000																											
T17C	0.853	0.913	0.653	1.000																										
T19C	0.887	0.848	0.671	0.814	1.000																									
T1C	0.848	0.897	0.589	0.889	0.771	1.000																								
T20C	0.800	0.756	0.706	0.718	0.843	0.743	1.000																							
T2C	0.860	0.786	0.765	0.767	0.899	0.699	0.835	1.000																						
T5C	0.860	0.865	0.625	0.804	0.842	0.835	0.812	0.822	1.000																					
T6C	0.879	0.875	0.662	0.857	0.830	0.885	0.786	0.793	0.843	1.000																				
T10D	0.836	0.851	0.671	0.793	0.902	0.792	0.821	0.852	0.889	0.910	1.000																			
T12D	0.767	0.743	0.782	0.789	0.764	0.750	0.813	0.833	0.803	0.845	0.831	1.000																		
T13D	0.776	0.831	0.489	0.761	0.800	0.765	0.700	0.710	0.794	0.769	0.754	0.609	1.000																	
T16D	0.723	0.750	0.646	0.746	0.721	0.771	0.733	0.783	0.734	0.813	0.758	0.902	0.655	1.000																
T17D	0.831	0.865	0.661	0.818	0.734	0.815	0.729	0.743	0.805	0.824	0.736	0.724	0.833	0.679	1.000															
T1D	0.926	0.939	0.617	0.881	0.868	0.855	0.746	0.814	0.909	0.841	0.825	0.707	0.792	0.696	0.862	1.000														
T20D	0.875	0.902	0.699	0.821	0.828	0.866	0.750	0.831	0.842	0.883	0.848	0.795	0.786	0.791	0.886	0.912	1.000													
T4D	0.856	0.886	0.632	0.859	0.798	0.841	0.729	0.774	0.856	0.865	0.830	0.794	0.769	0.779	0.844	0.866	0.894	1.000												
T6D	0.864	0.862	0.644	0.811	0.816	0.793	0.716	0.811	0.853	0.851	0.882	0.808	0.783	0.731	0.833	0.896	0.909	0.821	1.000											
T8D	0.848	0.874	0.721	0.867	0.766	0.844	0.738	0.793	0.802	0.878	0.784	0.824	0.742	0.825	0.851	0.825	0.874	0.878	0.821	1.000										
T10F	0.812	0.800	0.652	0.800	0.782	0.761	0.709	0.793	0.814	0.798	0.768	0.838	0.672	0.700	0.746	0.857	0.818	0.847	0.778	0.765	1.000									
T15F	0.857	0.866	0.581	0.821	0.793	0.775	0.671	0.795	0.786	0.843	0.827	0.790	0.763	0.768	0.857	0.881	0.920	0.857	0.921	0.833	0.772	1.000								
T16F	0.867	0.898	0.714	0.854	0.796	0.870	0.798	0.798	0.843	0.886	0.843	0.812	0.738	0.746	0.877	0.891	0.926	0.889	0.863	0.900	0.765	0.847	1.000							
T19F	0.872	0.857	0.738	0.814	0.854	0.787	0.790	0.871	0.787	0.826	0.812	0.788	0.698	0.767	0.767	0.903	0.835	0.773	0.802	0.831	0.747	0.790	0.802	1.000						
T20F	0.879	0.875	0.662	0.813	0.851	0.805	0.798	0.837	0.878	0.844	0.865	0.778	0.818	0.754	0.800	0.859	0.871	0.856	0.884	0.809	0.839	0.831	0.854	0.779	1.000					
T3F	0.872	0.821	0.727	0.826	0.809	0.822	0.775	0.831	0.770	0.837	0.824	0.843	0.705	0.763	0.778	0.813	0.867	0.788	0.835	0.849	0.778	0.835	0.847	0.928	0.805	1.000				
T6F	0.842	0.824	0.690	0.830	0.857	0.790	0.795	0.820	0.851	0.883	0.870	0.819	0.775	0.818	0.769	0.836	0.847	0.826	0.835	0.839	0.816	0.793	0.815	0.831	0.851	0.795	1.000			
T7F	0.888	0.923	0.552	0.852	0.763	0.790	0.690	0.753	0.813	0.821	0.816	0.661	0.783	0.682	0.781	0.887	0.854	0.859	0.829	0.818	0.806	0.833	0.816	0.795	0.846	0.784	0.768	1.000		
T8F	0.916	0.892	0.625	0.842	0.837	0.817	0.747	0.820	0.853	0.840	0.826	0.778	0.786	0.791	0.813	0.870	0.857	0.883	0.848	0.872	0.852	0.854	0.639	0.800	0.840	0.811	0.837	0.867	1.000	
TIF	0.923	0.936	0.571	0.859	0.813	0.861	0.714	0.792	0.857	0.862	0.840	0.741	0.818	0.797	0.800	0.906	0.888	0.922	0.841	0.895	0.861	0.871	0.868	0.806	0.844	0.811	0.823	0.911	0.951	1.000

APPENDIX B:

Solutions used for DNA extraction and electrophoresis (including staining).

DNA extraction

CTAB extraction buffer

20 g CTAB (hexadecyltrimethylammonium bromide)

280 ml 5 *M* HCl

50 ml 2 *M* Tris-HCl, pH 8.0

40 ml 0.5 *M* EDTA

0.5 M EDTA (ethylenediaminetetraacetic acid - disodium salt)

18.61 g disodium EDTA dissolved by heating gently in 100 ml distilled water.

pH adjusted with NaOH to pH 8.0

CI (chloroform:isoamyl alcohol)

Chloroform and isoamyl alcohol in the ratio 24:1

10 X Tris-EDTA (TE) buffer (pH 7.5) made in 1 L distilled water

0.01 *M* Tris-HCl

0.001 *M* EDTA

Electrophoresis and staining

30 % acrylamide solution

29 g Acrylamide

1 g N, N-methylenebisacrylamide

Distilled water to 100 ml

Solution heated to 37 °C to dissolve

100 ml of 8% polyacrylamide gel

6.65 ml of 30 % acrylamide

15.65 ml distilled water

2.5 ml 10X TBE

0.175 ml of 10 % ammonium persulphate (prepared freshly)

10 µl TEMED (N,N,N',N'-tetramethylenediamine)

10 % (w/v) ammonium persulphate

ammonium persulphate: 1 g

distilled water: 10 ml

10X Tris-borate EDTA (TBE) buffer (pH 8.3)

108 g Tris base

55 g Boric acid

9.3 g disodium EDTA

Add distilled water to 1 L

Gel loading dye

0.04 g bromophenol blue

12 g sucrose

20 ml 0.5 X TBE

Developer (prepared fresh with distilled water at 4 °C)

250 ml of 3 % anhydrous Na_2CO_3

125 μl of 40 % formalin

Appendix C: Primers used and their sequences

Primer	sequence
OPA 01	5'CAGGCCCTTC 3'
OPA 02	5'TGCCGAGCTG 3'
OPA 03	5'AGTCAGCCAC 3'
OPA 04	5'AATCGGGCTG 3'
OPA 06	5'GGTCCCTGAC 3'
OPA 08	5'GTGACGTAGG 3'
OPA 09	5'GGGTAACGCC 3'
OPA 12	5'TCGGCGATAG 3'
OPA 13	5'CAGCACCCAC 3'
OPA 18	5'AGGTGACCTG 3'

CHAPTER 5:

Aspects of post-shedding seed physiology and cryopreservation of the germplasm of three medicinal plants indigenous to Kenya and South Africa: an overview and future prospects

5.1 Introduction

The current state of the world's biological diversity is one of sustained quantitative and qualitative decline, with such decline being most severe in the diversity-rich tropical areas (WWF, 1999). In these areas, one of the most collectively vulnerable components of diversity comprises medicinal plants, utilised by both the pharmaceutical industry in the West (e.g. Husain, 1991), and the traditional herbal medicine practice in developing countries (e.g. Mander, 1998). The demand for medicinal plants, coupled with the collapse of traditional sustainable harvesting practices, has led to resource over-exploitation, and has contributed to the extinction of natural populations of many species such as *Mondia whitei* and *Siphonochilus natalensis* in South Africa (Cunningham, 2000); and the current vulnerability of others such as *Ocotea bullata, Bowiea volubilis, Haworthia limifolia, Scilla natalensis, Albizia suluensis* and *Begonia homonyma* (Scott-Shaw, 1999). The species under investigation in this study are utilised medicinally and are either endangered and virtually extinct in the wild in South Africa (*Warburgia salutaris* [Scott-Shaw, 1999]); or, as in the case of *Trichilia dregeana* and *T. emetica*, are not inventoried, and no wild populations could be found locally during the course of this study.

Of the various means available for the conservation of plant germplasm, seed storage offers the most efficient option (Withers, 1988). However, for plants indigenous to tropical Africa, such as those investigated in this study, seed storage is impeded by the almost complete lack of information on seed biology, and the equivocal and often inaccurate or contradicting nature of the meagre information available (e.g. compare Choinsky [1990] and Kioko *et al.* [1998]; Albrecht [1993] and Kioko *et al.* [2000]; and Msanga [1998] and Kioko *et al.* [2003]). Furthermore, many of the medicinal plants indigenous to tropical Africa produce large seeds that possess no adaptation to

chilling temperatures or desiccation, thus falling into the recalcitrant category of seeds (Roberts, 1973).

Research on recalcitrant seeds has been continuing for at least 30 years, and some of the most salient features to emerge have been the behavioural individuality of each recalcitrant seed type, and that not all seed-species fit neatly into distinct categories with clearly-defined boundaries, and consequently a continuum of seed behaviour has been proposed (Pammenter and Berjak, 1994). There are gradations even within the recalcitrant category, with seeds exhibiting high, moderate, or minimal recalcitrance (Farrant *et al.*, 1988; Berjak *et al.*, 1989).

Although recalcitrant seeds are diverse in morphological and physiological characteristics, they all share desiccation-sensitivity, a short lifespan, and unstorability (particularly in the long-term) by conventional means. Consequently, due to the existing need for the conservation of the germplasm of these species, it is necessary to develop and employ techniques for cryogenic storage, as this is presently considered to offer the only viable means of the long-term preservation of recalcitrant-seed germplasm.

5.2 The contribution of this study

This study has explored and revealed some important aspects with regard to the desiccation-sensitivity and storability of the seeds of *Trichilia dregeana*, *T. emetica* and *Warburgia salutaris*; and examined options for long-term cryogenic storage of the germplasm of these species. *Warburgia salutaris* was used as a model to investigate the effect that cryopreservation may have on genetic fidelity.

5.2.1 Desiccation-sensitivity: the critical rôle of drying rate

The response of recalcitrant-seed tissues to desiccation depends on not only the physiology, but also on a number of experimental variables such as the rate of drying and the temperature at which the dehydration occurs (reviewed by Berjak and Pammenter, 2001). In this study, rapid (flash-) drying of embryonic axes of *T. dregeana* and *T. emetica* facilitated their tolerance to water contents as low as 0.16 g g^{-1} and 0.26 g g^{-1}, respectively, in comparison to the loss of viability at axis values of 0.55 g g^{-1} (*T. dregeana*) and 0.42 g g^{-1} (*T. emetica*) when whole seeds of these species were dehydrated over several days (Chapter 2). Furthermore, while cells from flash-dried axes maintained ultrastructural integrity, those from axes of slow-dried seeds were ultrastructurally deranged at much higher water contents. This may be because, at the slower drying rates, the tissues were maintained at intermediate water contents (Vertucci, 1990) for extended periods, favouring the accumulation of harmful products of oxidative processes which take place at such water contents (Pammenter *et al.*, 1994; Smith and Berjak, 1995; Côme and Corbineau, 1996). Furthermore, during slow dehydration over a number of days, the seeds may have progressed further towards germination, becoming more desiccation-sensitive (Farrant *et al.*, 1896). In contrast, rapid dehydration minimised the period during which deleterious aqueous-based reactions occur (Berjak and Pammenter, 1997; Pritchard and Manger, 1998; Walters *et al.*, 2001), resulting in the tolerance of greater extents of desiccation.

In contrast to the increased capacity to withstand desiccation observed in rapidly-dehydrated embryonic axes of *T. dregeana* and *T. emetica*, the seeds of *W. salutaris*, dehydrated over six days tolerated a greater degree of desiccation (to less than 0.1 g g^{-1}) than rapidly-dried embryonic axes, which lost viability when dried to a similar water content within 45 min (Chapter 2). The response

of *W. salutaris* seeds may imply that these seeds possess protective mechanisms such as the deployment of amphipathic molecules (Hoekstra *et al.*, 1997; Golovina *et al.*, 1998) and/or production of appropriate proteins (Buitink, 2000) and/or sugars (Koster and Leopold, 1988), which might be induced during slow dehydration, but for which there is no sufficient time during rapid dehydration. While this response is the norm in desiccation-tolerant orthodox tissues (Kermode and Finch-Savage, 2002), dehydrated seeds of *W. salutaris* suffered desiccation damage if maintained at the reduced water contents (chapter 2), as has been demonstrated for the embryonic axes of other successfully dehydrated, but intrinsically desiccation-sensitive seeds (Pammenter *et al.*, 1998; Walters *et al.*, 2001). These seeds are therefore not orthodox *sensu* Roberts (1973), but may lie towards less recalcitrant end of the continuum of seed behaviour proposed by Pammenter and Berjak (1999), or they may fit somewhere into the intermediate category (*sensu* Ellis *et al.*, 1990).

Even though flash-drying enables recalcitrant tissues to tolerate relatively higher levels of dehydration than does slow drying (as observed for *T. dregeana* and *T. emetica*), there is still a limit to the degree of desiccation that can be withstood by recalcitrant seed tissues, and this is always higher than those tolerated by orthodox seeds or desiccation-tolerant vegetative tissues, the cells of which can become air-dry without losing viability (Hoekstra, 2002). Thus, recalcitrant tissues tolerate flash-drying only to 'intermediate' water contents (Vertucci, 1989; Vertucci and Farrant, 1995), and would lose viability under ambient and refrigerated conditions if stored at those water contents (Walters *et al.*, 2001). The efficiency of flash-drying may also be impeded by differential drying within the embryonic axes, with cortical cells losing water more rapidly than

cells within the procambium cylinder (Wesley-Smith *et al.*, 2001a), and the relatively exposed shoot meristem drying faster than the distal root meristem cells (Chapter 2).

Recent studies have highlighted the degree of desiccation damage that becomes evident during the rehydration phase of flash-dried embryonic axes, rather than during desiccation itself (e.g. Wesley-Smith, 2002). Those authors report that such damage is more severe during slow rehydration, as recalcitrant tissues may lack the effective spectrum of repair mechanisms that operates during similar rehydration of desiccation-tolerant tissues, while the added period at intermediate water contents may exacerbate any damage suffered as a result of dehydration. In this study, flash-dried axes were rehydrated slowly, by placing them on either moist filter paper or semi-solid germination medium (Chapter 2). It is therefore not possible to resolve how much of the damage suffered by the axes at the apparent limit of desiccation tolerance, was desiccation-, or rehydration-, induced. Subsequent investigations to resolve this would contribute to the achievement of ideal dehydration levels, as well as optimising the rehydration step.

5.2.2 Storability of recalcitrant seeds: the interaction of seed storage physiology, initial seed quality, and fungi.

Seed storage is conventionally carried out by placing seeds, dried to known low water contents, at low temperatures: conditions under which the seeds can be stored for predictably long periods (Ellis and Roberts, 1980). However, this holds only for orthodox seeds. Seeds exhibiting intermediate behaviour, although tolerant to relatively high levels of desiccation, lose viability relatively rapidly if maintained at low water contents, and are also often chilling-sensitive in the

dehydrated state (Ellis *et al.*, 1990; Hong and Ellis, 1996). For intact recalcitrant seeds, storage is only possible when they are fully hydrated, as the storage of such seeds following partial drying results in accelerated loss of viability (Corbineau and Côme, 1986, 1988; Drew *et al.*, 2000).

However, the achievable longevity is dependent on not only the post-harvest physiology of the seed, but on the initial seed quality and the level of microbial contamination. Seeds of *T. dregeana* were storable for at least five months without apparent loss of viability, only if microbial contamination was not prevalent. Seeds with a high internal fungal inoculum lost 50% germinability within two weeks unless treated with systemic fungicides, and lost total viability within 10 weeks even when treated with the fungicides (Chapter 2). The seeds of *T. emetica* used in this study were not obviously fungally contaminated and could be stored without any apparent adverse effects until they had all germinated in storage after about 60 d (Chapter 2). Extrapolation from the results for *T. dregeana* lead to the suggestion that their storage lifespan would have been significantly shorter, had the seeds carried more fungal inoculum at harvest. Such fungal contamination precluded trials on hydrated storage of *W. salutaris* seeds, which all lost viability, concomitant with fungal proliferation, within two weeks of hydrated storage (Chapter 2). The central contribution of fungi to the loss of viability in stored hydrated recalcitrant seeds has been demonstrated unequivocally (Motete *et al.*, 1997; Calistru *et al.*, 2000), and it is suggested that control of fungal contamination is a pivotal factor in successful short-term storage of seeds of the species such as those investigated in this study. Further investigations on fungal control in these seeds would provide useful insight about the possibility of attaining maximum longevity, and perhaps aid in providing a reliable practical means of seed storage, ideally bridging the period to the next planting season.

The fungal status of the seeds is likely to accentuate the effects of low the seed quality (Jalink *et al.*, 1999), and it may be impossible to distinguish between low seed quality and high fungal contamination in hydrated recalcitrant seeds. Nevertheless, in seeds that can be desiccated, such as those of *W. salutaris* (Chapter 2), the initial quality plays a critical rôle in the attainable longevity. For example, whereas seeds considered to be of high quality, obtained from Tanzania, could be stored in the dehydrated condition for 49 days before total loss of germinability, batches of poor-quality seed obtained from Silverglen, South Africa, dropped to 50% viability within 10 days (results not shown here) when stored under the same conditions (Chapter 2).

Thus, even though recalcitrant seeds have a short storage lifespan (reviewed by Berjak and Pammenter, 2001), the effects of fungi and seed quality often confound results obtained in storage trials, and deserve more attention than is often afforded in such studies. Even when all the intrinsic and extrinsic factors are optimised, however, long-term storage may be achievable only via cryopreservation.

5.2.3 Cryogenic storage depends on the utilisation of appropriate regeneration regimes, and avoidance of freezing injury

Cryopreservation has been widely utilised for the preservation of plant germplasm, and protocols have been developed for the cryopreservation of the orthodox seeds of over 200 agricultural and endangered native species (Stanwood, 1985; Pence, 1991; Touchell and Dixon, 1993; Cochrane *et al.*, 2002), and zygotic axes and somatic explants of many orthodox and recalcitrant-seed species (Table 1.5). Recalcitrant seeds are unsuitable for cryopreservation because they are not only

desiccation-sensitive, but are also too large to be effectively rapidly-dried (Berjak *et al.*, 1990) while retaining viability and, even if were not the case, they would cool too slowly (Wesley-Smith, 2002) for lethal ice formation to be avoided. Thus, cryopreservation of the germplasm of such species is necessarily restricted to excised embryonic axes, and this requires the development of appropriate excision techniques and suitable *in vitro* culture protocols for the production of plantlets from the axes. Even though broad generalisations can be made regarding cryopreservation procedures, the variability in the physiology of recalcitrant seeds demands that such protocols are determined empirically.

5.2.3.1 *in vitro* regeneration: the first critical step

An appropriate *in vitro* embryo germination medium must be established *before* the establishment of any cryopreservation procedures is attempted. The requirements for *in vitro* growth are often species-, or even genotype-specific, and cannot necessarily be generalised. This is well illustrated in this study, where it was found that embryonic axes of *T. dregeana* performed best on WPM (Lloyd and McCown, 1981), while those of *T. emetica* required full-strength MS (Murashige and Skoog, 1962) medium. Furthermore, while most *in vitro* cultures need to be supplemented with plant growth regulators in order to achieve the desired morphogenic response, excised axes of both *Trichilia* species produced plantlets most efficiently when no plant hormones were included in the culture media, presumably because the necessary hormones were synthesised *de novo*.

For both *Trichilia* species, the most critical step in *in vitro* axis germination was the excision of the axis, as any damage to the shoot apex precluded shoot formation regardless of the medium on to which the axes were cultured. However, in order to clarify whether shoot development depends

only on the avoidance of injury, or on a vital rôle played by the cotyledonary tissues, further studies need to be done, in which the size of the cotyledonary blocks is whittled down progressively to the situation where only the attachment sites remain.

Another vital aspect of *in vitro* plant culture is the elimination of microbial contamination, particularly in explants from recalcitrant seeds, which usually harbour high inoculum of a mixed microflora (e.g. Mycock and Berjak, 1990; Calistru *et al.*, 2000; Makhathini, 2001). When such microflora is located on the explant surface, surface sterilisation is generally sufficient. However, the appropriate type, concentration, and duration of application of surface-sterilants may need to be determined for each species under investigation. Thus, in this study (Chapter 3), axes of *T. dregeana* could be best sterilised with 1% sodium hypochlorite, applied for 20 minutes, while those of *T. emetica* were best surface-sterilised with 0.2% mercuric chloride applied for 1 min. On the other hand, the axes of *W. salutaris* were lethally harmed by all the surface-sterilants tested, and the microflora associated with the axes could be controlled (at least for two weeks) only by co-culturing the axes with a mixture of the fungicide Benlate (0.1 mg l^{-1}) and the antibiotics Rifampicin (20 μg ml^{-1}) and Trimethoprim (30 μg ml^{-1}). Similarly, in some seasons, axes of *T. dregeana* could be disinfected only by including the systemic fungicides, Previcur (2.5 ml l^{-1}) and Early Impact (0.2 ml l^{-1}), and the antibiotic, Kanamycin (50 μg ml^{-1}), in the axis germination medium. The high level of endogenous contamination of recalcitrant seeds presents a critical problem in the establishment of successful *in vitro* cultures, which is necessary following the cryopreservation of the germplasm of species producing recalcitrant seeds. It is an area that requires more intensive investigation than could feasibly be included in this study.

Another factor that appears to affect post-thaw recovery, and which would be appropriate to investigate, is the use of specific recovery media that might be different from germination media for excised embryonic axes or whole seeds following cryopreservation. Preliminary studies in our laboratory (results not presented) have indicated that D-fructose and D-glucose provide better sources of carbon for cryopreserved *T. dregeana* axes than does sucrose, the commonly-used source of carbon in plant tissue culture. In the case of *W. salutaris*, it may be suggested that the low vigour following cryopreservation may have contributed to fungal proliferation and low recovery rates. In forthcoming cryopreservation investigations, thawed seeds will be subjected to a priming treatment before sowing, which may improve vigour and uniformity of germination (Brocklehurst and Dearman, 1983; Hardegree, 1996).

5.2.3.2 Avoiding freezing injury: there is not a universal approach

Successful cryopreservation depends on the avoidance of lethal freezing injury during cooling and thawing. Such damage is primarily avoided either by minimising the amount of water available for ice-crystal formation via dehydration (e.g Berjak and Dumet, 1996; Wood *et al.*, 2000), by cryoprotection (e.g. Sakai, 1995, 1997) or by the application of appropriate cooling regimes (e.g. Wesley-Smith *et al.*, 1992, 2001b; Berjak *et al.* 1999). The injury-avoidance procedures may be used in appropriate combination. Dehydration of recalcitrant embryonic axes to water contents of 0.25 – 0.35 g g^{-1} facilitates cryopreservation probably because the remaining water is not freezable (Vertucci, 1989b,c; 1990; Pammenter *et al.*, 1991; Vertucci *et al.*, 1991; Berjak *et al.*, 1992; Wesley-Smith *et al.*, 1992; Pritchard *et al.*, 1995; Pritchard and Manger, 1998), but this provides only a narrow 'permissible' water-content window (Wesley-Smith, 2002), above which lethal ice crystal formation occurs, and below which the axes suffer desiccation injury. The axes of

T. dregeana, where drying responses of the shoot and root apices were markedly different, provide graphic demonstration of the difficulty of avoiding desiccation damage in the achievement of the required water content widow that would ensure the competence of seedling production after cryopreservation. Given the wide variability in the initial water contents of the embryonic axes (Chapter 2), it is likely that only a small number of axes were at the desired water content window at freezing; and, owing to the heterogeneity of the axis tissue, it is unlikely that this water content window pertained across all tissue types in any one axis. This could explain not only many of the present results, but also the generally low and partial axis survival after dehydration/cryopreservation of recalcitrant axes (Pritchard and Prendergast, 1986; Chandel *et al.*, 1995; Kioko *et al.*, 1998; Wesley-Smith *et al.*, 2001a).

An approach that is used to widen the range of water contents suitable for freezing, is to increase the cooling rate (Wesley-Smith *et al.*, 1992, 1995, 2001b; Berjak *et al.*, 1999, 2000), with cooling rates as high as 40 000 °C min^{-1} facilitating the cryopreservation of embryonic axes of *Aesculus hippocatanum* at water contents as high as 1.0 g g^{-1} (Wesley-Smith, *et al.*, 2001b). However, cooling rates are dependent on the thermal mass of the axis, and cooling rates in excess of 6 000 °C min^{-1} are difficult to attain in tissues with a dry mass of higher than 2 mg (Wesley-Smith, 2002). Therefore, the relatively large axes of *T. dregeana* and *T. emetica*, which necessarily were attached to parts of each cotyledon, may not have cooled at the ideal rates, and different parts of the axes may have been at different water contents, which could have resulted in the growth of ice crystals and the survival of only portions of the axis, such as the root-pole (Chapter 3).

Paradoxically however, there may be a maximum cooling rate suitable for the cryopreservation of embryonic axes of different species. Studies by Wesley-Smith (2002) showed that the survival of axes of *Poncirus trifoliata* decreased when cooling was at rates higher than 3 000 °C min^{-1}. Similarly, in this study, embryonic axes of *Trichilia* spp. did not survive cryopreservation, when cooled at the high rates attained when freezing is done in melting isopentane or nitrogen slush, but tolerated cryopreservation in plastic cryo-vials, in which cooling rates of about 1 200 °C min^{-1} are attained (Chapter 3).

Since ice crystal damage is obviated at very high cooling rates, the basis of the lack of survival of axes cooled at such rates is not clear, but may be influenced by physical changes such as lipid phase transitions (Franks, 1977), associated with low temperatures, or the thermal shock resulting from differential expansion/contraction of lipid and protein components in the plasmalemma (Meryman, 1956, 1966; Morris *et al.*, 1983). Definitive biophysical investigations into any injurious effects of high cooling rates would contribute to the development of appropriate cryopreservation regimes for the embryonic axes of recalcitrant seeds of tropical origin. For example, differential scanning calorimetry (DSC) on the axes of *T. emetica* and *T. dregeana* would reveal what fraction of water in the dehydrated axes was freezable, and which could have provided the basis for lethal ice crystallisation during the necessarily low cooling rates dictated by the relatively large thermal mass of the axis/cotyledonary block explants. Furthermore, DSC and other biophysical methods have the potential to reveal whether or not lipid phase transitions occur, and if so, the temperatures of such transitions.

In cases where extremely high cooling rates are unsuitable or unattainable for widening the water content window suitable for cryopreservation, cryoprotectants may serve this purpose. Such substances act by increasing the relative desiccation tolerance of the embryonic axes (e.g. Dumet and Berjak, 1996), or lowering the freezing point of the intracellular solution through colligative action (Storey and Storey, 1996; Popova, 1997; Santarius and Franks, 1998), or by interacting with, and stabilising, membranes (Meryman, 1966; Heber *et al*, 1971; Franks, 1977). By contributing to intracellular viscosity, the cryoprotectants also contribute to the avoidance of membranes making lateral contact with each other (Bryant *et al*., 2001). In this study, survival after the cryopreservation of embryonic axes of *T. dregeana* and *T. emetica* was attained only if the axes were cryoprotected and dehydrated to *c.* 0.3 g g^{-1} prior to the freezing step (Chapter 3), illustrating the joint efficacy of the two procedures.

Another factor that emerged as influencing survival after cryopreservation was the degree of development of the seeds or axes. Seeds of *W. salutaris* survived cryopreservation best if extracted from fruits at the green-yellow stage of maturity, while embryonic axes of *T. emetica* developed shoots if cryopreserved after a three-week period in hydrated storage. In the case of *W. salutaris*, cells of the embryonic axes of seeds at the appropriate maturity stage had the highest degree of organellar dedifferentiation and lipid body:organelle ratio; while embryonic axes of the stored *T. emetica* seeds had more organised shoot meristems, in which the cells were less vacuolated and had a higher cytoplasmic density compared with those from axes of newly-shed seeds. Even though there are a few studies addressing the effect of the degree of development on tolerance to cryopreservation (Chaudhury *et al*., 1991; Chandel *et al*., 1995; Chandel *et al*., 1996; Kioko *et*

al., 2003), this (developmental status) may play a more prominent rôle than often appreciated. Further studies are envisaged in this regard, for seeds of *T. dregeana* and *T. emetica*.

Recent studies have highlighted the rôle of calcium in organised *in vitro* plant growth following cryopreservation (Mycock, 1999; Berjak *et al*., 1999; Walker, 2000). This was also demonstrated in this study (Chapter 3), in the increased percentage survival of embryonic axes of *T. dregeana* and *T. emetica*, and shoot development in *T. emetica* axes, following thawing in a 1:1 solution of 1 µ*M* $CaCl_2.2H_2O$ and 1 m*M* $MgCl_2.6H_2O$ after cryopreservation. The Ca^{2+} and Mg^{2+} ions are involved in cytoskeletal assembly and in the gravitropic response (Sinclair *et al*., 1996; Haaf *et al*., 1998; Scott and Allen, 1999), as well as in many responses to stress in plant cells (Shacklock *et al*., 1992; Price *et al*., 1994; Knight *et al*., 1996, 1997; Gong *et al*., 1998) but the exact rôle in facilitating freezing tolerance is not understood. The most simple explanation is that after freezing, membranes may be transiently abnormally permeable to ions, thus permitting their massive loss when axes are thawed/rehydrated in water. However, this is presently only conjectural, and further studies on the nature of the efficacy of cation solutions in thawing/rehydrating media could help in designing cryopreservation protocols that would obviate the cause of unorganised or lack post-thaw growth.

5.2.4 Beyond zygotic axes

It is evident from this study that the applicability of cryopreservation as a means of zygotic germplasm conservation depends not only on variable experimental parameters (such as cooling rate and water contents), but also on the tissue type and organisation, ultrastructure, and specific handling of the zygotic explants. In cases where zygotic explants are not amenable to cryopreservation, such as embryonic axes of *T. dregeana* (Chapter 3), it may be useful to utilise

explants such as somatic embryos, suspension cultures and apical/axillary buds. To that end, preliminary investigations on *T. dregeana* in our laboratory have developed protocols for the induction and rooting of axillary buds, as well as the production of embryogenic callus from fruit-wall explants. However, the appropriate parameters for successful cryopreservation are likely to be different from those established for zygotic axes, and may need to be established afresh.

5.2.5 Genetic integrity following cryopreservation

A central aim of germplasm conservation is the preservation of genetic fidelity. While cryopreservation has been shown to maintain genetic fidelity in plant systems (Fretz and Lörz, 1994; Yoshimatsu *et al.*, 1996; Schäfer-Menuhr *et al.*, 1997), some genetic aberrations have been reported to result from cryopreservation of the explants of a number of species, such as seeds of mahogany, *Sweetenia macrophylla* (Harding *et al.*, 2000) and shoot-tips of potato (Perazzo *et al.*, 2001).

Among molecular techniques available to probe any genetic changes after cryopreservation, the PCR-RAPD technique is not only superior to others such as karyotyping (Campos *et al.*, 1994), isozyme studies (Orozco-Castillo, 1994) and RFLPs (Williams *et al.*, 1990), but is the cheapest (Ragot and Hoisington, 1993). Therefore this technique has been widely used in genetic studies of plant populations (Table 4.1), and is attractive in many developing countries, where germplasm conservation is imperative. Using PCR-RAPDs in this study, it was demonstrated that neither dehydration alone, nor dehydration followed by cryopreservation, had any effect on the genetic fidelity of the seeds of *W. salutaris*.

In assessing the maintenance of genetic stability, nucleotide diversity and divergence were used to determine genetic relatedness, because it was impossible to extract and compare DNA from individual seeds before, and after, cryopreservation. This approach, which would have been ideal, could not be attempted as the excision of tissue samples was found to be lethal to the seeds. Thus, the development of techniques for extraction of DNA from *W. salutaris* (or other, similarly sensitive) seeds without compromising viability or tolerance to desiccation and cryopreservation, would complement the results obtained in this study.

5.2.6 Aspects of taxonomy

During this study, there was little, if any, existing relevant information on the species investigated, a lack of information that extended to the taxonomy of the species. For example, in some literature, *Trichilia dregeana* and *T. emetica* were considered to be synonyms of a single species, *T. roka* (e.g. Wood Density Database, online), despite the two species being morphologically distinguishable and having different seeding seasons even in the same habitat (Pooley, 1993). A similar situation may exist for *Warburgia salutaris*, which is currently considered as a synonym of *W. ugandensis* and *W. breyeri* (Flora of Zimbabwe, online). It is instructive, however, that literature referring to the species as *W. ugandensis* emanates mainly from central and eastern Africa, whereas reference to *W. salutaris* is primarily used in southern Africa. Furthermore, genetic analysis indicates that the specimens from southern and central/eastern Africa may represent disparate species (Lamb[1], pers. comm.). In order to contribute to the resolution of the taxonomy of this species, it is envisaged that, as a follow-up to this study, controlled pollination experiments will be performed utilising plants raised during the course of the study

from seeds of Tanzanian, Kenyan and South African origin. This will have important implications for conservation and exchange of germplasm of this species, particularly since it is virtually extinct in parts of Africa, and material for investigation/conservation has to be obtained from other parts of the continent.

5.3 Conclusion

This study has established aspects of post-shedding physiology of the non-orthodox seeds of *Trichilia dregeana*, *T. emetica*, and *Warburgia salutaris*, that are pertinent to conservation of the germplasm of these species. It has highlighted the range of factors that must be considered and optimised for successful short-term storage and cryopreservation of the germplasm of indigenous medicinal plants which, together with other species in the tropical zone, face extinction at the rate of 120 species daily (Wynberg, 1993). In the absence of effective conservation measures, and if the current extinction rate is maintained, about 25% of the global species diversity could disappear in the next 50 years, and all species on earth would be lost within 300 years.

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