SOME INVESTIGATIONS OF THE RESPONSES OF *Quercus robur* AND *Ekebergia capensis* EMBRYONIC AXES TO DEHYDRATION AND CRYOPRESERVATION

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PREFACE

All experimental work accomplished in this thesis was carried out in the Department of Biology, University of Natal, Durban, from January 1998 to January 2000, under the supervision of Professor P. Berjak and Dr. P. Watt.

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

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ABSTRACT

Recalcitrant seeds are those that are shed at high water contents, are actively metabolic throughout development, when they are, and remain, desiccation-sensitive, and may also be chilling sensitive. These properties preclude their conventional storage. Because recalcitrant seeds lose viability rapidly (within a few days to several months depending on the species) the long-term storage of their germplasm is achievable only by cryopreservation [i.e. storage at very low temperatures, generally in or over, liquid nitrogen at -196°C or -150°C, respectively. Generally the seeds are far too large to be cryostored, thus explants - most conveniently, excised zygotic embryonic axes - are used. As the axes of recalcitrant seeds are highly hydrated, specific pre-treatments prior to freezing have to be applied in order to avoid lethal ice crystal formation.

During the course of this study, cryopreservation protocols were developed for excised zygotic embryonic axes of two different species (*Quercus robur* L. and *Ekebergia capensis* Sparrm.). Surface-sterilisation regimes were tested for axes of both species, with the use of a 1% sodium hypochlorite solution containing a wetting agent, emerging as the best. For both species, the vigour and viability of axes, assessed by *in vitro* germination performance, was tested after the implementation of four different rates of desiccation (achieved by a laminar-airflow; silica-gel-; flash- and fast flash-drying). The most rapid dehydration rate (fast flash-drying) facilitated the best germination rates (vigour) for both *Q. robur*

and *E. capensis* axes after 240 and 20 min, when water contents were reduced to 0.37 ± 0.04 and 0.39 ± 0.06 g g^{-1} (dmb), respectively.

Consequently, fast flash-drying was used in combination with three different freezing rates (slow, intermediate and ultra-rapid cooling). While axis viability was lost after slow or intermediate cooling, good survival was obtained for each species after ultra-rapid cooling. In addition to the optimisation of culture conditions, desiccation and freezing rates, the efficacy of different thawing media (distilled water, mannitol, sucrose, full-strength MS medium supplemented with sucrose and a 1 μM calcium/1 mM magnesium solution) was also assessed. The only thawing medium that ensured normal seedling production was the $Ca^{2+}Mg^{2+}$ solution, in which electrolyte leakage was significantly curtailed.

In addition to vigour and viability assessment the responses of the embryos to the various manipulations were monitored by light microscopy and/or transmission electron microscopy. The results of the various manipulations are discussed in terms of the stresses imposed on the excised axes, by each of the procedures. For axes of *Q. robur*, the outcome of the presently developed successful procedure and two unsuccessful protocols from the published literature are compared and contrasted.

It is concluded that while *in vitro* germination media need to be assessed on a species basis, use of the mildest effective surface-sterilant, in conjunction with

the most rapid means to achieve dehydration and cooling/freezing, are likely to underlie generally successful cryopreservation. Additionally, thawing parameters have emerged as being critically important.

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ABBREVIATIONS

min	minute(s)
h	hour(s)
NAA	naphthaleneactic acid
BA	benzylaminopurine
MS	Murashige and Skoog (19962) nutrient formulation
v/v	volume per volume
mg	milligram
l	litre
g	gram
WPM	Smith and McCown (1982/83) nutrient formulation
d	day(s)
°C	degrees celsius
Ca^{2+}	calcium (II) ions
Mg^{2+}	magnesium (II) ions
mm	millimetre
m/v	mass per volume
NaOCl	sodium hypochlorite
CaOCl	calcium hypochlorite
$HgCl_2$	mercuric chloride
pH	measure of hydrogen ion concentration
%	percent
Tween-20	polyoxyethylene sorbitan monolaurate
μg	microgram
μM	micromolar
mM	millimolar
H	hormones
n	number of specimens used per treatment
sec	second(s)
$g\ g^{-1}$	gram of water per gram of dry mass
fmb	fresh mass basis
dmb	dry mass basis

" To understand plant life

you must explore the obvious and the subtle,

as well as, all levels in between"

[unknown]

CHAPTER 1: CONSERVATION AND STORAGE OF PLANT GENETIC RESOURCES

Plant genetic resources for food and agriculture are the biological basis of world food security, and directly or indirectly, support every life form on earth. While plant genetic resources have been sought after, collected, used and improved for centuries, it has only been since the 1930s that official concern regarding the need for their conservation has been voiced. International efforts to promote conservation, exchange and utilization, initiated through the Food and Agriculture Organization of the United Nations (FAO), are more recent.

A panel of experts was created specifically for plant genetic resources in 1968 (FAO, 1996), one of which was the 'International Board for Plant Genetic Resources' (IBPGR) programme with the mission to coordinate an international plant genetic resources programme. The legal successor to IBPGR is now the International Plant Genetic Resources Institute (IPGRI). Collecting missions were accelerated, and genebanks were constructed and expanded at national, regional and international levels.

1.1 *In situ* AND *ex situ* CONSERVATION OF PLANT GENETIC RESOURCES

Plant genetic resources can be conserved either *in situ* or *ex situ*. *In situ* conservation has primarily been used to conserve forests and other sites containing threatened species, which are in their natural habitats, for example in natural parks or nature reserves. This allows normal evolution to continue with

no, or little disturbance. *Ex situ* conservation on the other hand, involves the removal of a plant species from its natural habitat (Krogstrup *et al.,* 1992).

Even though *in situ* conservation is perhaps the best way of preservation, it cannot work alone, but must operate hand-in-hand with *ex situ* conservation, not only because of capital outlay and lack of land availability, but also to allow plant material to be readily available for research breeding, *in vitro-* and long-term-conservation purposes. These *ex situ* collections ultimately consist of seed genebanks, field genebanks and *in vitro* collections.

1.1.1 METHODS OF *Ex Situ* CONSERVATION

Ex situ germplasm acquisitions are either 'active' or 'base' collections (Hawkes, 1987). Active collections are readily available for distribution as field or greenhouse collections. An active collection requires methods of storage that retain the viability of samples for short (a few weeks) to moderate periods (several years). The regular regeneration and multiplication should maintain genetic diversity. The disadvantage of maintaining active collections as living plants in either the greenhouse or field is the expense and labour intensity involved, pest problems and natural disasters (George, 1993; 1996).

A base collection, however, is for long-term conservation of germplasm by *in vitro* storage methods and is not intended for extensive distribution, but serves as a backup for the active collections. In addition, base collections reduce costs of regeneration and frequent viability testing (George, 1993; 1996).

1.2 *In Vitro* STORAGE METHODS

During the last twenty to thirty years *in vitro* culture techniques have been extensively developed and applied to more than a thousand different species (George, 1993; 1996). Culture of plant material *in vitro* induces morphogenic responses of meristematic tissue, including organised or differentiated growth via axillary or adventitious bud induction (directly as shoots and roots) or zygotic embryo culture. Alternatively, unorganised or undifferentiated cell division may be induced from tissues whether meristematic or not. This involves callus formation - the growth of an unorganised mass of plant cells which can be differentiated or modified into plantlets in the next phase of regeneration. The route of morphogenesis is dependent on factors such as: the size of the meristem-tips; the type of culture medium; the kind and concentration of growth hormones; the environmental conditions (light, temperature and photoperiod) and other factors such as seasonal fluctuations of donor plants and the type of containers used (George, 1993; 1996).

Storage of actively growing cultures, minimal growth storage and cryopreservation are the three basic approaches to *in vitro* storage of such *in vitro* cultures.

1.2.1 STORAGE OF ACTIVELY GROWING CULTURES

Storage of actively growing *in vitro* cultures requires a monthly transfer of such cultures to new media, to minimise the loss of material due to contamination or physiological decay. The benefit of this method is the rapid multiplication of the

plant tissue by micropropagation once retrieved from storage, thereby constituting a backup for a field grown active collection, supply to consumer or/and planting out (Krogstrup *et al.,* 1990; Krogstrup and Nørgaard, 1991).

1.2.2 MINIMAL GROWTH STORAGE

Minimal growth storage includes the exposure of tissue cultures (or young plants) to: growth limiting chemical and physical factors (growth retardants such as abscisic acid); reduced temperatures (2-3°C for temperate species and 14-18°C for tropical species); or reduced oxygen tension (Baucher *et al.,* 1989; Lizarraga *et al.,* 1989; Engelmann, 1990). Slow growth storage methods have been extremely successful for shoot cultures of a wide range of species including several root and tuber crops (potato, cassava, yam and sweet potato); fruits (banana, apple, pear, strawberry and kiwi fruit) and other horticultural and agricultural species (Withers, 1987).

Minimal growth may, however, impose definite selection pressures (*in vitro* selection) (Scowcroft, 1984) and environmental stresses which can ultimately cause genetic modifications. Minimal growth storage is therefore not necessarily the best long-term storage option and additionally, requires considerable space. This is a major reason for cryopreservation being considered as the best option for long-term conservation.

1.2.3 CRYOPRESERVATION

The ultimate base storage method, cryogenic storage, rests on the reduction and subsequent arrest of metabolic functions by placing the specimens into an ultra-low temperature (for instance, liquid nitrogen at -196°C). Due to the unavailability of liquid water, cellular metabolic activities cease, and consequently, genetic changes should be minimised (see section 1.3.6 for more details).

1.3 SEED STORAGE

Seed storage is the most common way of storing plant germplasm as seed bank facilities do not require sophisticated technology, and as a result are relatively cost effective. Unfortunately not all seeds behave in the same way and thus cannot all be stored using the same protocol. Roberts (1973) introduced the terms "orthodox" and "recalcitrant" to describe the storage behaviour of seeds, depending on their physiological response to desiccation and low temperature.

By definition, recalcitrant or desiccation-sensitive seeds have characteristics that do not conform to those of an orthodox or desiccation-tolerant propagule. For instance, the majority of orthodox seeds dry down naturally on the parent plant (maturation drying) to low water contents, and according to Roberts and King (1980), orthodox seeds can be further dehydrated to water contents of between 1-5% (0.01-0.05 g g^{-1} dmb) without loss of viability or damage. However, it should be noted that later work contests the assumption that ultra-dry conditions are invariably not damaging (Vertucci and Roos, 1993). In addition, orthodox seeds are able to be stored for long periods at water contents of 5-7% fmb (0.05-0.08 g

g^{-1} dmb), as metabolism is minimal and probably non-existent at -18°C or lower (IBPGR, 1976). Roberts and Ellis (1977) suggested that the lower the storage temperature, the longer these seeds could be stored.

The truly recalcitrant seeds, however, never dry out on the parent plant (do not undergo maturation drying) and are therefore shed at relatively high water contents (Chin, 1988). For example, *Avicennia marina* (which is highly recalcitrant) has a water content as high as 67% fmb (2.03 g g^{-1} dmb) on shedding (Berjak *et al.*, 1984). Another characteristic of truly recalcitrant seeds is their active metabolism throughout development to shedding. For example, after a small decline at the start of reserve accumulation, respiration in the seeds of *A. marina* remains relatively constant until abscission (shedding) according to Farrant *et al.* (1992b). In fact, relatively high respiration rates have been recorded for seeds of several recalcitrant species at shedding (Poulsen and Eriksen, 1992; Espindola *et al.*, 1994; Finch-Savage and Blake, 1994a). Another characteristic of many recalcitrant seeds particularly those of tropical species, seems to be their sensitivity to chilling (Chin and Roberts, 1980), although little systematic work has been done since then on this aspect.

Besides these two seed categories, Ellis *et al.* (1990; 1991a; b; c) and Hong and Ellis (1992), defined an "intermediate" category of seeds based also on post-harvest behaviour. Intermediate seeds were described by those authors to withstand dehydration to relatively low water contents [12-5% fmb (0.14-0.05 g

g^{-1} dmb)], depending on the species, but, in this state, are chilling-sensitive. The original examples of this behaviour were *Coffea* spp. reported by Ellis *et al.* (1990; 1991a), *Carica papaya* by Ellis *et al.* (1991b), and *Elaeis guineensis* by Ellis *et al.* (1991c). It should be noted, however, that Hong and Ellis (1996) have modified this definition.

Ongoing research, however, indicates that this strict categorisation of seeds may not be appropriate because of the wide range in the postharvest responses of seeds. It has been suggested that all seed behaviour will be found to constitute a continuum from the most orthodox to the most highly recalcitrant (Berjak and Pammenter, 1994). In fact a continuum across species is apparent already, within the recalcitrant category (Tompsett, 1987; Farrant *et al.*, 1988; Berjak *et al.*, 1989; Finch-Savage, 1995).

1.3.1 THE PHENOMENON OF RECALCITRANCE

Since the early definitions, much about the complexities of recalcitrant propagatory units has been discovered; and modified explanations of seed behaviour and requirements for storage of seeds, have been proposed. As evidence accumulated, it became clear that there are different degrees of desiccation-sensitivity among recalcitrant species at shedding (Farrant *et al.*, 1988; Berjak and Pammenter, 1994). This led to the suggestion that recalcitrant seed behaviour should be considered in the context of the natural habitat of, or extrinsic influences on, individual seed species, in order to better understand the phenomenon of recalcitrance (Berjak and Pammenter, 1994).

Hanelt (1977) proposed that recalcitrant seeds might be produced by trees and shrubs of the temperate and sub-tropical regions, which have been suggested to be minimally recalcitrant species (Farrant et al., 1988; Berjak et al., 1989), which might be interpreted as minimally desiccation-sensitive. Tropical rain forest, wet-land and aquatic plants were suggested to be likely to produce highly recalcitrant seeds by those authors, which could be interpreted as being highly desiccation-sensitive in the loose sense. Tropical recalcitrant seeds might be expected to show little, if any, dormancy, since those parent plants growing in naturally favourable wet habitats are not expected to dry out. Additionally, perennial plants produce seeds at regular intervals in relatively humid environments, which (theoretically) need not have long life-spans.

The intrinsic or inherent characteristics of the seed in its natural environment are important in the understanding of the recalcitrant seed and its degree of longevity, especially when compared with the desiccation tolerant or orthodox type of seed. For instance, according to Leprince et al. (1993), orthodox seeds acquire desiccation tolerance via three main so-called protective mechanisms: the accumulation of non-reducing sugars; the accumulation of dehydrins or Late Embryogenic Accumulating/Abundant (LEA) proteins that are inducible by abscisic acid (ABA) and; the ability to prevent, tolerate or repair free radical attack. It is possible that recalcitrant seeds are desiccation-sensitive due to inadequacies in these aspects. More recently, Pammenter and Berjak (1999) have extended the list of properties considered vital in the acquisition and

maintenance of desiccation tolerance. The list includes: "physical characteristics of cells and intracellular constituents; insoluble reserve accumulation; intracellular de-differentiation; metabolic 'switching off'; presence, and efficient operation, of anti-oxidant systems (mentioned in more detail later); accumulation of putatively protective substances including LEAs (Late Embryogenic Abundant Proteins), sucrose and other oligosaccharides, as well as amphipathic molecules; the presence and role of oleosins, and the presence and operation of repair systems during rehydration". Those authors have proposed that the absence or presence and degree of interaction of the many mechanisms/processes in this list, will confer widely differing behaviour of seeds across the spectrum of angiosperm and gymnosperm species.

Whatever the underlying causes, recalcitrant seeds deteriorate readily and special care needs to be taken with the methods adapted for harvesting, packaging and distribution and short and longer-term storage. For a storage method to be of any value for genetic conservation, seed vigor and viability must be maintained for the required period since loss in viability will almost certainly be accompanied by some change in the genetic constitution (Roberts, 1973). Consequently, any storage regime during which seed viability shows a significant decline at any stage, must either be changed or abandoned.

The only way recalcitrant seeds can presumably be stored, is in the fully hydrated condition, and this is only a short-term option. This is because

recalcitrant seeds are short-lived even under such conditions, with life spans ranging from a few weeks to a few months, depending on the species. This phenomenon has been suggested to be the outcome of their ongoing metabolism becoming deranged under storage conditions that supply no additional water to support these processes (Farrant *et al.*, 1986; Berjak *et al.*, 1989; Pammenter *et al.*, 1994).

Those recalcitrant seeds that are more actively metabolic at shedding will probably tolerate less water loss than those which are not as metabolically active. When germination ensues in hydrated storage, which occurs sooner or later depending on the species, metabolic rates become increasingly higher, as does desiccation sensitivity (Berjak *et al.,* 1993). This is correlated with the requirement for additional water (see later) which is not provided in storage.

1.3.2 SHORT-TERM WET STORAGE OF SEEDS

Recalcitrant seeds are highly metabolic and lack the ability to shut down this active state (Berjak, 1984; Pammenter and Berjak, 1999) which is the basis of their desiccation sensitivity (Pammenter and Berjak, 1999).

Thus, recalcitrant seeds must be stored under conditions that maintain their water content at, or only marginally below that characterizing the newly-shed state (King and Roberts, 1980a; Berjak *et al.*, 1989). This approach, termed 'wet-storage', is apparently useful for short-term storage of seeds of recalcitrant species in general (Berjak *et al.*, 1989), however, all become increasingly

desiccation-sensitive with time (Farrant *et al.*, 1986). Ultrastructural and biochemical investigations of a number of wet-stored recalcitrant species have shown that organisation and activity within the embryonic cells increases during wet-storage in line with the onset of germination, and a stage of cell division and extensive vacuolation is sooner or later reached, after which deterioration sets in (Berjak *et al.*, 1989; Farrant *et al.*, 1988; 1989). Because, under these wet-storage conditions, germination metabolism ensues and continues, what are essentially being stored are developing seedlings as opposed to ungerminated seeds. The more immediately and rapidly germination takes place after shedding, the sooner will cell division and vacuolation, followed by the onset of deterioration, these events being inversely related to the longevity of the seeds in hydrated storage (Farrant *et al.*, 1989).

Another seemingly inevitable problem associated with the maintenance of recalcitrant seeds in hydrated storage is microbial proliferation favoured by the moist, generally warm storage conditions (Berjak, 1996; Berjak *et al.*, 1990; Mycock and Berjak, 1990). A further limiting factor is that desiccation sensitivity is invariably found to increase once germination has proceeded to the stage of extensive vacuolation, as the minimum and lethal water content levels, increase (Farrant *et al.*, 1986, Berjak *et al.*, 1989; 1992), viability loss proceeds rapidly from this stage. The storage life-span can therefore vary from less than 2-3 weeks for some tropical species to 2-3 years for the more chilling-tolerant temperate species stored at lower temperatures (King and Roberts, 1980a;

Suska and Tylkowski, 1980; Pritchard *et al.*, 1986; Farrant *et al.*, 1989; Fu *et al.*, 1990; Tompsett, 1992), but is always curtailed.

Reference to that literature also shows that there is a wide variation in the water contents of seeds at which viability is lost and the proportion of water loss that can be tolerated. The most likely causes for loss of viability emanating from what is effectively a prolonged, mild water stress (Pammenter *et al.*, 1998) of wet-stored seeds recorded include: macromolecular conformational changes and impaired intracellular transport; changes in pH and changes in ion concentrations, all of which have the potential to disturb intracellular metabolism, which may lead to free radical generation (Senaratna and McKersie, 1986). Where those authors recorded these as likely events consequent upon dehydration, an internal water stress occurs due to the increased water level required for ongoing metabolism during hydrated storage of recalcitrant seeds, which would probably have similar deleterious effects (Pammenter *et al.*, 1994; 1998).

So, even though wet-storage is storage in a so-called hydrated condition, this method can only be used as a very short-term storage method, as seeds will not survive long periods of storage under these stressful conditions, as the vigour and viability of these seeds decline.

1.3.3 SHORT- TO MEDIUM-TERM STORAGE

One of the most important limitations to the storage of recalcitrant seeds is germination (Berjak *et al.*, 1989, Pammenter *et al.*, 1994, Finch-Savage, 1995) and in acorns it might be assumed that as long as germination is inhibited, recalcitrant seeds could be successfully stored. However, in practice this has not been satisfactorily achieved.

With regard to short- and medium-term storage of good quality seeds, the general and most economically viable ways for the package and distribution of whole recalcitrant seeds have included the use of a modified atmosphere. For example, this has been done by using carbon dioxide, to replace (wholly or partially) oxygen and any ethylene evolved from the seeds (Chin and Roberts, 1980). Although this concept is not new, the effectiveness of such treatments has not been confirmed. As early as 1914, Kidd prolonged the life of rubber seeds by sealing them in a 40-45% CO_2 atmosphere, and attributed this success to a narcotic induction of dormancy (Chin and Roberts, 1980). Certain workers have also investigated the effects of coating recalcitrant seeds with impermeable material to reduce moisture loss. For example, in 1964, Friend (Chin and Roberts, 1980) stored cocoa seeds coated with paraffin wax and found that the storage life of the seeds was doubled to 28 days. Storage was further enhanced by leaving an unwaxed central band on the seed, which presumably permitted respiratory exchange with only minimal water loss.

The most successful and most economical method so far, demonstrated by a number of workers, for storing recalcitrant seeds appears to be sealed storage within a thin polyethylene bag. Polyethylene bags appear to reduce water loss while permitting some gaseous diffusion and, provided microbial growth and germination in storage are kept to a minimum (both of which are unlikely to be achieved), lengthy storage may be possible. However, success with this approach is likely to be achieved only with non-orthodox seeds that are not truly recalcitrant (e.g. so-called imtermediate seeds) or those that are recalcitrant, but show dormancy. For example, coffee seeds (intermediate [Ellis *et al.*, 1990]) have been maintained in this manner for two and a half years by storing them at a 41% fmb (0.69 g g^{-1} dmb) water content at 15°C (Vossen, 1978). Pritchard *et al.* (1996) also showed that *A. hippocastanum* seeds which are recalcitrant but show dormancy have a storage life-span of 2-3 years if maintained hydrated at a temperature of 16°C.

Increasing the longevity of imbibed *Citrus aurantium* seeds in storage by way of decreasing seed respiration was suggested by Edwards and Mumford (1985). Evidence that this might work for some species was provided by Sowa *et al.*, (1991) who treated *Litchi chinensis* and *Dimocarpus longan* seeds with nitrous oxide (an anaesthetic able to reduce the respiration rates in seeds) which increased the storage life-span or both. Another and apparently very promising result was reported by Pammenter *et al.* (1997) and Motete *et al.* (1997) when a specially-prepared alginate gel increased the longevity of *Avicennia marina* by

curtailing fungal activity and slowing germinative metabolism in storage. The gel, an extract from the seaweed, *Ecklonia maxima* - was as effective alone as when abscisic acid was added to it. Bhargava (1988) used halogen and chlorine vapour treatments to enhance the storability of *Shorea robusta* seeds and to increase their tolerance to lower storage temperatures.

However, it must be stressed that the effects of none of these treatments at the physiological level of seeds, has been unequivocally demonstrated. Furthermore, longer-term effects of any such treatment on the ultimate production of normal plants, remains to be ascertained.

1.3.4 FREE RADICAL PRODUCTION

It has been hypothesised, and there is some evidence to substantiate, that free radicals are produced under conditions of water stress in desiccation-sensitive plant tissue. Free radicals (chemical species which have lost an electron) are, components of intracellular metabolism, but under normal conditions, produced and strictly controlled (Hendry, 1993). However, free radicals are potentially destructive in tissues, including those of seeds under stressed conditions, when control mechanisms may be ineffective, or even fail completely (McKersie *et al.*, 1988; Leprince *et al.*, 1990; Dhindsa, 1991; Arrigoni *et al.*, 1992; Hendry *et al.*, 1992; Cakmak *et al.*, 1993). In uncontrolled conditions, free radicals will abstract electrons from macromolecules, initiating a cascade of potentially lethal reactions (Smith and Berjak, 1995). Leprince *et al.* (1990) demonstrated the accumulation

of an organic radical (formed as a result of deranged electron transport) and the significant increase in lipid peroxidation in desiccation-sensitive maize seedlings. Similarly, damage initiated by free radicals occurred in axes/seeds of *Quercus robur* when dehydrated below 0.89 g g^{-1} dmb (47% fmb), and here too the evidence of free radical activity was obtained by the accumulation of peroxidised end-products (Hendry *et al.*, 1992). Free radical activity correlated with loss of viability during the drying of a number of desiccation-sensitive tree seeds was recorded by Finch-Savage *et al.* (1994c) and by Chaitanya and Naithani (1994) in their study on *Shorea robusta* seeds.

Protection against any oxidative attack is conferred primarily by the activity of anti-oxidants, e.g. superoxide dismutase (SOD) and glutathione reductase under unstressed conditions. However, there is evidence that anti-oxidant mechanisms may become impaired when desiccation-sensitive recalcitrant seed tissues become water stressed (Hendry *et al.*, 1992; Finch-Savage *et al.*, 1993).

A spectrum of inherent inadequacies of recalcitrant seeds has been discussed in the context of their desiccation-sensitivity (Pammenter and Berjak, 1999). Most of which only pertain indirectly to the present work (mentioned in section 1.3.1) and will not be fully discussed here. However, the potentially lethal effects of free radicals, generated as a consequence of deranged metabolism under prolonged water stress conditions, have been highlighted here, as they are central to the

argument in favour of very rapid dehydration as a prerequisite for successful cryopreservation (see later).

1.3.5 LOW TEMPERATURE STORAGE OF SEEDS

As the metabolic status of the seeds underlies their recalcitrance, brief mention must be made of other attempts to minimise developmental changes. There are a number of potential advantages associated with the storage of seeds at sub-ambient temperatures, such as decreased microbial growth and a lower seed metabolic rate, which should extend the hydrated storage lifespan.

Low temperature storage (chilling) was tried by Ryke as early as 1935 with cocoa seeds (*Theobroma cocoa* L.) which were killed at temperatures of 10°C and below (Chin and Roberts, 1980). It has subsequently been assumed from largely subjective observations (Chin and Roberts, 1980), that recalcitrant seeds of many species are chilling sensitive.

A more recent observation of chilling-sensitivity in recalcitrant seeds was done by Berjak *et al.* (1995) in a study to determine whether chilling (4 ± 2°C for ten days) had an adverse effect on African *Azadirachta indica* A. Juss (neem) axis cells. Those authors showed that a regression of subcellular development accompanied chilling, as many axis cells showed degenerative changes. Ezumah (1986) found that storage of dried neem seeds at 6-7°C was also

deleterious, irrespective of the water content. There therefore seems to be no doubt that neem seeds are chilling-sensitive

The most important thing to remember at this stage is that chilling (generally above 0°C) injury is not the same thing as freezing (below 0°C) injury. Also, chilling injury is thought to be another induction of unbalanced metabolism and therefore of uncontrolled free radical production.

1.3.6 LONG-TERM STORAGE - (CRYOPRESERVATION) - OF SEEDS AND SEED COMPONENTS

Conventional storage methods for chilling- and desiccation-sensitive (recalcitrant) seeds at sub-ambient temperatures are inappropriate at least for long-term storage. However, cryogenic techniques (e.g. liquid nitrogen storage at -196°C) similar to those found to be successful in the preservation of other biological materials (e.g. semen, mammalian embryos and plant meristems) had already (some years ago) been discussed as possibilities, for recalcitrant embryos and smaller recalcitrant seeds (Chin and Roberts, 1980). Cryogenic storage thus far appears to offer the only option for long-term conservation of the germplasm of species producing recalcitrant and intermediate seeds. Cryogenic storage in liquid nitrogen has the advantages of preventing germination and microbial growth and obviating physiological deterioration in biological tissues as all cell metabolic processes are halted. Theoretically, cryostorage (at least at -196°C) should facilitate storage for thousands of years (Ashwood-Smith and Friedman, 1979; Dumet *et al.*, 1997; Engelmann, 1997).

However, the potential for cryo-injury is very real. Mazur (1969; 1970; 1977) proposed that tissues and cells exposed to freezing would be susceptible to two very damaging factors: the toxic build-up of solutes and intracellular ice formation, specifically crystalline ice formation, which will inevitably destroy the tissue. Damage sustained during cryopreservation and inflicted by the necessary pre-treatments, will influence the capacity of injured plant cells to regain normal cellular and regenerative functions. In addition, Benson (1990) suggests that free radical generation still continues despite cryostorage temperatures, as does Hendry (1993).

The successful cryopreservation of plant cells, tissues and organs is dependent on a range of factors such as the size and ultrastructure of the tissue; the rates, conditions and duration of: dehydration (pre-freeze status); cryoprotection; cooling (freezing); thawing and post-thaw recovery. Indeed each component of a cryopreservation protocol can be stressful and injurious to the specimen in question. Ultimately, successful cryopreservation depends on the delicate balances that will be achieved only by experimentation with rate and extent of dehydration, and cooling conditions and rates as well as those involved after survival is achieved from the cryogen. Additionally, success can be claimed only if vigorously growing normal plants can ultimately be produced from most of the cryopreserved explants.

1.3.6.1 RECALCITRANT SEED SIZE AND ULTRASTRUCTURE

It would be ideal if intermediate and recalcitrant seeds could be stored whole, as this would be less labour-intensive and subject to less complication than the use of embryonic axes entails. Perusal of the literature suggests that suitably dehydrated, intact intermediate seeds can be successfully cryostored, for instance Berjak and Dumet (1996) recorded a 70-75% success of whole neem (*Azadirachta indica*) seeds. But this is seldom, if ever, the case for the more hydrated truly recalcitrant seeds. This is generally because of the relatively large size of these seeds. Large seeds have a lower surface to volume ratio than small ones and recalcitrant seeds have high water contents. Such seeds lose water at a lethally slow rate (Pammenter *et al.*, 1998) and would be dead well before reaching water contents sufficiently low to prevent lethal freezing injury. Work in our laboratory has repeatedly indicated that even in the case of smaller recalcitrant seeds, water is generally lost relatively slowly, with lethal injury occurring at relatively high water contents (which is not surprising considering that resistance to water loss must have an inherent survival value in the natural habitat). Thus, smaller sources of germplasm, such as the zygotic embryonic axes or somatic embryos must be used for cryopreservation.

Results of successful cryopreservation of zygotic embryonic axes, after partial desiccation, have been recorded for species producing recalcitrant (e.g. coconut) and intermediate (e.g. coffee, oil palm) seeds (Engelmann *et al.*, 1995) and for axes from various large-seeded temperate species (Pence, 1990). However, it is important to note that 'success' is generally reported as survival of cryostorage,

rather than by plant establishment, making it difficult to gauge true success. In addition, there has been extensive development of systems for *in vitro* propagation for species of economic importance (e.g. oil palm, date palm and coffee) to produce somatic embryos (Tisserat, 1984; Hatanaka *et al.*, 1991) which have shown the potential to be cryopreserved, probably because of their small size (Anandarajah and McKersie, 1990; Dumet *et al.*, 1993a; b) and tissue homogeneity.

At this point, it is important to realise that many intracellular characteristics are held to contribute to desiccation-tolerance of plant tissues. These characteristics have recently been reviewed by Pammenter and Berjak (1999).

Vacuolation and reserve deposition constitute one such mechanism, and perhaps one of the most important, as removal of water from plant cells is associated with shrinkage of fluid-filled vacuoles and volume reduction (Iljin, 1957). In desiccation-tolerant material, vacuoles divide into many small ones or become filled with insoluble reserve material. Neither appears to occur in recalcitrant seeds, linking the extent of vacuolation and lack or limitation of insoluble reserve accumulation, to the degree of desiccation-sensitivity (Berjak *et al.*, 1989; Farrant *et al.*, 1989; Farrant *et al.*, 1997; Pammenter and Berjak, 1999).

Farrant *et al.* (1997) carried out a study on three species of varying desiccation-sensitivity. *Avicennia marina* (highly desiccation-sensitive); *Aesculus hippocastanum* (moderately desiccation-sensitive) and *Phaseolus vulgaris* (orthodox i.e. desiccation-tolerant). It was found that *A. marina* embryo cells developed steadily larger vacuoles during development, so that 60 and 90% of the cell volume of axes and cotyledons, respectively, became occupied by vacuoles. In addition, little by way of insoluble reserves accumulated in *A. marina* tissues with the onset of seed maturity (Farrant *et al.*, 1992b; 1997). Those authors correlated the undiminishingly high water content, and degree of desiccation sensitivity of *A. marina* seeds with these characteristics.

Aesculus hippocastanum, on the other hand, was observed to have undergone a decrease in the vacuole size as development ensued, resulting in only small vacuoles at maturity, while *P. vulgaris* vacuoles *per se* disappeared with ongoing development becoming filled with what was described as insoluble, amorphous (probably protein) material (Farrant *et al.*, 1997). It appears therefore, that the larger the vacuoles and the less the insoluble reserve deposition, the greater the degree of desiccation sensitivity.

In terms of the cell (and therefore tissue) characteristics of zygotic embryos, some (e.g. *Trichilia dregeana*) have larger fluid-filled vacuoles within the (root) apical meristematic cells compared with others (e.g. *Quercus robur*) (Figure 1.1). This differing feature may be one of the reasons why no successful cryostorage

protocols have been able to be developed for. *T. dregeana* axes (Kioko pers. comm.[1]) while as the present work will show, significant success has been attained for those of *Q. robur.*
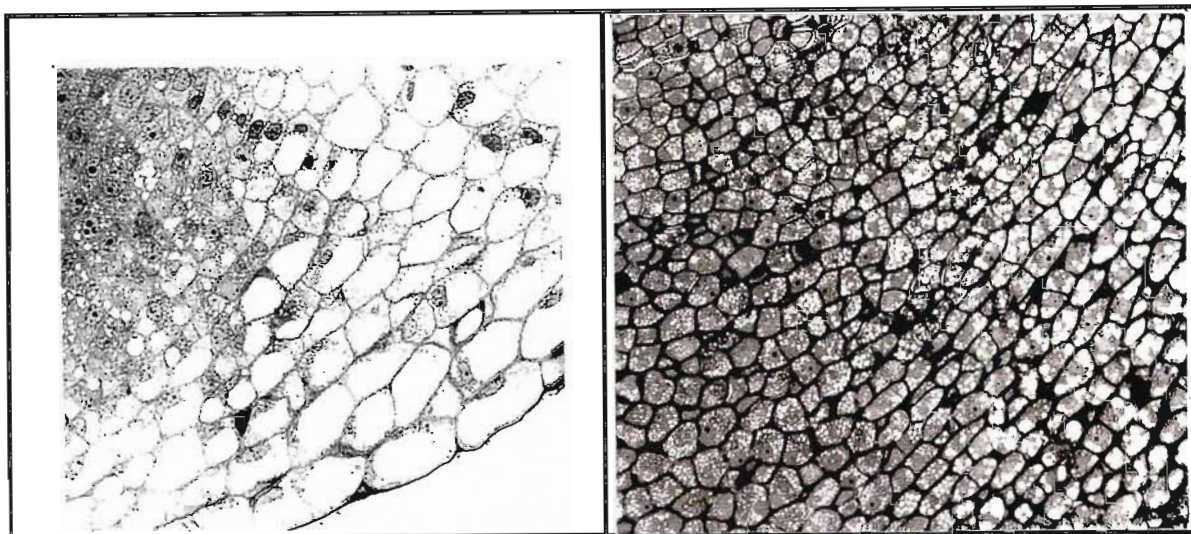
(a) x 150   (b) x 150
Figure 1.1: Light micrographs of (a) *Trichilia dregeana*, (compliments of J. Kioko) showing large vacuoles and (b) *Quercus robur*, showing less vacuolation in typical component cells of root caps.

The degree of vacuolation in embryonic axis cells must, therefore, be a primary consideration with regards to development of dehydration and freezing protocols for the long-term conservation of the germplasm of the species in question.

Farrant *et al.* (1997) also showed that mitochondria remained highly differentiated in the desiccation-sensitive species (*A. marina* and *A. hippocastanum*) while in the axes of the orthodox seeds, *P. vulgaris*, these organelles became completely de-differentiated prior to maturation drying. Lack of de-differentiation, which was accompanied by active respiration has been

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suggested to be a major factor contributing to desiccation-sensitivity in recalcitrant seeds in general (Pammenter and Berjak, 1999). *Phaseolus vulgaris* axes, as for all orthodox seeds, showed a de-differentiation of mitochondria which was accompanied by a decline in respiratory rate to unmeasurable levels, prior to maturation drying. This strategy of organelle de-differentiation is presumably another mechanism involved in the acquisition of desiccation-tolerance, as not only are the surface areas of vulnerable membranes reduced, but respiratory metabolism is potentially eliminated. This would significantly reduce free radical production under conditions of water stress during dehydration (Senaratna and McKersie, 1986).

The plant cytoskeleton (the major components of which are microtubules and microfilaments) is a vital component dictating intracellular organisation. Irreversible destruction of the cytoskeleton must result in death of that cell. According to Sargent *et al.* (1981), microtubule assembly may be markedly sensitive to desiccation stress, its derangement thus providing a further factor that might contribute to desiccation-sensitivity. It appears that disassembly and reassembly of the cytoskeleton is reversible in orthodox seeds, but irreversible for recalcitrant seeds (Mycock *et al.*, in press).

Thus, there are several categories of damage that may accompany dehydration of desiccation-sensitive axes from recalcitrant seeds, including: mechanical rupture or collapse and damage to supporting elements of the cyto- and

nucleoskeletons; metabolism-associated damage and, related to this, damage to organelles which includes membranes and macromolecules.

If explants of a suitable size are readily available (e.g. sufficiently small embryonic axes) or can be identified (e.g. meristems) or developed (e.g. somatic embryos), then the various parameters necessary for successful cryostorage used, need to be optimised. By this it is meant that the types of damage described above must be counteracted by appropriate procedures, before they can occur. Alternatively, if damage is inevitable then, as long as it is not lethal, measures to ensure remediation, must be developed. It must be realised, however, that notwithstanding optimisation of all known parameters, successful cryopreservation cannot be predicted.

1.3.6.2 WATER IN SEEDS

In order to understand the mechanisms of desiccation-tolerance better, one has to understand the nature of damage when water is removed from the cells of plant tissues. Five types of water have been distinguished by authors such as Rupley *et al.* (1983); Vertucci and Leopold, (1987); Vertucci (1990; 1993).

Type 1 water is described as very strongly structure-associated, i.e. water which primarily constitutes a mono-layer on proteins and other subcellular structures. According to Vertucci and Roos (1990), type 1 water occurs at water potentials less that -150 MPa or water contents less than about 0.08 g g^{-1} dmb (7% fmb). If this water is removed, destabilisation of structures such as membranes,

cytoskeletal elements and macromolecules could occur (Pammenter and Berjak, 1999). This is the reason type 1 water is also often called bound or non-freezable water (Pammenter et al., 1991; Pritchard, 1991; Berjak et al., 1993; Pritchard and Manger, 1998),: apparently the presence of this bound or non-freezable water has been observed in all desiccation-tolerant plant tissues examined by Vertucci and Leopold (1987), although, using sorption isotherms, those authors could not ascertain the situation in desiccation-sensitive material at that stage. According to Meryman and Williams (1985), more than 20% of total cell water may in fact be termed non-freezable.

Type 2 water is known to be more weakly associated, and has glassy characteristics. It is believed to interact with polar surfaces of macromolecules or hydroxyl groups of solutes. Type 2 water is detected at water potentials between -12 and -150 MPa or water contents of 0.25 and 0.08 g g^{-1} dmb (20%-7% fmb) Type 3 water has been suggested to be that water which interacts with hydrophobic groups. It is detected at water potentials between -4 and -11 MPa or water contents between 0.45 and 0.25 g g^{-1} dmb (31%-20% fmb). Type 4 water is believed to be a concentrated solution or capillary water and is detected at water potentials between -2 and -4 MPa, or water contents between 0.7 and 0.45 g g^{-1}dmb (41%-31% fmb). Type 5 water, on the other hand, is dilute solution water or 'bulk water' and is detected at water potentials greater than -2 MPa or water contents ranging from 0.6 to 0.9 g g^{-1} dmb (38%-47% fmb).

If dehydration is achieved rapidly, from axes excised from mature desiccation-sensitive seeds of a variety of species, viability is generally maintained to water contents in the range 0.45 - 0.28 g g^{-1} dmb (31%-22% fmb). However, below this water content, the water which is all structure-associated, not only becomes more resistant to removal, but its removal is lethal. Vertucci and Farrant (1995) have therefore suggested that many recalcitrant seeds can survive drying to within the third level of hydration, but cannot however survive prolonged periods at this level. Vertucci (1992) suggested that this is possibly because of the unregulated catabolic activities that lead to the degradation of macromolecules as well as the accumulation of toxins. In fact, various authors have suggested that with the complete removal of type 3 water, associated membrane structural changes occur (Crowe et al., 1987; 1992; Wolfe, 1987; Bryant and Wolfe, 1989; 1992; Steponkus and Webb, 1992b).

It seems that proportions of water of different types do not actually differ from recalcitrant seeds to orthodox seeds, as previously suggested by Berjak et al. (1989) and Vertucci (1990). Desiccation tolerance is rather suggested not to be a result of the amount of structured water, but rather the ability of that seed type to lose a considerable proportion of hydration water without damage (Vertucci and Farrant, 1995).

Pammenter et al. (1991) concluded that while desiccation-tolerant seeds naturally lose freezable water, desiccation-sensitive seeds can only lose this

water when it is removed very rapidly. Thus, rapidly dried tissue can survive when dried to lower water contents because the tissue spends insufficient time at intermediate water contents (hydration levels 3 and 4) therefore not allowing time for deleterious reactions to occur (see below).

1.3.6.3 DEHYDRATION (PRE-FREEZE STATUS)

The most critical step needed to achieve survival before freezing can be implemented, is dehydration. Seven dehydration procedures have been identified over the years, including encapsulation-dehydration; vitrification; encapsulation-vitrification; desiccation; pre-growth; pre-growth desiccation and droplet freezing (Engelmann, 1997). For the purposes of the present investigation, success was attained using desiccation, thus the other more complex, dehydration regimes, were not attempted.

Desiccation involves a simple procedure of removing water from the plant material before freezing. Desiccation has been applied mainly to zygotic embryonic axes of various species, including numerous tropical forest trees (Normah and Marzalina, 1995) as well as to somatic embryos of several species and to shoot tips of mulberry (Engelmann, 1997).

In many cases, zygotic embryos are potentially the easiest and most accessible means of conserving seed germplasm where whole seeds cannot be used. On excision from fresh recalcitrant seeds, the excised zygotic axes are highly hydrated, and have to be desiccated to suitable water contents before freezing

can be implemented. This is primarily to protect them from the damage caused

by the crystallization of intracellular water to ice (Meryman, 1966; Mazur, 1969;

1970; 1984). It has been stated that recalcitrant seeds undergo serious,

frequently lethal cellular damage when their water content is reduced below a so-

called 'critical' water content suggested to be species-specific pertaining to the

specific seed in question (Chin and Roberts, 1980; Engelmann, 1992; 1997).

However, many characteristics, some of them inherent, (e.g. the developmental

stage of the embryos at the time of harvest [Berjak *et al.*, 1992; 1993]) and

others, experimental - particularly drying rate (see below), as illustrated by Berjak

et al. (1993) and Pammenter *et al.* (1998). For instance, mature coffee embryos

displayed a higher survival rate than immature ones (Abdelnour-Esquivel *et al.*,

1992).

Dehydration has frequently been carried out by placing embryonic axes of

recalcitrant and intermediate seeds, or small seeds themselves in a laminar-flow

cabinet, for example: for oil palm (Grout *et al.*, 1983); coffee (Abdelnour-Esquivel

et al., 1992); black pepper (Chaudhury and Chandel, 1994); hazelnut (Gonzales-

Benito and Perez, 1994). Pritchard and Prendergast (1986) achieved

unorganised growth within the root meristem of embryos of *Araucaria hunsteinii*

after laminar airflow desiccation and cryopreservation. Normah *et al.* (1986)

indicated that 20-69% fmb (0.25-2.23 g g^{-1} dmb) of the embryonic axes of *Hevea*

brasiliensis could survive storage in liquid nitrogen after partial desiccation to

water contents of 14-20% fmb (0.16-0.25 g g^{-1} dmb) and developed into

complete plants. Among other authors who used laminar-airflow for the dehydration of seed material were Chaudhury *et al.* (1991) and Chandel *et al.* (1994) for *Camellia sinensis*; Assy-bah and Engelmann (1992) for mature axes of *Cocos nucifera*; Gonzales-Benito and Perez-Ruiz (1992) for *Quercus faginea*; Poulsen and Eriksen (1992) for *Quercus robur* and Chandel *et al.* (1994) for both *Theobroma cocao* and *Artocarpus heterophyllus*. However, success in terms of normal seedling development was not attained following cryopreservation in several of these cases.

But, the more rapidly dehydration can be achieved, the greater the water loss that the embryonic axes can withstand (Berjak *et al.*, 1989; 1993; Pammenter *et al.*, 1998). Potential for germination after cryopreservation in liquid nitrogen at -196°C of very rapidly dried axes is theoretically much higher, as long as desiccation damage *sensu stricto* (Pammenter *et al.*, 1998) has not occurred. In the context of manipulation of axes for cryostorage, it must be stressed that the advantage of very rapid dehydration lies, not in achieving maximum water contents commensurate with viability retention, but in achieving the devised water content as quickly as possible. The less time, desiccation-sensitive axes are maintained in the water-stressed conditions imposed by dehydration prior to freezing, the shorter the period during which metabolism-related, particularly lethal, damage can occur (Pammenter *et al.*, 1998) According to those authors one of the most serious consequences of metabolism-related damage, is free radical generation, which given adequate time, would be lethal. There is little

doubt that the type, and the degree, of stress-related damage inflicted on the specimen before, and during, the actual freezing processes will determine the ultimate vigour and viability of that specimen: metabolism-related damage during dehydration is considered to be a major consideration. The present work focuses on the critical importance of the parameter relating to the desiccation of zygotic axes in the establishment of freezing protocols (see Chapters 2 and 3).

More rapid drying techniques such as placing axes in airtight containers with silica-gel (Engelmann, 1997), and another, termed flash-drying, has been developed (Berjak et al., 1990). Flash-drying involves placing excised axes in a stream of compressed air. The theory behind flash-drying is that it facilitates greater retention of viability with minimal trauma, as the time to attain suitable water contents is curtailed (Berjak et al., 1990; Pammenter et al., 1991). Dehydration has been shown to be reduced to that of the level of structure-associated, non-freezable water in a matter of approximately 15 min to 3 h, depending on the species, size and initial water content of the axis and its developmental status (e.g. Pammenter et al., 1991; Berjak et al., 1992; 1993). It has been shown that excised embryonic axes of desiccation-sensitive seeds can withstand considerable dehydration if flash-dried (Berjak et al., 1990; 1992; 1993; Pammenter et al., 1991; Vertucci et al., 1991) thus making the cryopreservation of such dried axes a possibility (Wesley-Smith et al., 1992).

In recent studies on *Ekebergia capensis* (Pammenter *et al.*, 1998), the data confirmed that the seeds showed a response to the drying rate itself. In fact, axes from rapidly dried seeds showed little sign of intracellular damage, unlike the excessive degradation that took place in the axes of slowly dried seeds.

Wesley-Smith (1999) has improved on the original flash-drying apparatus, thus achieving increased dehydration rates. This technique, described as fast flash-drying is described in relation to the relevant experiments in chapters 2 and 3. Because of the faster dehydration rate, fast-flash drying should go further to obviate the metabolism-related damaging reactions that result in viability loss at relatively high water contents when dehydration proceeds slowly (Pammenter *et al.*, 1991; 1998; Berjak and Pammenter, 1997 and Pritchard and Manger, 1998).

It is also important to note that the rate at which desiccation-sensitive seeds/axes lose water and the proportional amount of water lost before lethal damage occurs, is species-dependent. No matter how rapidly desiccation-sensitive seed tissue is dried, the 'critical' or lowest water content to which it will still be viable will always be higher than the 'critical' water contents of desiccation-tolerant seed tissue (Pammenter and Berjak, 1999). However, the objective of flash-drying or fast flash-drying in the context of cryopreservation, is to attain damage-free dehydration to the highest water content allowing successful cooling/freezing, and not the lowest.

1.3.6.4 CRYOPROTECTION

A further consideration in cryostorage studies is whether or not to use cryoprotectants [e.g. sorbitol, mannitol, proline, glycerol, sucrose, dimethyl sulphoxide (DMSO), methanol, polyethylene glycol or combinations thereof (Withers, 1985a; Engelmann, 1997)]. Cryoprotectants, which themselves may effect considerable dehydration, are used as a form of protection for biological material before drying and eventual immersion into liquid nitrogen. The theory behind the use of cryoprotectants is basically to increase the concentration of the cytoplasm and reduce the amount of ice formed at any given temperature (Meryman and Williams, 1985). The nature and the timing of cryoprotectant application can be critical to the survival of the biological material in question.

Thus, cryoprotection can be advantageous as it can prevent water from freezing in the form of crystalline ice. Some non-cytotoxic cryoprotectants may rapidly penetrate cells, and act as solvents for electrolytes (Meryman, 1966). For example, pre-treatment with high concentrations of sucrose has been used to promote desiccation and/or freezing tolerance of some zygotic and somatic embryos, as reported by Monier and Leddet (1978), Engelmann (1986), Anandarajah and McKersie (1990), Assay-Bah and Engelmann (1992), Dumet *et al.* (1993b), and Thierry *et al.* (1997). Cryoprotectants are also thought to be beneficial in the stabilisation of membranes and macromolecules through various mechanisms, for instance free radical scavenging (Benson and Withers, 1987; Benson, 1988).

However, use of cryoprotectants may also lead to complications, as the more parameters involved in a procedure, the more complicated and variable conservation of seed germplasm will be in terms of stress and injury to the specimen. Furthermore, cryoprotectant pre-treatment may actually be injurious: for example, cytotoxicity, dehydration injury and mechanical injury during the freezing and thawing steps have been ascribed to such procedures (Kartha, 1985; Engelmann, 1991; Steponkus et al., 1992a). There is evidence, for instance, of DMSO interfering with microfibrils, microtubules and the cell division process (Withers, 1987).

1.3.6.5 FREEZING

According to Withers (1980; 1982; 1985a), however, cryopreserved material is presumed to remain genetically stable, thus minimising genotypic changes that occur during conservation by the use of standard techniques, including tissue culture manipulations. This is of vital importance in germplasm storage as the primary aim is to conserve the pre-existing genotype (Mycock et al, 1989). A limited assessment of genetic stability in plants has been done (Engelmann, 1997). While studies indicating maintenance of genetic stability have been carried out for long-stored in vitro shoot cultures (of Cassava [CATIE-IPGRI, 1997], none appears to date to have been undertaken as cryostored material. It is a priori requirement that for cryopreservation to be of any value for the long-term conservation of genetic material, the genetic stability of the regenerating plants after cryopreservation must be identical to the initial starting material.

Related studies focussing on this vital aspect of the procedure are ongoing in our laboratory, although they are not a feature of the present work.

1.3.6.5.1 CLASSICAL CRYOPRESERVATION TECHNIQUES

Cryopreservation is generally understood as storage between -79 and -196°C (-196°C being the temperature of liquid nitrogen). The rate of cooling (more usually termed freezing), however, may have significant implications for the success/failure of survival (Engelmann, 1997). The two rates of freezing discussed under this heading include the classical slow and rapid cooling. Slow cooling was thought to be the most effective in preventing ice formation and cellular injury (Kartha, 1987).

Slow cooling involves regulated cooling at a constant rate of 0.5 to 2°C min^{-1} to temperatures between -30 to -40°C, using programmable freezing devices which achieve precise freezing conditions followed by transfer of the specimens to liquid nitrogen (Withers, 1985b; Kartha, 1987). However, success has been reported by several authors who achieved slow cooling using deep-freezers (Lecouteux *et al.*, 1991; Sakai *et al.*, 1991; Nishizawa *et al.*, 1992; Engelmann *et al.*, 1994; Tessereau *et al.*, 1994; Engelmann, 1997). During the slow cooling process, the plasmalemma/plasma membrane appears to act as a physical barrier preventing the ice from seeding the cell interior, which as a result, becomes supercooled, remaining unfrozen.

As the temperature is further decreased, an increasing amount of cell water is drawn out to the extracellular ice nucleation centres, resulting in the concentration of intracellular solutes (Merryman et al., 1977). This process has been suggested to occur in a variety of plants (Burke et al., 1976). Success with this slow cooling has been revealed for a number of tropical forest tree species (Chai et al., 1994; Normah and Marzalina, 1995). However, according to Krishnapillay (1989), major damage is likely to occur on slow cooling as a result of osmotic cell dehydration causing cell shrinkage and toxic concentrations of intracellular solutes.

Rapid cooling (also known as classical cooling), on the other hand, is accomplished when axis material enclosed in cryotubes, is plunged directly into liquid nitrogen, at cooling rates of several hundred degrees per minute (Kartha, 1987). It has been assumed that the intracellular fluids do not have time to equilibriate with the external ice, and lethal intracellular ice formation occurs. It has also been suggested by Kartha (1987) and Grout (1990) that the rapidity with which the cells go through the temperature zone in which lethal ice formation occurs may in fact prevent intracellular ice crystal development both during cooling and thawing, as long as this is carried out sufficiently rapidly. According to Steponkus et al. (1992a) and Sakai (1995), rapid cooling, which they suggest involves vitrification-based procedures, offers more practical advantages than classical slow cooling, since a programmable freezer is not required.

Vitrification is defined as the formation of a glassy, non-crystalline, highly viscous metastable state: for this to be achieved, direct plunging into liquid nitrogen would be better than slow cooling. It is suggested that such vitrification technology would be advantageous for more complex organs such as apices/embryos which contain a variety of cell types (Withers and Engelmann, 1997). This rapid cooling regime was also demonstrated by Kartha in 1981 to be successful for cryopreservation of shoot-tips of a few species. Langis et al. (1989) successfully vitrified cell suspension cultures of Brassica, while in 1990, Langis and Steponkus reported success with rye protoplasts. Mycock et al. (1991) managed to conserve hydrated embryonic axes of Pisum sativum again using this rapid cooling technique.

In terms of relative success, the type of rapid cooling, described above is not necessarily the optimal or most rapid approach to freezing axis material. The cryotube can impede efficient heat transfer, and liquid nitrogen itself is not the best of cryogens for rapid cooling (Wesley-Smith et al., 1999).

1.3.6.5.2 ULTRA-RAPID COOLING

Wesley-Smith et al. (1992) investigated the possibility that the principles used in cryo-electron microscopy might be used to minimize freeze-related damage and enhance viability retention in cryostored embryonic axes of Camellia sinensis. These principles embody the theory that only at sufficiently high rates of freezing, can the formation of crystalline ice be avoided, allowing the intracellular solution to become vitrified. Under such conditions, if any ice crystals formed, they should

be sufficiently small to preclude physical intracellular damage. If vitrification can be achieved, this should be the optimal situation favouring survival.

Extension of the 1992 investigations of Wesley-Smith et al. resulted in the design of the cryopreservation of embryonic axes at ultra-rapid cooling rates, equipment, and use of alternate cryogens, Wesley-Smith et al. (1999). For optimal ultra-rapid cooling, it is essential to reduce the latent heat and thermal mass of the material to achieve higher cooling rates. Ultra-rapid cooling widens the range of water contents for successful cryopreservation, such that axes no longer need to be dehydrated to levels near the limit of desiccation tolerance (Wesley-Smith et al., 1999).

It was suggested by Wesley-Smith et al. (1995; 1999), that even though liquid nitrogen is a good cryogen, it is a poor acceptor of heat, thus a secondary cryogen was needed. The safest, easiest and most non-toxic secondary cryogen currently used for ultra-rapid cooling of embryonic axes is iso-pentane (Wesley-Smith et al., 1999).

This ultra-rapid cooling, freezing or rapid entry of the axes into iso-pentane contained within a reservoir of liquid nitrogen itself, should therefore aid in the heat exchange process. In addition, sub-cooling the secondary cryogen (iso-pentane) prevents the formation of a gaseous envelope around the specimen

that would otherwise reduce the freezing rate (Wesley-Smith *et al.*, 1995). This will be fully discussed in Chapters 3 and 4.

1.3.6.6 THAWING AND POST-THAW RECOVERY

In all cases, thawing must be carried out rapidly to avoid ice re-crystallisation damage. Ice re-crystallisation could occur on slowly warming the intracellular water above the re-crystallisation point where minute crystals start to melt and re-freeze, resulting in larger ice-crystal formation. Depending on the size of the ice-crystals, lethal damage could result (Farrant, 1980). Therefore, according to that author, rapid warming of cells and tissues affords better survival potential than slow warming. A number of rapid warming protocols have been used. For instance, Meryman and Williams (1985) suggested that cells could be rapidly thawed by microwave heating, Wesley-Smith (1999) suggested warming ultra-rapid cooled specimens in distilled water at 35-40°C. Whatever the thawing procedure, once newly thawed, the specimen is very vulnerable.

According to Benson (1995), the storage in, and recovery of plant cells and tissues from, ultra-low temperatures could potentially lead to: loss of structural integrity; cell wall damage; organelle damage; membrane damage; disruptions in metabolism; disruptions in cellular communications (signalling); loss of regenerative capability and disruption of genetic processes. Unfortunately, all these different levels of injury are interrelated. For instance, structural damage can lead to disruptions in membrane/cell wall-dependent communications, which in turn would impair the cell signalling events that regulate genome expression.

For these reasons, studies on the structural/metabolic situation and genetic integrity are very important in evaluating cryopreservation and its use as a tool to conserve plant genetic resources.

1.3.6.6.1 *In Vitro* RECOVERY

The formulation of germination media for the successful recovery of any cryopreserved material via *in vitro* culture procedures is a major consideration. In order for isolated plant tissues to grow successfully *in vitro*, the explant must be provided with all the essential macro- and micronutrients, a carbohydrate source, amino acids, vitamins and growth regulators. However, success is often elusive. In some cases, composition of the recovery media have encouraged abnormal development patterns, such as the non-development of the haustorium in the case of *Howea* and *Veitchia* (Chin *et al.*, 1988); or callusing and/or incomplete development with *Hevea* (Normah *et al.*, 1986), *Castanea* and *Quercus* (Pence, 1990), and oil palm (Engelmann *et al.*, 1995). All parameters vary considerably on a species-basis and the outcome of their combined usage is unpredictable.

In summary, freezing may involve damage from either dehydration or ice formation or both. Thawing and recovery will ultimately reveal either the achievement of producing organised plant structures such as roots and shoots or damage such as deplasmolysis (i.e. expansion-induced injury to cells on thawing) (Withers, 1987). Another point is that the cellular heterogeneity in plant tissues and organs, hinders the optimisation of a specific cryopreservation strategy for all explant types and species.

Some cryopreservation procedures have been successful. Procedures have been developed for explants of about 100 different species cultured in various different ways, including: cell suspensions, calluses, apices, zygotic and somatic embryos (Withers, 1982; Kartha and Engelmann, 1994; Engelmann *et al.*, 1995; Withers and Engelmann, 1997). Ideally, routine application should be implemented for all of these, as has been done for oil palm, where 80 clones of somatic embryos are stored in liquid nitrogen and samples thawed upon request for plant production (Dumet, 1997).

1.4 THE PRESENT INVESTIGATION

The present investigation therefore aims at: optimising on culture media for the growth of *Q. robur* and *E. capensis* zygotic axes; manipulating drying and freezing/cooling rates; as well as optimising recovery on different thawing media with the objective of minimising structural damage as much as possible. The main idea was therefore to gain success in the long-term storage (cryostorage) of these two recalcitrant-type species.

CHAPTER 2: MATERIALS AND METHODS

2.1 ESTABLISHMENT OF GERMINATION AND CULTURE PROTOCOLS FOR *Quercus robur* AND *Ekebergia capensis* WHOLE SEEDS AND ZYGOTIC AXES

The way a plant grows and develops, whether from a whole seed or a zygotic embryo (in this case), results from complicated interactions among three levels of control - the intracellular level, the intercellular level and the environmental level.

Control over growth and development of the organism occurs at the intracellular level by the production of hormones and other substances that are transported between plant tissues (i.e. intercellular level). The environmental level involves the control and development of that organism by factors such as direction or intensity of light, amount of moisture, acidity of rain, extremes in temperature, availability of minerals from the soil, etc. Basically the environment plays a critical role in moulding plant growth and development (Brum and McKane, 1989).

The outcome of cryopreservation depends on factors at three levels of control of growth and development. These include the habitat of the parent plant; the time of seed harvest relative to full pre-shedding development (physiological age); the initial water content; initial germination rate and totality (i.e. vigour and viability); as well as the size and ultrastructural characteristics of the structure(s) to be cryopreserved.

Other critical factors contributing to ultimate success include harvesting and transport of the seeds, and equally importantly, seed health. Once recalcitrant seeds have been hand-harvested (preferably off the parent plant or at least very soon after shedding), preparation of the material for eventual cryopreservation should be immediate to prevent dehydration, fungal infection and proliferation, and to obviate the deteriorative changes which inevitably accompany hydrated storage (Pammenter *et al.*, 1994; Smith and Berjak, 1995). These ideals may not always be achievable, however, as was the case for *Q. robur* in the present study. Those *Q. robur* seeds transported from overseas (see below), were not only consigned in large batches, but were also immature upon receipt; they were germinable, but did not, however, develop into seedlings (see Chapter 3). To achieve post-harvest maturation, these seeds were cold-stored (6 ± 2°C) hydrated and intact. Ultimately, it is preferable to hand-harvest (more) mature seeds, but this is not always possible partly because of reliance on other collectors, and as problems are posed by birds, mammals and insects making early harvesting desirable. Fruits of *Ekebergia capensis,* the other species used for the present study, could be harvested once mature, as trees grow in close proximity to the University of Natal, in Durban, and therefore could be monitored frequently.

2.1.1 PROVENANCES AND INITIAL TREATMENT OF MATERIAL

2.1.1.1 *Quercus robur*

Acorns were translocated under moisture retaining conditions from a number of provenances, namely: the United Kingdom[1]; Humlebaek, Denmark as well as

locations in South Africa: Stellenbosch[2]; Grahamstown[3]; and in KwaZulu Natal from Underberg and Himeville. After transportation by air (under controlled temperature and pressure conditions from abroad) or road, acorns were kept intact for varying periods at $6 \pm 2°C$ in open mesh bags, raised on a grid, (Finch-Savage, pers. comm.[1]). The acorns were periodically sprayed with a liquid form of the fungicide, Previcur N[®] (propamocarb - HCl - 722 g l^{-1}) [AgrEvo S. A. (Pty) Ltd]. Axis water contents were determined gravimetrically to constant mass after drying at 90°C (17-24 h). All water contents were expressed on a dry mass basis [gram of water per gram of dry mass (g g^{-1})].

Fresh seeds or those seeds sampled from storage were set out in moistened vermiculite under greenhouse conditions. Twenty seeds were used per germination trial. Before planting out, the pericarp and testa was removed from each seed, after which the seeds were soaked in tap water overnight. The next day all the seeds were cut transversely, the distal halves of the cotyledons were planted out into moistened vermiculite with distal ends facing upwards and cut ends facing downwards (Finch-Savage, pers comm[1].). Germination was monitored daily.

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2.1.1.2 *Ekebergia capensis*

Deep-red, ripe fruits were hand-harvested from local trees. Upon arrival to the laboratory, a batch of twenty seeds (per germination trial) was removed from the fruits, and after the testa was removed from each seed to expose the cotyledons, they were soaked overnight in tap water. The next day, these seeds were set out on filter paper moistened with a 1 g l^{-1} (m/v) MS solution. Germination was assessed daily.

2.1.2 SEED GERMINATION PERFORMANCE AND INITIAL WATER CONTENTS

Prior to experimentation, seed germination performance and initial water contents of *Q. robur* and *E. capensis* embryonic axes were assessed to ascertain the maturity status of several surface-sterilant treatments and media on which to grow *Q. robur* and *E. capensis* embryonic axes, were then tested. Percentage survival and growth rates (the rate of root elongation and rate of shoot production) were measured at constant intervals.

2.1.3 GERMINATION ASSESSMENT

Radicle protrusion and intensified shoot greening leading to the production of leaves were used as the criteria for germination and the ability for seedling production in the case of both whole seeds and embryonic axes, of both *Q. robur* and *E. capensis.*

2.1.4 MANIPULATION OF EMBRYONIC AXES

Prior to any manipulation, embryonic axes of *Q. robur* and *E. capensis* (approximately 3 and 2 mm, respectively) were excised, placed onto moist filter paper within Petri dishes and covered to prevent drying, until the required number had been excised.

2.1.5 ESTABLISHMENT OF THE MOST SUITABLE SURFACE-STERILANT

Different surface-sterilants were tested to ascertain the most effective and least injurious procedure to remove fungal propagules from the embryonic axes.

The surface-sterilants used were 1% (v/v) sodium hypochlorite containing a wetting agent, 1 g l^{-1} (m/v) mercuric chloride (Chmielarz, 1997) and a step-wise combination of 70% (v/v) alcohol, 6% (v/v) calcium hypochlorite, 5% (v/v) sodium hypochlorite (Poulsen, 1992).

2.1.5.1 SODIUM HYPOCHLORITE, 1%

Embryonic axes were submerged in a 1% (v/v) sodium hypochlorite solution containing Tween-20 (1 drop) for 10 min, after which the axes were briefly rinsed three times in sterile distilled water under sterile laminar-flow conditions.

2.1.5.2 MERCURIC CHLORIDE, 0.1% (Chmielarz, 1997)

Embryonic axes were surface-sterilised using 1 g l^{-1} (m/v) mercuric chloride for 2.5 min, and then rinsed four times in sterile distilled water under sterile laminar-flow conditions.

2.1.5.3 COMBINATION SURFACE-STERILISATION - (used only for *Q. robur*) (Poulsen, 1992)

For this method, acorns were briefly soaked in 70% (v/v) alcohol, after which the pericarps were removed. Whole acorns (minus the pericarps) were then swabbed with 70% (v/v) alcohol, and then, under a laminar airflow, soaked in 6% (v/v) calcium hypochlorite for 15 min followed by three washes in sterile distilled water. Embryonic axes were then excised, and immediately plunged into a filter-sterilised solution of anti-oxidant [10 mg l^{-1} (m/v) ascorbic acid and 5 mg l^{-1} (m/v) citric acid]. Axes were then sterilised in 5% (v/v) sodium hypochlorite for 3 min, and washed three times in sterile distilled water and finally soaked for approximately 30 min in the ascorbic acid/citric acid anti-oxidant solution.

2.1.6 ESTABLISHMENT OF THE OPTIMAL *in vitro* CULTURE MEDIA FOR *Q. robur*

To determine the optimal medium for *Q. robur* embryonic axes, a number of media were tried. We have found from embryonic axis propagation, different strengths of Murashige and Skoog (1962) medium (MS) produce good, but varying results depending on the species. Thus, initially, embryonic axes were plated out horizontally on full-, half- and quarter-strength MS media. All media were supplemented with 30 g l^{-1} (m/v) sucrose and solidified 10 g l^{-1} (m/v) agar. In addition, a half-strength MS medium supplemented with 0.1 mg l^{-1} (m/v) NAA and 2 mg l^{-1} (m/v) BA and containing 30 g l^{-1} (m/v) sucrose and 10 g l^{-1} (m/v) agar has been previously successful for a number of species in our laboratory, and was therefore considered for *Q. robur*. Furthermore, a medium employed by Chmielarz (1997) specifically for *Q. robur* was also used. This consisted of the

macronutrient formulation of Quoirin and Lepoivre (1977), MS micronutrients and vitamins (Murashige and Skoog, 1962) and 1 mg l^{-1} (m/v) BA, 0.25 mg l^{-1} (m/v) zeatin, 30 g l^{-1} (m/v) sucrose and 6 g l^{-1} (m/v) agar. Propagation of Q. robur axes was also carried out on Woody Plant Medium (WPM) (Smith and McCown, 1982/83) supplemented with the hormones NAA (0.01 mg l^{-1}) and BA (0.3 mg l^{-1}), and containing 5 g l^{-1} (m/v) polyvinylpyrrolidone (PVP), to reduce tissue browning (Poulsen, 1992). The seventh medium assessed, again because it had previously been used for Q. robur, was that described by Vieitez and Vieitez (1983) consisting of 6 g l^{-1} (m/v) agar, 30 g l^{-1} (m/v) sucrose and 1 mg l^{-1} (m/v) BA.

The pH of all media was adjusted to 5.6-5.8 prior to autoclaving for 30 min in a TOMY autoclave (temperature = 120-125 °C, pressure = 1.0-1.5 kg cm^{-2}).

Four hundred and twenty axes were excised: 140 of which were surface-sterilised with 1% (v/v) sodium hypochlorite, a further 140 with 1 g l^{-1} mercuric chloride (Chmielarz, 1997), and the remaining 140 with the 'combination surface-sterilisation' procedure of Poulsen (1992). Twenty axes from each different surface-sterilisation procedure were plated out on each of the seven media described above. All cultures were then incubated at 26 ± 2°C in 16/8 hour light/dark photoperiod (200 $\mu E\ m^{-2}\ s^{-1}$).

2.1.7 ESTABLISHMENT OF THE OPTIMAL *in vitro* CULTURE MEDIA FOR *E. capensis*

After surface-sterilisation, *E. capensis* embryonic axes were cultured horizontally on full-, half- and quarter-strength MS (Murashige and Skoog, 1962) media, as well as on half-strength MS medium supplemented with 0.1 mg l^{-1} (m/v) NAA and 2 mg l^{-1} (m/v) BA and containing 30 g l^{-1} (m/v) sucrose and 10 g l^{-1} (m/v) agar. Parameters such as pH and culture conditions were as described for *Q. robur*.

2.1.8 HARDENING-OFF AND ACCLIMATISATION OF ALL PLANTLETS

After 30 d for *Q. robur* and 15 d for *E. capensis* axes *in vitro*, all plantlets were transferred to hydrated Peat Moss (Jiffy-7 peat pellet, Hummert International Catalogue, 1997) that had been sterilised in closed, Magenta™ boxes (Sigma); and watered at weekly intervals with sterile distilled water using a sterile Pasteur pipette.

These Jiffy-7 peat pellets (approximately 7 mm in height when dry) are encased with a thin netting. The pellets consist of compressed peat with N, P and K plus minor nutrients such as B, Cu, Fe, Mn, Mo, Mg, S, Ca and Zn. After hydrating in sterile distilled water for approximately 10 min, each pellet swells to 35 mm in height. The pH averages from 5.5 to 5.8. Seedlings planted directly into these hydrated pellets were incubated at $26 \pm 2°C$ in 16/8 hour light/dark photoperiod (200 μE m^{-2} s^{-1}).

After seedlings reached the full height of the Magenta™ boxes (Sigma)[1], the entire Jiffy 'pot' was transferred to a small conventional plastic plant pot containing sterile soil. Once planted, a polyethylene bag was placed over individual seedlings and taped to the base of the pot. The covered pots were placed in the greenhouse for 7 d, after which, the polyethylene bags were pierced (using a lighted cigarette) to start the process of equilibriation of the atmosphere in the bag, with the ambient atmosphere. Bags were increasingly perforated until their removal after three months.

2.2 THE EFFECTS OF DIFFERENTIAL DRYING RATES ON THE VIABILITY OF *Quercus robur* AND *Ekebergia capensis* EMBRYONIC AXES

The long-term storage of desiccation-sensitive seed germplasm is not straightforward and cryostorage offers the only means of conservation. However, because of their high water contents, the embryonic axes of recalcitrant seeds must be subjected to certain pre-treatments such as dehydration to prevent any ice-crystal formation during cryostorage (see Chapter 1). In this study, embryonic axes of both species were subjected to rigorous desiccation pre-treatments to prepare them for the ultimate stress of cryostorage.

The aim of these trials was to determine an optimal drying rate that would reduce the water contents of embryonic axes of *Q. robur* and *E. capensis* without

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[1] As the hydrated 'pots' swelled to an almost similar diammeter as the culture vessels, their removal for subsequent planting was very difficult. Consequently, smaller pellets (Jiffy-9) have been used.

affecting the vigour and viability of these specimens. All drying experiments took place at ambient temperature.

2.2.1 DRYING METHODS USED

2.2.1.1 LAMINAR AIRFLOW DRYING

This drying method is considered a relatively slow procedure which is implemented by placing embryonic axes onto sterilised aluminium foil trays and allowing the laminar (sterile) air to flow over the specimens, which are turned frequently to promote uniform drying (this has the disadvantage that the axes may well be bruised or even perforated during turning). The laminar-flow hood is designed so that a curtain of filter-sterilised air continually covers the front opening, which should prevent contaminants from entering. The air in the curtain is re-circulated through filters, which remove particulate material, including microbes (Ketchum, 1988).

2.2.1.2 SILICA-GEL DRYING

This method is considered faster than laminar airflow drying, and is implemented by placing a mono-layer of 30 embryonic axes onto sterile filter paper supported on 100 g of sterilised activated silica-gel within 100 g flint ointment jars (Lasec) enclosed with polycarbonate lids (National Botanic Gardens, Kirstenbosch).

2.2.1.3 FLASH-DRYING

This method was developed by Berjak et al. (1989), and is considered faster than silica-gel drying. This technique involves the enclosure within a small plastic

container (120 mm x 95 mm), of embryonic axes upon a mesh under which a rapidly-flowing air stream ($\pm$ 9-10 l min^{-1}) is introduced from a compressed air source. The air-stream is diffused from below the axes and vented from the container through small holes in the lid. The principle of flash-drying is to reduce axis hydration to any level down to that of the structure associated, non-freezable water in a matter of 15 min to 3 h, depending on the species, and developmental status, size and initial water content of the axes.

2.2.1.4 FAST FLASH-DRYING

This method is essentially a combination of the silica-gel method and the flash-drying technique of Berjak *et al.* (1989), and affords the most rapid method of dehydration (Wesley-Smith *et al.*, 1999). The apparatus utilises a 12V PC fan fitted to the inside of the lid of a closed 1800 ml container, in which the axes are elevated on a grid above a measured quantity of activated silica-gel (Wesley-Smith *et al.*, 1999). The principle is that the fan removes water from the specimens, and is absorbed by the silica-gel. Batches of 60 axes were subjected to fast flash-drying for varying periods.

2.3 CRYOPRESERVATION AND POST-THAW RECOVERY OF *Q. robur* AND *E. capensis* EMBRYONIC AXES

Cryopreservation has been shown to be the most effective method of conserving germplasm (Haskins and Kartha, 1980) and is probably the only way to preserve recalcitrant seed germplasm viz. zygotic embryonic axes, or other explants. One

has to be circumspect, however, after all, cryopreservation of axes is literally in its earlier phase of development.

Liquid nitrogen is most commonly used to achieve cryopreservation, the major advantage being the extremely low temperature (-196°C) which should halt all metabolic processes and retard metabolism-related deterioration to immeasurably low levels. However, to achieve successful cryopreservation involves further optimisation of variables, including cooling (freezing) rate. Although it is an over-simplification, rates can be broadly described as slow, intermediate, rapid or ultra-rapid.

Thus, in this phase of the investigation, freshly excised embryonic axes of *Q. robur* and *E. capensis*, fast flash-dried to various water contents, were used to determine the effects of different freezing rates on viability.

2.3.1 PREPARATION OF EMBRYOS FOR COOLING/FREEZING

After surface-sterilisation in 1% (v/v) sodium hypochlorite for 10 min and rinsing in distilled water, fast flash-drying was implemented for various time intervals to dehydrate the specimens to various water contents.

2.3.1.1 CONVENTIONAL COOLING

Quercus robur axes were dried and slow cooled by reducing temperatures at a constant rate of 1°C min^{-1} to -38°C, using the subambient head of a Perkin-

Elmer 7 differential scanning calorimeter (DSC), and placed in this state in liquid nitrogen (Poulsen, 1992) or cooled at 2°C min^{-1} to 0°C, then at 1°C min^{-1} to -20°C, using the DSC and introduced into liquid nitrogen (in 1.8 ml sterile plastic cryovials) (Chmielarz, 1997).

In addition, a more rapid method of cooling (intermediate rate) was used. This involved placing already dried batches of 5 axes into 1.8 ml cryovials which were then plunged directly into liquid nitrogen achieving cooling rates of several hundred degrees per minute (Kartha, 1987). The specimens were kept in liquid nitrogen for 48 h.

The cryovials containing frozen specimens were ultimately rapidly transferred to a water bath at 40°C where they were maintained for 2 min to effect thawing of the embryonic axes. Success was scored by onwards growth of axes to the seedling stage, in vitro (see 2.1.6 and 2.1.7).

2.3.1.2 ULTRA-RAPID COOLING

The principle behind this approach to cryopreservation is to maximise the rate of heat loss from the specimen to a cryogen (in this case isopentane) in order to minimise ice-crystal growth. Fast flash-drying (see 2.2.1.4) was first implemented to lower axis water content and in so doing, reducing the thermal mass of the material, which consequently facilitates faster cooling rates (Wesley-Smith et al., 1999). Specimens were then individually plunged into the cryogen, isopentane,

which was cooled in liquid nitrogen, to just above its freezing point (-160°C), which maximises the thermal gradient between axis and cryogen. This optimally cooled isopentane bath was raised to the mouth of the liquid nitrogen flask to prevent harmful pre-chilling of the axes while travelling through cold gas layers. The wooden rod bearing an individual axis terminally, was poised approximately 10 mm above the cryogen and the specimen was immediately plunged into the isopentane to an average plunging distance of 145 mm. Axes were then retrieved directly from a stainless steel mesh basket held in liquid nitrogen for ± 1 h to standardise the final storage temperature of all treatments at -196°C prior to thawing. Warming was performed by rapidly plunging the specimens to a depth of 150 mm in a deep beaker of sterile distilled water at 35-40°C (Wesley-Smith *et al.*, 1999). Axes were retrieved immediately, placed onto filter paper to remove the surface water, and then plated onto the appropriate germination medium (see 2.1.6 and 2.1.7).

2.3.2 OTHER THAWING MEDIA

Other thawing media used were: a 50:50 solution of $1\mu M$ $CaCl_2$. $2 H_2O$ and 1 mM $MgCl_2$. $6 H_2O$ (Mycock, 1999); full-strength MS medium, supplemented with 4 g l^{-1} sucrose at 37°C for 30 min on a rotary shaker at 120 rpm; sucrose (Ψ -2 Mpa) and mannitol (Ψ -2 Mpa).

2.3.3 ELECTROLYTE LEAKAGE

A study was undertaken to compare the effect of distilled water and the $Ca^{2+}Mg^{2+}$ solution after axes were dried, frozen and thawed in the two media.

Measurements of conductivity of the leachate, were done to indicate the quality of each embryonic axis, and was performed on 20 individual axes for each treatment (distilled water and $Ca^{2+}Mg^{2+}$ solution, respectively), each in 1 ml distilled water, using a CM 100 conductivity meter (Reid and Associates, Durban, S. A.) Electrolyte leakage was recorded over 6 h at 5 min intervals.

2.3.4 SPECIMEN PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

Specimens (root and shoot apices separately) were trimmed to $\pm$ 1 x 1 x 1 mm and placed into 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight. Specimens were then washed in a 0.1 M phosphate buffer (pH 7.2) (3 x 5 min), post-fixed in 0.5% (m/v) osmium tetroxide for 1 h, and washed again in phosphate buffer (3 x 5 min). Subsequent dehydration of the specimens was carried out in a graded series of acetone [30%, 50%, 75% and 100% (v/v)]. The 100% (v/v) acetone was then replaced with a 1:1 mixture of epoxy resin:acetone and left for 4 h in a turn-table at room temperature, after which the material was immersed in full resin overnight at room temperature for infiltration. Polymerisation of individual specimens in fresh resin in silicone wells was carried out in a 70°C oven for 10 h.

2.3.5 MICROTOMY AND MICROSCOPY

Embedded specimens were sectioned using a Reichert-Jung Ultracut E microtome, post-stained with lead (for TEM) or toludine blue solution (pH 9.6) and viewed using both the Jeol JEM 1010 transmission electron microscope and

the Nikon Biophot light microscope. Images were recorded photographically on Kodak TEM film or Pan-F 35 mm film for light microscopy.

2.3.6 DATA ANALYSIS

Data were analysed using a number of techniques. Where appropriate, Univariate Multifactorial Analysis of Variance (ANOVA) (SPSS, version 9, 1998) and Scheffe's test at 95% confidence test interval and multiple range test (Statsgraphics Plus version 7) as well as descriptive statistics in Microsoft Excel (1997) were used.

CHAPTER 3: RESULTS AND DISCUSSION, *Quercus robur*

Quercus robur L. (Fagaceae), is commonly known as the English Oak, even though its natural habitat includes not only Europe but also northern and western Africa and western Asia. *Quercus robur*, also called the pedunculate oak, is one of the most economically important deciduous forest-tree species producing recalcitrant seeds. The seeds called acorns, are borne singly or in clusters on a 20 to 50 mm peduncle. These $\pm$ 65 x 35 mm brown propagules are actually simple fruits, each developed from an individual ovary. The outer brown layer of the structure is the dry pericarp, the inner brown layer is the testa and the cup-shaped involucre covers the basal third of each acorn. The two massive cotyledons (about 98-99% of the seed consists of the cotyledons) enclose the small embryonic axis, which, in the average-sized acorn, is about 3 mm x 1.5 mm (Pence, 1990 and Gracan, 1998).

It has been recorded that *Q. robur* seeds can be cold-stored for up to 2-3 years (Chin and Roberts, 1980). However, our observations (unpublished) are that the quality declines more or less rapidly depending on the initial condition, particularly as the seeds appear invariably to harbour an active, internal mycoflora. It is therefore necessary to conserve the germplasm of this economically-important species by means other than seed storage. The most obvious way to achieve this, is by cryopreservation of the zygotic embryonic axes. Until recently, *Q. robur* axis cryopreservation, with the outcome of successful seedling production, has been seemingly impossible (Pence, 1990;

Poulsen, 1992; Chmielarz, 1997). The objectives of the present study were to employ improved techniques of drying and freezing (Wesley-Smith *et al.*, 1999) as well as the rationalisation of the thawing regime, to attain successful cryostorage in terms of the ultimate production of vigorous, hardened-off young plants.

The results presented here detail the development of a successful protocol, which considers the issues of: the physiological age and water content of the seeds; the importance of optimal culture conditions; drying- and freezing-rates; and thawing parameters. Axis survival correlated with ultrastructure monitored at key points during the procedures used by Poulsen (1992) and Chmielarz (1997) is compared with that obtained following very rapid dehydration and cooling as rationalised by Wesley-Smith *et al.* (1999) and detailed by ourselves for *Q. robur* (Berjak *et al.*, 1999).

3.1 WATER CONTENTS BEFORE AND AFTER SHORT-TERM STORAGE

Batches of ten *Q. robur* axes from each of four South African and two European provenances (Stellenbosch, Grahamstown, Himeville, Underberg [S. A.] and the United Kingdom and Denmark) were taken for water content analysis immediately on arrival as well as after ten weeks in cold storage. Figure 3.1 shows that there was marked variability in axis water content among the various seed batches, both from single provenances, but different seasons, and from the different provences before (shaded bars) and after 10 weeks cold storage (clear bars). Water contents were taken after 10 weeks in storage to ascertain the
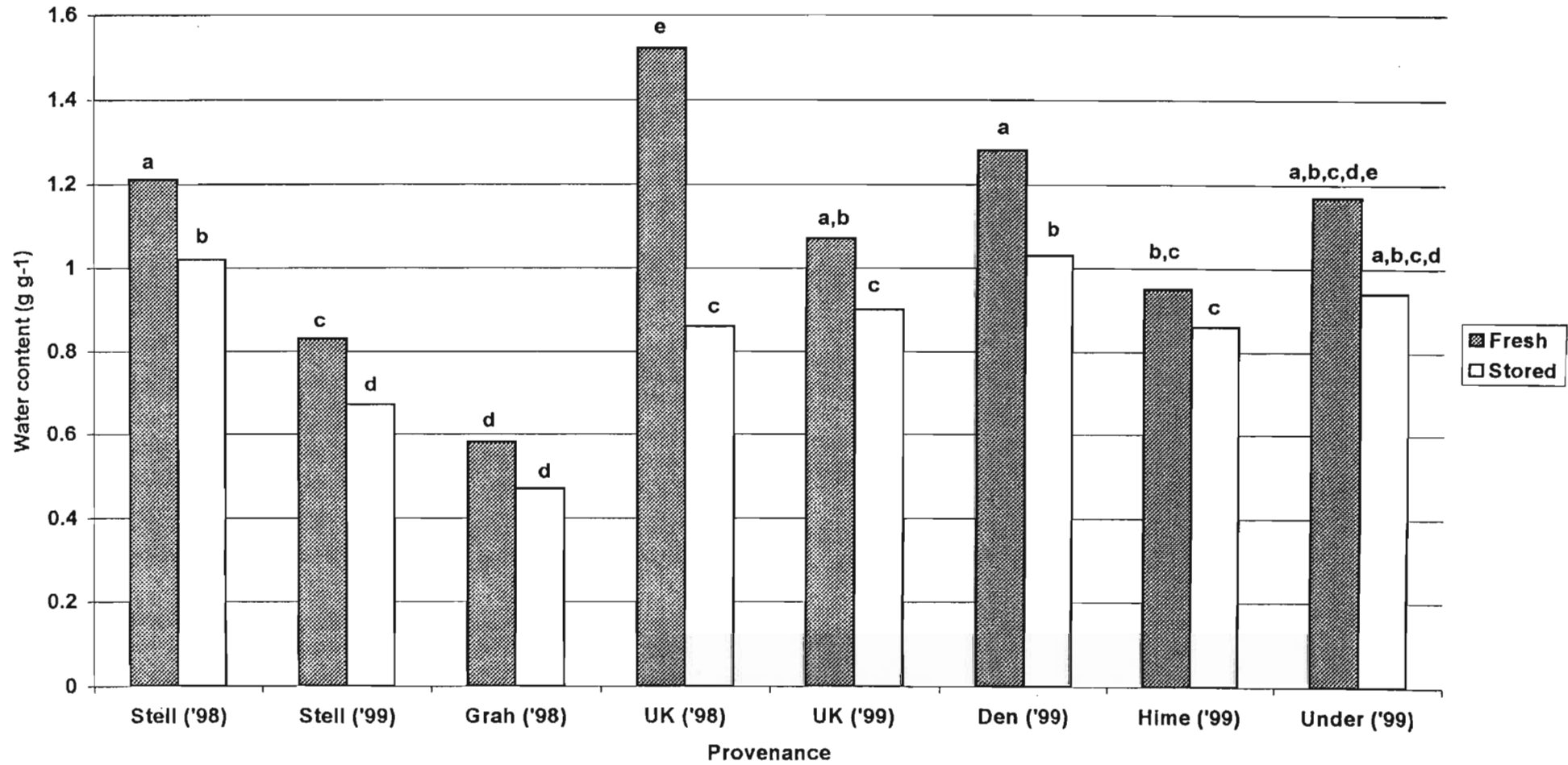
Figure 3.1: Water content variation (g H_2O g^{-1} dry mass) of *Q. robur* axes taken from seeds of six different provenances, immediately on arrival (shaded bars) and after ten weeks in storage (clear bars). Data points with different letters (a - e) are significantly different (P < 0.05).

trend(s) in water loss over time. It was observed that embryonic axes from all the seed batches, lost a considerable amount of water during storage, irrespective of the provenance, with the apparent exception of those from Grahamstown (1998). However, even in this case, the mean axis water content was lower after storage than before.

The water contents were not consistent for individual provenances over 1998 and 1999 (Figure 3.1). For instance axes from those fresh acorns from the United Kingdom from the 1998 batch had a mean axis water content of 1.44 g g^{-1} and those from the 1999 batch had a significantly different mean water content of 1.02 g g^{-1} ($P < 0.05$, ANOVA), despite being collected from the same tree. Similarly, mean water contents of the axes from fresh 1998 Stellenbosch acorns were significantly different from those of the fresh 1999 batch from the same provenance (1.21 g g^{-1} ± 0.08 and 0.79 g g^{-1} ± 0.02 respectively). Published data by Finch-Savage and Blake (1994) concerning fruit and seed development in *Q. robur* from a single tree over five consecutive seasons reflects similar significant variations in water content among seasons. Additionally, those authors observed a seasonal difference in the growth patterns in both the cotyledons and embryonic axes, which differed among years and resulted in seeds of different sizes.

The variability in the axis water contents of the acorns (Figure 3.1) was probably due, in part, to differences in habitat, geography, soil conditions, temperature and

seasonal cycles in the different provenances. Furthermore, it is possible that seeds from the various harvests were collected at different physiological ages and/or from different trees after being naturally shed. In this regard, Berjak *et al.* (1992; 1993) have shown water content differences to characterise the developmental progression of axes of recalcitrant seeds of other species, but in the present case, nothing accurate can be said because of the non-standardised collection.

Furthermore, it was observed that the seed quality differed amongst the provenances. The differences in mean water content are thought to be related not only to initial, but also to ultimate quality. In the case of the material from Grahamstown, indiscriminate collecting could have accelerated seed deterioration already before, and during, storage, as indicated by the lower mean water contents (before 0.58 ± 0.06 and after 0.47 ± 0.19 storage) of the fresh seeds and poor germination (80% before and 60% after storage).

In general, those acorns harvested from the northern hemisphere, had higher mean water contents than those from the southern hemisphere, which may be related to climatic and soil conditions. Additionally, while Finch-Savage and Blake (1994) had personally harvested their material, there is no guarantee that acorns harvested by others for the present study, would have been collected with meticulous attention to their newly-shed state.

3.2 WHOLE SEED VIABILITY

All fresh *Q. robur* seeds (except those from Grahamstown), irrespective of water content variation, achieved 100% germination after 4 weeks in vermiculite at $\pm$ 23-25°C. The same germination procedure was carried out with all seeds after 10 weeks in cold storage (6 $\pm$ 2°C), when it was observed that they germinated more rapidly, with seedling production occuring in approximately 2 weeks compared with 4-5 weeks for the fresh seeds. Similar results which have been shown for a variety of recalcitrant seed species, were first discussed for *Avicennia marina* (Pammenter *et al.*, 1984; Farrant *et al.* 1986; Berjak *et al.*, 1989).

Those observations, coupled with ultrastructural and biochemical analyses, led to the conclusion by those authors that, in the case of the highly recalcitrant seeds of *A. marina*, there was ongoing germinative metabolism during hydrated storage. Thus, when planted out, the stored seeds were further along the germination pathway than were those newly-harvested, therefore showed an ostensibly more rapid rate of germination. The difference was, however, obviated if the first day in storage (equivalent to the day of harvest, for *A. marina*) was taken as the datum point for all the seeds (Pammenter *et al.*, 1984). In a fine-tuning of the model developed by Farrant *et al.* (1986), Berjak *et al.* (1989) considered the situation of recalcitrant seed types that are shed prior to full embryo development (i. e. before physiological maturity). Such seeds were

described as having to complete development in the post-harvest condition, before germination could be initiated.

It was also interesting to note in the present study that even when it was known that all seeds of the same batch were simultaneously hand-harvested, some germinated and established seedlings considerably more rapidly than others, indicating a variability in the developmental status of the seeds at harvest. This variability may be correlated with different stress tolerance among the seeds to treatments such as dehydration and/or freezing even within a single harvest of seemingly similarly mature seeds.

3.3 *Quercus robur* EMBRYO CULTURES FROM SEEDS STORED FOR TEN WEEKS

For all experiments, the most visibly unblemished embryonic axes of standard size and appearance were excised and divided into three batches. Each batch was surface-sterilised with one of three surface-sterilants (sodium hypochlorite, mercuric chloride and Poulsen's combination surface-sterilant), and plated out onto seven different media (Figure 3.2 - see key). The embryonic axes chosen, not only appeared vigorous at excision, but proved to be 100% viable in culture, regardless of surface sterilant or medium used. However, the best possible combination of surface-sterilant and medium had to be selected to maximise the chances of survival after the imposition of the dehydration and freezing stresses (see later).
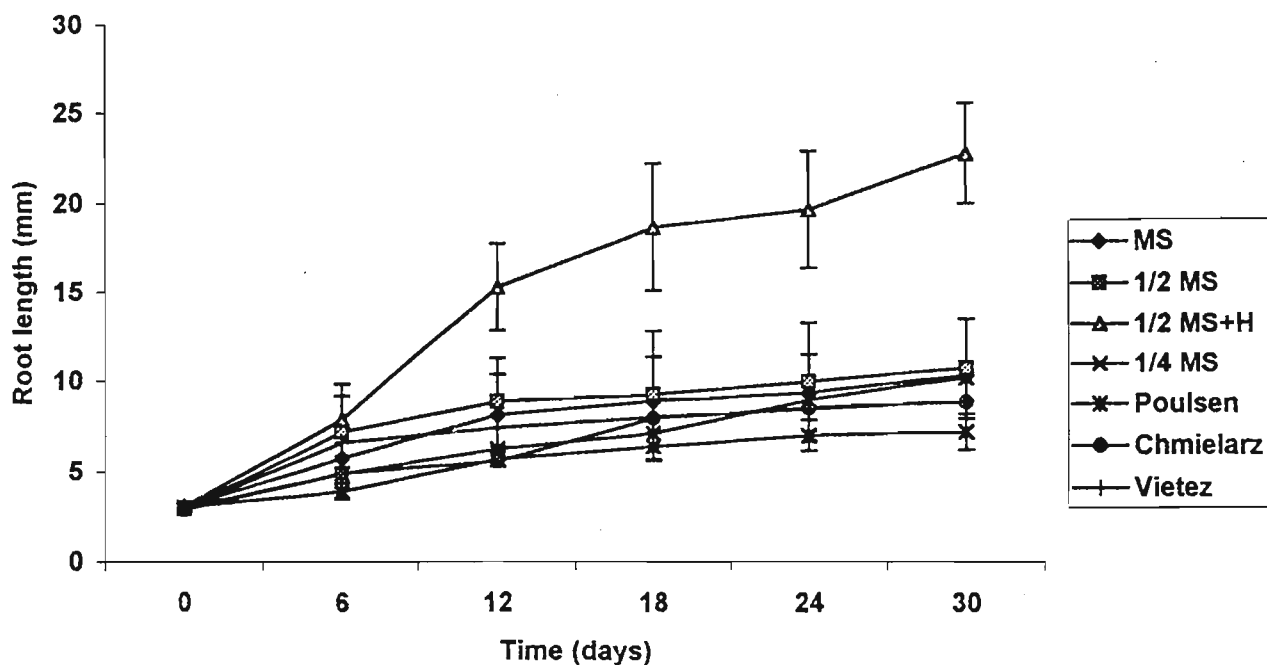
Figure 3.2a: Root length of *Q. robur* axes germinated on various media (see key) after sodium hypochlorite surface sterilisation (n = 20). Bar = mean ±SE.
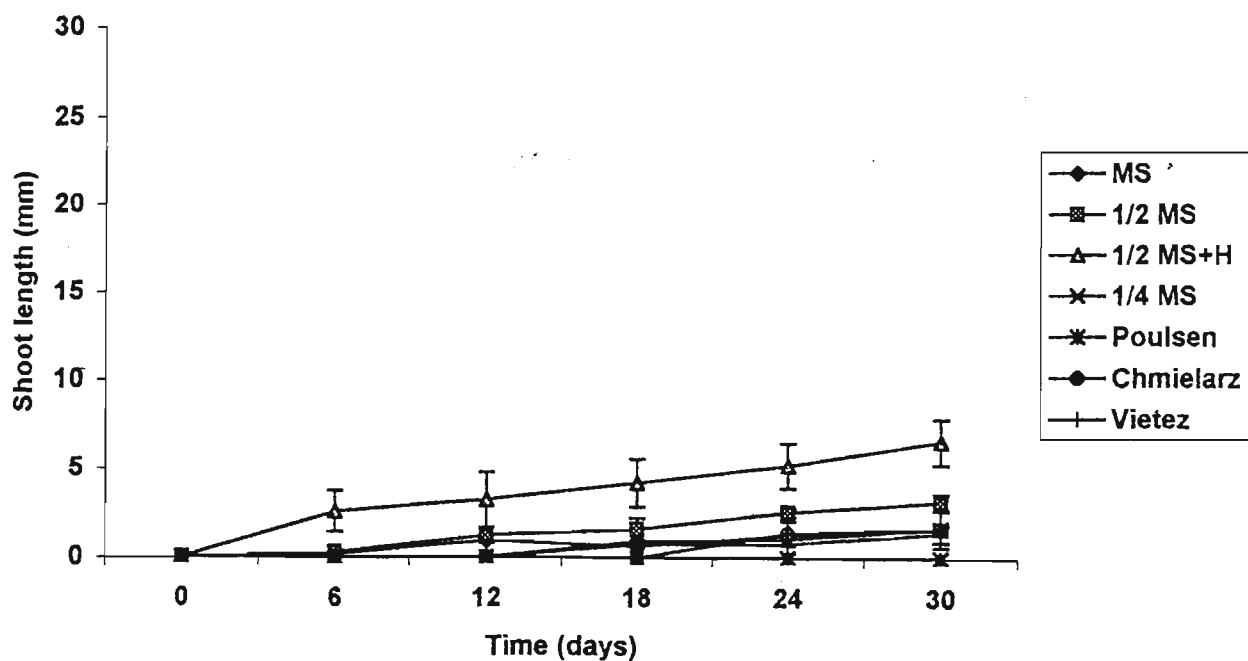
Figure 3.2b: Shoot length of *Q. robur* axes germinated on various media (see key) after sodium hypochlorite surface sterilisation (n = 20). Bar = mean ±SE.
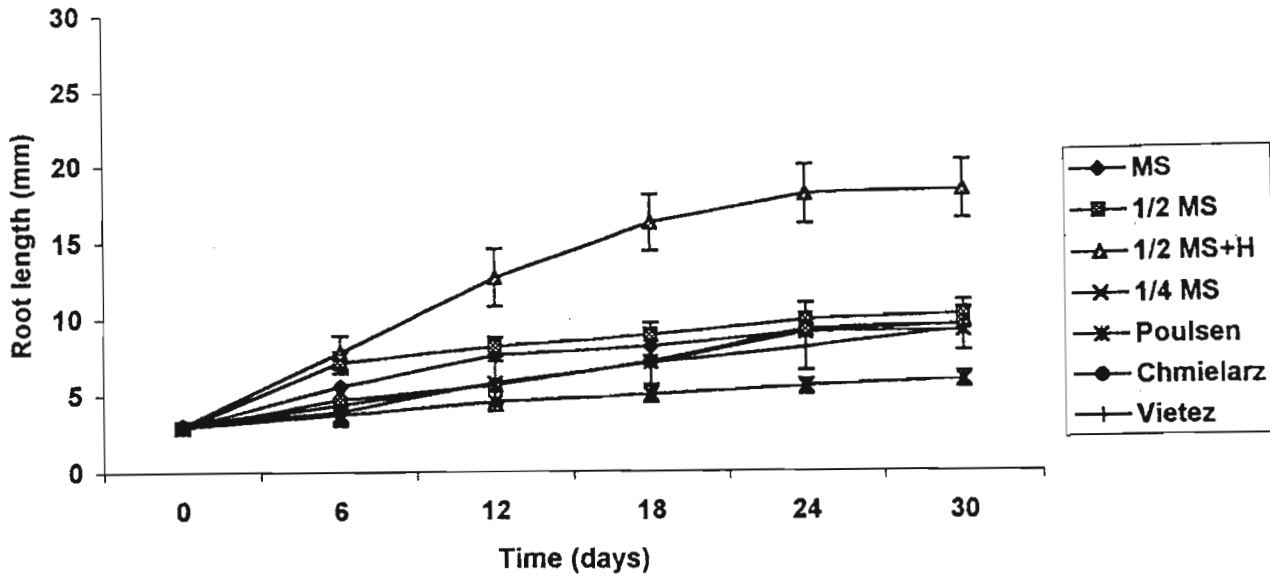
Figure 3.2c: Root length of *Q. robur* axes germinated on various media (see key) after mercuric chloride surface sterilisation (n = 20). Bar = mean ±SE.
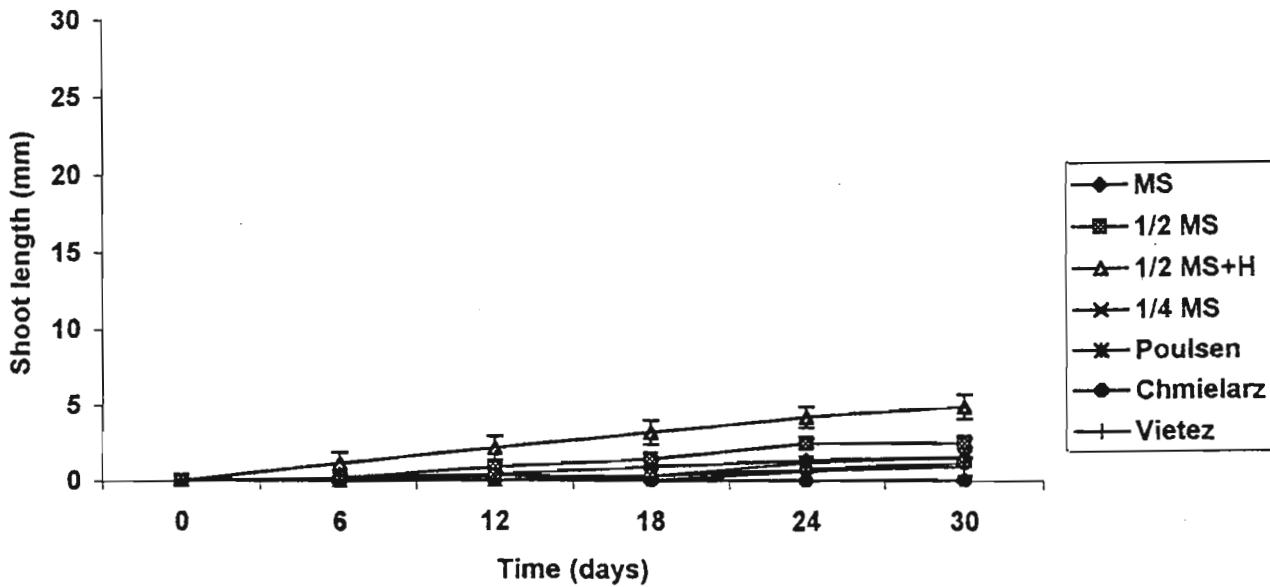
Figure 3.2d: Shoot length of *Q. robur* axes germinated on various media (see key) after mercuric chloride surface sterilisation (n = 20). Bar = mean ±SE.
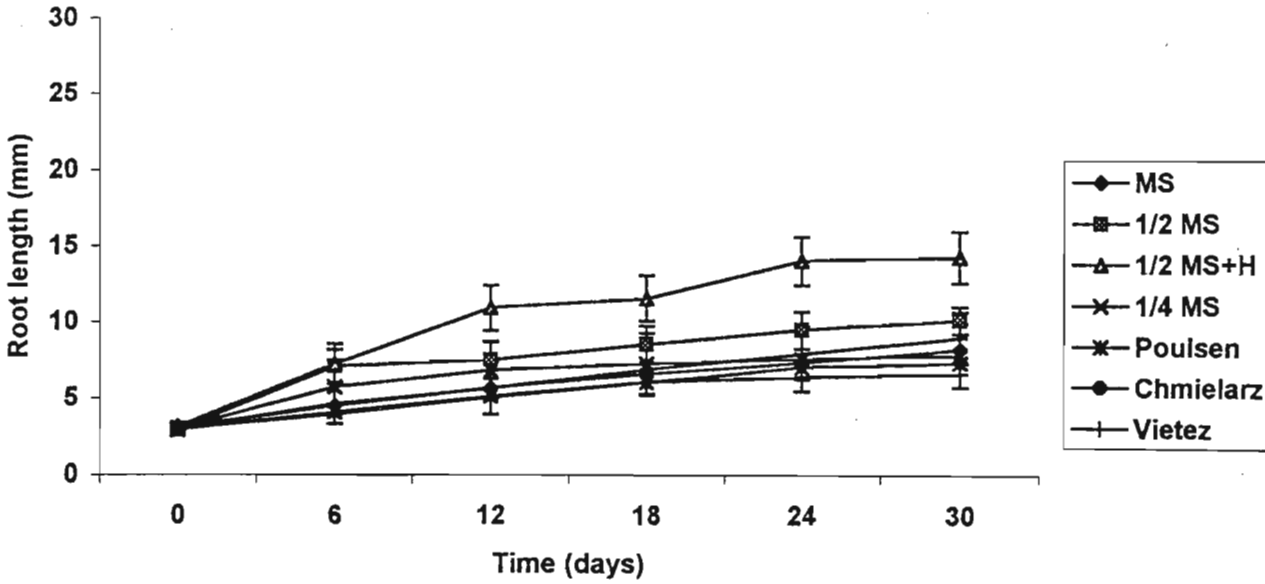
Figure 3.2e: Root length of *Q. robur* axes germinated on various media (see key) after Poulsen's combination surface-sterilisation (n = 20). Bar = mean ±SE.
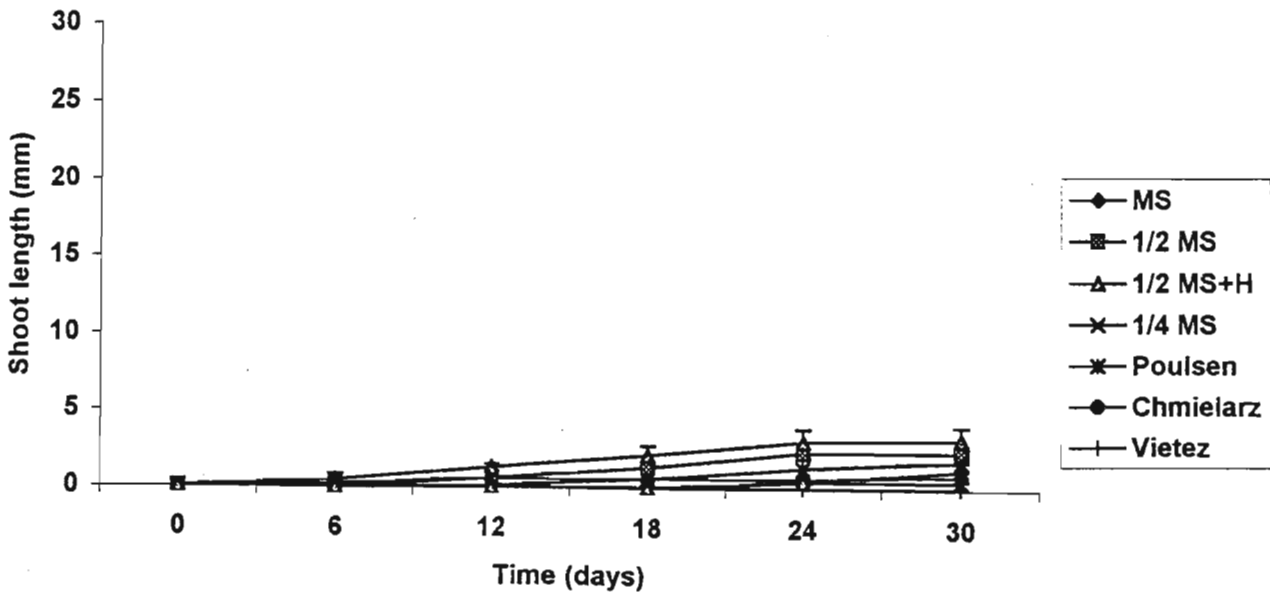
Figure 3.2f: Shoot length of *Q. robur* axes germinated on various media (see key) after Poulsen's combination surface-sterilisation (n = 20). Bar = mean ±SE.

It is important for the *in vitro* culture protocol to be optimised before cryopreservation is attempted in order to optimise the germination rate of the seeds and to ensure recovery after the stress of any cryopreservation procedure, as the *in vitro* generation of seedlings from embryos is crucial. The *in vitro* medium must therefore provide what the plant tissue is lacking, which, in the case of zygotic axes, is what would have been available from cotyledonary or endosperm reserves. The formulation of the medium is thus pivotal to survival success.

The data displayed in Figure 3.2 show the rate of germination measured in terms of root length (Figure 3.2a, c and e) and shoot length of *Q. robur* zygotic axes (Fig. 3.2b, d and f) on seven different media (see keys) using sodium hypochlorite (Figure 3.2a and b), mercuric chloride (Chmielarz, 1997: Figure 3.2c and´d) and Poulsen's (1992) combination surface-sterilant (Figure 3.2e and f).

After 30 d in culture on half-strength MS medium plus hormones (½ MS + H; Figure 3.3) following surface-sterilisation with sodium hypochlorite, mean root and shoot lengths of 22.64 mm and 6.55 mm, respectively, were achieved. Lower mean root and shoot lengths (18.24 mm and 4.84 mm, respectively) were recorded at day 30 after mercuric chloride was used on axes then grown on the same medium. After axes were exposed to Poulsen's combination surface-sterilant, the mean root and shoot lengths were 14.25 mm and 3.22 mm, respectively.

The half-strength MS medium (½ MS) was observed to produce the next best root and shoot growth in all cases, irrespective of the surface-sterilant used, but the values were not significantly different to those obtained on the other five media (Figure 3.2, see key). Thus, the medium chosen for later work was the half-strength MS medium supplemented with the hormones, BA (2 mg l^{-1}) and NAA (0.1 mg l^{-1}) which, in all cases, facilitated the best growth rate of both roots and shoots regardless of the surface-sterilant used.

A major extraneous problem with *in vitro* plant cultures is that of contamination. There is little doubt that all recalcitrant seeds (and in general, all seeds) harbour a microflora, generally dominated by fungi (Mycock and Berjak, 1990; Berjak, 1996). Microflora flourishes under *in vitro* conditions, rapidly out-competing and over-growing axes in culture. Hence, elimination of the propagules of all the microflora associated with the seed tissue, is absolutely essential. This is achieved by means of surface-sterilisation. However, although the range of surface-sterilants commonly used is restricted, all are not invariably suitable for zygotic axes (or indeed, any type of explant) of an individual species. Thus, experimentation on a tissue-specific basis, and a species basis, is generally recognised as a pre-requisite to optimise surface-sterilisation.

It was observed that sodium hypochlorite was the best of the three surface-sterilants presently used. This is clearly shown by both root and shoot lengths (Figure 3.3a and b respectively). Figure 3.3 also shows that both the mean root
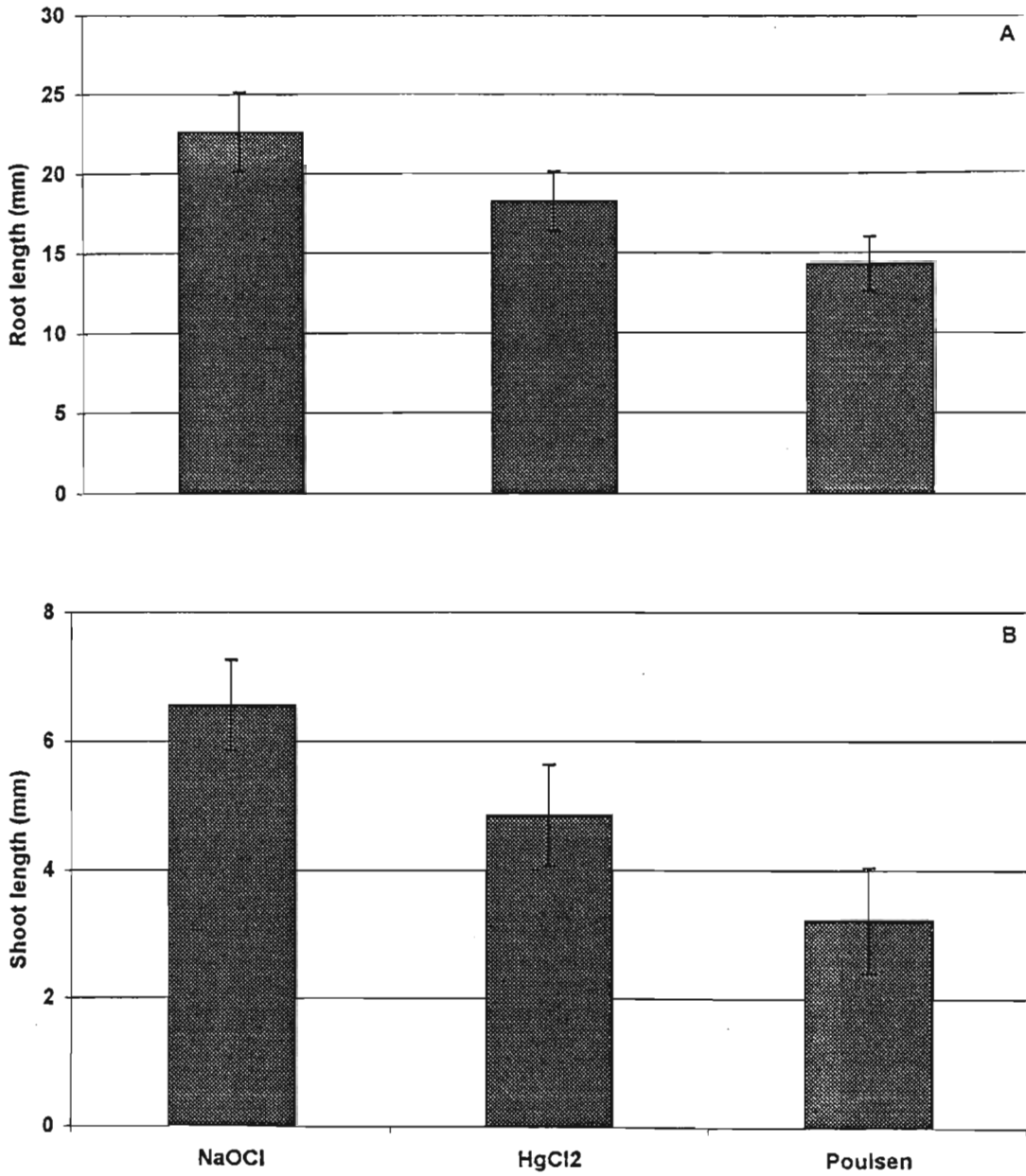
Figure 3.3: Root (A) and shoot (B) length of *Q. robur* axes germinated after 30 d on ½ MS + H (the optimal medium) after pre-treatment with surface-sterilants (n = 20). Note different scales on y-axes.

And shoot lengths after surface sterilisation with sodium hypochlorite are significantly different to those achieved after use with the other two surface-sterilants, whereas there is no significant difference in the mean lengths (root and shoot) achieved after use of mercuric chloride or Poulsen's combination surface-sterilants.

Furthermore, 100% geotropic curvature was established by day 12 when sodium hypochlorite was used, by day 18, when mercuric chloride was used and only by day 24 when Poulsen's (1992) combination surface-sterilant was used. These observations argue for more deleterious effects of inappropriate surface sterilants on the columella cells of the root cap which are primarily involved in gravitropism (Brum and McKane, 1989). Resultant damage was, however, able to be overcome, as graviperception ultimately occurred irrespective of the surface-sterilant used.

Thus, for excised axes of *Q. robur*, the surface sterilant, sodium hypochlorite, inhibited fungal contamination adequately and facilitated survival of embryonic axes into the seedling stage of development. Mercuric chloride (Chmielarz, 1997) and the combination surface-sterilant (Poulsen, 1992) appeared injurious to *Q. robur* embryonic axes, the growth and gravitropic response of which was somewhat slower than when sodium hypochlorite was used. Thus, from this point onwards, all experimental procedures utilised sodium hypochlorite and ½ MS + H medium, to allow for the best growth rates.

3.3.1 MICROSCOPICAL EXAMINATION AFTER SURFACE-STERILISATION

Roots and shoots of *Q. robur* embryonic axes from those seeds cold-stored for ten weeks, were examined microscopically following surface-sterilisation. The light and transmission electron micrographs that follow show control material from 6 d germinated seeds and embryonic axes cultured for 6 d on ½ MS + H medium using one of three surface-sterilants [NaOCl, $HgCl_2$ (Chmielarz, 1997), Poulsen's (1992) combination surface-sterilant].

KEY FOR ALL MICROGRAPHS

Co = columella
Me = root meristematic region
 S = statolith
 C = corpus
 T = tunica
 M = mitochondrion
 G = Golgi body
 V = vacuole
 N = nucleus
Nu = nucleolus
ER = Rough endoplasmic reticulum
 St = starch grain
 P = plastid profiles

(a) x 120   (b) x 10 400

(c) x 130   (d) x 15 700
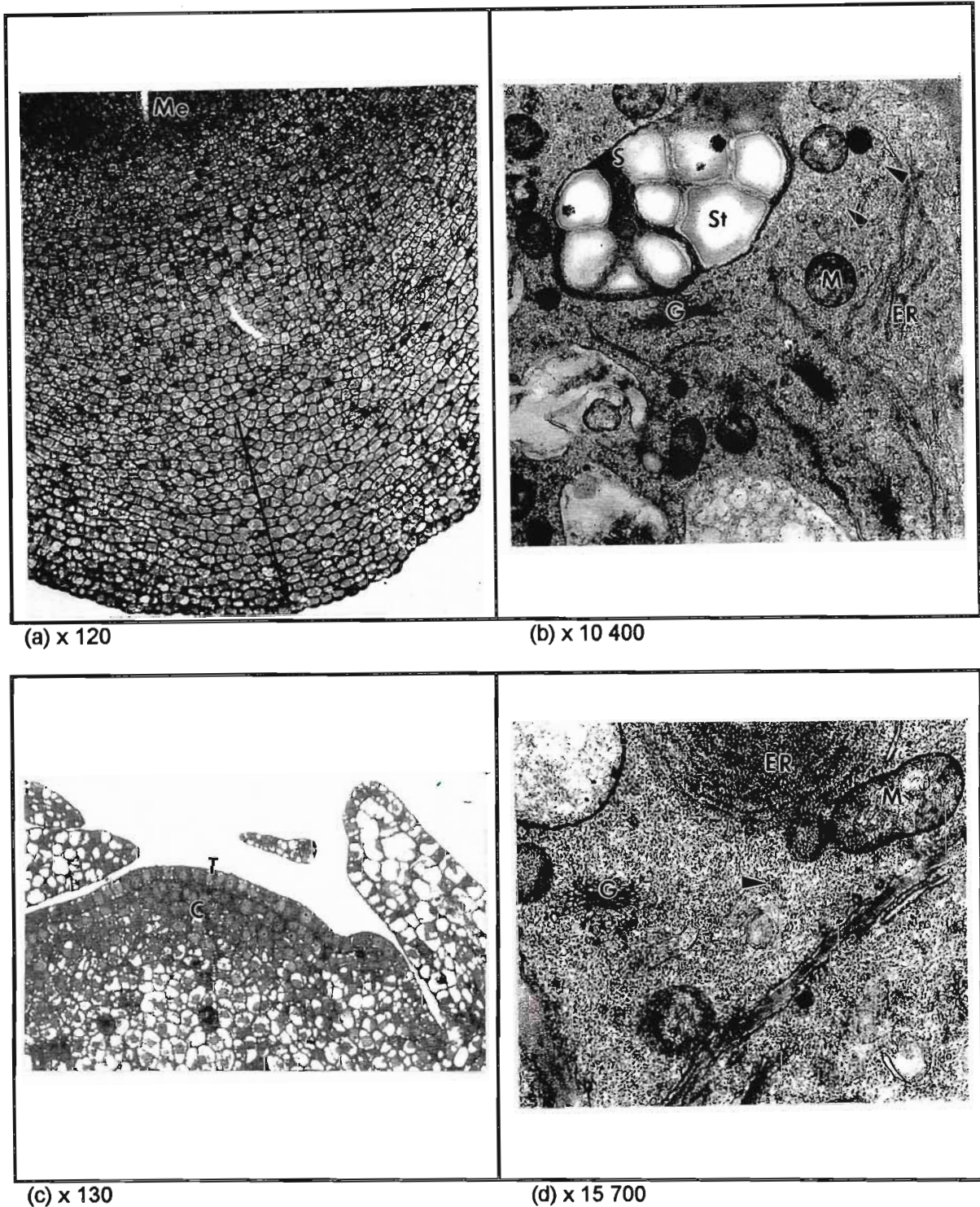
Figure 3.4: Fresh (control) *Q. robur* embryonic axes excised from seeds set out to germinate for 6d. Fig. 3.4a: Root cap (arrow) and root apical meristem (me). Fig. 3.4b: An example of a root cap columella cell containing a statolith, mitochondria, profiles of ER and prominent Golgi bodies. Fig: 3.4c: A typical shoot apical meristem, showing tunica and corpus cells. Fig 3.4d: Shoot apical meristem showing evidence of metabolic activity in the presence of Golgi bodies, mitochondria and concentric profiles of ER. An abundance of polysomes (arrow-heads) can be seen in both root cap and shoot apex cells.

(a) x 150

(b) x 10 500

(c) x 130

(d) x 7 500
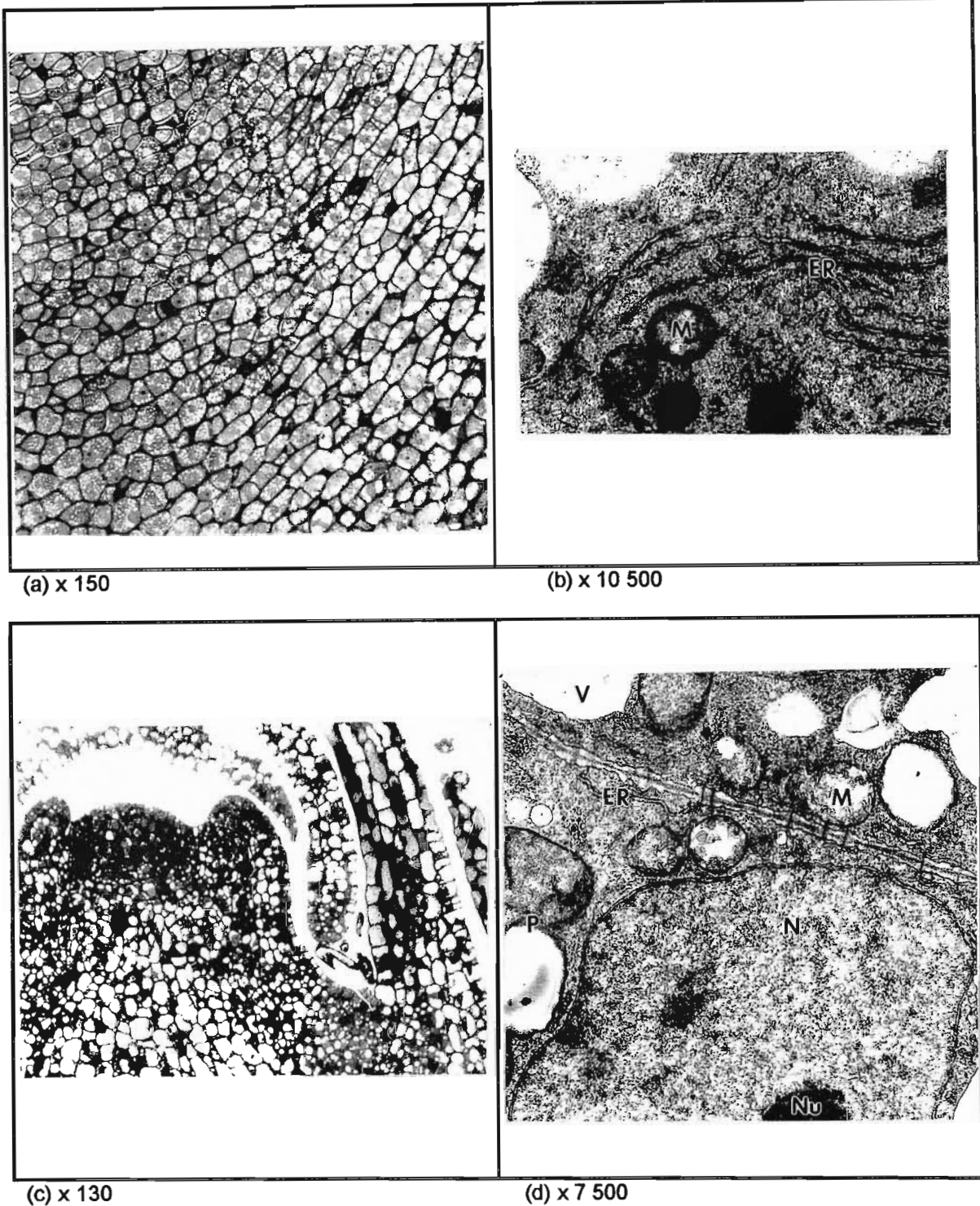
Figure 3.5: NaOCl surface-sterilised *Q. robur* embryonic axes, prepared for microscopy following a 6 d *in vitro* recovery period. Fig. 3.5a: No necrosis was observed, indicating no gross damage to the root cap. Fig. 3.5b: Portion of a typically metabolic root cap cell showing profiles of rER polysomes and mitochondria. Fig. 3.5c: The general state of organisation typical of metabolic activity within the shoot apex cells. Fig. 3.5d: Showing the prominent nucleus, nucleolus, mitochondria, plastids, small vacuoles, as well as profiles of rER.

(a) x 130   (b) x 6 500
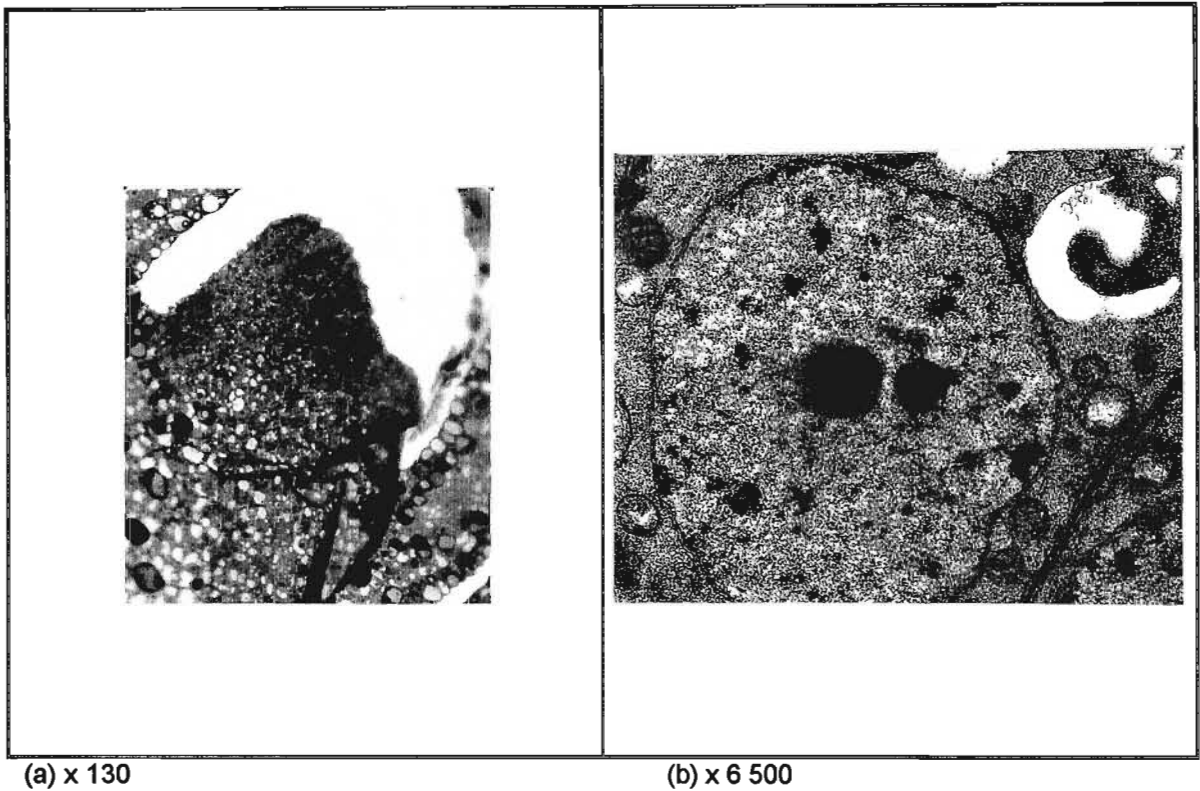
Figure 3.6: HgCl$_2$ surface-sterilised *Q. robur* embryonic axes. Fig 3.6a: Tunica and corpus of shoot apical meristem. Fig. 3.6b: An example of corpus cells, appearing relatively metabolically active, showing no obvious damage.
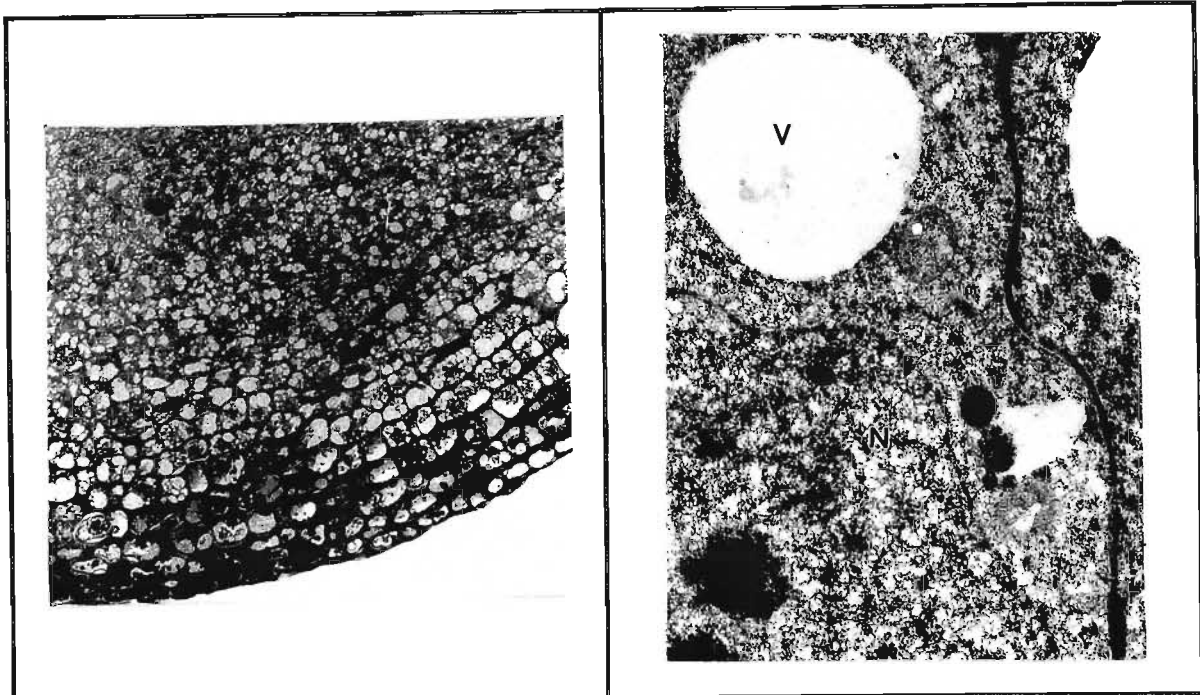
(a) x 130

(b) x 7 200
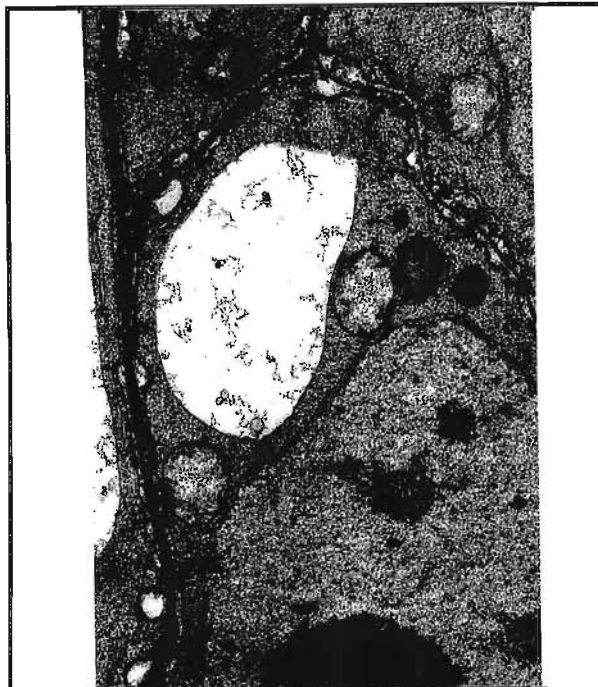
(c) x 16 000

Figure 3.7: *Quercus robur* embryonic axes after Poulsen's combination surface-sterilisation. Fig 3.7a: Necrosis was observed in several ranks of the outer root cap cells. Fig. 3.7b: Relatively large vacuoles (in root cap cells) were observed, as well as typical organelle deterioration and nuclear abnormality. Fig. 3.7c: Cell wall deterioration (in shoot apical meristem cells) indicated by the sinuous buckling and a marked localised inwards withdrawal of the plasmalemma is shown, as is the somewhat irregular nuclear profile. There is little evidence of polysomes.

The root apical meristem (Figure 3.4a) is protected by the substantial root cap. The latter is a stratified tissue, the cells of which are continuously regenerated distally from the apical meristem, and sloughed at the surface. As a result, the cap protects the root apical meristem from peripheral abrasion by soil particles. In *Q. robur*, the cap cells are relatively unvacuolated (Figure 3.4a), with many cell layers providing a considerable barrier between the apical meristem and the external environment. The central region of the cap, termed the columella, is critical to graviperception. Columella cells (Figure 3.4b) typically, had large amyloplasts containing many spherical starch granules; these specialised organelles are the statoliths (Figure 3.4 b) and are implicated in graviperception (Brum and McKane, 1989). The columella cells in the control material could be seen to be highly active, from the well developed rER, frequent Golgi bodies, many mitochondria with prominent cristae and incidence of polysomes (Figure 3.4b).

Shoot apical meristems, consisting typically of the tunica and corpus regions, are entirely superficial (Figure 3.4c) and, although loosely surrounded by leaf bases, are essentially unprotected, unlike cells of the root cap and apex. Those of the shoot tip showed an ultrastructure characteristic of considerable metabolic activity (Figure 3.4d). This is illustrated by the many polysomes that are evidence of protein synthesis, ranks of long parallel, or concentric, rER profiles and frequent Golgi bodies, which attest to endomembrane activity, and well-developed mitochondria typified by relatively short cristae and dense matrices

(Figure 3.4d). The subcellular situation was similar in tunica and corpus cells. It should be noted that the control axes of Q. *robur,* used presently, were excised from seeds that had been set to germinate for 6 d, to achieve a developmental state comparable to experimental material, that was afforded a 6 d *in vitro* recovery period after each manipulation. Because of the fungal contamination, axes of Q. *robur* that had not been surface-sterilised could not be cultivated *in vitro.*

Surface-sterilisation is an essential procedure used to eliminate any surface fungal spores present on the specimen to be cultured, so that fungi do not contaminate and eventually dominate the culture. In this study, three different surface-sterilisation procedures were used (see Chapter 2). Root and shoot apices were then examined microscopically to determine whether or not these various surface-sterilants had damaging (or other) effects on the specimens exposed.

The situation shown in both the shoot and root apex cells (cap and meristem) in material that had been surface-sterilised with sodium hypochlorite (NaOCl) (Figures 3.5a-d) was essentially similar to that shown in the control axes (Figures 3.4a-d). There was no obvious damage to be seen in root cap cells at the light microscope level (Figure 3.5a), a situation borne out by their organised ultrastructure (Figure 3.5b).

It was found that when NaOCl was used, only the most peripheral root cap cells (already poised to slough off naturally) were adversely affected. Figure 3.5a shows the bulk of the root cap which was essentially no different from that of the fresh control material (Figure 3.5a). Root apex cells (cap or meristem) appeared essentially unchanged both ultrastructurally and, by implication, in their apparent activity, as evidenced by the many mitochondria, profiles of rER and plastids containing starch grains (Fig. 3.5a).

The shoot apical meristem also did not show any obvious damage after surface-sterilisation with NaOCl (Figure 3.5c). Figure 3.5d presents a typical appearance of a highly active cell containing well-developed mitochondria, rER, Golgi bodies, polysomes and a prominent nucleus showing a dense nucleolus which is indicative of active ribosome subunit assembly.

Mercuric chloride (Chmielarz, 1997) did not seem to impose any obvious damage on *Q. robur* axes either, and it was effective in removing contaminants. The shoot apical meristem (Figure 3.6a) appeared undamaged at the light microscope level, which was borne out by the ultrastructure (Figure 3.6b) showing a prominent nucleus with nucleolus and well developed mitochondria, and often essentially normal features.

In contrast to the situation after the use of either NaOCl or $HgCl_2$, those axes exposed to Poulsen's surface sterilisation protocol, showed damage to several

layers of the root cap (Figure 3.7a), indicating that over-rigorous surface-sterilisation, while removing surface contaminants can be markedly deleterious. The damage was not confined only to the peripheral ranks of root cells, as many of the columella cells retained damage even after the 6d *in vitro* recovery period. This can be seen particularly from the buckled walls (Figure 3.7c). Damage retention and the relatively slow growth (Figures 3.2e and f and 3.3) indicated that although all the axes would ultimately germinate, they had been severely set back by the harsh surface-sterilisation.

The shoot apical meristematic cells had abnormally thin, buckled walls (Figure 3.7c) and a localised withdrawal of the plasmalemma from the cell wall. Some nuclei in the shoot apex were somewhat irregular (Figure 3.7c) suggesting perhaps impaired nucleo- or cytoskeletal formations, but organelle disposition appeared essentially normal.

The surface sterilant, sodium hypochlorite ultimately inhibited fungal contamination adequately and allowed the survival of embryonic axes well into the seedling stage of development on ½ MS + H medium. This treatment also did not cause tissue/cell abnormality. Its use was also preferable to that of $HgCl_2$ when germination parameters were monitored. The harsh combination of conditions inherent in the method of Poulsen (1992) was presently shown to cause a significant amount of damage. Hence surface-sterilisation with a 1% (v/v) concentration of sodium hypochlorite was adopted as the best of those

treatments presently tested. Pre-treatments, such as differential drying rates, for the eventual cryopreservation of these axes, were then evaluated.

3.4 EXPERIMENTATION WITH DRYING: *Q. robur*

In this comparative phase of the study, excised, surface-sterilised (NaOCl) axes were dehydrated by one of each the four methods described in Chapter 2. They were laminar airflow [L-F (Poulsen, 1992)], silica-gel [S-G (Chmielarz, 1997)] and the two developed in Durban, viz. flash-drying [F-D (Berjak *et al.*, 1990) and fast flash-drying (Wesley-Smith *et al.*, 1999)]. *Quercus robur* root and shoot growth *in vitro* was assessed after axes were subjected to one of the four different drying methods, as shown in Table 3.1. In all cases, embryonic axes were exposed to the experimental conditions for a total of 360 min and sampled at zero time and then at 8 intervals for water content and *in vitro* germination performance.

Table 3.1: Percentage root (R) and shoot (S) survival of *Q. robur* embryonic axes at 30 d, after exposure to four different drying rates and nine different time intervals, respectively (n = 20).

DESICCATION	DRYING METHOD USED VS % SURVIVAL							
TIME (mins)	L-F		S-G		F-D		FF-D	
	% R	% S	% R	% S	% R	% S	% R	% S
0	100	100	100	100	100	100	100	100
30	100	100	100	100	100	100	100	100
60	100	100	100	100	100	100	100	100
90	100	100	100	100	100	100	100	100
120	100	100	100	100	100	100	100	100
180	100	100	100	100	100	100	100	100
240	100	80	100	100	100	100	100	100
330	100	35	100	70	100	100	100	100
360	100	30	100	40	100	100	100	100

Data shown in Table 3.1 indicate that, regardless of the drying technique used and of the time taken, 100% root survival was achieved after 30 d in culture on the selected medium (½ MS + H). However, shoot survival from axes dried for 240 min (0.54 ± 0.09 g g^{-1}) under laminar airflow conditions, had declined to 80%. The trend of decreased shoot survival continued over time to 35% after 330 min (0.50 ± 0.07 g g^{-1}) and 30% after 360 min (0.47 ± 0.09 g g^{-1}) of drying. Similarly, a 330 min (0.44 ± 0.08 g g^{-1}) exposure to silica gel affected the shoot apices resulting in 70% survival of shoots, and, after 360 min (0.41 ± 0.14 g g^{-1}) in the same desiccant, only 40% of the shoots survived.

This trend did not, however, occur with those axes exposed to the two faster methods of drying [flash drying (F-D) and fast flash-drying (FF-D)]. Water contents as low as 0.37 ± 0.11 g g^{-1} and 0.28 ± 0.09 g g^{-1} were achieved with flash-dried and fast flash-dried axes, respectively, after 360 min (Figure 3.8). Rates of root growth after laminar airflow and silica-gel drying, declined after peaking at water contents $> 0.7 - 0.8$ g g^{-1}. In contrast, root growth rate continued to accelerate following dehydration down to *c*. 0.4 g g^{-1}, by flash drying and fast flash-drying.

Adverse effects on shoot growth with the two slower drying rates were noted at water contents from < 1.0 g g^{-1} and 0.8 g g^{-1} for laminar airflow and silica-gel dehydration, respectively (Figure 3.8b). Furthermore, some shoots were lethally affected (0.46 ± 0.09 g g^{-1} and 0.41 ± 0.14 g g^{-1}, Table 3.1; Figure 3.8) after

laminar airflow and silica-gel drying, respectively. In contrast, for axes dehydrated by flash-drying or fast flash-drying, as the objective in the present study was to achieve water contents that would maintain full axis potential as well as, facilitating cryopreservation, no further degrees of dehydration were imposed (see section 3.5).

The efficiency of the dehydration methods emerged as laminar airflow < silica gel < flash-drying < fast flash-drying. However, there was no significant difference between the two slowest methods of drying (S-G and L-F), nor between the two most rapid methods (F-D and FF-D). There were, however, significant differences in growth parameters between the slowest (L-F) and the most rapid (FF-D) modes of drying, particularly at, 0.6 g g^{-1}.

It is important to note that all *Q. robur* embryonic axes, irrespective of their mode of drying and the time taken between 30 min and 360 min, maintained 100% root formation. However, even though 100% root growth occurred subsequent to laminar airflow and silica-gel drying, a gradual and relatively uniform decrease in the rate of root growth was observed after 30 min of dehydration (to $1.04 \pm 0.06 \text{ g g}^{-1}$ and $0.88 \pm 0.14 \text{ g g}^{-1}$, respectively; Figure 3.8a. At every time interval, there was no significant difference in root length achieved after 30 d between these slow drying procedures.
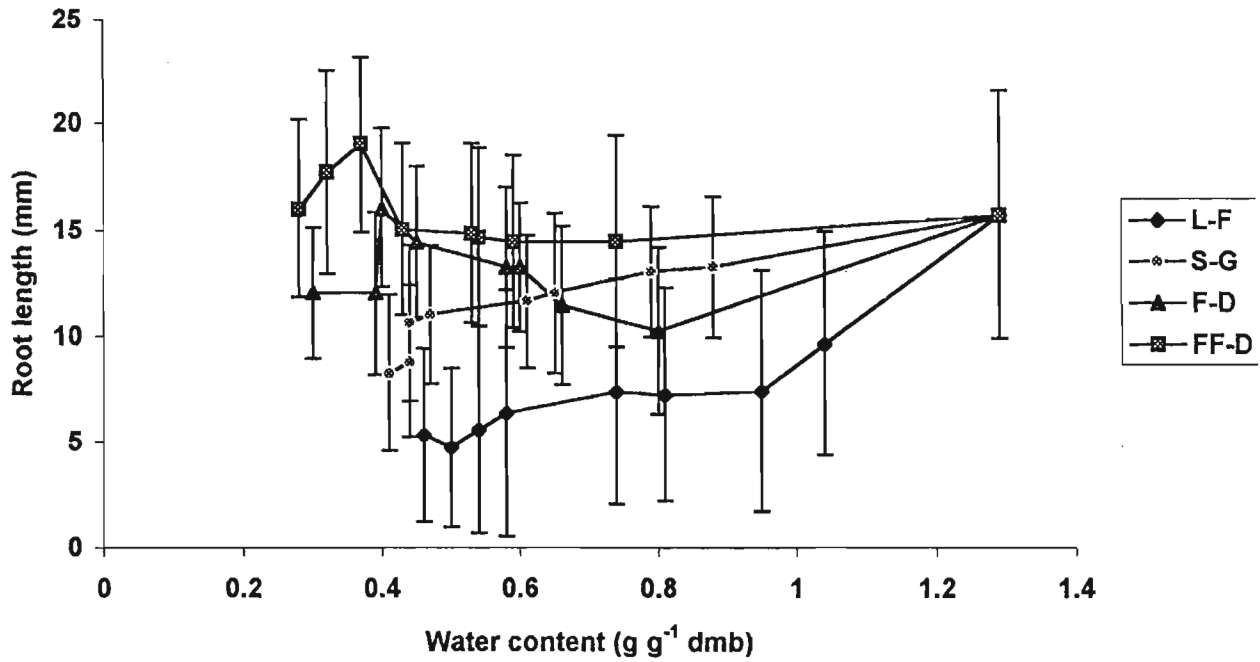
Figure 3.8a: Water content after exposure to differential drying rates (see key) versus root growth of *Q. robur* axes after 30 d. Bars = mean ±SE.
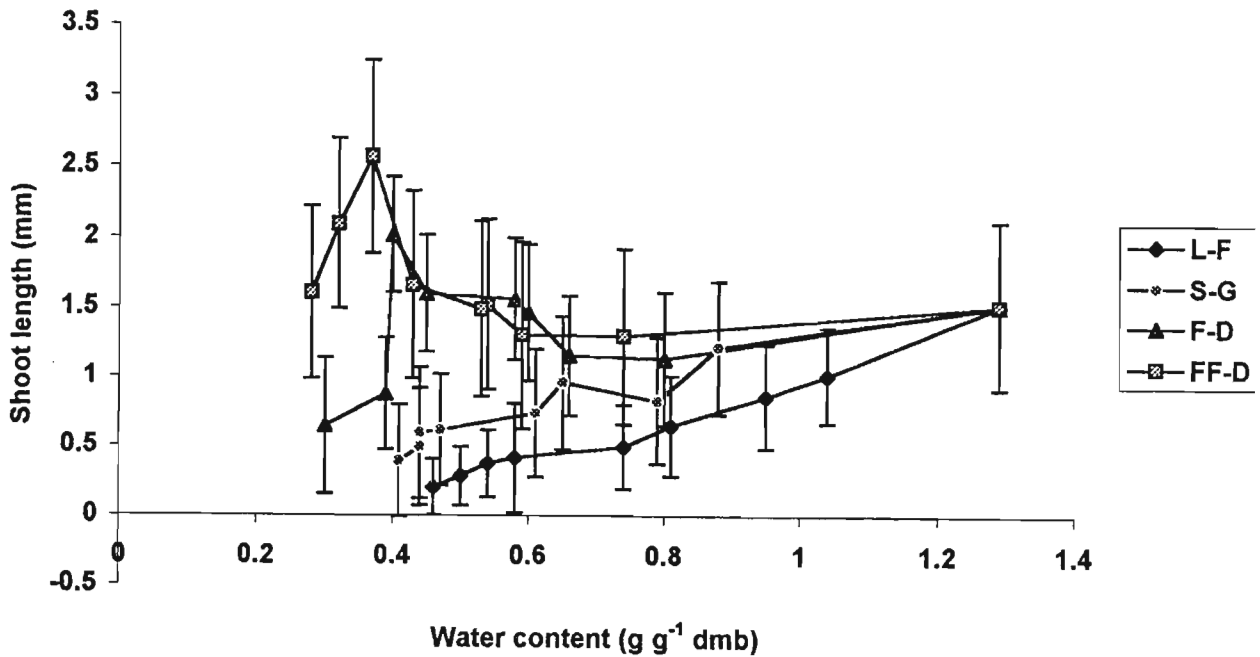
Figure 3.8b: Water content after exposure to differential drying rates (see key) versus shoot growth of *Q. robur* axes after 30 d. Bars = mean ±SE.

Those axes that were flash-dried, showed a similar trend to those that had been fast flash-dried, where a stimulation in root growth was demonstrated as the desiccation time increased from 30 to 240 min (0.8 ± 0.09 g g^{-1}; 0.74 ± 0.13 g g^{-1} to 0.4 ± 0.1 g g^{-1}; 0.37 ± 0.04 g g^{-1}, respectively). The significance of this stimulatory effect of rapid drying should be noted, but its exploration is beyond the scope of the present work. From the data on *Q. robur*, it can be seen that if dehydration occurs more rapidly, viability (in terms of root survival) will be retained to lower water contents, as has been demonstrated previously for other species (Berjak *et al.* 1990). It has been suggested by Berjak *et al.* (1990) and Pritchard (1991) that it is likely that very rapid drying of excised axes precludes not only germination-associated metabolism, but curtails other metabolic, and also deteriorative, reactions. Beyond dehydration by F-D or FF-D for 240 min (0.4 ± 0.1 g g^{-1} and 0.37 ± 0.04 g g^{-1}, respectively) the root growth rate declined, but not significantly. After 300 min (0.32 ± 0.08 g g^{-1}) and 360 min (0.28 ± 0.09 g g^{-1}), of drying, a decline in root length occurred, but again not significant.

The variability in the growth rate of shoots (Figure 3.8b) was observed to be as equally variable as for the roots (Figure 3.8a). As desiccation time increased, so the shoot length decreased in a relatively uniform and consistent rate after exposure to laminar airflow. Those axes exposed to silica-gel showed a decreasing, but erratic shoot growth rate.

After flash and fast flash-drying, however, the same trend as seen for the roots was observed, viz. an increasingly stimulatory effect of dehydration up to 240 min (0.37 ± 0.04 g g^{-1}; Figure 3.8b. It should be noted that although this was not statistically significant between flash and fast flash-drying, the effect was clearly demonstrated. After 300 min (0.32 ± 0.08 g g^{-1}) and 360 min (0.28 ± 0.09 g g^{-1}), of drying, a decline in shoot length occurred, even though this was not significant, this trend is similar as that described for the roots.

Those shoots that lost water more rapidly via the fast-flash drying method therefore withstood dehydration to lower water contents better than those exposed to slower rates of drying. However, in terms of the slower dehydration regimes, the shoots were more sensitive to water loss than were the roots (Table 3.1 and Figure 3.8a). This could be because shoot apices are not protected as are the roots (by the root caps) and are consequently more vulnerable to potentially injurious stress, such as that imposed by prolonged slow dehydration.

The more rapid the rate of water removal therefore, the more efficiently is the time factor reduced and thereby the injurious effects of stress imposed upon the axes. Furthermore, up to a point, this stress actually promoted the growth rate of roots and shoots (Figure 3.8).

In an attempt to explain the better seedling formation of those more rapidly dried axes as opposed to that of those slower dried axes, Pammenter *et al.* (1991) indicted that under natural (i.e. slow dehydration) conditions, desiccation-

sensitive seeds lose their viability when solution (freezable) water is decreased below a relatively high water content. The results of the present study show a similar trend, whereby the slow dehydration or loss of solution water below a relatively high level disrupts some vital aspect(s) of the cellular metabolism within the shoot apical meristem.

3.4.1 MICROSCOPICAL EXAMINATIONS AFTER IMPLEMENTATION OF DIFFERENTIAL DRYING RATES

The following microscopical examinations were undertaken after exposure of axes from the 10 week cold-stored seed batch to three different drying procedures, followed by 6d *in vitro* recovery both roots and shoots were examined microscopically (LM and TEM). The 3 different drying procedures were: the dehydration of *Q. robur* embryonic axes by fast flash-drying for 240 min (i.e. the optimal drying regime, even though not statistically significant, taken from results previously obtained - Figure 3.8); after 8 h of laminar airflow drying (the apparent optimal drying rate and time for *Q. robur* axes given by Poulsen, 1992 and after Chmielarz's (1997) apparent optimal drying rate(s) and time for *Q. robur* axes (i.e. use of cryoprotection, laminar airflow and silica-gel).

(a) x 7 500   (b) x 17 000
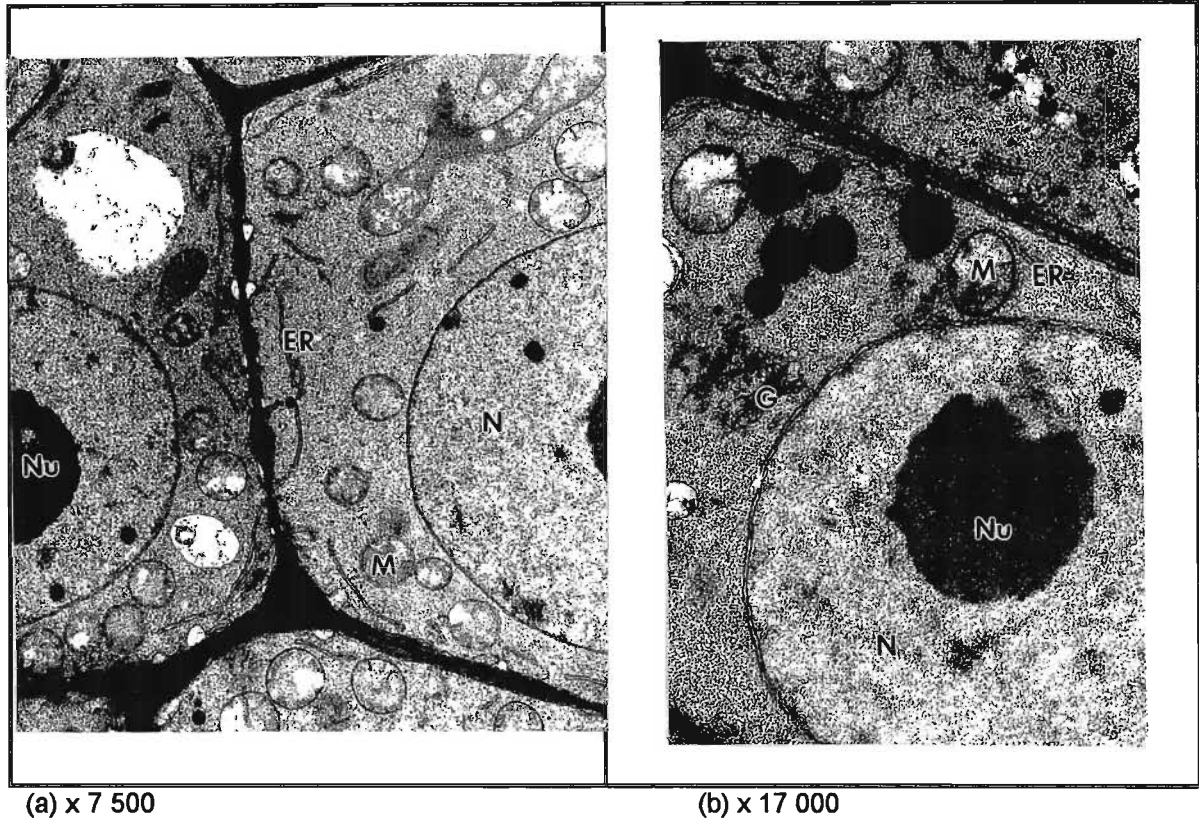
Figure 3.9: Dehydration of *Q. robur* embryonic axes by fast flash-drying for 240 min. After the 6 d recovery period, root apex (Fig. 3.9a; illustrating the meristem) and cap cells showed evidence of enhanced activity compared with undried material, as did the cells of the shoot apical meristem (3.9b). Cells were dominated by large, spherical nuclei with well-defined nucleoli; the essentially spherical mitochondria were plentiful, and rER profiles and Golgi bodies were common features.

(a) x 150

(b) x 12 800

(c) x 150

(d) x 9 600
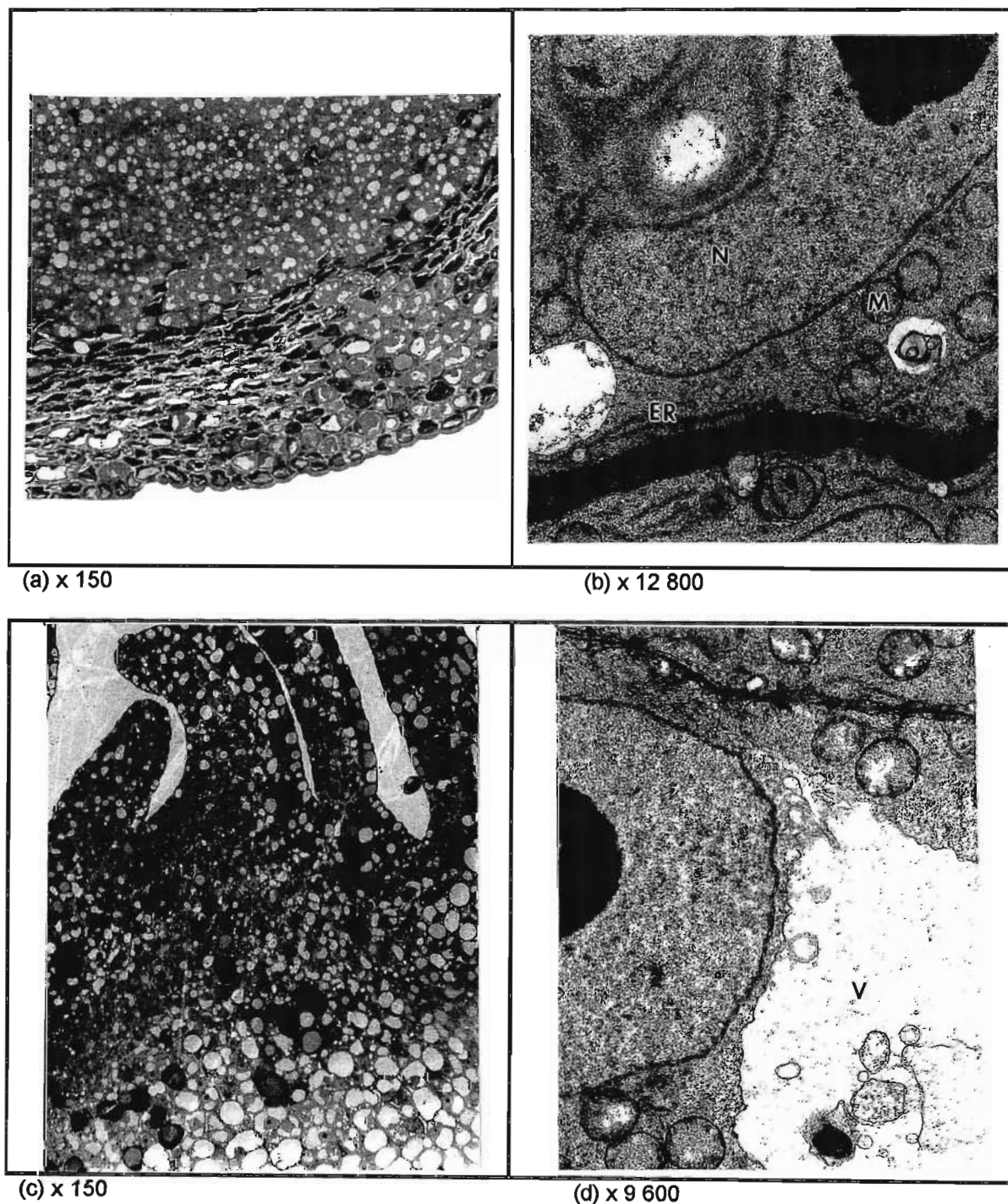
Figure 3.10: After 8 h of laminar airflow drying (Poulsen, 1992) of *Q. robur* axes, and despite the 6-d recovery period, there was evidence of persisting damage, especially in the root cap (3.10a), where multiple cell ranks were necrotic. Fig. 3.10b: Although cells deep within the cap retained signs of damage (e.g. highly abnormal nuclear shape and buckled walls) evidence of activity was provided by the many, well-developed mitochondria and ER proliferation. Fig. 3.10c: The typical appearance of the shoot apex included a scattering of darkly-stained degenerated cells. Fig. 3.10d: There was a marked degree of vacuolation in shoot apical meristem cells, the intravacuolar contents, including membranous material, indicated autophagic activity. Cell walls retained a slightly irregular appearance.

(a) x 150   (b) x 10 700

(c) x 150   (d) x 6 500
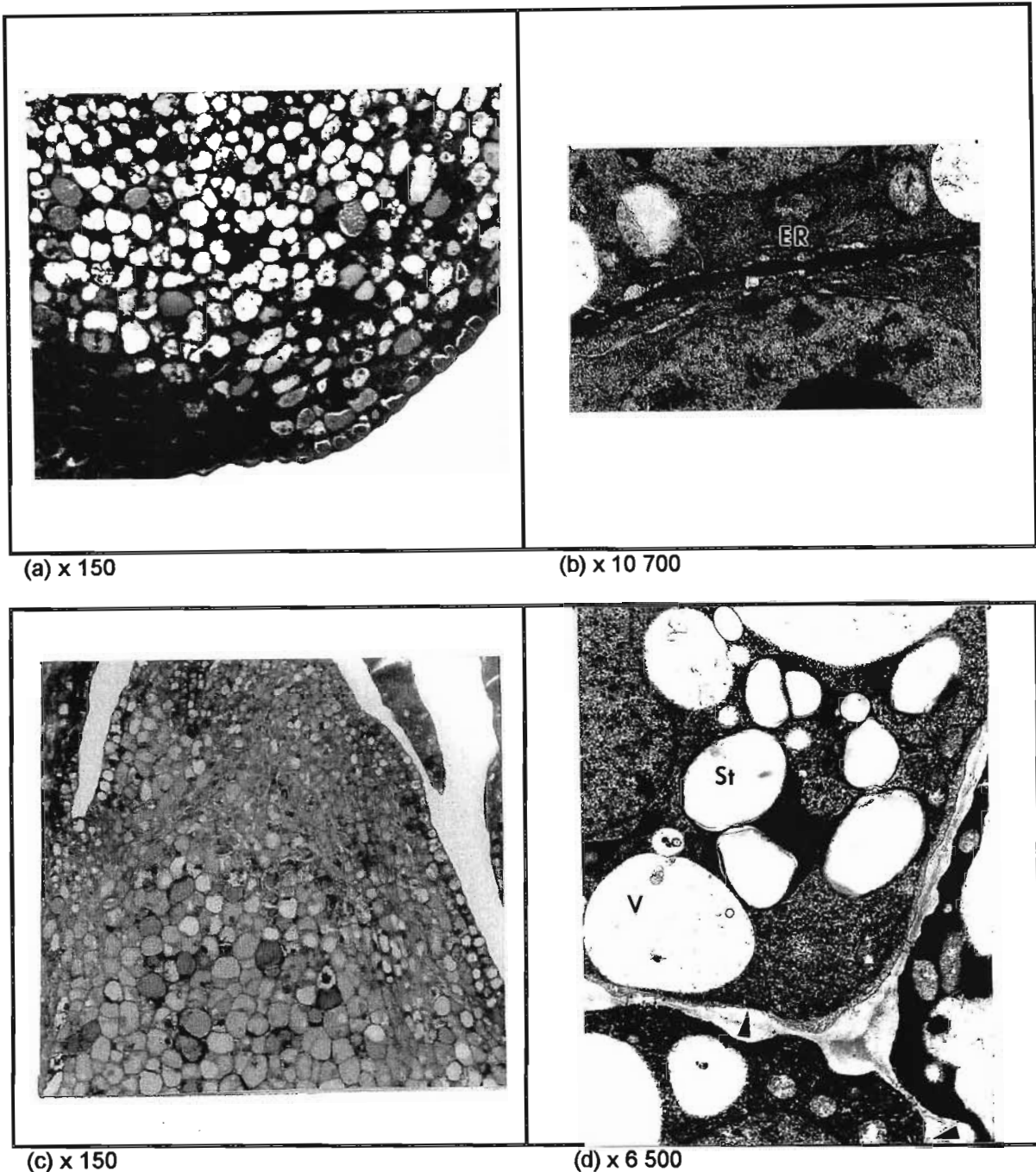
Figure 3.11: Dehydration of *Q. robur* embryonic axes using Chmielarz's (1997) method (cryoprotectants, laminar airflow and silica-gel). Fig. 3.11a: Extensive degeneration of the root cap was observed after this dehydration protocol was implemented. Essentially, the whole cap became necrotic - a persisting symptom, even after the recovery period. Fig. 3.11b: In the root apical meristem, cells retained some signs of ultrastructural abnormality, e. g. dilated rER cisternae, some irregularity of the nuclear envelope, and frequent, small plasmalemma vesiculations. Fig. 3.11c: Extensive vacuolation was a feature of the shoot apex. Fig. 3.11d: While many of the shoot apical meristem cells appeared necrotic (e.g. upper left) or degenerating (top) there was evidence of marked starch accumulation and periodic cell wall hypertrophy (arrows). Both these features are abnormal and could be correlated with the sugar provided during the cryoprotectant treatment.

Following fast flash-drying for 4 h, and a recovery period of 6 d in culture, no damage seemed evident. Apical meristem cells (Figure 3.9a) and those of the root cap were dominated by spherical nuclei, each with a prominent nucleolus. Other organelles such as active mitochondria, small vacuoles and profiles of rER were present. In addition, the cell wall to which the plasmalemma was closely applied, had remained regular.

In the shoot apex, the tunica and corpus cells of those fast flash-dried *Q. robur* axes (Figure 3.9b) were dominated by their prominent nuclei. These cells were also highly active, indicated by the frequent occurrence of Golgi bodies and the appearance of the mitochondria, presumably associated with preparation for cell division.

Those roots dried using Poulsen's (1992) technique (8 h laminar airflow), showed detrimental effects from the exterior to the deep-lying cells of the cap (see arrows) (Figure 3.10a). In these cells, this drying technique was associated with detrimental changes - e.g. to some nuclei, where the shape (form) had become markedly abnormal (Figure 3.10b). Additionally, cell walls showed some irregularity. Some similarly affected cells also occurred in the apex of the root proper (not illustrated). These abnormalities, although obviously not lethal in themselves (as all axes were capable of root growth) had persisted despite the 6 d recovery period after dehydration.

The shoots, after exposure to laminar airflow, showed scattered necrotic cells or patches of such cells. The typical ultrastructure of shoot apical meristem cells is shown in Figure 3.10d. Nuclear profiles were slightly distorted which could indicate some irregularity of the nuclear lamina of the nucleoskeleton. Intensive vacuolation had occurred in many tunica and corpus cells associated with the slow-drying regime imposed on these axes. The large vacuole illustrated (Figure 3.10d) shows evidence of removal and lysis of presumably damaged intracellular structures.

Chmielarz's (1997) dehydration protocol was more complex than the other two regimes and involved not only 23 h of cryoprotection, followed by encapsulation in calcium alginate, but also vigorous dehydration procedures comprising 1 h of laminar airflow and 20 h of drying in silica-gel. In this experiment, axes reached a very low water content (0.19 g g^{-1}) which was nevertheless higher than the 0.15 g g^{-1} achieved by Chmielarz (1997). This treatment resulted in extensive degeneration of the root cap (Figure 3.11a). Ultrastructurally, root apical meristem cells retained signs of damage even after the 6 d recovery period. Persistent abnormalities included dilation of the rER cisternae, some nuclear envelope irregularity and plasmalemma separation from the cell wall (Figure 3.11b).

All peripheral tunica and some corpus cell layers were also considerably damaged after Chmielarz's (1997) dehydration procedure was implemented

(Figure 3.11c), in fact the tunica cells could be seen to be highly vacuolated, even at the LM level. Figure 3.11d shows vacuolation in three contiguous cells. Additionally, apparent cell wall hypertrophy had occurred and large starch grains had formed within the plastids. This probably resulted from the high sucrose pre-treatment, the effects of which had persisted despite the 6 d recovery period, indicating that the rate of recovery-associated metabolism might have been very slow.

This study on differential drying rates therefore indicated that the more rapid the drying rate, the less the cell and tissue damage. From this point onwards therefore, fast flash-drying was used as the drying pre-treatment before freezing.

3.5 CRYOPRESERVATION OF *Q. robur* EMBRYONIC AXES

It was interesting that all those 'physiologically younger' *Q. robur* axes (i.e. those flash-dried or fast flash-dried and frozen, regardless of the method of freezing used, immediately on arrival) were not all killed, but never reached the full seedling stage of development, despite the use of the Ca^{2+} Mg^{2+} solution after freezing, used as a thawing agent. Many of those *Q. robur* axes sampled from the seeds stored for 10 weeks or longer, however, survived preliminary cryopreservation trials, after freezing and thawing in the Ca^{2+} Mg^{2+} solution. For this study, therefore, only those 'physiologically older' (i.e. those axes stored for 10 weeks or longer) acorns were used.

Table 3.2: Percentage survival, after 30d in culture, of Q. _robur_ axes which had been subjected to fast flash-drying to various water contents and frozen at two rates, intermediate and ultra-rapid cooling, for cryopreservation (n=20).

		COOLING RATE	
DRYING TIME (h)	**WATER CONTENT (g g^{-1})**	**INTERMEDIATE**	**ULTRA-RAPID**
< 3	1.44 ± 0.05 - 0.53 ± 0.06	0	0
3	0.37 ± 0.04	0	0
6	0.32 ± 0.08	0	36
9	0.21 ± 0.05	0	40
12	0.18 ± 0.07	0	50
15	0.12 ± 0.09	60	50

Results of the preliminary work reported in Table 3.2 show the range of water contents [1.44 ± 0.05 g g^{-1} to 0.12 ± 0.09 g g^{-1}] achieved over 15 h using the fast flash-drying protocol. It was found that using the conventional (intermediate) method of freezing (i.e. direct plunging into liquid nitrogen), root growth was observed for 60% of the Q. _robur_ axes only at very low water contents (0.12 ± 0.09 g g^{-1}). When ultra-rapid cooling was implemented it was found that 36% of the embryonic axes survived freezing at the considerably higher water content of 0.32 ± 0.08g g^{-1}. Although, only a 50% survival was achieved at 0.12 ± 0.09 g g^{-1} after ultra-rapid freezing, and was lower than that obtained by the conventional 'intermediate-freezing' method. The former showed far more potential, facilitating axis survival over a range of water contents from 0.32 ± 0.08 g g^{-1}. As the primary objective is not to dehydrate the axes more than is absolutely necessary for them to survive freezing, ultra-rapid cooling emerged as the method of choice.

In this preliminary investigation, where material retrieved from liquid nitrogen was thawed by direct immersion in distilled water (40°C), survival was scored by root growth only (Table 3.2). The main objective of this study, however, was to achieve seedling establishment, but after 30, 60 and 90 days of observation, and despite periodic transfer of the specimen onto fresh medium, no shoots developed.

The following investigation involved the use of the three drying regimes detailed above [Poulsen (1992), Chmielarz (1997) and fast flash-drying] and the ultra-rapid cooling of all the specimens. The results are shown in Table 3.3.

Table 3.3: Percentage survival after three different methods of drying, and ultra-rapid cooling. (n = 20).

REFERENCE	PERCENTAGE SURVIVAL	
	Roots	shoots
Poulsen (1992)	0	0
Chmielarz (1997)	0	0
Fast flash drying	66	0

Results indicated that the only acceptable dehydration regime was fast flash-drying, achieving 66% axis survival, although no shoot development occurred. For both the Poulsen (1992) and Chmielarz (1997) techniques, no axis survival occurred. These data were further substantiated when specimens were examined microscopically.

3.5.1 MICROSCOPICAL EXAMINATION AFTER DRYING AND ULTRA-RAPID COOLING

Roots and shoots, from those axes excised from seeds cold-stored for 10 weeks,

were examined microscopically following ultra-rapid cooling after dehydration by

fast flash-drying and Poulsen's (1992) and Chmielarz's (1997) methods.

(a) x 130

(b) x 20 800

(c) x 150

(d) x 16 000
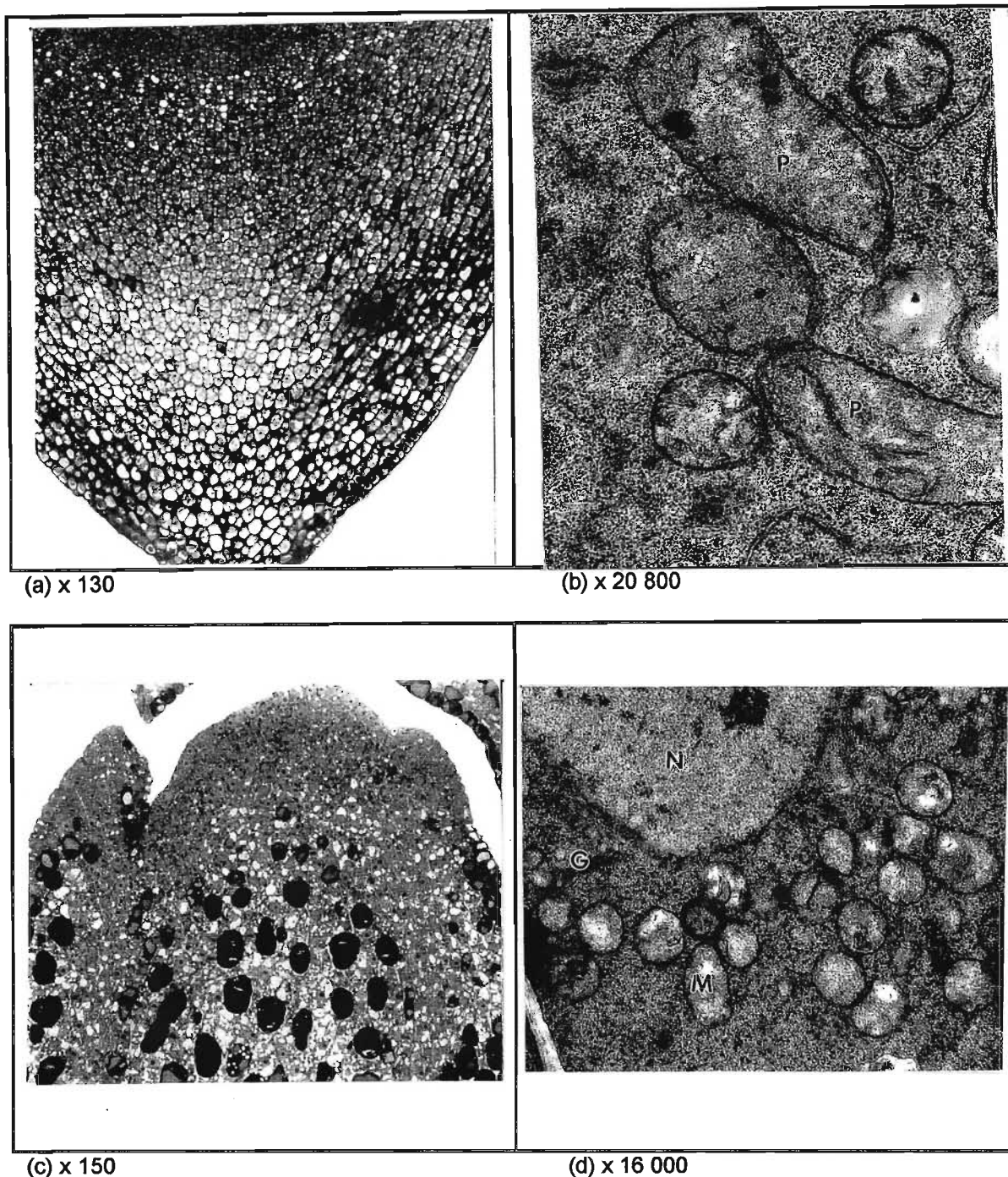
Figure 3.12: *Quercus robur* embryonic axes after fast flash-drying, ultra-rapid cooling and thawing in distilled water. Fig. 3.12a: Deterioration was confined to peripheral root cap cells and only the occasional internal cell appeared adversely affected Fig. 3.12b: Cap cells were generally well organised and showed all the signs of considerable metabolic activity. Note particularly, the elongated plastid profiles and absence of starch (cf. Fig. 3.15a). Fig 3.12c: Shoot apical meristems, did not appear to be adversly affected, although hypertrophied, presumably necrotic cells were scattered throughout the underlying parenchyma. Fig. 3.12d: Cells of the shoot apical meristem were not disrupted: many mitochondria, plastids occurred and Golgi bodies were evident. However, note the peri-nuclear orientation of organelles and the featurelessness of the nucleus and lack of definition of the nuclear envelope.

(a) x 150

(b) x 3 300

(c) x 150

(d) x 6 500
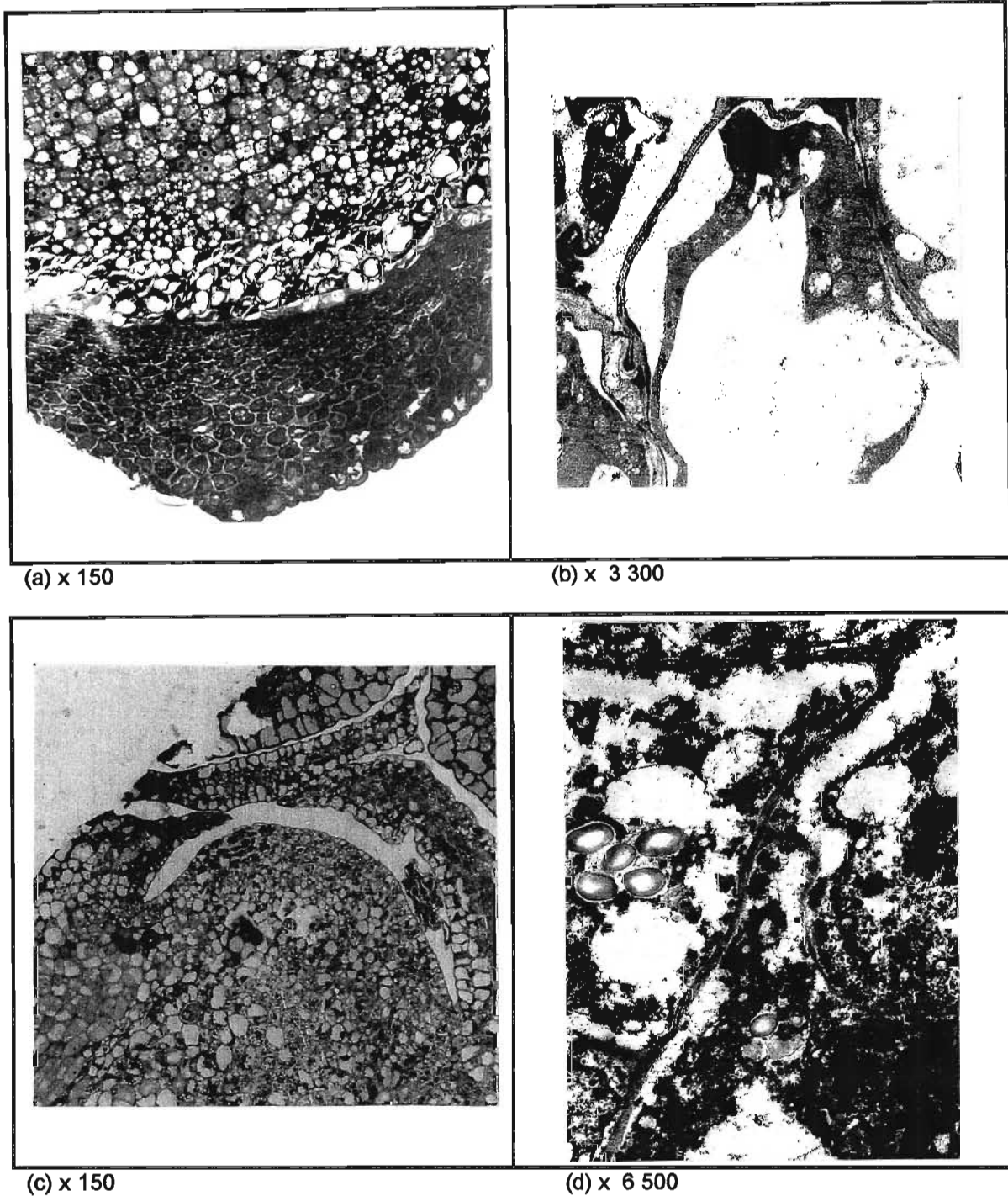
Figure 3.13: *Quercus robur* embryonic axes exposed to Poulsen's (1992) dehydration procedure, but then subjected to ultra-rapid cooling and thawing in distilled water. Fig. 3.13a: Extensive deterioration had occurred and persisted from the exterior to the deep-lying cells in the columella of the root cap. Fig 3.13b: Illustrates the extensive intracellular deterioration characteristic of most inner cap cells and the root apical meristem. Fig 3.13c: The shoot apical meristems were extensively damaged, as is obvious even at the light microscope level. Fig. 3.13d: Absolute disarray had occurred in the shoot apical meristems of axes exposed to these treatments.

(a) x 120   (b) x 8 100

(c) x 130   (d) x 8 600
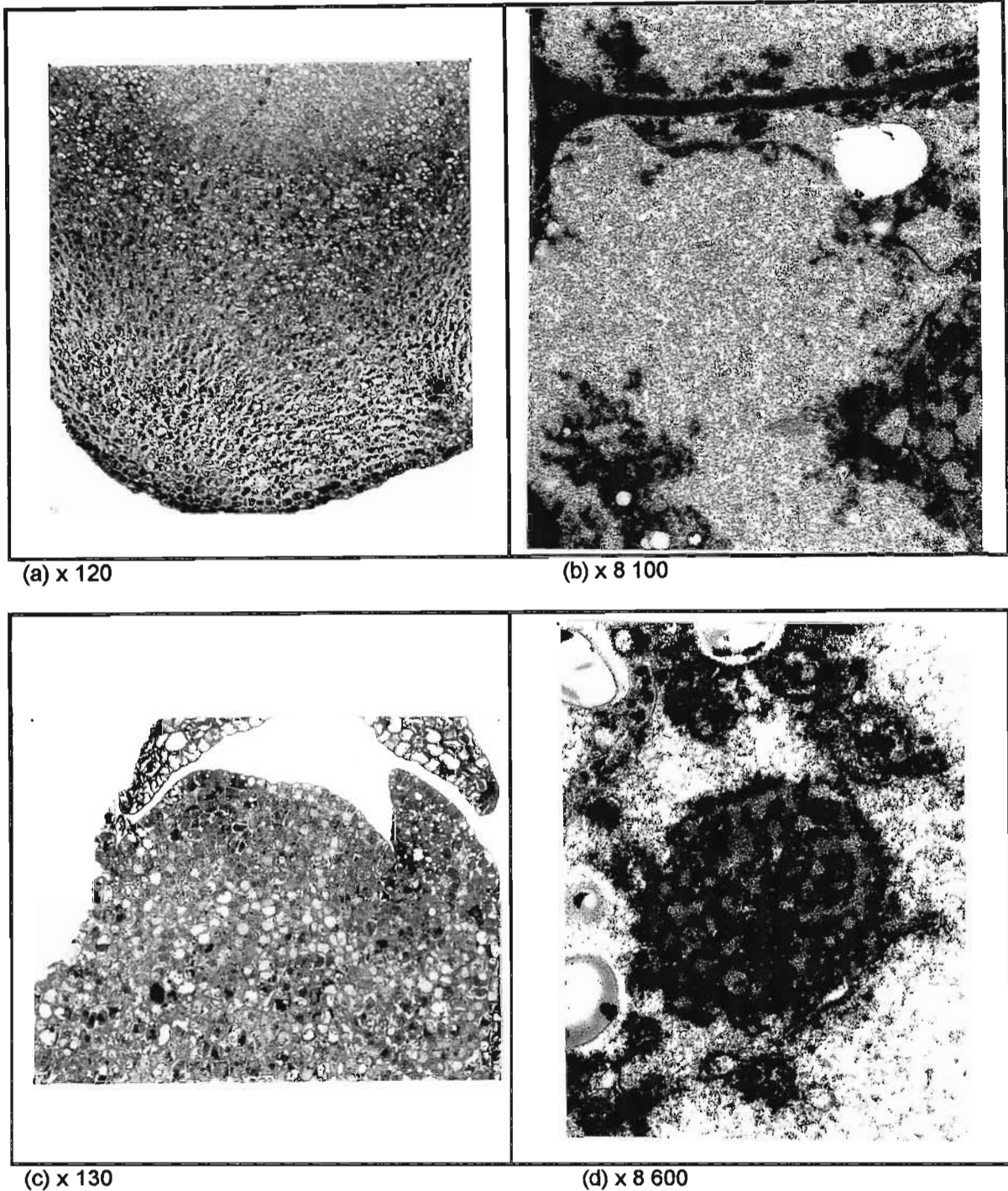
Figure 3.14: *Quercus robur* embryonic axes after exposure to Chmielarz's (1997) dehydration protocol, but followed by ultra-rapid cooling and thawing in distilled water. The root cap and shoot apices of these treated axes were disorganised and necrotic, as could be seen even at the light microscope level. (Fig. 3.14a and c). Examination with the TEM revealed that all the cells of both the root and shoot apex were extensively degraded (Fig. 3.14b and d).

(a) x 20 000

(b) x 20 800

(c) x 9 000

(d) x 40 000
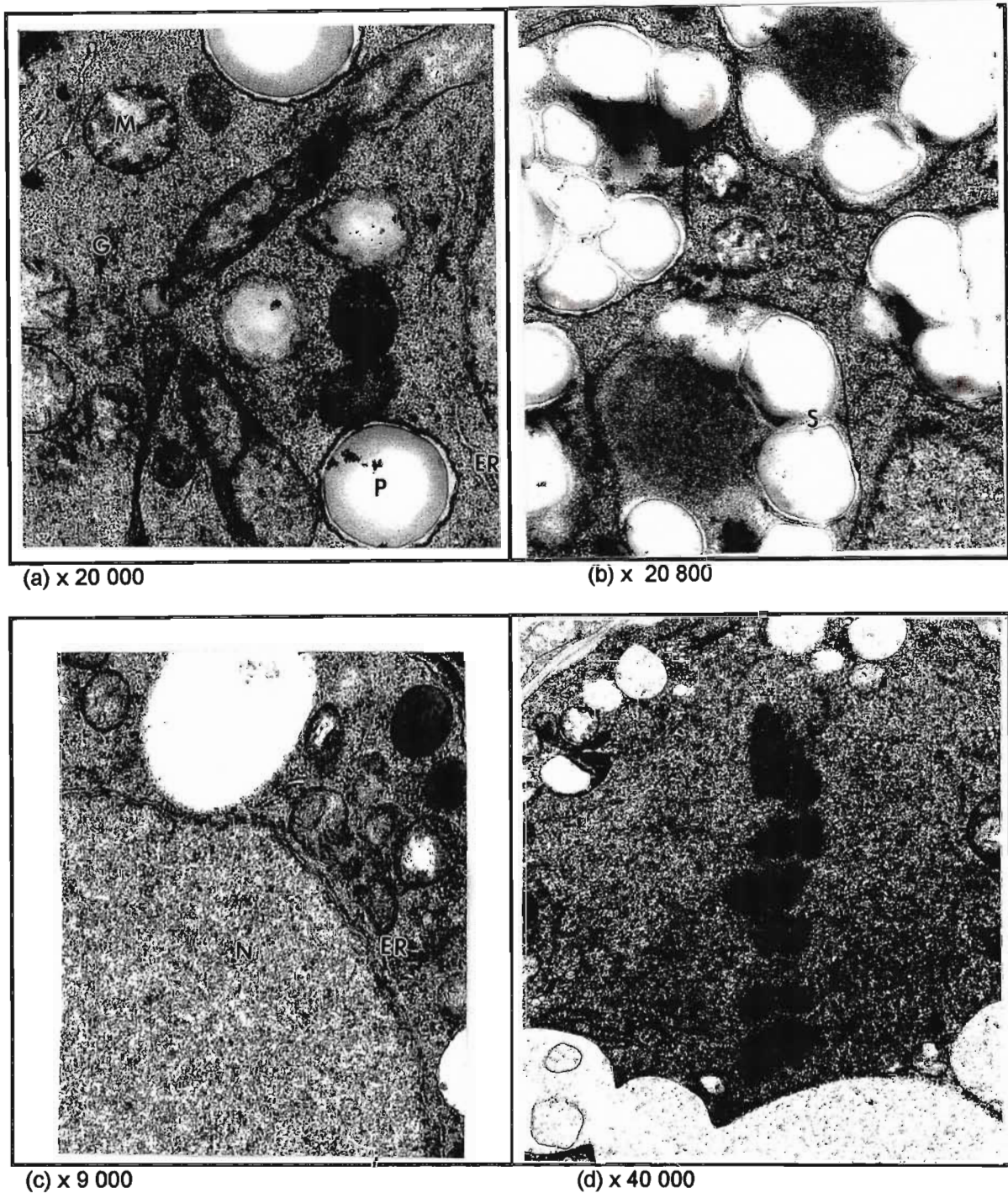
Figure 3.15: *Quercus robur* embryonic axes after fast flash-drying, ultra-rapid cooling and thawing in a $Ca^{2+}Mg^{2+}$ solution. Fig. 3.15a: A highly metabolically active root cap cell showing plastids, many developed mitochondria, a Golgi body and profiles of ER. Fig. 3.15b: Showing statoliths in a columella cell. Fig. 3.15c: Apical shoot cells well characterised by well-developed organelles, rER development and polysomes, normally disposed in the cytomatrix; the nuclear envelope is sharply defined, and the nucleus is spherical. Fig 3.15d: Mitotic activity was characteristic of shoot apical meristems after the 6 d *in vitro* recovery period.

Following fast flash-drying, ultra-rapid cooling and thawing in distilled water, only peripheral necrosis was observed in the root cap (Figure 3.12a), with most of this protective tissue not deleteriously affected. While the necrotic outermost cells could reflect damage that occurred during the manipulations, these cells are known to autolyse normally shortly before they are sloughed. From the ultrastructure, it could be seen that nuclear morphology was normal (not illustrated) and many well-developed mitochondria were observed, indicating that a high level of metabolic activity was occurring, polysomes and rER were also features of these cells (Figure 3.12b). Figure 3.12b shows the typical ultrastructure of the columella cells: although there were many plastids, no statoliths were present in any of these cells (see later).

After this same procedure, however, cells of the shoot apical meristem did not maintain the same high level of organisation as the root cells. Figure 3.12c shows a shoot apex in which a scattering of hypertrophied (swollen) necrotic cells, although not in the meristem. At the TEM level (Figure 3.12d) although the nuclear envelope sometimes appeared ill-defined, the cells showed evidence of metabolic activity following the 6 d recovery period. There were many polysomes and mitochondria, as well as Golgi bodies and some rER. In many cases, however, organelles of the shoot apical meristem cells were not evenly distributed, tending instead to a perinuclear orientation. Shoot cells remained static in culture, no shoot growth occurred, and the shoot apices became necrotic.

After exposure to Poulsen's (1992) pre-treatments, severe necrosis, from the exterior to the deep-lying cells in the columella of the root cap occurred (Figure 3.13a). Proximal to the obviously necrotic zone, cells were markedly and abnormally vacuolated. This phenomenon, in virtually all the columella cells, was associated with tonoplast dissolution and intracellular lysis (Figure 3.13b), indicating that even those cells not appearing necrotic at the LM level, were in fact, dead. Similarly in the shoot apical meristem, increased vacuolation was observed and some cells were obviously necrotic (Figure 3.13c). Figure 3.13d, which is representative of many of the shoot apical meristem cells, shows the extensive intracellular lysis that had occurred, with few components remaining identifiable.

The most severely degradative response, however, was after Chmielarz's (1997) pre-treatment despite ultra-rapid cooling, as shown in Figures 3.14a and c, where even at the LM level, both the root and shoot apex cells could be seen to be extensively damaged. The ultrastructure (Figures 3.14b and d) revealed complete intracellular degradation and collapse. It is concluded that the severe pre-freeze treatments both here and that embodied in the Poulsen (1992) approach, had pre-disposed the axes to lethal damage during cooling (freezing) and/or thawing.

Among surviving axes that had been fast flash-dried and cryostored, vigorous root growth occurred, but the roots remained horizontal. As gravitropism occurred

in control and dried axes, the freezing and/or thawing steps were identified as being imperfect. Gravitropism in roots causes growth towards the earth's gravitational field, while stems grow away from it. This root curvature results from differential growth on upper and lower sides. In roots, the cap detects the stimulus of gravity, whereas actual bending occurs in the elongation zone behind the root cap. Detection of gravity appears to involve the sedimentation of specialised amyloplasts, the statoliths (Chen *et al.*, 1999).

Thus, in order to achieve the ultimate objective, that of the normal seedling production after cryostorage of *Q. robur* embryonic axes, the thawing step in distilled water was examined. The thawing procedure was modified using media that included sucrose, or mannitol and an MS medium (see Chapter 2). However, despite the use of these thawing agents, the integrity of the embryonic roots was maintained, but still no gravitropic curvature occurred. Initially shoot apices remained static, but soon became necrotic.

Two observations prompted the use of a solution containing calcium and magnesium in concentrations known to promote cytoskeleton assembly (Wolfe, 1995). These were the disorientation of organelles seen in some shoot apical meristem cells (e. g. Figure 3.12d) and the lack of the gravitropic response. Mycock (1999) had used a 50:50 solution of $1 \mu M$ $CaCl_2. 2 H_2O$ and 1 mM $MgCl_2. 6 H_2O$ both as a pre-treatment and a thawing medium, with very beneficial effects on cryopreserved somatic embryos.

Thawing in the $Ca^{2+}Mg^{2+}$ solution at 40°C facilitated both the gravitropic response and shoot production in a significant proportion of axes retrieved from cryostorage. Comparative results are presented in Table 3.4. All freezing experiments were done in triplicate (n =20), for a total of 60 axes per treatment.

Table 3.4: The effect of two different thawing regimes (distilled water and the $Ca^{2+}Mg^{2+}$ solution) on percentage *Q. robur* root and shoot survival assessed after 30 d in culture. All experiments were done in triplicate (n = 20).

THAWING AGENTS

	Distilled water			$Ca^{2+}Mg^{2+}$		
	% Roots	G/r	% Shoots	% Roots	G/r	% Shoots
1st TRIAL	35	−	0	100	+	70
2nd TRIAL	66	−	0	60	+	40
3rd TRIAL	40	−	0	80	+	50

G/r = Gravitropic response

Comparison of shoot apical meristem cells after thawing in distilled water (Figure 3.12d) and the $Ca^{2+}Mg^{2+}$ solution (Figure 3.15c) shows that use of the cation solution promoted the orderly orientation of highly differentiated organelles, and that many of the cells were actively mitotic (Figure 3.15d). These observations support the idea that the availability of calcium and magnesium promoted normal cyto- and nucleoskeleton assembly after cryopreservation. The cytoskeleton, a sub-cellular proteinaceous filament system, is responsible for the position and movements of organelles. The principle components of the plant cytoskeleton are the microfilaments and microtubules (McNulty and Sanders, 1992; Wolfe, 1993). The major difference in root cap columella cells after thawing in the divalent cation solution, was the promotion of statolith formation (Figure 3.15b). Because

of the use of TEM alone, there could be no direct evidence implicating the cytoskeleton in the absence or presence of the gravitropic response.

The disassembly or re-assembly of the plant cytoskeleton is regulated by numerous signalling agents, including calcium and magnesium, which are considered to be key controlling elements (Hepler and Hush, 1996). Calcium promotes microtubule assembly at low concentrations, and at high concentrations causes disassembly of microtubules (Wolfe, 1995).

Magnesium, on the other hand, binds to actin before assembly into microfilaments. According to Wolfe (1995), between 0.1 and 10 mM $MgCl_2$ promotes rapid and stable microfilament assembly. The use in this present study, of the $Ca^{2+}Mg^{2+}$ solution was on the basis that the cations might promote re-assembly of the cytoskeletal components, not only within the shoots, but also to induce root gravitropism after cryostorage, and thereby normal seedlings. The results presented in Table 3.4 show that this was the case, although presently there is little (or no) information that explains the effect of the cations on statolith formation.

3.6 THE EFFECT OF THAWING AGENTS ON MEMBRANE ELECTROLYTE LEAKAGE

The rates and levels of electrolyte leakage indicate the degree of tissue damage that has occurred and have been found to correlate well with the viability of seeds (Vertucci and Leopold, 1987; Vertucci, 1989; Pammenter *et al.,* 1991). The

increase in leakage in orthodox seeds was originally attributed to a loss of cell membrane integrity by Ching and Schoolcroft (1968) and Matthews (1971) and has remained a favoured theory.

According to McKersie and Stinson (1980), water uptake by desiccation-tolerant seeds reinstates the original structure of the cellular membranes, whereas the membranes of desiccation-sensitive seeds that have been dehydrated are unable to reform completely.

Table 3.5 shows the difference in electrolyte leakage rates after submerging $Q.$ *robur* axes either into the $Ca^{2+}Mg^{2+}$ solution or distilled water, respectively for 180 min after fast flash-drying and ultra-rapid cooling.

Table 3.5: The difference between the leachate conductivity after $Q.$ *robur* axes were exposed to distilled water or the $Ca^{2+}Mg^{2+}$ solution, for 3 h.

	ELECTROLYTE CONDUCTIVITY (μ S cm^{-1})		
	SOLUTION ALONE	SOLUTION PLUS AXES	DIFFERENCE
WATER	2	304	302
$Ca^{2+}Mg^{2+}$	246	309	63

The data in Table 3.5 indicate a far higher electrolyte leakage after thawing in distilled water than those axes exposed to the $Ca^{2+}Mg^{2+}$ solution, clearly indicating that the better thawing agent is the cation solution.

After fast flash-drying and ultra-rapid cooling (using the $Ca^{2+}Mg^{2+}$ solution as the thawing agent), an acceptable proportion of axes did survive cryostorage and developed into seedlings (Table 3.4). However, not all survived, which was puzzling, as all axes were from the same batch of seeds as well as having been subjected to exactly the same treatments. It is, however, well-established that there is marked seed-to-seed variability at any one harvest (e.g. Berjak *et al.*, 1996) although presently there is no information on this aspect for the *Q. robur* seeds used in these experiments. However, Kioko *et al.* (1999) have indicated that the state of axis development in *Warburgia salutaris*, may well be critical to survival of cryostorage.

From these thawing trials, use of the $Ca^{2+}Mg^{2+}$ solution seems to offer a means to alleviate injury that occurs after cryopreservation. Use of this divalent cation solution is therefore recommended as a post-freezing (and perhaps also a pre-freezing) treatment for axes of other species, as Chapter 4 will show for *Ekebergia capensis*.

CHAPTER 4: RESULTS AND DISCUSSION, *Ekebergia capensis*

Ekebergia capensis Sparrm. (Meliaceae) is a semi-deciduous to evergreen tree of eastern tropical and subtropical Africa. When ripe, just before shedding, the fruit turns deep red. These fruits have a fleshy mesocarp and a hard, fairly impermeable endocarp. The ex-endospermous seeds are 6-8 mm x 4 mm, and the embryonic axes are approximately 2 x 1 mm. *Ekebergia capensis* is used in traditional medicine as an emetic and to treat diarrhoea and dysentery (Pooley, 1993). Thus, because of its medicinal use and availability locally, it was chosen as one of the two species for this study. *Ekebergia capensis* was treated in a similar manner to *Q. robur* (Chapter 2), based on the promising protocol developed for that species (Chapter 3).

The results presented here emphasise the issues of: the importance of optimal culture conditions; drying- and freezing-rates; thawing and post-thaw recovery.

4.1 WHOLE SEED VIABILITY

Ekebergia capensis (Cape Ash) fruits were hand-harvested when red/burgundy (mature) from one provenance, a group of Durban trees planted by the Parks Department in the city. After removal of pericarp and testa, all seeds were set out to germinate on filter paper (moistened with 1% MS salts). After 2-3 weeks, 100% viability was recorded. Embryonic axes were from newly-harvested seeds taken for water content determination, which emerged as 1.13 ± 0.16 g g^{-1}.

It was observed that even though all seeds set out for germination were from simultaneously collected fruits of similar size and colour, some germinated and established seedlings much more rapidly than others, indicating a range in the developmental status of the seeds at harvest. This variability may ultimately, as suggested for *Q. robur*, be correlated with the different stress tolerance among the embryonic axes of these seeds to treatments such as dehydration and/or freezing, even within a single harvest of seemingly similar mature seeds.

4.2 *Ekebergia capensis* EMBRYO CULTURES

In the laboratory, *E. capensis* seeds were extracted and the endocarps removed. The axes immediately excised, were maintained on barely moist filter paper within Petri dishes until a sufficient number had been accumulated and then surface-sterilised in either sodium hypochlorite (containing a drop of Tween-20) or mercuric chloride and plated out onto four different sucrose-containing media (MS, ½ MS, ½ MS + H, ¼ MS, Chapter 2). Embryonic axes of similar size were carefully selected for *in vitro* culture, with any appearing in less than optimal condition being discarded. The results (Table 4.1) show that while 100% germination was achieved on all media after 15 d when embryonic axes were cultured on ¼ MS after surface sterilising with sodium hypochlorite, this was not the case for axes treated with mercuric chloride where all axes germinated only on ¼ MS medium. This suggested that ¼ MS was the best medium of the four, and that sodium hypochlorite was the best surface sterilant to use for all subsequent experimental procedures as the combination of mercuric chloride

surface sterilisation with the non osmotically-challenging media appeared toxic for the *E. capensis* axes.

Table 4.1: Percentage survival, after 15 d, of *E. capensis* zygotic axes on four different media after surface-sterilisation with 1% (v/v) sodium hypochlorite and 0.1% (m/v) mercuric chloride, respectively (n = 20).

MEDIUM	PERCENTAGE SEEDLING SURVIVAL	
	1 % NaOCl	0.1 % HgCl$_2$
MS	100	60
½ MS	100	70
½ MS + H	100	90
¼ MS	100	100

However, the growth rate of both roots (Figures 4.1a and c) and shoots (Figures 4.1b and d) was slowest on ¼ MS medium. When root lengths achieved after NaOCl surface-sterilisation on ¼ MS medium were compared with those on the other media at day 15, results showed, ½ MS (26.53 mm) > MS (24.49 mm) > ½ MS + H (20.79 mm) > ¼ MS (20.59 mm). Even though these results were not significantly different from one another (P < 0.05, ANOVA) for either of the surface-sterilisation procedures, general growth rate on ¼ MS medium was the slowest, as was the case for the shoots. Root growth rates were however, significantly lower after HgCl$_2$ surface sterilisation when compared with NaOCl surface sterilisation (Figures 4.1a and c).
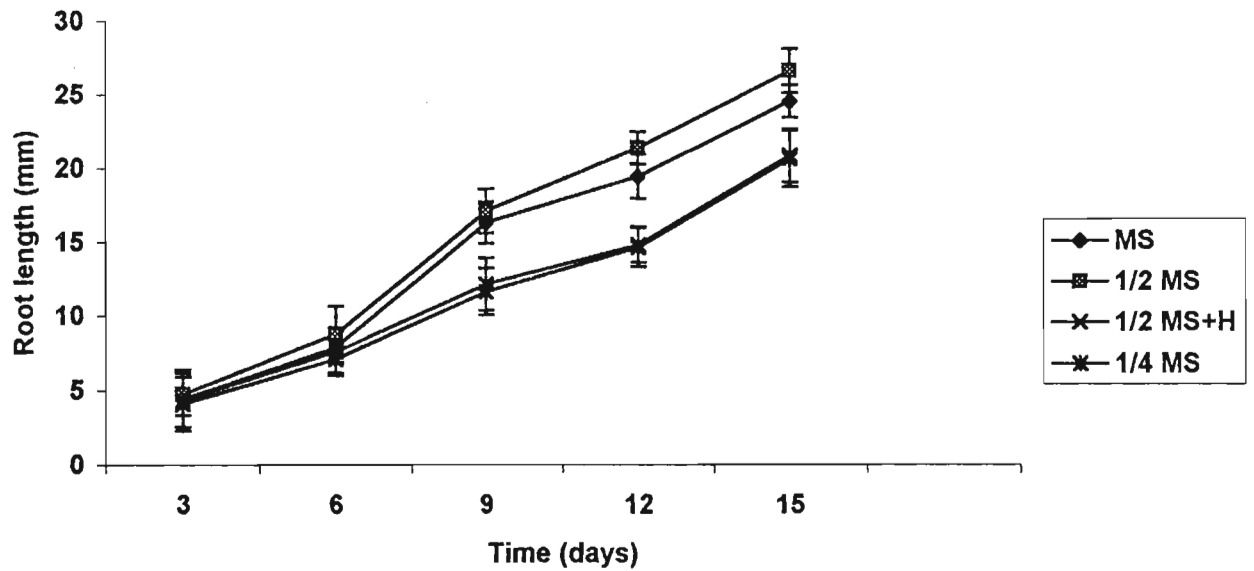
Figure 4.1a: Root length of *E. capensis* axes germinated on various media (see key) after sodium hypochlorite surface-sterilisation (n = 20). Bar = mean ±SE.
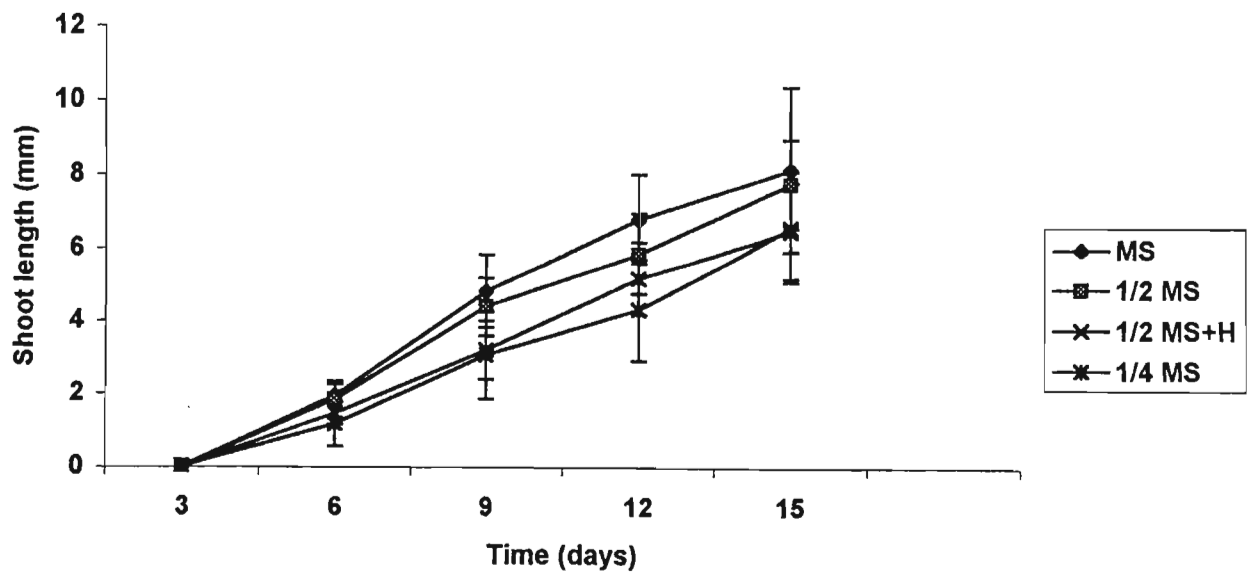
Figure 4.1b: Shoot length of *E. capensis* axes germinated on various media (see key) after sodium hypochlorite surface-sterilisation (n = 20). Bar = mean ±SE.
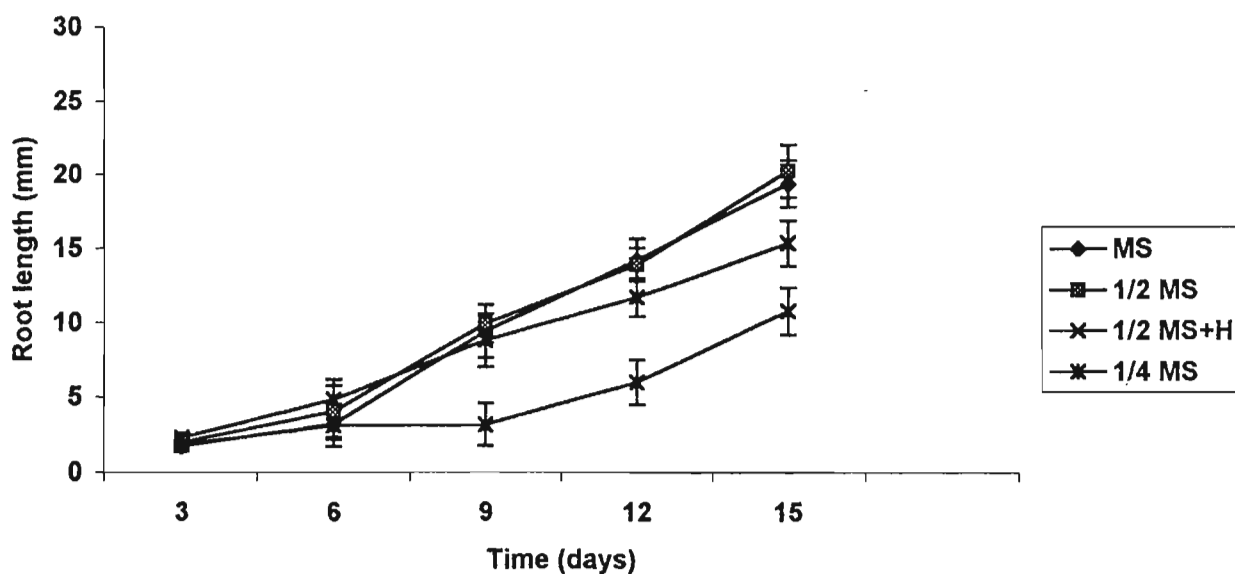
Figure 4.1c: Root length of *E. capensis* axes germinated on various media (see key) after mercuric chloride surface sterilisation (n = 20). Bar = mean ±SE.
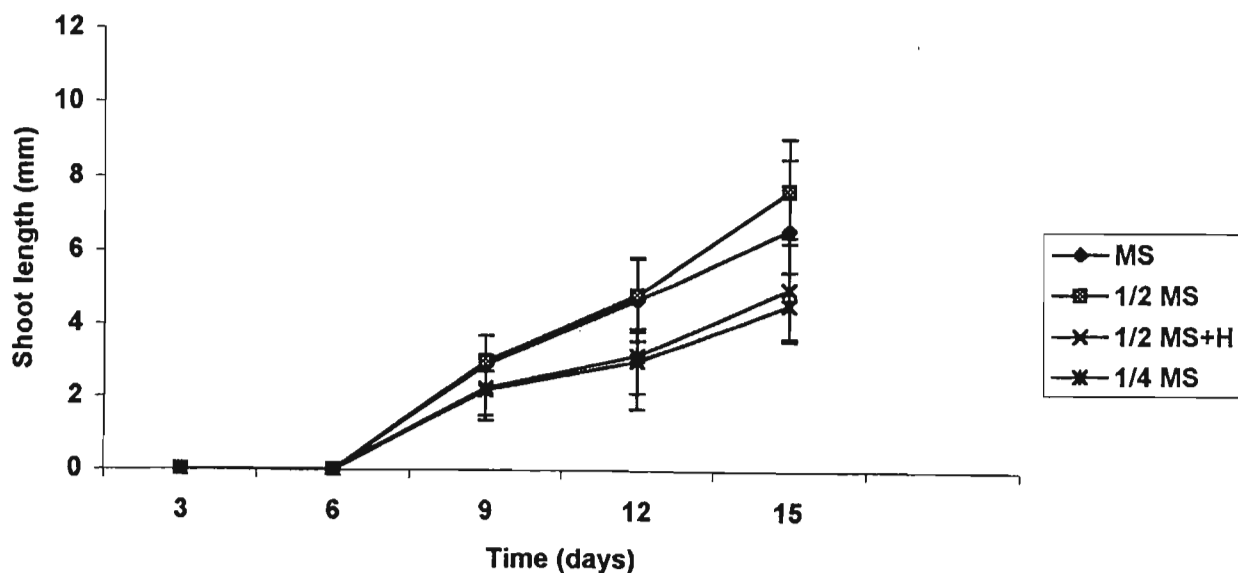
Figure 4.1d: Shoot length of *E. capensis* axes germinated on the various media (see key) after mercuric chloride surface-sterilisation (n = 20). Bar = mean ±SE.

However, from the trials with HgCl$_2$ surface sterilisation, the more concentrated the media, the lower the survival. In view of the fact that use of NaOCl eliminated all fungal contaminants and that growth rates on ¼ MS maintained 100% viability, despite the surface-sterilant used, this combination was chosen for all subsequent investigations with *E. capensis* axes.

4.3 EXPERIMENTATION WITH DRYING: *E. capensis*

Desiccation trials were implemented to assess the viability retention of *E. capensis* axes after exposure to four different methods of drying (laminar airflow-, silica-gel-, flash- and fast flash-drying). Samples of 30 axes were taken at 5 min intervals over 30 min of each drying regime. Ten axes were used for water content determination, and 20 for *in vitro* culture. Thus, 750 *E. capensis* axes were used for this preliminary desiccation trial. Before any drying was implemented, all axes were surface-sterilised in a solution of sodium hypochlorite containing Tween-20, rinsed 3 times in sterile distilled water (see Chapter 2) and then dried. Those axes taken for assessment of survival were plated out *in vitro* onto ¼ MS medium supplemented with 30 g l^{-1} sucrose and solidified with 10 g l^{-1} agar.

The water contents achieved for *E. capensis* axes after 30 min dehydration were laminar airflow > silica-gel > flash-dried > fast flash-dried (Figure 4.2). It should be noted that the water contents achieved with the use of laminar airflow were significantly higher for the first 20 min (0.74 ± 0.04 g g^{-1}) than those axes
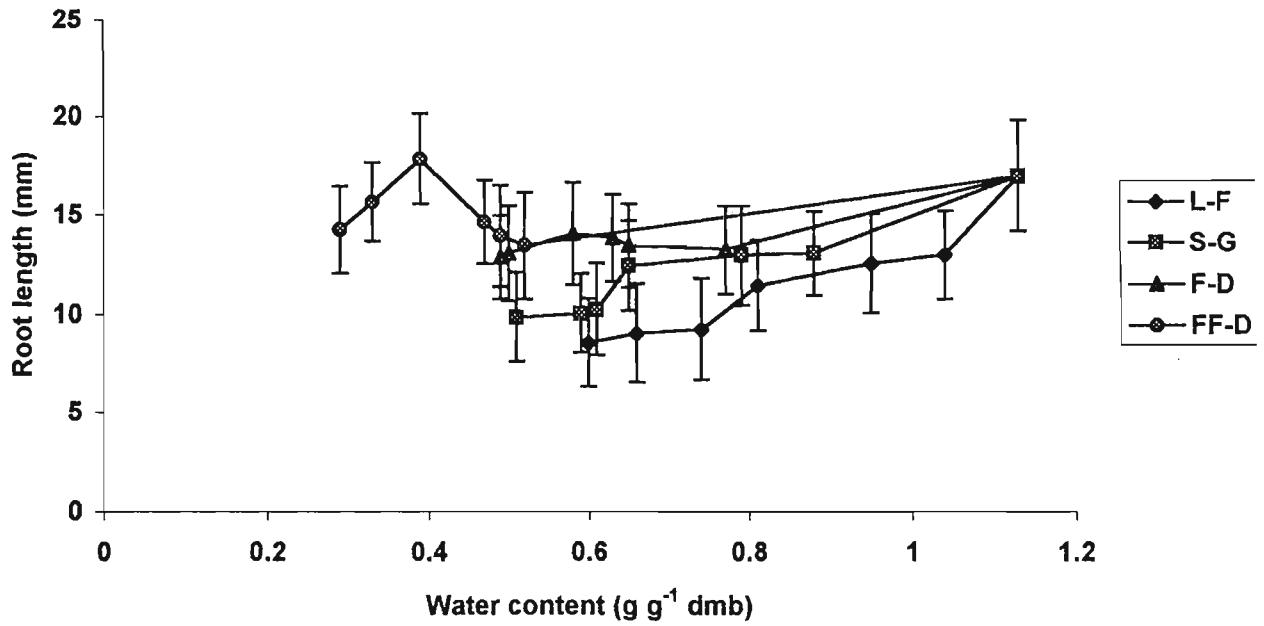
Figure 4.2a: Water contents and root growth of *E. capensis* axes after exposure to differential drying rates. Bars = mean ±SE.
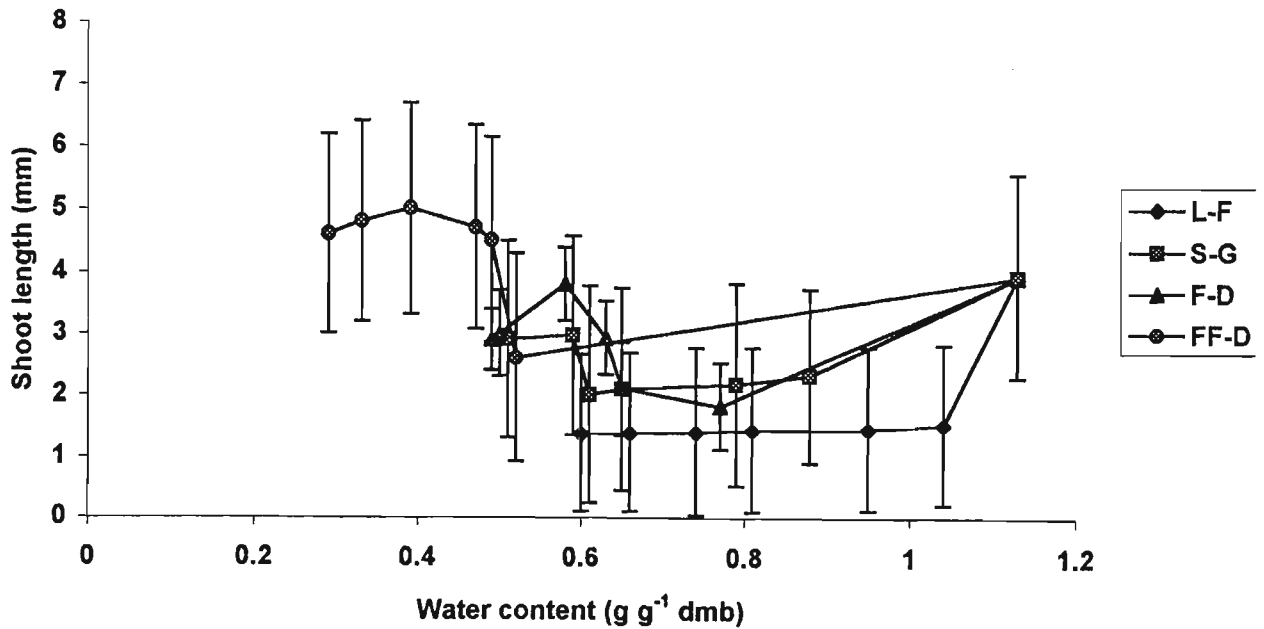
Figure 4.2b: Water contents and shoot growth of *E. capensis* axes after exposure to differential drying rates. Bars = mean ±SE.

exposed to fast flash-drying (0.39 $\pm$ 0.06 g g^{-1}) for all embryonic axes. Furthermore, after 30 min, those axes exposed to the most rapid form of drying (FF-D) (0.29 $\pm$ 0.04 g g^{-1}) had significantly lower water contents than laminar-flow (L-F) (0.6 $\pm$ 0.05 g g^{-1}) dried for both roots and shoots (Figure 4.2).

Ekebergia capensis embryonic axes, irrespective of the mode of drying and the time taken (5 min - 30 min), maintained the ability for 100% root development. However, in terms of root, and especially shoot growth, *E. capensis* axes did not tolerate a slow removal of water (L-F) as well as those exposed to fast-flash drying. Figure 4.2a indicates that even though 100% root development occurred subsequent to laminar airflow (L-F) drying, a gradual and relatively uniform decrease in the rate of root growth was observed. Silica-gel (S-G) dried axes, also showed a slight decrease in root growth rate over the desiccation period. This supports the contention of Pammenter *et al.* (1998) that if embryonic axes of a recalcitrant species are dehydrated slowly, time is afforded for damaging processes to occur while the water content is relatively high.

In terms of root development, those axes that were flash-dried, showed an essentially similar response to the fast flash-dried axes. The latter, particularly, showed a stimulation of root growth after water contents had been reduced to less than *c.* 0.4 g g^{-1} and this response was particularly marked for the shoots, even though not shown as statistically significant, (Figure 4.2b). This suggests firstly that a certain degree of rapid dehydration of desiccation-sensitive material

is stimulatory (Pammenter *et al.*, 1998) and that there is no loss of viability until structure-associated water is perturbed (Pammenter *et al.*, 1991). It has been suggested by Berjak *et al.* (1990), Pritchard (1991) and Pammenter *et al.* (1998) that it is more likely that very rapid drying of excised axes rapidly precludes not only germination-associated metabolism, but most metabolism, which implies a stasis on deteriorative reactions as well. Presently, a gradual but not significant, decrease in growth rate of both roots and shoots occurred below 0.3 g g^{-1} (possibly by the lower limit of freezable water) suggesting that a measure of desiccation damage *sensu stricto*, as opposed to metabolism-related deteriorative events (Pammenter *et al.*, 1998) had occurred.

A further interesting, although subjective observation, was that after fast flash-drying, the developing roots showed the most acute gravitropic response, compared with developing seedlings from axes dehydrated by the other methods.

The root responses were mirrored by the shoots (Figure 4.2b) subjected to the various drying regimes. After exposure to flash-drying, shoot length increased although showing no significant difference from one interval in time to the next. A gradual increase was observed after drying to *c.* 0.6 g g^{-1}, after which time, shoot length decreased, but again not significantly (P < 0.05, ANOVA).

Fast flash-drying resulted in a marked enhancement of this trend, with a significant increase in shoot growth from axes dehydrated below *c.* 0.5 g g^{-1} (20 min). This trend was maintained in axes dehydrated to *c.* 0.4 g g^{-1}, after which growth rate declined slightly, but not significantly (P < 0.05, ANOVA).

The effects of differential drying rates on the viability retention of recalcitrant seeds of *E. capensis* were also investigated by Pammenter *et al.* (1998). The difference between the study of those authors and the present one was the drying of whole seeds as opposed to the drying of embryonic axes. The results of both studies, however, show similar trends. The implementation of short-term rapid drying by those authors was associated with an enhanced germination rate, until a stationary phase, associated with degree of dehydration, was recorded. In the current investigation, where individual roots and shoots were measured, these did decrease, albeit not significantly, after 20 min of drying to water contents a little below 0.4 g g^{-1}, although a stationary phase, *per se*, was not presently recorded.

Other factors such as the effect of time course on variability of water content could not, however, be strictly compared between these two studies, as the whole seeds were dried for hours, and naked axes for minutes. Whatever the case, responses of both whole seeds and axes to different drying rates implied that it is not possible to determine a 'critical water content' for the loss of viability (Pammenter *et al.* 1998). Ultimately, however, it can be deduced that the more

rapid the drying rate, the higher the survival rate and the lower the water content which can be achieved over a shorter time. From this point, fast flash-drying was used as the drying pre-treatment before freezing. It should be noted, however, that the variability within this particular batch of axes was vast, and more intensive studies at different physiological ages should be carried out to determine optimal drying rates and times.

4.4 FREEZING OF *E. capensis* AXES

Ekebergia capensis axes were frozen at two different rates (Chapter 2): intermediate (direct plunging of cryovials containing axes, into liquid nitrogen) and ultra-rapid cooling (naked axes, individually plunged into isopentane and then immediately into liquid nitrogen). All axes died when contained in the cryovials that were plunged directly into liquid nitrogen. After ultra-rapid cooling, although some axis survival was achieved when distilled water was used as the thawing agent, only roots developed *in vitro*. When the $Ca^{2+}Mg^{2+}$ solution was used, not only did up to two-thirds of the axes survive, but almost all produced shoots (Table 4.2).

Table 4.2: The effects of thawing in distilled water or the $Ca^{2+}Mg^{2+}$ solution on percentage root and shoot survival, assessed after 15 d in culture. All experiments were done in triplicate (n = 30).

THAWING AGENTS

	DISTILLED WATER		$Ca^{2+}Mg^{2+}$	
	% ROOTS	% SHOOTS	% ROOTS	% SHOOTS
1st TRIAL	33	0	60	57
2nd TRIAL	20	0	40	40
3rd TRIAL	30	0	65	60

The results obtained here for *E. capensis* substantiate those for *Q. robur*, in demonstrating that liquid nitrogen plunging of axes within cryovials is likely to be lethal, but that isopentane held just above its freezing point (-150°C) is a far superior cryogen. Secondly, and most importantly, these results strengthen the contention that thawing is also a critical step, and that when rapid axes are immersed in distilled water, survival rates are low and shoot development does not occur. Finally, the survival obtained when the *E. capensis* axes were thawed in the divalent cation solution was not only acceptable, but provision of Ca^{2+} and Mg^{2+} in the medium promoted shoot development. Composition of the cation solution was based on the rationalé of Mycock (1999), in terms of promoting cyto-and nucleoskeleton re-assembly during manipulation for cryostorage, and after retrieval from the cryogen. Calcium and magnesium are the key controlling ions in the dynamics of the skeletal elements (Hepler and Hush, 1996) and although there is no direct evidence in the present study, it is highly likely that their efficacy in the thawing step is based on this rôle.

4.5 MICROSCOPICAL EXAMINATION OF *E. capensis* AXES

When observing root and shoot cells which had been surface-sterilised in sodium hypochlorite, it was clear that those cells were metabolically active as in both the root and shoot, cells contained large central spherical nuclei, small mitochondria, and vacuoles as well as Golgi bodies and many profiles of rER. The presence of the compounds of the endomembrane, particularly of the Golgi bodies, indicated the metabolically-active status of the cells. Furthermore, the cell wall was regular, with the plasmalemma closely associated. In addition, Figure 4.3b shows a few of the many statoliths (gravity-perceptors) in the root cap columella cells that developed in the surface-sterilised material after 6 d *in vitro*, the rôle of which was indicated by the pronounced geotropic curvature.

(a) x 9 800   (b) x 25 300
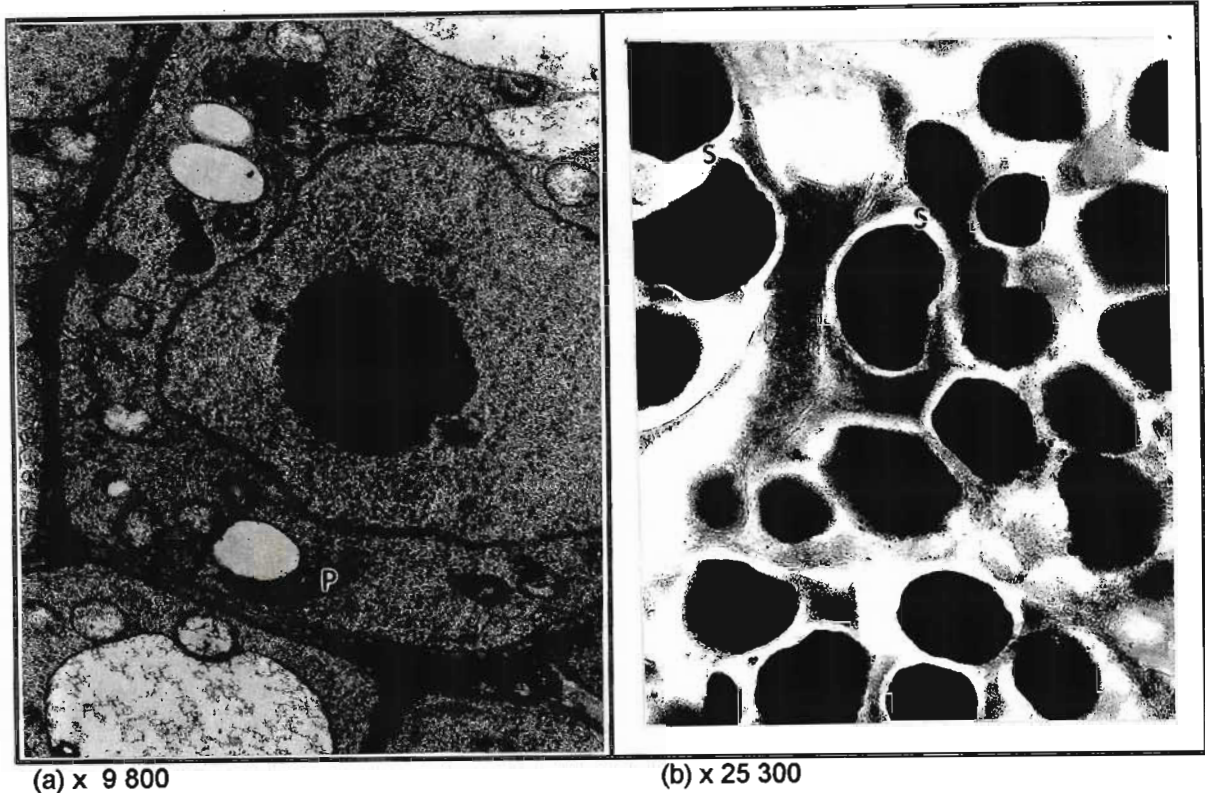
Figure 4.3a: Ultrastructural aspects of typical *E. capensis* axis cells. The ultrastructure is consistent with cells that are hydrated, show some metabolic activity and have not suffered any obvious adverse effects from the surface sterilisation procedure. Fig 4.3b: Details of a cluster of statoliths, which were prominent features of the root cap columella cells, in material after 6 d *in vitro* following surface-sterilisation.

Both shoot and root apex cells of axes that had been subjected to fast flash-drying, appeared normal (Figure 4.4). Cells of both axis apices contained large spherical nuclei as were seen in the control material. Mitochondria (not as spherical as those in the control axes, in fact the mitochondria appeared relatively undifferentiated compared with control shoots), small Golgi bodies and regular cell walls with closely adpressed plasmalemmas were characteristic. However, there seemed to be an increase in the degree of vacuolation in the shoot apical cells (Figure 4.4a). The vacuoles contained degraded remains of material that appeared to have been internalised from the cytomatrix. The

columella root cap cells (Figure 4.4b) exhibited well-developed statoliths correlating with the gravitropic response shown by the axes in culture.

(a) x 5 000   (b) x 23 400
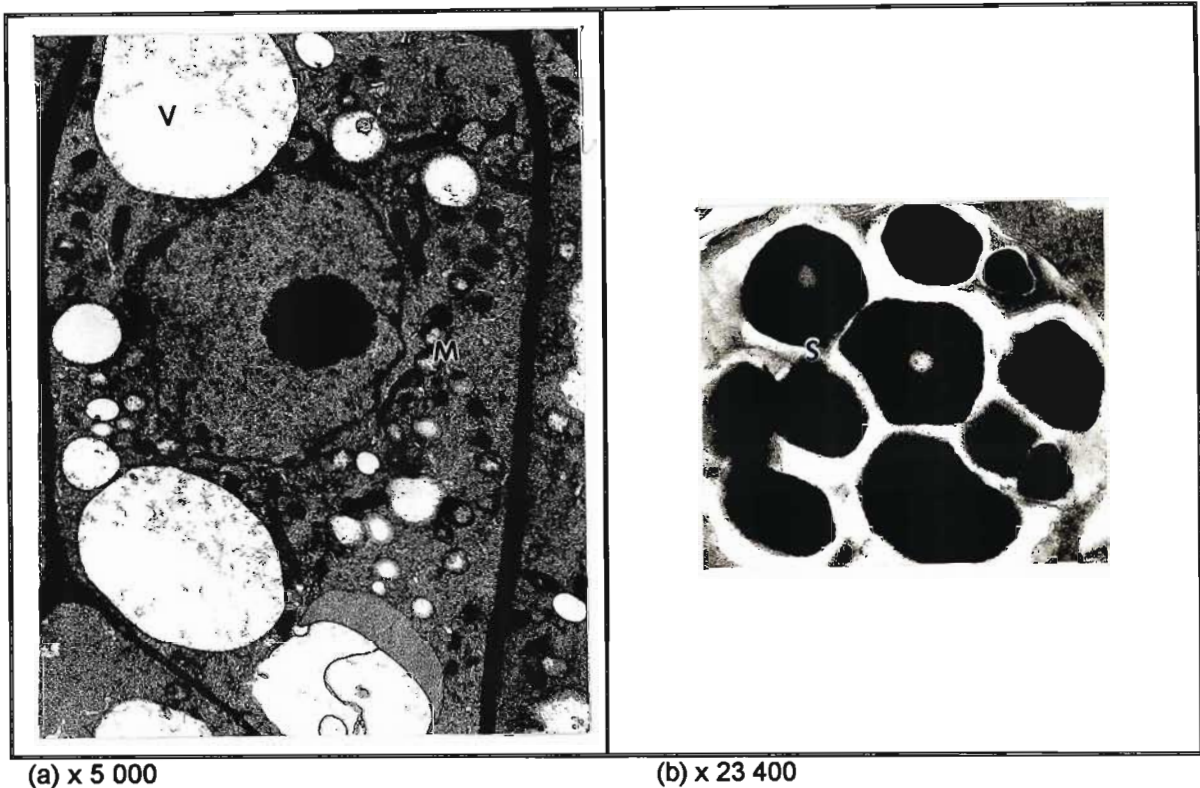
Figure 4.4: Ultrastructural aspects of *E. capensis* axes after fast flash-drying. Fig. 4.4a: Even though the mitochondria were less differentiated than in the control, fast flash-drying appeared to have no adverse effect on the shoot apical meristem cells, judging from plastid development. The degree of vacuolation did, however, increase after drying, this might have been stress-related. Fig 4.4b: The occurrence and structure of cap columella statoliths seemed unchanged by dehydration.

After fast flash-drying, ultra-rapid cooling and thawing in distilled water (Wesley-Smith *et al.* 1999), most shoot apex cells (Figure 4.5a), showed advanced degradation, with extensive internal lysis. The ultrastructure of these cells indicated that the shoot apices had rapidly become necrotic. Shoot apices generally, have no external protective structures, and, in fact, constitute the apical meristems. Their degeneration is basic to the lack of any shoot development following water thawing. Figure 4.5b illustrates the typical

ultrastructure of the root cap columella cells. These cells were obviously active, showing mitochondria and Golgi bodies, but no statoliths had developed, indicating the basis for the lack of the gravitropic response.

(a) x 19 000　　　　　　　　　　　　　　　(b) x 18 500
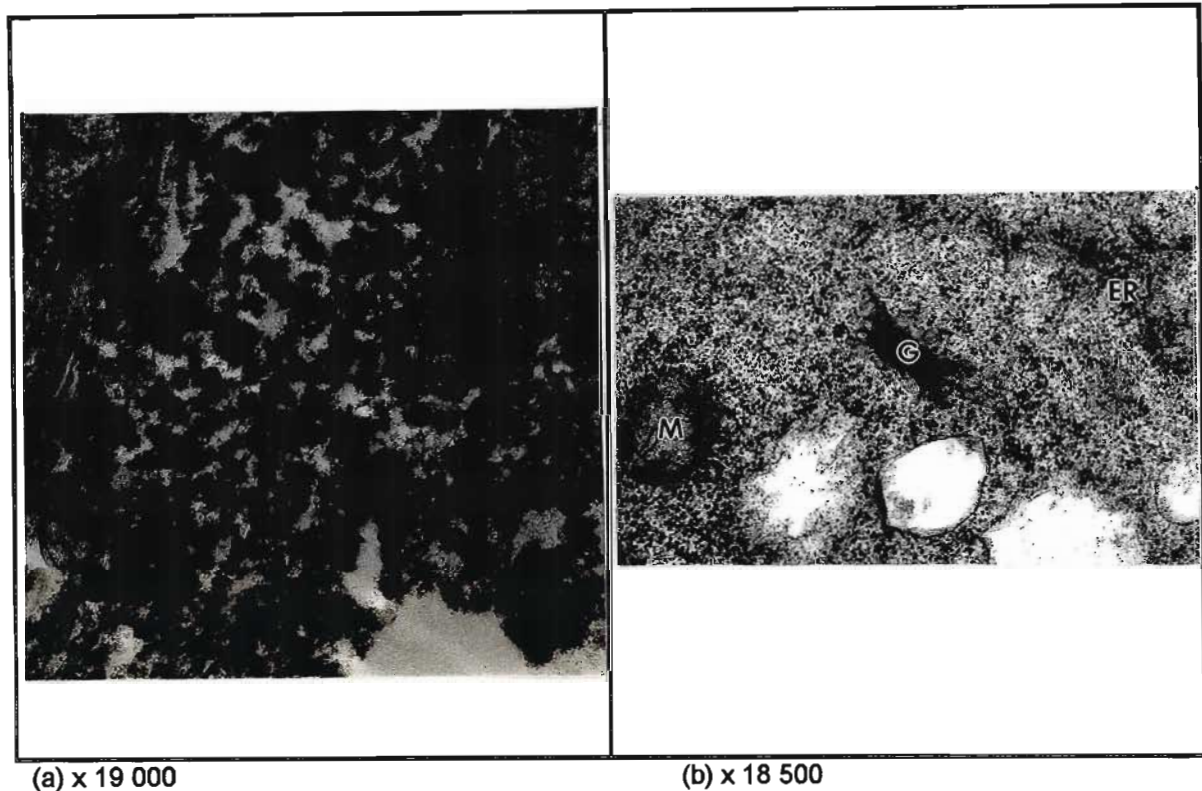
Figure 4.5: *Ekebergia capensis* embryonic axes after fast flash-drying, ultra-rapid cooling and thawing in distilled water. Fig. 4.5a: Many of the shoot apex cells of these treated axes were in disarray, where organelles were difficult to identify among the intracellular débris Fig. 4.5b: Root cap columella cells showed signs of greatly enhanced activity, in the production of many polysomes, short rER profiles, Golgi bodies and active mitochondria. However, no statoliths were present.

Those root cells that were fast flash-dried, ultra-rapidly cooled and thawed in the $Ca^{2+}Mg^{2+}$ solution, on the other hand, showed a high level of ultrastructural organisation. The shoot cells were typified by small vacuoles, mitochondria, Golgi bodies and profiles of ER. Additionally the integrity of the plasmamembrane seemed to have been retained. Significantly, statolith formation had been

promoted after thawing in the cation solution, this correlated with gravitropic curvature displayed with the 6 d *in vitro* growth period.

(a) x 6 300   (b) x 23 200
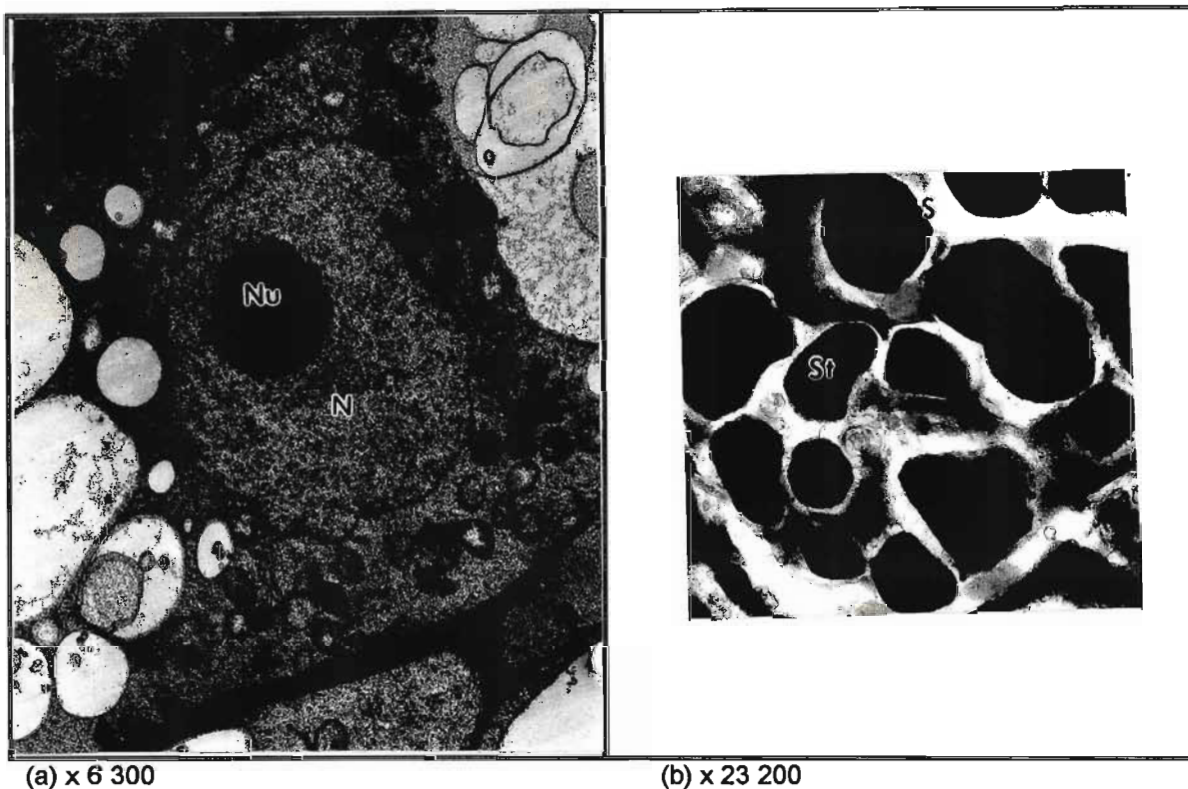
Figure 4.6: Ultrastructural aspects of *E. capensis* embryonic axes after fast-flash drying, ultra-rapid cooling and thawing in $Ca^{2+}Mg^{2+}$ solution. Fig. 4.6a: Cells of the shoot apex were in good condition, although relatively more vacuolated than in the control material. Organelle disposition appeared normal, as did the nuclei, which contained prominent nucleoli. Fig. 4.6b: Statoliths, containing the deeply-stained starch grains typical of this species, occurred in root cap columella cells.

Figure 4.7 shows the difference in general appearance of the typical *E. capensis* axes exposed to fast flash-drying only and fast flash-drying, ultra-rapid cooling and thawing in distilled water or the $Ca^{2+}Mg^{2+}$ solution, respectively. Axes had been maintained *in vitro* for 15 d.

Figure 4.7: Actual size.

The typical appearance of *E. capensis* axes after thawing in either distilled water or the $Ca^{2+}Mg^{2+}$ solution, respectively, after exposure to fast flash-drying and ultra-rapid cooling. The dried axis on the left of the photograph indicates a typical example of an axis fast flash-dried only.

Those axes fast flash-dried, ultra-rapid cooled (frozen) and thawed in distilled water, showed greening, indicating that some cells had survived, but with only minimal root growth and no shoot formation. The shoot apices became necrotic relatively rapidly, as borne out by the ultrastructural studies. Those axes dried and frozen in the same way, but thawed in the $Ca^{2+}Mg^{2+}$ solution, however, did maintain good seedling formation for some axes, and even though not as vigorous as those dried only axes, appeared somewhat normal.

Figure 4.8 shows a typical *E. capensis* seedling after removal from Jiffy-7 pots (peat moss) (see Chapter 2), and then into potting soil.
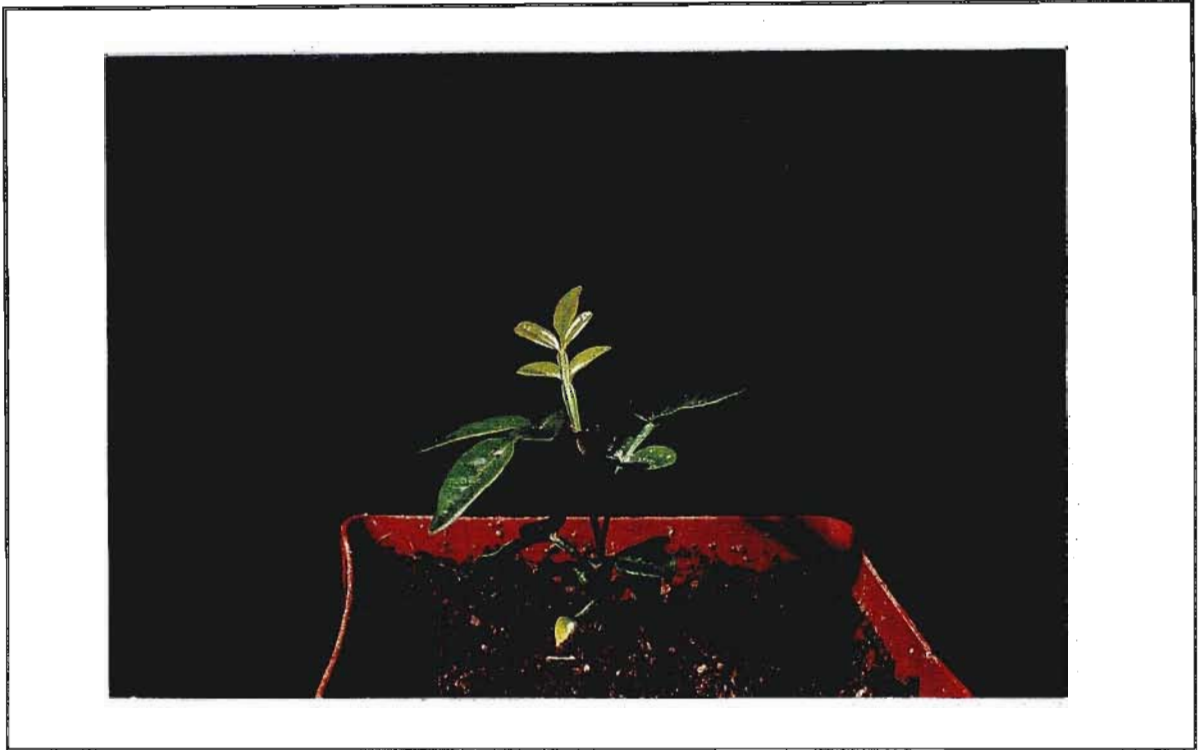
Figure 4.8: Actual size.

Ekebergia capensis 3 month old plant, in potting soil, grown from an axis after fast flash-drying, ultra-rapid cooling, and thawing in the $Ca^{2+}Mg^{2+}$ solution.

The results obtained for axes and seedlings of *E. capensis*, like those for *Q. robur*, indicate that besides optimisation of dehydration and cooling (freezing), the parameters of the thawing step are vitally important in facilitating successful seedling development.

OVERVIEW

From the outcome of the investigations described in preceding chapters, it is clear that embryonic axes of *Q. robur* and *E. capensis* are desiccation-sensitive. Not only are they shed at high water contents, but are actively metabolic throughout development and in the case of *E. capensis*, may be chilling sensitive, but they are also prone to microbial contamination (Berjak, 1996; Berjak *et al.*, 1990; Mycock and Berjak, 1990). These features, in combination, demand that the axes of both species be taken through the procedures of surface-sterilisation, controlled dehydration and cooling (freezing) in order for them to be successfully cryopreserved.

The different surface-sterilants tested, showed that the best, most effective and least injurious procedure to remove the propagules of contaminating microorganisms from the embryonic axes of both *Q. robur* and *E. capensis* was 1% (v/v) sodium hypochlorite, containing a wetting agent (Tween-20). The present investigation on axes of *Q. robur* clearly demonstrated that tissue injury can be caused by over-rigorous, prolonged surface-sterilisation. It was concluded that the damage caused to the root cap and shoot apical meristem at this initial stage of axis manipulation, predisposed the tissues to fatal injury when the axes were frozen and/or thawed. In this regard, the surface-sterilisation procedure of Poulsen (1992) was apparently a major factor in the lack of successful cryopreservation of *Q. robur* embryonic axes.

With respect to optimal culture conditions, *Q. robur* axes germinated, best judged by their performance after 30 d, on a half-strength MS medium supplemented with 0.1 mg l^{-1} (m/v) NAA, 2 mg l^{-1} (m/v) BA and 30 g l^{-1} (m/v) sucrose and solidified with 10 g l^{-1} (m/v) agar. The results for *E. capensis* suggested that quarter-strength MS medium supplemented with 30 g l^{-1} (m/v) sucrose but not containing any hormones and solidified with 10 g l^{-1} (m/v) agar was preferable for the culture of embryonic axes of this species. Comparison of the culture conditions best favouring axis germination for the two species presently investigated, indicates a fundamentally important principle in *in vitro* embryo culture. That is, the axes of different species may be expected to differ markedly in their requirements for good germination in culture. In the present investigation, a 50% reduction of MS salts from the original formulation (Murashige and Skoog, 1962) promoted *Q. robur* axis development, while a further halving of the MS salts was best for those of *E. capensis*. The fact that inclusion of plant growth regulators in the case of *Q. robur* and the exclusion for *E. capensis* afforded a better performance for the two species, respectively, further emphasises the need for a matrix of parameters to be tested for each species being considered for axis cryopreservation.

It was shown that for both of the desiccation-sensitive species, *Q. robur and E. capensis*, axis viability was best conserved if water was removed rapidly. The retention of viability to relatively low water contents upon flash and fast flash-drying of axes from both species reinforces observations~ made for other

recalcitrant or desiccation-sensitive species (Berjak *et al.*, 1990; 1992; 1993; Pammenter *et al.*, 1991; Vertucci *et al.*, 1991; Wesley-Smith *et al.*, 1999). Viability was, however, lost after slower dehydration (via laminar airflow and silica-gel drying) to similar water contents as has been reported by other authors (Berjak *et al.*, 1993; Pammenter *et al.*, 1998).

Additionally, when flash or fast flash-dried, axes from both *Q. robur* and *E. capensis* could tolerate a greater amount of water loss and thus survive to lower water contents than those axes dried using slower drying regimes (laminar airflow and silica-gel). This supports the contention (Pammenter *et al.*, 1998) that flash and fast flash-drying imposes a stasis on the deteriorative biochemical reactions in the cells, enabling axes to maintain viability to relatively low water contents. Pammenter *et al.* (1998) showed that marked intracellular damage occurred at relatively high water contents in axes of *E. capensis* when whole seeds were relatively slowly dehydrated, and that those seeds lost vigour and viability at correspondingly high water contents. Those authors showed, in contrast, that when the whole seeds were rapidly dehydrated, far lower water contents, commisurate with vigour and viability retention, were achieved. That work indicates that irrespective of the presence of the cotyledons, rapid water removal facilitates dehydration to significanty lower axis water contents without serious damage, as in the case for the excised axes presently used. It is not that rapidly-dried axes have become desiccation-tolerant but, as emphasised by Pammenter *et al.*, (1998) and Pammenter and Berjak (1999), that during slow

dehydration, there is adequate time for damage to occur and accumulate as a result of deranged metabolism under conditions of prolonged and increasing water stress.

It is apparently impossible to remove all the water from desiccation-sensitive plant tissues, including those of recalcitrant seeds, or in this case, embryonic axes even when this material is equilibrated to very low relative humidities (Leopold and Vertucci, 1986; Vertucci and Leopold, 1987, Berjak et al., 1990). Berjak et al. (1990) contended that there is a fraction of persistent water that is very strongly membrane bound and which cannot be removed by conventional dehydration. Those authors suggested that this water fraction might stabilise membranes and macromolecules transiently when axes from recalcitrant seeds are very rapidly dehydrated, by flash or fast flash-drying. In fact, Pammenter et al. (1990) went further in contending that a major difference between orthodox (desiccation-telerant) and recalcitrant seeds is that the former are able to lose a considerable proporton of this strongly-bound water without damage, whereas desiccation-sensitive seeds cannot.

Metabolic activity was maintained throughout a 10 week cold-storage of Q. robur seeds, as these acorns were observed to germinate more vigorously and rapidly than fresh, newly-harvested seeds. Ultrastructural studies of the embryonic axes of 10 week cold-stored seeds revealed that in fast flash-dried axes, cellular integrity was well maintained after drying to water contents of 0.37 ± 0.04 g g^{-1} for

Q. robur, as opposed to the situation in slow dried axes from the same stored population where there had been extensive loss of membrane integrity and cellular organisation (Poulsen, 1992; Chmielarz, 1997). It is interesting that axes from the 10 week cold-stored, relatively more mature seeds, provided better starting material for cryopreservation than did those from fresh seeds. This emphasises that comparative developmental status is a further critical parameter for successful cryostorage.

When fast flash-dried axes of both *Q. robur* and *E. capensis* were cooled/frozen at different rates, it was noted that irrespective of optimised pre-treatments, those axes frozen at slower rates died. Survival occurred only after ultra-rapid cooling/ freezing (Wesley-Smith *et al.*, 1999) was implemented. Ultra-rapid cooling/freezing facilitates passage of the axes extremely rapidly through the temperature range at which vitrelline ice forms. In this regard, should there have been freezable water remaining after fast flash-drying (to 0.37 ± 0.04 g g^{-1} and 0.39 ± 0.06 g g^{-1}) for *Q. robur* and *E. capensis*, respectively) then either only minute (non-injurious) ice crystals or vitrelline ice, should have resulted (Wesley-Smith *et al.*, 1999). It can be deduced that this was the case, judging from viability retention of axes of both species, after cryopreservation. It seems possible that, for both *Q. robur* and *E. capensis* axes, some freezable water must have remained within the cells after fast flash-drying. This is suggested to be the basis of viability loss ofter slower cooling/freezing, as a consequence of lethal ice crystal damage, in the present studies.

It is common practice to rapidly warm embryonic axes in distilled water (40°C for a few min) after retrieval from cryostorage (e.g. Wesley-Smith *et al.*, 1999). While rapid warming is held to obviate ice crystal formation, only some root survival was presently achieved, and in all cases, shoots were adversely affected. After rapid warming was carried out in a cation solution ($Ca^{2+}Mg^{2+}$), based on the rationalé of Mycock (1999) in terms of promoting cyto- and nucleoskeleton re-assembly after cryostorage, the roots and some shoots of a significant proportion of axes survived, facilitating production in both species. Another important deficiency of thawing in distilled water was that root geoperception did not occur. This too, was successfully overcome by thawing in the divalent cation solution.

Shoot apices of water-thawed axes became necrotic within 30 d *in vitro*. Ultrastructural analysis of shoot apical meristems of *Q. robur* 6 d after water-thawing, showed symptoms suggestive of spatial disorganisation of both the cytomatrix and the interior of the nucleus. In contrast, after a similar *in vitro* recovery period, shoot apical meristem cells of axes had been thawed in the cation solution were actively mitotic and highly organised. These observations support the contention made that water-thawing results in irreversible disorganisation, whereas provision of $Ca^{2+}Mg^{2+}$ in appropriate concentrations rapidly restores intra-cellular homeostasis. The brief study on leakage thawing indicates that a massive solute efflux from axes in distilled water, whereas those thawed in $Ca^{2+}Mg^{2+}$ leaked far less. While it is tempting to adduce plasmalemma damage (e. g. Simon, 1974) having been counteracted by thawing in the cation

solution. The present study did not reveal ultrastructurally-visibe defects of this vital cell boundary. Presently, therefore, the beneficial effects of the cations are conjectural.

In terms of the promotion of graviperception by $Ca^{2+}Mg^{2+}$ thawing, it is relevant that these conditions facilitated statolith formation in root cap columella cells of axes of both species, while after water thawing, statolith formation was conspicuously absent. It is concluded that calcium (or its loss) is the central issue here: loss of cell homeostasis as a consequence of calcium loss during water-thawing is conjectured to have been counteracted by provision of this cation. Calcium is known to be normally sequestered in amyloplasts (Chandra et al., 1982) and may well be involved in activation of certain enzymes of the starch synthesis pathway (Sturm and Tang, 1999). Absence of this cation by its loss during thawing could have prevented the synthesis of starch grains that are essential features of these specialised amyloplasts called statoliths, in the root cap columella cells of both species. As a consequence, water-thawed axes could not show any graviperception.

As yet, achievement of successful cryopreservation for desiccation-sensitive germplasm is in the experimental stages. The preservation of viability to low water contents by flash or fast flash-drying offers possibilities for the crystorage of such axes, as does careful control of cooling/freezing and warming parameters.

Rapid or ultra-rapid cooling/freezing (theoretically at thousands of degrees sec^{-1}) at relatively high water contents combines the possibility of cryopreservation with the advantage of only moderate dehydration to water contents well above those at which intracellular damage and the necessity of extensive repair, occur. This practice would minimise the *in vitro* germination lag during which recovery occurs. Thus a rapid rate of freezing of embryonic axes of recalcitrant seed species, following partial flash or fast flash-drying of these axes, appears to offer a favourable approach for the long-term storage of such desiccation-sensitive species, by cryopreservation.

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