

THE DEVELOPMENT OF SHORT-TO-MEDIUM AND LONG-TERM
GERMPLASM STORAGE PROTOCOLS
FOR *Eucalyptus* spp.

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, under the supervision of Dr Paula Watt and co-supervision of Dr David Mycock.

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

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ABSTRACT

Eucalyptus trees are a significant source of fuelwood, timber and raw material for the paper and pulp industry. In South Africa, *Eucalyptus grandis* and its hybrids are in high demand due to their fast growth and suitability of their timber for a wide range of products. Breeding and selection for superior quality eucalypts could sustain this high demand through selection and subsequent multiplication of superior genotypes and their use in controlled crosses. However, for this to be successful, a wide gene pool must be available and maintained. Germplasm conservation of both vegetative and sexual material is therefore an integral part of such activities. However, in the case of trees, *in vivo* conservation practices are expensive and hazardous. The aim of this project was therefore to establish alternative conservation strategies for short-to-medium and long-term use of *Eucalyptus* spp. to facilitate on-going breeding and clonal programmes.

For short-to-medium term storage, shoot cultures were subjected to various minimal growth conditions. Of the investigated treatments, reducing nutrients was the best storage method and shoots were maintained for 10 months with multiplication rates of 13.75 ± 7.05 shoots/explant. Encapsulated axillary buds were stored in jars at 10 °C or 28 °C. Of these two treatments, viability was sustained for longer (6 months) at 4 °C.

Before establishing pollen storage regimes, viability assessment methods were evaluated and these consisted of *in vitro* germination on a BK (Brewbaker and Kwack, 1963) medium for 24 hours ($26 \pm 3.0\%$) and staining with two tetrazolium salts. Medium-term storage of pollen was best achieved by maintenance in the fridge (4 °C) without any desiccating substance (8 months at $6.73 \pm 1.21\%$).

Cryopreservation protocols were investigated for axillary buds and pollen. Buds that were 2 mm long were pretreated with chemical cryoprotectants, and a mixture of DMSO (dimethylsulphoxide) and glycerol was found to induce high survival rates (63%) after washing with MS (Murashige and Skoog, 1962) and 4 g.l⁻¹ sucrose solution. Explants precultured in 1M sucrose showed increased tolerance (explant retained high survival

rates of 80%) when desiccated to 20% moisture content fresh weight basis (fwb). Although pretreatments were successfully established, explants did not survive storage in liquid nitrogen indicating the need for further optimization of protocols. Pollen was successfully cryopreserved for 12 months with 23% survival rates.

Applications and future research strategies of the developed protocols to *Eucalyptus* breeding programmes are discussed.

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LIST OF ABBREVIATIONS

%	percent
μ	micro
°C	degrees Celsius
μE.m ² .s ⁻¹	micro Einsteins per metre square per second
ABA	Abscisic acid
ANOVA	One Way Analysis of Variance
BAP	6-Benzylaminopurine
BEN	Benlate®
BK	Brewbaker and Kwack (1963) pollen media formulation
CCC	dimethyl succinamic acid
Ca(NO ₃) ₂ .4H ₂ O	calcium nitrate
CaCl ₂	calcium chloride
CIAT	International Centre of Tropical Agriculture
cm	centimetre
cm ²	square centimetre
DMSO	dimethylsulphoxide
fwb	fresh weight basis
g	grams
h	hours
H ₃ BO ₃	boric acid
ha	hectares
IAA	indole-3-acetic acid
IBA	indole butyric acid
IBA	indole-3-acetic acid
ISTA	International Seed Testing Association
KIN	kinetin
KNO ₃	potassium nitrate
l	litre
m	metre

M	Molar (mole.l ⁻¹)
m ²	square metre
mg	milligrams
MgSO ₄ .7H ₂ O	magnesium sulphate
min	minutes
ml	millilitre
mm	millimetre
mm ²	square millimetre
MS	Murashige and Skoog (1962) nutrient formulation
MTT	3(4-5-dimethylthiazoyl) 2,5-diphenyl tetrazolium bromide
Na ₂ HPO ₄	sodium hydrogen phosphate
NAA	1-naphthylacetic acid
PEG	poly)ethylene glycol
pH	hydrogen ion concentration
PPFD	photosynthetic photon flux density
rpm	revolutions per minute
s	seconds
spp.	species (plural)
TTC	2,3,5-triphenyl tetrazolium chloride
Tween 20	polyoxyethylenesorbite monolaurate
v/v	volume per volume
w/v	weight per volume
ZEA	zeatin

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

1.1.1 The genus *Eucalyptus* and its uses

The genus *Eucalyptus* belongs to the family Myrtaceae. It has an estimated number of species varying from 450 to more than 700 depending on the source of information (Hora, 1979). The genus is subdivided into three subgenera viz. Symphomyrtus, Monocalyptus and Corymbia. *Eucalyptus* derives its name from the characteristic lid or operculum which covers the unopened flowers (Muralidharan and Mascarenhas, 1995). *Eucalyptus grandis* is specifically characterised by a hemispherical operculum, an umbel inflorescence and a peduncle that is distinctly flattened while fruits are capsules between 5-11 mm in diameter. Mature leaves of this species are lanceolate with the adaxial often darker than the abaxial side (Watt, Blakeway and Jain, 1998a).

The natural distribution of *Eucalyptus* is largely in Australia, but eucalypts are widely grown as exotics in most other parts of the world. At least 23 countries have areas of 30 000 ha or more planted with *Eucalyptus* and among those with more than 100 000 ha are South Africa, USA, Brazil and Uruguay (van Wyk, 1990). In those countries where they are grown, *Eucalyptus* trees are a significant source of fuelwood, timber and raw material for the paper and pulp industry (van Wyk, 1990; 1997; Watt, Blakeway, Cresswell and Herman, 1991) and to a lesser extent for honey, tannins and essential oils (Gupta and Mascarenhas, 1987). In South Africa, *E. grandis* is the most commonly grown eucalypt occupying approximately 29% of all commercial forestry land (Anon, 1993). The fast growth and the general suitability of its timber for a wide range of products are the main reasons for the large demand of that species. The already high demand of *E. grandis* is projected to be likely to increase sharply in the next century, thus the need to optimise breeding for improved yields and quality cannot be underestimated. The use of biotechnology and other associated disciplines could be implemented as tools in addressing tree improvement, selection of superior genotypes, mass propagation and germplasm conservation to facilitate production of better quality eucalypts (Watt, Blakeway, Herman and Denison, 1997).

1.1.2 Breeding and propagation methods

Because of their desirable properties for fuelwood, pulp and paper, breeding in eucalypts has attracted world-wide interest. Although genetic improvement programmes in *Eucalyptus* were reportedly still in their infancy in the mid-seventies (Eldridge, 1978), significant progress has so far been achieved, especially in countries like Brazil (Ikemori, Penchel and Bertolucci, 1994). Breeding for genetic improvement can be achieved through the selection of better families and/or individuals and subsequently multiplying those superior genomes for future planting using vegetative propagation techniques. Alternatively, those genotypes with desirable traits can be sexually hybridized through controlled breeding to produce better individuals (Palmberg-Lerche, 1993; Dewald and Mahalovich, 1997). Both vegetative propagation and controlled breeding, therefore, form an integral part of tree improvement and should be combined to form a comprehensive system. However, for the implementation of effective breeding broad genetic variations must exist to allow for selection of 'potentially useful' genotypes. In this regard, integration of genetic conservation strategies into tree breeding programmes is essential so that both immediate and future demands of eucalypt genotypes are addressed (Haines, 1994). In order to have a complete germplasm storage programme that supports tree genetic improvement efforts, two types of storage material must be considered, namely vegetative (shoots) and sexual (pollen). The former is important in clonal breeding while the later is useful in hybridization or controlled breeding.

Sexual recombination/hybridisation is a long and expensive process which requires the identification of superior genotypes and subsequent combination of difficult crosses to produce progeny for the next generation (Burley, 1987). In South Africa, hybrids are becoming increasingly popular especially those of subtropical eucalypts, the most common hybrid combinations being *Eucalyptus grandis* crossed with either *E. camaldulensis*, *E. urophylla* or *E. tereticornis* (Denison and Kietzka, 1993). The wood and growth properties of hybrids is generally intermediate between both parent species, but superior growth to both parents is common (Denison and Kietzka, 1993). Hybridization has therefore greatly enhanced tree planting in South Africa because areas that were traditionally off-site can now be planted with hybrids.

Controlled breeding techniques play a significant role in the production of hybrids of superior genotypes. Apart from hybrid production, several other gains can be derived from those techniques. For example, information on individual and population genetic parameters is enhanced and specific combining abilities can be estimated in addition to the general combining abilities (Eldridge, Davidson, Harwood and van Wyk, 1993). Moreover, as the parentage of each progeny is known, crosses resulting in superior offspring can be repeated with ease (Wilkes, 1983). Furthermore, both open-pollination and/or controlled crossing populations meet several objectives such as maintenance of genetic variability for future generations of selection thus improving the technical qualities of wood material produced.

Having discussed the significance of hybridisation in tree breeding, it must be noted that one of the most important factors that affects successful hybrid seed production is the establishment of sound methods of collecting, storing and testing of pollen. Subsequently, methods of applying pollen to female plants and seed collection are also essential to establish (Kantha, 1985). It is, therefore, important to stress the significance of pollen storage for any conservation strategy, especially if it is with the view of enhancing tree improvement programmes.

Traditional and recent techniques in vegetative propagation have greatly facilitated the work of plant breeders (Namkoog, 1989; Miller, 1993). In some cases, vegetative propagation is, in addition to being a tool for tree breeding, a means of multiplying for the direct planting of high genetic quality material (Ahuja, 1993). In any germplasm collection or restoration program, replication is an important insurance against loss (Haines, 1994). The use of clonal or vegetative propagation, therefore, offers the ability to preserve and replicate exact genotypes without sexual recombination (Namkoog, 1986; 1989). As a consequence, this gives greater control over genetic diversity, which is a critical tool for germplasm conservation. In addition to capturing otherwise unavailable genotypes, clonal propagation affords a security measure for conservation. Vegetative propagation is also valuable for the international distribution and use of conserved germplasm. Similarly it

offers an alternative method of conservation in cases where seeds may not be physically available, are difficult to store or, for various reasons, where they may be unavailable at the time of collection (Hamilton, 1994). Moreover, clonal propagation is especially valued when the genetic integrity of seeds is suspect.

Despite the discussed advantages of clonal propagation in tree breeding, vegetative/clonal propagation should be handled with care particularly in cases where genetic diversity is to be conserved (Hammatt, 1992). With the ability to replicate exact genotypes comes the potential to reduce species diversity, due to the planting of many copies of one or a few genotypes (Miller, 1993; Palmberg-Lerch, 1993). However, if such problems are avoided, clonal propagation plays an important role in germplasm conservation and should therefore be part of all on-going tree breeding programmes.

Several conventional methods of cloning *Eucalyptus* trees are available. However, due to the rising demand of high quality wood, there is need to incorporate both conventional and modern methods of propagation (Campinhos and Ikemori, 1977; Delwaulle, 1985; Zobel, 1993). Conventionally, most forest trees can be propagated by leaving a few good quality trees to re-seed a cleared area after felling (McComb and Bennett, 1986). Despite the fact that *E. grandis* and many other *Eucalyptus* spp. flower abundantly and seeds can be stored satisfactorily, propagation by seeding is not ideal where rapid propagation is required. This is due to difficulties associated with the relatively long life cycles of the species before maturity (Ahuja, 1993). Moreover, seeds give rise to new progeny that are different from parents and thus the genetic traits of interest may be lost (McComb and Bennett, 1986).

Seeds:

Grafting is an alternative method of propagation possible for many important timber trees such as *Eucalyptus* (McComb and Bennett, 1986). However, care must be taken to select the appropriate kind of graft and the condition of the stock (Hartney, 1980). Due to the possibility of incompatibilities that may occur, it is also important to use a seedling of the same species if not of the same tree as stock material. Grafting is not always recommended for propagation because of the associated expenses (McComb and Bennett, 1986).

Vegetative propagation through rooted cuttings has been successfully used for a number of eucalypts (McComb and Bennett, 1986; Zobel, 1993; Campinhos and Ikemori, 1995). However, for many species, this approach is not efficient to produce enough plants for progeny testing as only a limited number of shoots can be produced from each plant and rooting is inadequate (McComb and Bennett, 1986; von Arnold, Clapman and Ekberg, 1990). Moreover, there is no standard universal method of selecting cuttings for individual species or even clones, thus making this route of propagation highly inefficient. ~~_____~~

Due to the various problems presented by conventional vegetative propagation techniques, micropropagation may be used as an alternative to conventional methods. Micropropagation is a process that involves the multiplication and maintenance of selected genotypes *in vitro* under sterile conditions. It has advantages over conventional propagation techniques in that it is often much more rapid and it may be used in cases where traditional methods are difficult or impossible (George, 1993; Watt *et al.*, 1998a). Micropropagation of trees has the highest multiplication rate estimated to be 100 000 plants per year from a single bud in eucalypts (Gupta, Mascarenhas and Jagannathan, 1981). *In vitro* propagation of *E. grandis* has been discussed in several articles (McComb and Bennett, 1986; Lakshmi-Sita and Rani, 1985; Muralidharan and Mascarenhas, 1987; Le Roux and van Staden, 1991; Watt *et al.*, 1991; Watt, Duncan, Ing, Blakeway and Hermant, 1995). Routes of propagation in that species include direct organ culture from seedling and embryonic material (Cresswell and De Fossard, 1974; Cresswell and Nitsch, 1975; Warrag, Ortega, Lesney and Rockwood, 1987; MacRae, 1991; Watt, Gauntlett and Blakeway, 1996) and shoot induction from nodal meristem (axillary buds) (De Fossard, Barker and Bourne, 1977; Durand-Cresswell and Nitsch, 1977; Gupta *et al.*, 1981; Furze and Cresswell, 1985; Warrag, Lesney and Rockwood, 1990). The production of adventitious shoots via callus has also been documented for *E. camaldulensis* (Muralidharan and Mascarenhas, 1989) and *E. citriodora* (Lakshmi-Sita, 1979), amongst others. Of these routes of propagation, micropropagation via axillary bud production is most useful regarding germplasm preservation as it ensures that the characteristics of the source plant are conserved and thus there is a strong likelihood of producing physiologically uniform and genetically stable plants. Propagation via axillary buds has been reviewed in several papers (Durand-Cresswell, Boulay and Franclet, 1982;

McComb and Bennett, 1986; Le Roux and Van Staden, 1991).

1.2 Conventional methods of genetic conservation

While it has so far been mentioned that novel techniques of tree manipulations and breeding will meet many future needs for specific applications in many *Eucalyptus* breeding programmes, the reality of the situation is that such techniques are only likely to modify and move genes rather than create them (Kantha, 1985; Withers, 1986). Even if it should become possible to design genes *de novo*, it would be absurd to disregard those of potential value already existing in the natural genepool. The best insurance, therefore, lies in conserving maximum diversity of what is available to facilitate on-going research aimed at meeting future timber requirements.

Two methods of conserving germplasm have been identified, *viz.*, *in situ* and *ex situ* (Wilkins, Bengochea and Dodds, 1982; Withers, 1988; Krogstrup, Baldursson and Norg, 1992). Depending on the objective, *ex situ* germplasm acquisitions can either be active or base collections. An active collection requires methods of storage that retain the viability of samples for short-to-medium periods while base collections are intended for long-term conservation of germplasm (Withers, 1988; Ford-Lloyd and Jackson, 1990). Applications and limitations of each of these storage techniques are discussed in sections 2.1.4 and 3.1.4, respectively. However, it should be noted that the two systems complement each other and hence should operate in parallel. For instance, base collections are intended for retaining viability over long periods and thus serving as back ups for active collections.

Conventionally, active collections are kept as field or greenhouse collections where they are readily available for distribution, evaluation and documentation (Wilkins *et al.*, 1982; Krogstrup *et al.*, 1992). However, maintaining active collections in the field or greenhouse is labour intensive and expensive (Withers, 1988; George, 1993). Moreover, the requirement of technical personnel is very high. This type of storage is also highly susceptible to environmental changes such as climate, pests and pathogens, making it extremely risky for conservation (Wilkins *et al.*, 1982; Blakesley, Monzrooei and Henshaw, 1995; Ashmore, 1997; Engelmann, 1997). As a result, plantation managers are

faced with problems of monitoring impacts presented to plantation stands by soil, climate, weed cover, pests and diseases (Atkinson, 1997). For forestry, loss of any conservation field collection would be catastrophic as such collections may constitute selection material that is counted in thousands of years (Anon., 1996).

The other problem facing the South African Forest Industry, and other forestry-based industries world-wide, regarding field collections is the decrease of land available for plantations (Denison and Quale, 1987; van Wyk, 1990; Watt *et al.*, 1997). This is due to pressures on land being impacted by such activities as population and agricultural growth, as well as the rising concerns for preservation of indigenous flora. Due to the large gene pool of forest trees, a number of individual genotypes are necessary in order to conserve the genetic variations within the population (DeWald and Mahalovich, 1997). This results in the use of large amounts of land, putting a further constraint on land. *Ex situ* methods of conservation are therefore highly impractical for conservation of most plantation species including eucalypts. An alternative method that is theoretically risk-proof and uses minimal amount of space is therefore a necessity in germplasm storage of forest species.

Base collections of genetic material have traditionally been maintained using seed storage techniques (Kantha, 1985; Krogstrup *et al.*, 1992). Seed viability can be maintained for long periods by storing seeds in sealed containers at -18 °C or less and at a moisture content of between 5 and 7% (Roberts, 1973; 1975). Seed storage might appear an ideal method of long-term germplasm conservation as it is cheap and requires low-tech equipment. However, this may not be true for many forest species (Ford-Lloyd and Jackson, 1990). Although most *Eucalyptus* species produce orthodox seeds, the seeds exhibit such a high degree of heterozygosity that they are of limited interest (McComb and Bennett, 1986). Moreover, the technical difficulties related to obtaining hybrid seed present a problem with seed storage as a means of germplasm conservation (Denison and Kietzka, 1993). Another problem mentioned earlier is that some *Eucalyptus* spp. such as *E. dunnii* produce seeds inconsistently and only periodically due to poor flowering. Conventional seed storage techniques are also generally not possible for hybrids because

many of them are sterile or have limited fertility (Krogstrup *et al.*, 1992; Withers and Engelmann, 1996). Although numerous efforts have been made to maximise the quality of conservation offered by field genebanks, it is obvious that alternative methods and approaches to genetic conservation are needed.

1.3 *In vitro* methods of genetic conservation

As has been mentioned above, because of the hazards associated with field-banks *in vitro* storage is most commonly applied to vegetatively propagated species as it offers greater security to germplasm collections. This is a great advantage especially in *Eucalyptus* where vegetative propagation is such an integral part of the tree production system.

During the past 30 years, *in vitro* culture storage techniques have been extensively developed and applied to more than 1000 species (Benson, 1995; Bull, 1996; George, 1996). Different *in vitro* conservation methods are employed depending on the storage duration required (Engelmann, 1996). Germplasm preservation *in vitro* may thus be divided into two categories: short-to-medium term (minimal growth storage) as active collections, and long-term preservation (cryopreservation) as base collections (Wilkins *et al.*, 1982; Wilkins and Dodds, 1983; Engelmann, 1996; Ashmore, 1997). The objectives of short-to-medium term storage are to reduce growth thereby increasing intervals between subcultures (Blakesley, Pask, Henshaw and Fay, 1996). Long-term storage, on the other hand, involves storage at an ultra-low temperature, usually that of liquid nitrogen [-196 °C] (Kantha, 1985). Cryopreservation is currently the only method available for long-term preservation of germplasm *in vitro*. A more detailed discussion of these methods follows in subsequent chapters.

1.4 Aims of the investigation

Earlier discussions in this chapter have brought to attention the fact that the demand for good quality wood in the world is increasing rapidly. It is also evident that improved methods of breeding and production of superior quality wood would have to be undertaken in order to meet this high demand. However, for this to occur, a wide genepool has to be available from which superior genotypes could be selected and subsequently propagated or

used in hybridisation. Traditional methods of storing propagated material are often prone to environmental and other hazards, thus the need for new storage methods that can provide a risk-proof germplasm storage is paramount. This study therefore undertook to investigate and develop germplasm storage protocols to complement on-going *Eucalyptus* sp. breeding and production programmes, particularly those at Mondi Forests. Two approaches were taken: firstly, the development of short-to-medium term storage protocols of shoot cultures, axillary buds and pollen (Chapter 2) for direct use in clonal and breeding programmes, respectively and secondly, development of long-term storage protocols through cryopreservation techniques for both pollen and axillary buds (Chapter 3), as a back-up storage for long-term conservation of important and/or 'potentially useful' genotypes.

CHAPTER 2: ESTABLISHMENT OF SHORT-TO-MEDIUM-TERM STORAGE TECHNIQUES OF *Eucalyptus* spp. MATERIAL: *In Vitro* SHOOT CULTURES, ENCAPSULATED BUDS AND POLLEN

2.1 Introduction and Literature review

2.1.1 General aspects of *in vitro* tissue and cell culture

2.1.1.1 Types of cultures

Plant tissue culture has been described as the 'art' of growing plant cells, tissues or organs from the mother plant on an artificial medium (George, 1996). This technique is made possible by the unique ability of cells to grow, divide and regenerate into an entire organism (totipotency) (Lowe, Davey and Power, 1996). According to George (1996), tissue culture strictly refers to cultures of unorganised aggregates of cells, although it is now more commonly used to describe all kinds of *in vitro* plant cultures.

Tissue cultures comprise two types of growth, organised and unorganised. The following types of cultures are recognised as exhibiting unorganised growth: callus, cell suspension, protoplast and anther/ovule cultures. Callus cultures consist of a mass of undifferentiated cells (Duncan and Widholm, 1986; Collin and Dix, 1990; Allan, 1991), which are formed by aseptically transferring a sterile explant onto a nutrient medium supplemented with plant growth regulators. A cell suspension culture comprises a population of isolated plant cells and small clumps of cells dispersed in an agitated liquid medium (Allan, 1991; Collin and Dix, 1990), whereas protoplast cultures consist of isolated cells without a cell wall (Scarpa, Pupilli, Damiani and Arcioni, 1993). Anther and ovule cultures consist of immature pollen microspores and ovules respectively (George, 1996).

Organ culture generally refers to aseptic isolation of whole plant parts with a definite structure, for example, meristem, shoot tip or shoot cultures, node, embryo and isolated root to form cultures (Collin and Dix, 1990; Allan, 1991). The growing points of shoots may be cultured in such a way that they continue uninterrupted growth giving rise to small organised shoots which can be rooted to form plantlets (Duncan and Widholm,

1986; Collin and Dix, 1990). Meristem, shoot tip and node cultures are essentially similar, the only variation being the size and position of explant on the mother plant. Culturing of the extreme tip of the shoot gives rise to meristem culture, the larger shoot apices lead to the formation of shoot cultures while node cultures are merely an adaptation of shoot cultures (George, 1996). Embryos dissected from seeds when cultured *in vitro* to form seedlings are referred to as embryo cultures, while in order to obtain root cultures, isolated roots may be induced to grow and form branched roots. In relation to germplasm conservation, organised cultures have an advantage over unorganised ones because of the reduced possibility of somaclonal variation occurrence during culture. Somaclonal variation arises from genetic instabilities as a result of callus mediated pathways, that is, from unorganised growth (Dodds, 1991). Although somaclonal variation may be an advantage as a new source of genetic variability for plant improvement, it presents a hazard where clonal fidelity and conservation are required. Organised cultures were thus of more interest in the present study.

2.1.1.2 The explant and its preparation

In vitro systems are initiated from pieces of whole plants called explants. The plant part from which explants are obtained determines the type of culture to be initiated which, in turn, has influence on the purpose of the proposed culture (Warren, 1991). Successful culture initiation of the explant depends on a number of factors such as the absence of microbial contaminants, composition of the medium on which it is cultured and its environment.

Since plants growing in the external environment are invariably contaminated with microorganisms, sterilisation of explants prior to culturing is of absolute necessity. Surface sterilants such as sodium hypochlorite are usually sufficient to eliminate superficial contaminants (Warren, 1991). De Fossard *et al.* (1977) suggested that pre-treatment with fungicide, bactericide and insecticide prior to explant selection for *in vitro* culture would possibly contribute to greater success in eliminating respective contaminations in culture. To circumvent surface contamination, an alternative route would be obtaining explants from *in vitro* grown cultures. However, it is becoming

increasingly obvious that even *in vitro* cultures are rarely ever totally aseptic (Herman, 1996). *In vitro* cultured plant tissue may only temporarily suppress the growth of microbial contaminants, which might become evident after several subcultures. Because *in vitro* grown cultures are administered under sterile conditions, the risk of contaminated explants is still much less than those obtained from the external environment. Further, as explants are grown on nutritive media which are also favourable for the growth of microorganisms, strict maintenance of sterile conditions while initiating cultures *in vitro* must be exercised. This includes manipulations such as autoclaving media and utensils, as well as performing culture techniques in a laminar flow hood (De Fossard *et al.*, 1977; Gupta *et al.*, 1981; Dodds and Roberts, 1985; Le Roux and Van Staden, 1991; Warren, 1991; Gamborg and Phillips, 1995).

2.1.1.3 Routes of regeneration

On condition that the factors affecting explant development *in vitro* are satisfactory, explant regeneration can occur in one of two ways, organ development (organogenesis) or through the formation of somatic embryos (somatic embryogenesis). Both routes can occur either indirectly, via a callus stage or directly from the explant (Fig 2.1). Phillips and Hubstenberger (1995) describe plant propagation in the form of axillary bud development as one of the simplest types of propagation *in vitro*, as it follows a direct route of plantlet regeneration. Axillary buds are treated with hormones to break dormancy and produce shoot branches. The shoots are then elongated, separated and rooted to produce plants. This method of propagation has been found to be particularly suitable for commercial propagation of woody plant species (Mantell, Matthews and McKee, 1985; Pierik, 1987; Chu, 1992) and is the procedure used routinely in the commercial laboratory of Mondi Forests. Axillary bud development and propagation were of particular interest in this study for reasons already discussed (section 1.1.2).

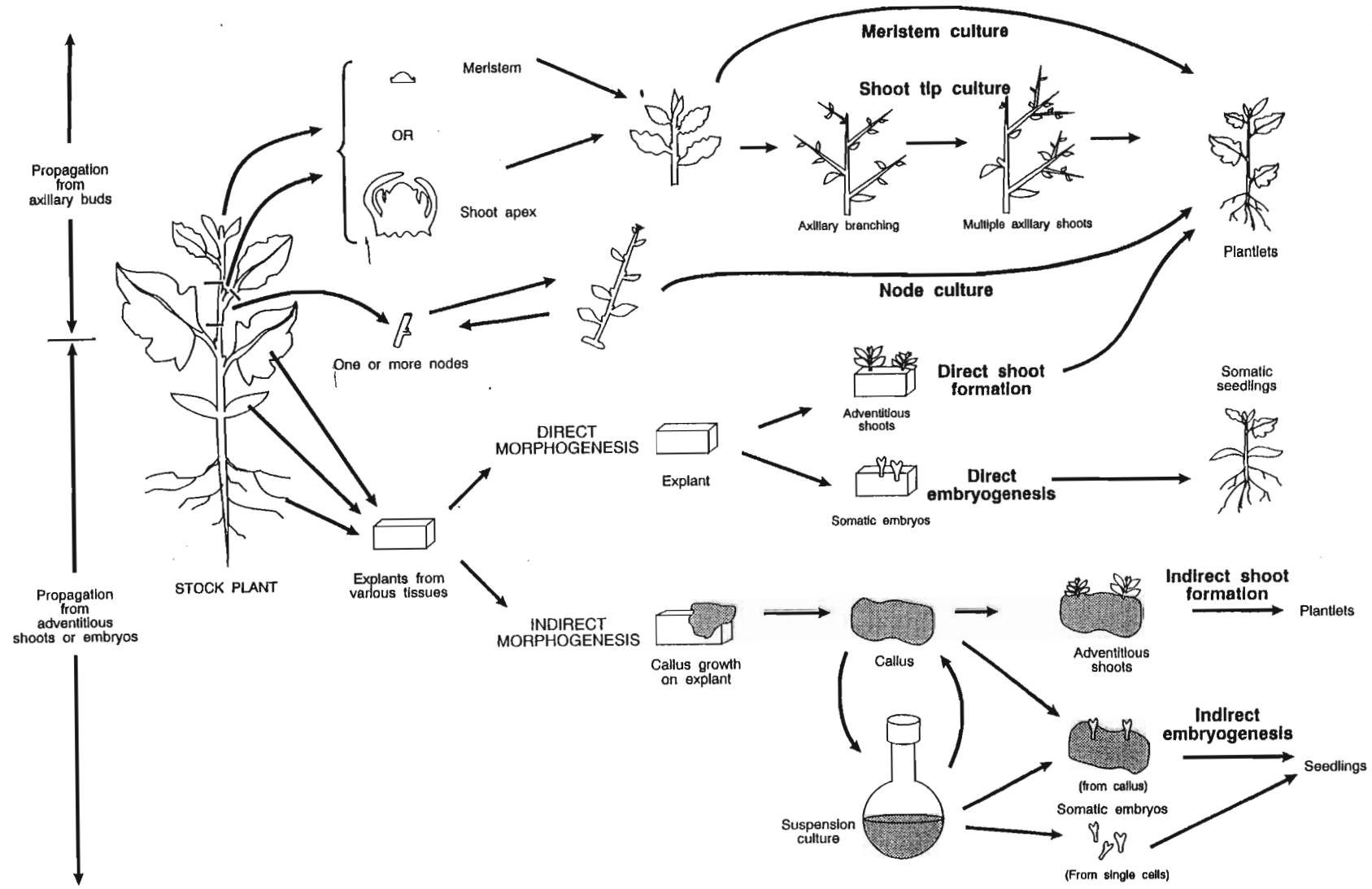


Fig 2.1 The principal routes of plant propagation (George, 1996).

2.1.1.4 Factors that affect *in vitro* explant development

An explant will grow *in vitro* when provided with a special medium formulation (George, 1996). Plant cell culture media usually consist of a solution of salts supplying all the major and minor elements necessary for plant growth, various vitamins, amino acids, a carbon source and appropriate plant growth regulators. Several media formulations are available, some of the common ones being Murashige and Skoog (MS) (Murashige and Skoog, 1962), Gamborg's B5 (Gamborg, Miller and Ojima, 1968) and White's media (White, 1943).

Since most culture systems are heterotrophic, carbon is necessary for plants to develop *in vitro* (Herman, 1996). A more commonly used source of carbon is sucrose but the type of carbon used may vary, depending on the species and purpose of experiment. Sugar alcohols such as sorbitol and mannitol have been used extensively as growth retardants (Zandvoort, Hulshof and Staritsky, 1994; Grout, 1995), while glucose and other sugars have been used to supplement sucrose (Atanassov and Brown, 1984; Sundberg and Glimelius, 1986; Watt *et al.*, 1991).

Plant growth regulators may not necessarily be nutritive but they play an important role in the induction and control of morphogenesis (Ammirato, 1986; George, 1996). The more commonly used plant growth regulators in tissue culture include auxins [indole acetic acid (IAA) and naphthylacetic acid (NAA)] and cytokinins [benzyl aminopurine (BAP), zeatin (ZEA) and kinetin (KIN)] (Thorpe, 1983; Ammirato, 1986; Minocha, 1987; Reynolds, 1987). Gibberellins, ethylene and abscisic acid (ABA) are also used for specific responses. Abscisic acid is often regarded as being a plant growth inhibitor, partly because it can control bud and seed dormancy, but more importantly because it inhibits the auxin-promoted cell wall acidification and loosening, which permits cell elongation (Ammirato, 1983; Warren, 1991; George, 1996). Because of its ability to retard growth, ABA has played an important role towards short-to-medium-term storage of plant material as discussed (sections 2.1.2.2c, 2.3.1.4).

Browning of culture media, accompanied by death of explants, has been a particularly

serious problem in shoot cultures of woody plants including those of *Eucalyptus* spp. (MacRae and van Staden, 1990; Warrag *et al.*, 1990; Herman, 1995). This phenomenon is believed to be due to oxidation of phenolic compounds released from the cut ends of explants, resulting in phytotoxic products. Consequently, antioxidants have been used frequently to prevent browning in the initial stages of *in vitro* culture (De Fossard, 1974; Furze and Cresswell, 1985; Tulecke, 1987). The use of antioxidants such as polyvinylpyrrolidone (Tulecke, 1987; Warren, 1991), ascorbic (Grewal, Ahuja and Atal, 1980; George, 1993) citric and boric acid (Creemers-Molenaar and van Oort, 1990; Watt *et al.*, 1991) has led to successful prevention of the problem.

Besides medium influence, factors such as atmospheric gaseous composition, light and temperature also play a significant role on *in vitro* development and morphogenesis (Thorpe, 1980; Hughes, 1981; Ammirato, 1986). Plant growth and development are dependent on respiration, which in turn is dependent on the availability of oxygen. Increased supply of oxygen was found to increase the rate of respiration and growth in callus cultures of potato (Forward, 1965; van der Plas and Wagner, 1986). Conversely, the rate of plant growth can be diminished by limiting oxygen supply (Kozai, 1991; de Goes, 1993).

The growth and development of plants is dependent on light for photosynthesis, photomorphogenesis and phototropism (George, 1996). To induce any of those processes, not only is the presence or absence of light important but also the photoperiod, intensity, quality and wavelength (Ammirato, 1986; Kozai, 1991). Although the light requirements necessary for photomorphogenesis and phototropism may be ignored (Thorpe, 1980; George, 1993), photosynthetic light is essential to maintain plant growth.

Temperature effects have not been thoroughly evaluated in tissue culture. However, the general practice has been to maintain cultures at a constant temperature of approximately 25 °C (Thorpe, 1980). Although it has been observed that the optimum temperature for growth varies from species to species, it has also been noted that, generally, growth will decline as temperatures are reduced from the optimum (Grout, 1995; George, 1996).

2.1.1.5 Acclimatisation and hardening-off

Acclimatisation, acclimation and 'hardening-off' are often used synonymously for the final process of transferring plantlets from aseptic culture environment to free-living environment such as the greenhouse and ultimately to the field. In this study, acclimatisation is used to denote hardening-off, while acclimation is used in conjunction with pretreatment of explants before freezing (sections 3.1.2.2).

The clonal propagation of plant species may be achieved by explant establishment, multiplication and development followed by acclimatisation and eventually establishment of plants in the field (Murashige, 1974). Prior to hardening-off, plantlets rooted *in vitro* should have well-proportioned shoots and roots that are capable of supporting each other (Sommer and Caldas, 1984). In the laboratory, acclimatisation is usually achieved by covering potted-out plants with plastic bags and either removing bags for progressively longer periods or by punching an increasing number of holes on the bags (Warren, 1991). Alternatively, mist houses can be used, especially for acclimatisation on a commercial scale. It is important to understand that the ultimate success of any form of plant regeneration *in vitro* lies in the ability to transfer plants out of culture with high survival rates and good performance (Bhojwani and Dhawan, 1989; Bhojwani, 1990).

2.1.2 Techniques for minimal-growth storage of shoot cultures

The term 'minimal-growth' is used here to refer to 'growth inhibition', 'limitation', and/or other similar terms which imply modification of the culture conditions resulting in 'slow growth' of material *in vitro* (Withers, 1987). Minimal growth storage is the most direct way of restricting growth and development of *in vitro* materials and is applied to differentiated plantlets and developing meristem cultures (Grout, 1995). Provided that due precautions are taken, this kind of storage can be used to preserve genetic combinations of plants propagated by meristem or shoot cultures, without change, over a long period (1-8 years) (Henshaw and Blakesley, 1996; Taylor, Pone and Palupe, 1996). Minimal-growth techniques have a distinct advantage over normal culture conditions as they avoid the high costs and labour intensive procedures that arise as a result of frequent subculturing (George, 1993).

The concept of conserving valuable genotypes *in vitro* was first propounded in the mid 1970s (Henshaw, 1975; Morel, 1975), by which time some limited success had been achieved in the storage of cultures using slow-growth techniques (e.g. Galzy, 1969) or freezing (e.g. Nag and Street, 1973). In the subsequent decades, techniques of *in vitro* storage were applied with varying degrees of success to a wide range of species and culture systems (Withers, 1986). Although species vary in the length of time during which their cultures can be stored, periods between 6 and 96 months have been cited in the literature (Table 2.1) (George, 1993). The type of explant used also contributes towards storage time (Kantha, 1985; Withers, 1986; Grout, 1995). Single shoots derived from meristematic tissue have been found to be most suitable for storage due to the genetic stability of the tissue from which they are obtained (Henshaw, 1975; Kantha, 1985; Blakesley *et al.*, 1996). Unrooted shoot clusters, somatic embryos or rooted plantlets have also been found to be suitable for storage, with plantlets or shoots frequently being the most successful material in this regard (George, 1993). There are various ways in which the life span of cultured tissue or organs have been extended, such methods may be categorised into alteration of the physical or the chemical environment of cultures as per discussions that follow.

2.1.2.1 Alteration of the physical environment of cultures

a) Light and temperature

The simplest and most obvious way of influencing the rate of *in vitro* plant growth is by reducing temperature and light from optimum conditions for active growth (Grout, 1995; Kozai, Kubato and Jeong, 1997). Although intervals between transfers can often be greatly reduced by keeping cultures in weak light (or in the dark) and low temperatures, suitable temperatures for storage vary significantly with species, variety, and often with specific lines of plants (Kantha, 1985).

Table 2.1 Examples of species which have been stored as *in vitro* shoot cultures or plantlets by incubation under different minimal-growth conditions

Species	Storage conditions	Time (months)	Reference(s)
<i>Actinidae chinensis</i>	8 °C	12	Monette (1986)
<i>Allium sativum</i>	100 g.l ⁻¹ sucrose	10	El-Gizawy and Ford-Lloyd (1986)
<i>Caraway</i> sp.	0.5 °C	20	Ammirato (1974)
<i>Centarium rigualii</i>	5 °C, IBA	36	Iriondo and Perez (1996)
<i>Coffea arabica</i>	16 °C	30	Kartha (1981) Dussert, Chabrilange, Anthony, Engelmann, Recalt and Hamon (1997)
	16 °C	36	
<i>Colocasia esculanta</i>	9 °C, total darkness	96	Bessembinder, Staritsky and Zandvoort. (1993)
<i>Dioscorea rotunda</i>	16 - 22 °C	12	Ng and Hann (1985)
<i>Eucalyptus citriodera</i>	Reduced nutrients	36	Mandal and Chandel (1996) Mascarenhas and Agrawal (1991)
	10 °C	10	
<i>Fragaria</i> sp.	4 °C	72	Vysotskaya (1994) Reed (1991)
	1 °C	21	
<i>Ipomea</i> sp.	1/2 MS, no sucrose	3	Kartha, Gamborg, Shyluk and Constabel (1976)
<i>Lilium</i> sp.	-2 °C	60	Bonnier, Hoekstra, Vos and Vantuyl (1997)
<i>Malus domestica</i>	BAP	12	Lundergen and Janick (1979)
<i>Miscanthus ogiformis</i>	8 °C	7	Hansen and Kristiansen (1997)
<i>Musa</i> sp.	ABA	9	Van de houwe, De Smet, du Montcel and Swennen (1995)
<i>Panax ginseng</i>	reduction of O ₂ partial pressure	6	Mannonen, Toivonene and Kauppinen (1990)
<i>Populus</i> sp.	4 °C	60	Son, Chun and Hall (1991); Hausman, Neys, Kevers and Gasper (1994)
		8	
<i>Prunus</i> sp.	-3 °C	8	Marino, Rosati and Sagratti (1985)
<i>Pyrus</i> sp.	8 °C, ABA and ABA	12	Moriguchi (1995)
<i>Solanum</i> sp.	6 - 12 °C mannitol	24	Wescott (1981a) Mandal and Chandel (1996)
		36	
<i>Trifolium repens</i>	5 °C	10	Bhojwani (1981)
<i>Vitis vinifera</i>	Oxygen deprivation MS no hormones	12	Moriguchi and Yamada (1989) Ciccotti and Malossini (1997)
		6	
<i>Xanthosoma</i> spp.	Mannitol	36	Zandvoort <i>et al.</i> (1994)

Storage temperature is chosen with regard to optimal growth temperature and susceptibility to chilling injuries of the species concerned (Blakesley *et al.*, 1996). Typically, shoots and plantlets of species that are maintained at 20 - 28 °C *in vitro* were reported to store successfully at temperatures between 0 and 10 °C (Son *et al.*, 1991; Hausman *et al.*, 1994; Blakesley *et al.*, 1996; Bonnier *et al.*, 1997). The tips of apple shoots, for instance, were stored at 1 °C and 4 °C for one year in the dark and gave good rates of shoot proliferation when returned to 26 °C (Mullin and Schlegel, 1976). Bessembinder and colleagues (1993) reported that, at 9 °C in total darkness, cultures of *Colocasia esculenta* (taro) could be stored for more than 8 years with transfers at 3 year intervals. Temperatures from 0-5 °C have since been found by most researchers to be suitable for conserving shoot tips of woody species (Lundergen and Janick, 1979; Chun and Hall, 1986; Aitken-Christie and Singh, 1987; Hausman *et al.*, 1994). For species normally maintained at higher temperatures and those that are chilling-sensitive (Staritsky, 1986; Withers, 1986; Mascarenhas and Agrawal, 1991; Ng and Ng, 1991), a more elevated storage temperature appears to be appropriate. Successful storage of *Dioscorea rotundata* (Ng and Hann, 1985) and that of *Musa* spp. (Van de houwe *et al.*, 1995) has been achieved at temperatures between 16 and 22 °C, for longer than 12 months and 11 months, respectively (Table 2.1).

b) Oxygen

Placing cultures in an environment with a low partial pressure of oxygen seems to have potential for limiting *in vitro* growth (George, 1993). Caplin (1959) used mineral oil overlay to conserve callus cultures and attributed the observed decrease in growth rates to lack of oxygen. However, the contaminating and toxic effects of oils have been found to be disadvantageous and, as a result, Moriguchi, Kozaki, Matsuta and Yamada (1988) suggested that autoclaved silica gel (which dissolves ten times the amount of oxygen that is solubilised in water) may be more effective in regulating oxygen supply. Those authors found that *Vitis vinifera* callus cultures, which had been stored at 10 and 15 °C covered with silica gel to a depth of 90 mm, were still embryogenic after one year. Other published methods of reducing oxygen supply include a direct reduction of oxygen partial pressure surrounding the tissue or replacing oxygen with an inert gas (Bridgen and Staby,

1981; 1983). Example of studies where reduction of oxygen partial pressure was used to minimise growth in culture are that of *Panax ginseng* callus (Mannonen *et al.*, 1990) and shoot cultures of *Spiraea nipponica* (Norton and Norton, 1988).

A simple way of reducing the supply of oxygen reaching plant tissues and organs is by placing them in a long narrow vessel in which the distance between cultures and the point of gaseous exchange with the external atmosphere is maximum (Grout, 1991). As the diffusion distance is increased, the rate of growth decreases and this allows for the frequency of subculturing to be reduced.

2.1.2.2 Alteration of chemical environment of cultures

a) Culture medium and its constituents

Cultures can be left untouched on the shelf of the growth room, leading to a storage condition known as “laissez-faire” (George, 1993). As the medium is depleted, shoot growth slows until eventually shoots become desiccated. In this way, shoots of some species remained viable without subculturing for up to 12 months (Redenbaugh, Fujii and Slade, 1991). However, ‘spent’ medium may contain toxins or be at unfavourable pH, which may negatively affect shoot growth. Alternatively, plant material can be transferred onto fresh, but a lesser nutrient or sucrose concentration to reduce their growth rate. This approach was employed by Kartha, Mroginski, Pahl and Leung (1981a; 1981b) who stored coffee shoot cultures effectively for 2 years at 26 °C, by keeping them on a medium with either low sucrose concentrations or no sucrose. Similarly, *Vitis ripensis* plantlets stored for 11 months survived better in 3 g.l⁻¹ than 15 g.l⁻¹ (Galzy and Compan, 1988). Decreasing the supply of inorganic nutrients may also result in restricted growth. Schnapp and Preece (1986) showed that tomato shoot growth was reduced significantly (four times) when shoots cultured on a 3/4 strength MS nutrient medium containing 5% (w/v) sucrose compared with those grown on full strength MS nutrients (See Table 2.1).

b) Osmotic regulators

According to George (1993), changing the osmotic potential of cells in culture results in reduced growth rates and consequently less subculture manipulations. Negative osmotic potential is the result of increasing the concentration of most sugars above normal. At such high concentrations, sugars begin to have an inhibitory but non-toxic effect on plant cell growth. Osmotic potential regulators (often referred to as osmoregulators in this study) used for minimal-growth storage include mannitol, sorbitol and dimethyl succinamic acid (CCC). For example, CCC has been successfully used to reduce growth of *Solanum* spp. cultures (Wescott, 1981b; Withers, 1986; Ng and Ng, 1991) and 3% (w/v) mannitol on *Xanthosoma* spp. (Zandvoort *et al.*, 1994). More examples are listed in Table 2.1

c) Plant growth regulators

Growth regulators are normally added to culture media to promote and regulate plant growth *in vitro* (Grout, 1995; George, 1993; 1996). They may also help to slow down growth of *in vitro* cultures by acting directly to induce organ dormancy, reduce cellular metabolism, or prevent cell nuclear division. For example, Ammirato (1974) used ABA, which induces dormancy in plant meristems, to arrest growth of late stage somatic embryos in *Carum* sp (caraway). Similarly, Jarret and Gawel (1991) used 10 mg.l⁻¹ ABA to reduce growth in sweet potato. Withdrawal of these chemicals can also assist in retarding growth for *in vitro* storage purposes (Taylor *et al.*, 1996). Strawberry cultures remained viable for over five months when stored on a medium without growth regulators (Reed, 1991).

2.1.2.3 Treatments in combination

Whilst little systematic work has been published, there is evidence supported by common sense, suggesting that combinations of the treatments outlined above may be of benefit in the implementation of minimal-growth strategies. For example, reduced temperature in combination with ABA (5 - 10 mg.l⁻¹), high sucrose (up to 8% [w/v]) and mannitol (between 3 - 6% [w/v]) enhanced survival of potato shoots by 57 months (Henshaw, Stamp and Wescott 1980; Wescott, 1981b). Reduced temperature combined with low

sucrose concentration were found to enable storage of *Coffea* spp. for up to three years (Bertrand-Desbrunais, Nacrot and Charrier, 1992; Dussert *et al.*, 1997) and a combination of low indole-3-butyric acid (IBA) and 5 °C enhanced storage of *Centarium rigualii* Esteve for three years (Iriondo and Perez, 1996).

2.1.2.4 Storage of encapsulated buds

In early definitions, the only explant type that was considered for synthetic seeds (synseed) production was somatic embryo (Redenbaugh, 1993). However, more recently, synseeds have been defined as artificially encapsulated somatic embryos, including shoots, axillary buds and other tissues that can be used for sowing under *in vitro* or *ex vitro* conditions (Aitken-Christie, Kozai and Smith, 1995; Piccioni, 1997). The idea of production and storage of somatic embryos as synthetic seeds was first proposed more than 15 years ago. Methods of storing encapsulated somatic embryos include; ABA pretreatment (Senaratna, Bryan, McKersie and Bowley, 1989; Anandarajah and McKersie, 1990; Lecouteux, Lai and McKersie, 1993; Pliegoalfaro, Litz, Moon and Gray, 1996), desiccation (McKersie, Senaratna, Bowley, Brown and Krochko and Brewley, 1989; Anandarajah, Kott, Beversdorf and McKersie, 1991), reduction of light and temperature (Pliegoalfaro *et al.*, 1996) or a combination of treatments (Bapat, 1993; Piccioni, 1997; Castillo, Smith and Yadava, 1998). Unlike their counterparts, storage of encapsulated buds has not been used until recently.

Maruyama, Kinoshita, Ishii, Shigenaga, Ohba and Saito (1997) have used alginate-encapsulated explants for long-term storage of commercially valuable forest tree germplasm (*Cedrela odorata* L., *Guazuma crinita* Mart. and *Jacaranda mimosaeifolia* Don, D.). Those authors used a combination of two treatments for storage of encapsulated buds, namely, temperature and media composition. They stored encapsulated shoot tips of the above-mentioned tropical forest trees at temperatures varying from 12 - 25 °C in water, on media consisting of 2% (w/v) sucrose and on agar solidified culture media. Those authors found that non-nutritive water based medium was the best substrate for suppressing growth. With regard to temperature, those authors found that the lower the storage temperature, the longer the storage period. In another study, *Jacaranda*

mimosaeifolia shoot tips remained viable for 12 months at 12 °C as opposed to 6 months at 20 °C. Other species on which storage of encapsulated buds has been carried out include mulberry (Bapat, 1993) and yam (Hasan and Tagaki, 1995). This fairly recently developed technique offers a method of storage for clonally propagated species and is an advantage where clonal propagation and subsequent multiplication of superior genotypes is required.

2.1.2.5 Viability assessment

The most convincing method of testing viability of shoot cultures or axillary buds after storage is their subculture and subsequent regeneration and multiplication under optimal conditions (Kantha, 1981; Withers, 1986; Grout, 1995; George, 1996). Often, shoot cultures are stored with the aim of recapturing and micropropagating them in the future. Their ability to regenerate and multiply is, therefore, the ideal method of assessing viability. Alternatively, subcultured shoot cultures can be evaluated by growth rates on the basis of fresh or dry weight (e.g. Monette, 1986). Other evaluation methods include determination of the ultrastructural state of tissues as an indication of the extent of the physiological damage due to storage (e.g. Engelmann, Assy-Bah, Bagniol, Dumet and Michaux-Ferriere, 1995; Jekkel, Gyulai and Heszky, 1995).

2.1.3 Techniques for medium-term storage of pollen

The longevity of pollen for most agronomic species is normally measured in minutes or hours and occasionally days or weeks, as is the case for most woody species (Collins, Vatcharee and Jones, 1973). If longevity could be prolonged substantially, pollen could be collected and stored until needed in tree improvement programmes. The major factors that affect pollen longevity are the same as those for seed storage, *viz.*, temperature and moisture content (Stanley and Linskens, 1974; Kantha, 1985; Barnabas, Kovacs, Abrany and Pfahler, 1988). Although other factors such as the composition and pressure of the gas phase surrounding pollen are known to affect longevity, they are rarely manipulated for optimum storage except in the case of freeze- or vacuum-dried pollen (e.g. Hanna, 1994; Inagaki, Nagamine and Mujeebkazi, 1997). Successful storage of pollen has often been achieved through manipulation of two major factors, temperature and moisture

content. Examples include tomato (Sacks and Stclair, 1996), maize (Georgieva and Kruleva, 1993; Broglia and Brunori, 1994), rye and wheat (Kovacs and Barnabas, 1993). With the short longevity of fresh pollen of many woody species, including *Eucalyptus*, the understanding of the factors that influence pollen viability during storage is, therefore, the key to prolonged longevity.

2.1.3.1 Moisture content

Pollen from different species may be classified into two categories with regard to desiccation tolerance (Roberts, 1975). Binucleate pollens, which exhibit an orthodox storage behaviour, are those that have a thick exine enabling them to withstand considerable desiccation and, therefore, have longer longevity. Desiccation-tolerant or binucleate pollen can survive longer storage periods if the pollen is first dried to a relatively low moisture content (<15% to 20% moisture). Orthodox pollen of species such as *Brassica* spp. (Brown and Dyer, 1991), pea (Layne and Hagedorn, 1963) and tomato (Visser, 1955) have been reported to survive drying to 10% moisture.

Trinucleate pollens generally have a thinner exine and are sensitive to desiccation and they are therefore short-lived. Freshly-shed Gramineous pollen, which is typically recalcitrant in terms of storage behaviour, can be kept alive only for a few days at 2-10 °C under 90 - 100% moisture content (Goss, 1968; Broglia and Brunori, 1994). Due to the high sensitivity to desiccation in this family, drying often results in irrecoverable damage. A classical example is that of maize pollen, which was reported to have undergone detrimental changes due to drying to a moisture content of about 20% (Webber, 1995).

It is clear, therefore, that the most crucial factor affecting individual species' pollen viability during storage is the degree of drying that it can withstand. A hypothesis has been put forward by Goss (1968) to explain the resistance or tolerance of pollen to desiccation damage. That author proposed that as a consequence of dehydrating mature pollen, metabolic processes are severely retarded and respiration is reduced. The inhibition of biochemical processes involved in respiration and dehydration of some

proteins may occur leading to reduced enzyme activity. That author further suggested that induced dehydration during storage would probably damage male cell nuclear components thus reducing the viability of trinucleate or desiccation-sensitive species.

2.1.3.2 Temperature

Another important environmental factor which affects stored pollen is temperature. Pollen storage treatments can be subdivided into two categories, with regard to temperature manipulation. Storage above zero (0 - 30 °C) or sub-zero (-10 - 0°C) but excluding ultra-low (cryogenic) temperatures (Connor and Towill, 1993). For medium-term storage, pollen can be stored at either sub-zero or above-zero temperatures (Towill, 1985; Grout and Robert, 1995). However, unlike cryogenic storage, storage at these temperatures usually cannot maintain viability for longer than a year or two (Stanley and Linskens, 1974). Therefore, it is often essential for a breeding programme to have two methods of storage operating in parallel. Medium-term for day-to-day requirements and cryogenic storage for the longer-term storage of germplasm.

Besides members of the Graminaeae family, pollen of most species can be stored for only a few days to weeks at temperatures above 0 °C (Goss, 1968; Kindiger and Dewald, 1993; Broglia and Brunori, 1994). Khosh-Khui, Bassrii and Niknejad (1976) found that rose pollen viability declined within nine weeks if stored at temperatures above 0 °C. According to Collins *et al.* (1973), storage at sub-zero temperatures increased pollen longevity of many woody species and other agronomic crops such as alfalfa (up to 11 years) and pea (2 years). Work with *Eucalyptus* spp. has also shown that sub-zero storage temperatures generally prolong the period over which pollen viability can be retained (Janick and Moore, 1975). But, occasionally, both sub- and above 0 °C storage regimes have been cited in storage of *Eucalyptus* spp. pollen. For example, Boden (1958) suggested storage of *Eucalyptus* in a deep freeze (-16 °C), while Borges, Da Silva and Ferreira (1973) favoured storage at 4 °C.

2.1.3.3 Methods of testing viability

After pollen has been collected and removed from storage, an assessment of the capacity to germinate and grow normally is usually essential (Stanley and Linskens, 1974). Since considerable investment in time and money is involved in preparing and pollinating field plantings, precautions must be taken so that pollen retrieved from storage for use will grow reasonably well. The main problem faced by any breeder is, therefore, how to measure pollen viability accurately after storage before attempting to use it in the field. Three methods of testing viability are available: a) pollen staining/fluorescence, b) pollen germination and c) fruit or seed set (Kartha, 1985). The present discussion focuses on the first two tests because of their relevance to this particular study.

a) Pollen staining/fluorescence

Assays involving staining or fluorescence are diverse and include starch, aceto carmine, peroxidase and fluorochromasia measurements. The starch viability assay uses iodine-potassium iodide (I-KI) to stain starch-containing pollen (Kartha, 1985). According to that author, this method lacks correlation and is highly unreliable. Thick walled pollen shows little staining with I-KI while with other species such as cotton, viable pollen does not stain at all (Sarvella, 1964). Aceto carmine is used to stain chromosome and nuclei. While this method may be simple and easy to follow, correlation with germination has been found to be very low (Janssen and Hermsen, 1976). These two methods were thus neglected in this study.

The fluorochromasia and tetrazolium assays, on the other hand, have been found to be more reliable than iodine-potassium iodide assay (Kartha, 1985; Heslop-Harrison and Heslop-Harrison, 1970; Enikeev, Vysotskaya, Leonova and Gamburg, 1995). There are up to 12 tetrazolium salts and their action is based on the uptake of the compound and its subsequent reduction to produce a coloured product (Enikeev *et al.*, 1995). Membrane integrity of the tissue the viability of which is being tested is, therefore, necessary to prevent loss of the concentrated reduction product. Cells with ruptured membranes or dead cells will not take in the stain and therefore will remain unstained.

While the use of tetrazolium stain appears to be fairly accurate in determining viability, pollen of different species may respond differently to any of the 12 salts. The selection of an appropriate salt is, therefore, very important. Werner and Chang (1981) found that staining of stored peach pollen with 3(4-5-dimethylthiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) correlated with *in vitro* germination better than when using triphenyl-tetrazolium chloride (TTC). Khatun and Flowers (1995) also made a similar observation with rice. However, TTC has been used to obtain reliable viability for several other species, such as grape (Iborra, Guardiola, Montaner, Canovas and Majon, 1992) and pea (Enikeev *et al.*, 1995).

b) Pollen germination

Testing the ability of pollen to germination *in vitro* has long been used as a gauge of pollen viability and has been found to correlate well with *in vivo* germination (Stanley and Linskens, 1974; Kartha, 1985). However, one of the disadvantages of this approach is that a near-optimum growth medium that allows maximum germination and minimum pollen-tube bursting is difficult to establish. Another problem is that most pollen tubes cultured *in vitro* stop growing before the size normally attained in the style and the rate of tube growth is seldom as rapid as *in vivo*. This suggests that optimum growth conditions are often hard to establish in *in vitro* media, even though several studies have established near-optimum conditions for a number of species, for example, *Capsicum* sp. (Mercado, Fernandezmazon and Quesada, 1994), *Eucalyptus* (Boden, 1958) and Douglas fir (Webber and Bonnet-Masimbert, 1993).

The major components of pollen germination medium are sucrose (10-40% (w/v)) boric acid (0.05-0.2 g.l⁻¹), calcium (0-0.3 g.l⁻¹) and magnesium (0-0.2 g.l⁻¹). Many researchers (Potts and Marsden-Smedley, 1989; Heslop-Harrison and Heslop-Harrison, 1970; Marien, 1988; Loupassaki, Vasilakakis and Androulakis, 1997) have obtained high germination rates using the medium described by Brewbaker and Kwack (1963) which consists of 0.1 g.l⁻¹ H₃BO₃, 0.3 g.l⁻¹ Ca(NO₃)₂.4H₂O, 0.2 g.l⁻¹ MgSO₄.7H₂O and 0.1 g.l⁻¹ KNO₃.

2.1.4 Applications and limitations of medium-term storage

2.1.4.1 Shoot cultures

For reasons discussed in section 1.2, clonal propagation forms an integral part of *Eucalyptus* spp. breeding programmes. Storage of such propagated material is often *ex situ* in field banks. However, *ex situ* storage materials are often prone to damage through viruses, pests, natural disasters or climatic hazards hence an alternative risk-proof storage method is a necessity (Engelmann, 1997). Therefore *in vitro* conservation approaches can be applied to supplement *ex situ* techniques because of the reduced chances of natural disasters and other hazards. Moreover, plants produced *in vitro* are mostly disease-free; because they are often kept in a protected environment, *in vitro* conserved plants are less likely to be damaged or killed by pathogens and predators (Krostrup *et al.*, 1992; Hamilton, 1994). There is also the potential for virus elimination from contaminated tissue through the use of meristem cultures (Withers and Alderson, 1986). In addition to their implementation as a tool for conservation, *in vitro* cultures allow easy and safer distribution across borders in terms of germplasm health status (Ashmore, 1997; Withers and Engelmann, 1996).

Generally, conventional seed storage strategies are not recommended for clonal material because of the accompanying sterility or heterozygosity of seeds leading to genotypes that are not true-to-type. *In vitro* storage techniques therefore play an important role in the preservation of clones which are required as stocks for continued propagation *in vivo* or *in vitro*, or those required as parents in plant breeding programmes (Grout, 1995; Ashmore, 1997). Once in storage, clones can be easily propagated when stock is required using micropropagation techniques. This form of storage therefore enables tissue culture laboratories to carry out year-round production of some plants required for seasonal planting (George, 1993).

The field space saved by utilisation of *in vitro* methods of germplasm storage can be considerable (George, 1993) especially in the case of woody species, which require large amounts of land for field banks. Morel (1975) described how nodal cuttings of grapevines

could be multiplied and cultured at a reduced temperature so that six plants of each of 800 varieties could be preserved in 2 m² of controlled environment room space. According to that author, if the plants had been grown in the field, about 1 ha of suitable land would be required with high labour and management costs. Similarly *Pinus radiata* shoot cultures stored in 2000 culture tubes which occupied 3.83 m² of the cold room would require 4 ha of field space, if planted as hedged ramets (Aitken-Christie and Singh, 1987). In Columbia, the *in vitro* genetic bank at CIAT currently comprises nearly 6000 cassava clones from 23 countries occupying 50 m² storage space (Escobar, Mafia and Roca, 1997).

Encapsulated buds (synthetic seeds) offer an additional dimension to storage of shoot cultures for the conservation of commercially valuable forest trees such as *Eucalyptus* spp (Ahuja, 1993). Because of their small size and ease of handling, encapsulated buds could be useful in the exchange of sterile material among laboratories both nationally and internationally. As mentioned, because buds are genetically stable, when propagated they give rise to true-to-type plantlets thus ensuring genotype conservation. Furthermore, synthetic seeds offer the opportunity to store genetically heterozygous plants, or hybrids with unique gene combination. The preservation of alginate coated buds also offers an attractive benefit for conservation of forest species, which are often intolerant to conventional reduced-temperature storage systems (e.g. cryopreservation) as an alternative method of germplasm conservation (Ashmore, 1997). Moreover, compared with cryopreservation, medium-term storage of encapsulated buds is easier and does not require sophisticated and expensive equipment. Like shoot cultures, storage of encapsulated buds under growth retarding conditions reduces the numbers of subcultures and this in turn reduces contamination risks associated with subculturing, cost of maintenance and subsequent loss of material.

In spite of the obvious advantages, storage of shoots has a number of limitations. Problems may arise mainly due to loss of vigour in plantlets as a result of prolonged storage time (Withers and Alderson, 1986). Ensuring genetic stability following the use of growth retardants is another distinct difficulty associated with minimal-growth

techniques (Withers, 1988; Ashmore, 1997). Moreover, the potential tendency to reduce the genetic base of plant populations, where clonal propagation is used, can be an added hazard of *in vitro* storage techniques (Fahy, 1986; Arakawa, Carpenter, Kita and Crowe, 1990). There is also an additional disadvantage due to time and effort spent developing routes of propagation for each species. Another problem is the requirement of equipment specific for tissue culture and the on-going expense of well-trained staff to manage germplasm banks. Thus *in vitro* germplasm storage methods still have to be optimised to make them more practicable for most poor countries.

2.1.4.2 Pollen banks

The value of stored pollen as an efficient way of conserving plant genes *in vitro* has been widely recognised as part of a broader strategy for the conservation of plant genetic resources (Withers, 1991; Grout and Roberts, 1995). Stored pollen, if recovered with acceptable pollination competence, is of great value in breeding and production programmes. The constant availability of pollen in tree improvement is important for the hybridisation between plants that flower at different times or those that grow at different and distant places and also for those species with short lived pollen (Kantha, 1985; Bajaj, 1995). Moreover, the use of stored pollen also reduces the spread of diseases transferred by pollination vectors. The increasing success of *in vitro* technology for pollen culture leading to tissue culture and plant regeneration has advantages for industrial and agricultural biotechnology and consequently, generates a further need for pollen storage. Figure 2.2 summarises the advantages associated with the development of pollen germplasm banks.

Due to the global concern for crop improvement and for germplasm collection, there is an increased need for the transport of materials to many countries, pollen storage can present an efficient, economical and space saving method of conserving genetic diversity in forest species and fruit trees as well as for international germplasm exchange. When carried out from seeds, propagation of *Eucalyptus* spp. is very time consuming (5-7 years to flowering) and heterozygosity leads to variations between trees (Lakshmi-Sita, 1986). The distribution of pollen, rather than seed, can therefore reduce the time taken to cross

two individuals with desired traits in that species. Although pollen shipment is a desirable method of germplasm exchange locally between laboratories or internationally, there should be safeguards against the introduction and spread of disease by this exchange. This follows from the fact that pollen-borne viruses and mixtures of pollen and fungal spores are common (Grout, 1995).

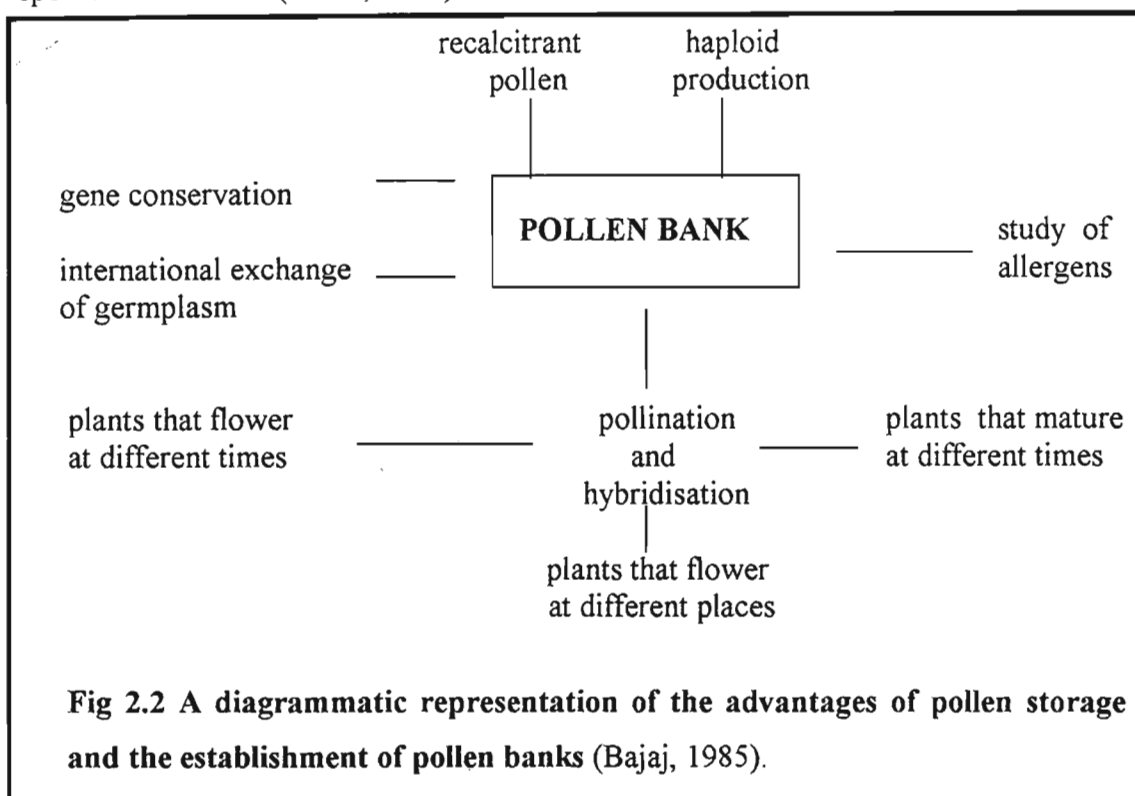


Fig 2.2 A diagrammatic representation of the advantages of pollen storage and the establishment of pollen banks (Bajaj, 1985).

2.1.5 Aims of the investigation

Two types of material are required for clonal propagation and breeding programmes of *Eucalyptus* spp. namely, vegetative (e.g. shoot cultures and buds) and sexual (e.g. pollen) materials, respectively. Both programmes would therefore benefit from short-to-medium term storage of such material. Hence the initiation of this study. In order to achieve methods of storage for buds and shoot cultures, various growth limiting factors such as temperature, light and media composition were investigated. For short-to-medium term storage of buds, an encapsulation method was established, followed by an investigation of growth-retarding factors. Pollen storage was attempted through manipulation of temperature and moisture content. However, before establishing pollen storage methods, viability assessment protocols had to be developed.

2.2 Materials and Methods

2.2.1 Plant material

Seeds of *Eucalyptus grandis* M5684 were obtained from Mountain Home, Mondi Forests (Hilton) in sealed plastic bags and stored at 10 °C. Pollen was harvested from Mondi Forests provenance trials and delivered to the University of Natal, in tubes wrapped in brown-paper envelopes. It was then tested for viability and germination ability and stored under various conditions.

2.2.1.1 Seed germination

Seeds were germinated according to Hope (1994). They were separated from husks manually and surface sterilised for 15 min in 3.5% (v/v) sodium hypochlorite containing a drop (approximately 0.2 ml.l⁻¹) Tween 20. After three washes in sterile water, they were immersed in 4% (v/v) hydrogen peroxide for 10 min and a drop of Tween 20, rinsed thoroughly in sterile distilled water and placed in solutions of 50 mg.l⁻¹ each of penicillin and streptomycin (Highveld Biologicals, South Africa) for 10 min. Then they were germinated on a medium consisting of MS (Murashige and Skoog, 1962), 10 g.l⁻¹ sucrose, 1 g.l⁻¹ casein hydrolysate, 4 g.l⁻¹ Gelrite and 1 g.l⁻¹ benomyl (Benlate®) at pH 5.7. Seeds were thereafter incubated in the dark for 6 days after which they were placed under a 16 h light and 8 h dark photoperiod at 200 µEm⁻².s⁻¹ photosynthetic photon flux density (PPFD) for two weeks.

2.2.1.2 Multiplication, elongation and acclimatisation of shoot cultures

After two weeks, 5 cm-long *in vitro* seedlings were immersed in a solution of 50 mg.l⁻¹ each penicillin and streptomycin for 20 min. Their roots were cut and the shoots were placed on a multiplication medium containing MS salts and vitamins supplemented with 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.2 mg.l⁻¹ benzyl amino purine (BAP), 30 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite (pH 5.6 - 5.8) for 4 weeks. Clumps of multiplied shoots were then separated and transferred to an elongation medium consisting of MS, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.35 mg.l⁻¹ naphthylacetic acid (NAA), 0.1 mg.l⁻¹ kinetin and 0.1 mg.l⁻¹ Indole-3-butyric acid (IBA), 30 g.l⁻¹ sucrose, and 3.5 g.l⁻¹

Gelrite (pH 5.6 - 5.8). When shoots were about 25 mm long (four weeks), they were separated and transferred to a hormone-free medium consisting of MS, 30 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite (pH 5.6 - 5.8) to allow them to acclimatise for two weeks before being subjected to different storage treatments as described in 2.2.2. All cultures were maintained under a 16 hour light/8 hour dark photoperiod at 200 $\mu\text{E.m}^{-2}.\text{s}^{-1}$ at 28 °C.

2.2.1.3 Bud encapsulation

Seedlings were obtained from seeds germinated *in vitro*, multiplied, separated and elongated (section 2.2.1.2). Individual shoots were then transferred to a multiplication medium that consisted of MS, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.2 mg.l⁻¹ BAP and 30 g.l⁻¹ sucrose (pH 5.6 -5.8) for buds to grow to a length of approximately 2 mm (3 weeks). Shoots were then transferred to the laminar flow, where buds were excised under aseptic conditions for encapsulation.

Sterile buds were placed on a sterile filter paper in a Petri dish to remove excess water. Using a pair of forceps, each bud was immersed in 0.3M sucrose and 3% (w/v) sodium alginate (Sigma) dissolved in distilled water. After 2 min, buds were dispensed with a 3 ml plastic pipette into a complexing solution of MS and 11 g.l⁻¹ CaCl₂ for 25 - 30 min to form spherical beads (5 mm diameter). The CaCl₂ solution was then decanted and replaced with distilled water for 10 min to wash away calcium chloride residues. Encapsulated buds were thereafter transferred to their respective storage conditions.

2.2.2 Storage of vegetative material

2.2.2.1 Storage of shoot cultures

After two weeks on a hormone-free medium, shoots (25 mm) were transferred to different storage conditions (2.2.2.2). They were stored in culture bottles (100 ml capacity), each culture bottle containing 25 ml of culture medium. Culture bottles were tightly sealed with well fitting lids to prevent evaporation and subsequent dehydration. Each treatment consisted of 400 shoots, with 5 shoots/culture bottle. Four types of storage conditions were investigated: reduced light and temperature, reduced nutrients, ABA and mannitol (Table 2.2).

Table 2.2 Different minimal-growth conditions tested to establish protocols for *in vitro* storage of shoot cultures. Unless otherwise stated, all cultures were stored at 24 - 28 °C under a 16 h light and 8 h dark photoperiod at 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. All media also contained 3.5 $\text{g}\cdot\text{l}^{-1}$ Gelrite and 1 $\text{g}\cdot\text{l}^{-1}$ Benlate.

Treatment	Concentration of MS nutrients	Sucrose ($\text{g}\cdot\text{l}^{-1}$)
10 °C and 4 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	0.5	30
60 $\text{g}\cdot\text{l}^{-1}$ mannitol	0.5	0.5
25 $\text{mg}\cdot\text{l}^{-1}$ ABA	0.5	0.5
10 $\text{mg}\cdot\text{l}^{-1}$ ABA	1.0	30
reduced nutrients	0.5	30

2.2.2.2 Storage of encapsulated buds

Buds encapsulated in alginate beads were placed in jars (100 ml) containing 5 ml sterile water to prevent dehydration of buds during storage. Two storage conditions were tested: storage at 1) 10 °C under continuous light of 4 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 2) 28 °C under 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with a 16 h light and 8 h dark photoperiod. Each treatment consisted of 100 buds.

2.2.2.3 Assessment of viability and multiplication rates

Sampling from the different shoot storage conditions was done every second month for 8 - 10 months. Each sample consisted of 50 shoots. To assess multiplication and survival rates, shoots were transferred to the multiplication medium (section 2.2.1.2) under growth room conditions. After four weeks, contamination, survival and the rate of multiplication per explant were recorded. For encapsulated buds, samples of 15 buds were retrieved and tested for viability initially after two weeks, thereafter after every month. Viability was assessed by culturing buds in tubes on a multiplication medium consisting of MS, 30 $\text{g}\cdot\text{l}^{-1}$ sucrose, 3.5 $\text{g}\cdot\text{l}^{-1}$ Gelrite, 0.01 $\text{g}\cdot\text{l}^{-1}$ ascorbic acid and 0.01 $\text{g}\cdot\text{l}^{-1}$ citric acid in the growth

room at a 16 h light/8 h dark photoperiod between 24 - 28 °C.

2.2.3 Pollen storage

2.2.3.1 Storage conditions

For purposes of short-to-medium-term storage, or active collection, storage conditions for pollen were as follows:

a) Storage over silica gel

Two aluminium foil weighing boats containing 0.445 g of pollen (after 3 months of storage) each were placed in 100 ml jars containing 60 g of activated silica gel. The jar was closed and samples from one of the boats were collected at 2 hours intervals to assess pollen viability using 2,3,5-triphenyl tetrazolium chloride (TTC) (section 2.2.3.2). Samples collected from the second boat were weighed at 2 hourly intervals and recorded to monitor the change in fresh weight of pollen. Storage over silica gel was undertaken over a period of 24h (1440 min).

b) Storage at 28 °C

Approximately 0.02 g of pollen was placed in Eppendorf tubes (1.5 ml) which were closed and stored in 1) a desiccator with activated silica gel, 2) a 100 ml jar without silica gel, and 3) Eppendorf tubes containing 1:1 ratio of pollen and talc (Johnson and Johnson baby powder) in 100 ml jars without silica gel.

c) Storage at 4 °C

Samples of pollen (0.02 g) in Eppendorf tubes (1.5 ml) were stored as follows: 1) in 100 ml jars with 60 g of silica gel, 2) in 100 ml jars without silica gel, and 3) in Eppendorf tubes containing 1:1 ratio of pollen to talc in 100 ml jar without silica gel.

Each storage treatment was for a period of 10 months with an overall sample size of 40 Eppendorf tubes per treatment each containing 0.02 g pollen. A sample containing 0.02 g, was randomly selected every second month for viability testing with TTC (sections 2.2.3.2a).

2.2.3.2 Viability assessments

a) Staining techniques

Two tetrazolium salts were investigated, 2,3,5-triphenyl tetrazolium chloride (TTC) and 3-{4,5-dimethyl-thiazolyl 1-2-}-2,5-diphenyl monotetrazolium bromide (MTT or thiazolyl blue) (Norton, 1966). Tetrazolium chloride salt was prepared by dissolving 1% (w/v) of 2,3,5-triphenyl tetrazolium chloride in a 3:2 solution of Na_2HPO_4 and KH_2PO_4 (ISTA¹ regulations). Stock staining solution was stored in opaque containers in the dark at 4 °C. To test viability using TTC assay, 1 ml of 1% (w/v) TTC was added to approximately 0.02 g of pollen in Eppendorf tubes and incubated in the dark at 28 °C for 12 h. The MTT stain was prepared as 0.9% (w/v) MTT dissolved in 54% (w/v) sucrose solution. For viability analysis, 1 ml of MTT stain was added to 0.02 g of dry pollen in an Eppendorf tube and incubated in the dark at 28 °C for a maximum period of 30 min. Two drops of the resulting pollen/stain mixtures were mounted on a haemocytometer slide (0.0625 mm²) and viewed with a Zeiss light microscope. Each evaluation consisted of two samples, each of which was mounted on a slide and percentage viability determined at three different areas on the slide. Pollen grains were classified according to colour intensity. For the TTC assay, light red to red was considered viable while yellow or non-stained pollen was recorded as non-viable (Norton, 1966). For the MTT assay, red or black signified viability while yellow colour designated non-viability (Norton, 1966).

i. Determination of optimal period of incubation for MTT and TTC assays

Pollen samples (0.02 g) contained in two Eppendorf tubes were mixed with fresh TTC or MTT staining solutions (in the manner described in section 2.2.3.2a) and incubated at 28 °C. Colour changes were monitored at the following time intervals; every 6 h up to 48 h for TTC, and every 15 min for a maximum of 60 min for the MTT (Norton, 1966). Both tests were performed using three different pollen collections (harvested in February, May and June 1996).

¹ ISTA - International Seed Testing Association

ii. Comparison of performance between MTT and TTC assays

To determine the effectiveness of the two stains, two Eppendorf tubes with equal amounts of fresh pollen (0.02 g) were placed in a hot water bath, (80 °C) for 24 h followed by staining with either TTC or MTT (2.2.3.2a). The resulting staining differences were recorded in both cases.

b) *In vitro* germination

i. Determination of a sterilisation protocol

Due to problems pertaining to fungal contamination encountered during preliminary investigations into *in vitro* germination of pollen, it was essential to develop a protocol for pollen sterilisation. The following treatments were thus investigated: 1) pollen was imbibed in 1, 0.2, 0.1, 0.05 and 0.01% (v/v) sodium hypochlorite with 0.02 ml.l⁻¹ Tween 20 for 3 min, 2) pollen was imbibed in sterile water for 1 hour prior to sterilisation to allow hydration of fungi and treated as in 1) and 3) pollen was germinated on BK pollen germinating medium (Brewbaker and Kwack, 1963), which consisted of 10 mg.l⁻¹ H₃BO₃, 30 mg.l⁻¹ Ca(NO₃)₂.4H₂O, 20 mg.l⁻¹ MgSO₄.7H₂O, 10 mg.l⁻¹ KNO₃ and 200 g.l⁻¹ sucrose with 100 mg.l⁻¹ Benlate (pH 5.6 -5.8) as described in (2.2.3.2a ii). Hydrated pollen was centrifuged for 5 minutes to remove water. The resulting pellet was resuspended in different sodium hypochlorite concentrations (treatment 1 and 2 above) and centrifuged immediately for 3 minutes. The sterilant was thereafter decanted and pollen resuspended three times in sterile water followed by centrifugation. All centrifugations were done in a microcentrifuge at 9000 rpm for 3 min.

ii. Pollen germination protocol

Pollen was germinated on liquid BK medium (Brewbaker and Kwack, 1963), 200 g.l⁻¹ sucrose and 100 mg.l⁻¹ Benlate (pH 5.6 -5.8), as described for *Eucalyptus* pollen by Boden (1958). A sterile filter paper was moistened with sterile water then placed in a sterile Petri dish, four sterile cover slips were then placed on the filter paper. A drop of BK medium (0.5 ml) was placed on each cover slip, onto which

approximately 0.01 g pollen grains were slowly tapped. Petri dishes were then sealed with Parafilm and incubated at 28 °C and 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ 16 h light/8 h dark photoperiod for 48 h. To determine percentage germination, a cover slip with germinating pollen was placed (pollen side down) on a haemocytometer slide (0.0625 mm²) and viewed with a light microscope. Each evaluation consisted of two samples on cover slips, each of which was placed on the slide and percentage viability determined at three different areas on the slide. Pollen was scored as germinating if the pollen tube length was greater than one half the diameter of the pollen grain (Boden, 1958). Clumps of pollen grains were avoided when scoring germination in accordance with Brewbaker and Kwack (1963).

2.2.4 Photography and Microscopy

The effects of the different storage treatments on shoots and encapsulated buds were recorded using a Nikon FM2 camera with a 60 mm Mikro Nikon macro lens. Stained and germinating pollen were viewed using a Olympus Vanox AHBS3 light microscope and results were recorded using a an Olympus C-35AD-4 camera.

2.2.5 Data Analysis

Average values with standard error were recorded for the different staining techniques at various stages of storage and for multiplication rates of shoots during storage. Where appropriate, One Way Analysis of Variance (ANOVA) (SAS, 1982) was used to assess differences in the recorded mean values of the variables investigated. Alphabetical values were assigned to the mean values recorded per treatment. Mean values that did not share the same letter were recognised as being significantly different.

2.3 Results and Discussions

2.3.1 Minimal growth storage of shoot cultures

2.3.1.1 *In vitro* production of shoots from seeds

One of the major problems associated with *in vitro* storage of shoots is microbial contamination (George, 1996). According to McComb and Bennett (1986), the greatest limitation of micropropagation of eucalypts is the problems associated with sterilization of field-grown material. That author pointed out that it is very difficult to obtain 'clean' viable material for production of shoot cultures if field-grown explants are used. Further, on-going research in our laboratory with *Eucalyptus* spp. has also proved that the use of explants from field-grown material is often associated with intensive contamination (Watt *et al.*, 1995; 1996). In this study, the use of aseptically-produced seedlings was adopted to circumvent such problems. The seed germination protocol that was followed was that established in our laboratory (Watt *et al.*, 1996; 1998a), and seed germination was approximately 80% (Fig 2.3A). Approximately 20% of the germinating jars were infested with both fungi and bacteria, these were discarded and only 'clean' shoot cultures were multiplied (Fig 2.3B). The rates of multiplication per shoot were found to be 6.20 ± 1.12 standard error (SE).

The use of *in vitro* produced plantlets in this study had immense advantages. As has been pointed out, *in vitro* culture of field-grown tissue is constantly hampered by microbial contaminations (Durand-Cresswell *et al.*, 1982; Furze and Cresswell, 1985; Warrag *et al.*, 1990). Although protocols for surface sterilisation of *Eucalyptus* spp. explants have been established (e.g. Durand-Cresswell and Nitsch, 1977; Hartney, 1980; Laine and David, 1994; Muralidharan and Mascarenhas, 1995), problems of endogenous contaminants and phytotoxicity cannot be eliminated completely. The use of *in vitro* produced plantlets offered the possibility of screening out contaminated material and all subsequent studies were thus carried out with 'clean' cultures. Moreover, with the use of micropropagation techniques and the high multiplication rates obtained (6.20 ± 1.12), large quantities of plantlets required for the various storage treatments were obtained within a relatively short period (approximately 3 months).

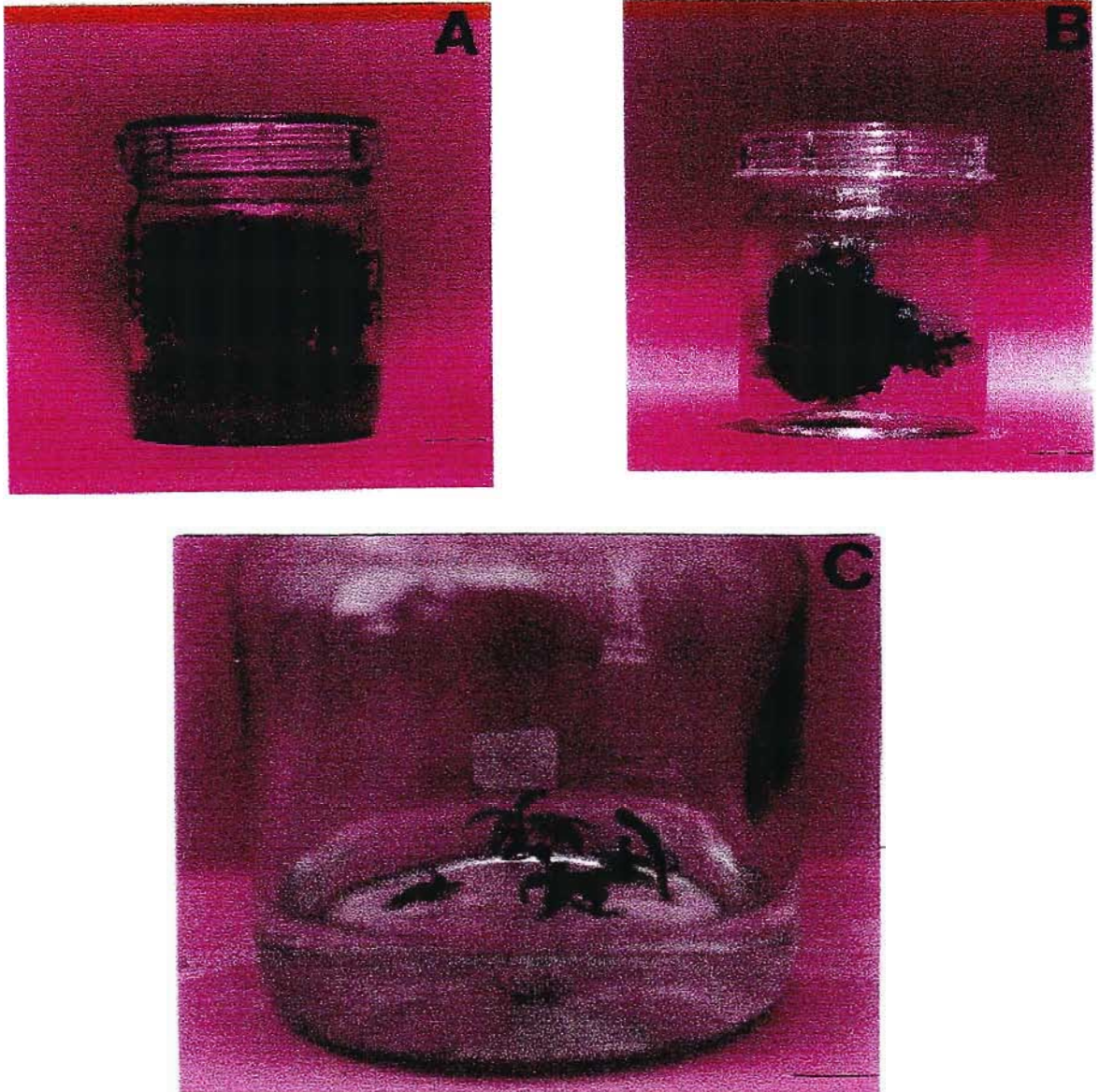


Fig 2.3: *In vitro* production of sterile shoots for medium-term storage A) Seedlings from seeds germinated *in vitro* [bar = 16.3 mm]. B) Clumps of shoots on the multiplication medium [bar = 16.8 mm]. C) Shoots on an acclimatisation medium before storage [bar = 0.08 mm].

After four weeks on a multiplication medium shoots were placed on an elongation medium. Four weeks later, shoots had grown to a height of about 2.5 cm (Fig 2.3C) and they were transferred to an acclimatisation medium consisting of MS nutrients, 30 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite. The duration of any minimal-growth storage technique has been shown to increase as a result of two factors, explant size (Barlass and Skene, 1983) and shoot density (Aitken-Christie and Singh, 1987). From the literature reviewed, explant sizes between 2.5 cm - 3.0 cm often resulted in better survival after storage (e.g. Monette, 1986; Son *et al.*, 1991) compared with smaller explants of less than 2 cm (Dale, 1980; Iriando and Perez, 1996). From this information, it was thus decided to use explant of 2.5 cm for storage in this study.

After two weeks on acclimatisation, shoots were transferred to their respective storage conditions (section 2.2.2.1). Acclimatisation is essential due to the fact that freshly transferred cultures have less chances of survival (George, 1993). Several researchers have studied the effect of acclimatisation before explants could be transferred to storage and a period of one to two weeks appears to result in better survival rates. Examples where delaying transfer by two weeks resulted in longer storage periods include work on *Populus* spp (Hausman *et al.*, 1994), *Miscanthus x ogiformis* Honda (Hansen and Kristiansen, 1997) and *Prunus* sp. (Marino *et al.*, 1985). It was in view of the evident advantages of subjecting explants to a few weeks of acclimatisation that *Eucalyptus* sp. shoots were cultured on a hormone-free medium for two weeks prior to storage.

2.3.1.2 Storage of shoots under low light intensity and low temperature

In this treatment, shoots were retrieved from acclimatisation after two weeks and transferred to a medium consisting of MS, 3.5 g.l⁻¹ Gelrite and 30 g.l⁻¹ sucrose and placed at 10 °C and continuous low light intensity (4 μE.m⁻².s⁻¹) for 8 months. Throughout the tested period, shoots showed stunted growth with no root development and no chlorosis was observed during this period. A relatively low level of contamination was encountered. Total contamination recorded was approximately 20% of explants infested by both fungi and bacteria.

Shoots that are stored *in vitro* are in aseptic conditions and theoretically, no contamination is expected to occur. For instance, Dale (1980) indicated that storage of mulberry shoots in culture for 3 years was not affected by contamination. In this study, contamination was not expected to occur during *in vitro* storage because of the fact that only clean cultures had been selected for storage. However, it is possible that contamination could have occurred due to handling during transfer of shoots to their respective storage media. Taylor *et al.* (1996) lost a high percentage of *Colocasia* cultures as a result of *in vitro* contamination reducing storage to only 12 weeks. On the other hand, there are many reports of 'latent' contamination, that is, contamination that appears only after a few subcultures (Long, Curtin and Cassells, 1989; Gunson and Spencer-Phillips, 1994; Herman, 1996).

Every two months, shoots were subcultured onto a multiplication medium and contamination, multiplication and survival rates were recorded, as shown in Table 2.3. When shoots were subcultured, a significant amount of brown phenolic exudate was observed on the culture media after about a week in culture. Marino *et al.* (1985) and Son *et al.* (1991), working on *Prunus* sp. and *Populus alba* respectively, previously reported a similar observation. According to those authors, such results may be due to phenolic and/or oxidative substances accumulating in the media in response to physiological damage and defoliation just after storage.

During the 8 months of low light and temperature storage, shoots showed no signs of chlorosis (Fig 2.4A). However, shoots subcultured from the 4 and 6 months storage batches started exhibiting increased yellowing (Table 2.3), although this did not seem to have a significant effect on the rates of survival and multiplication. By 8 months, shoots had completely lost multiplication and survival potential. All subcultures (for 2 up to 6 months) were accompanied by an average contamination rate of 20%. For the purpose of commercial production, the loss of material as a result of contamination has to be taken into consideration in order to meet target values.



Fig 2.4 Survival and growth of shoots stored under four different treatments after various storage periods. A) shoots retrieved from reduced light and temperature storage conditions which involved storage on a medium that consisted of 0.5 MS nutrients, 0.5 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite at 10 °C with a consistent light intensity of 4 μE.m⁻².s⁻¹ for 6 months [**bar = 0.07 mm**]. **B)** shoots cultured in the presence of an osmoregulator for 6 months on a medium consisted of 0.5 MS, 0.5 g.l⁻¹ sucrose, 3.5 g.l⁻¹ Gelrite and 60 g.l⁻¹ mannitol [**bar = 0.07 mm**]. **C)** shoots stored under the influence of a plant growth inhibitor for 10 months on a medium that consisted of MS, 0.5 g.l⁻¹ sucrose, 3.5 g.l⁻¹ Gelrite and 10 mg.l⁻¹ ABA [**bar = 11.5 mm**]. **D)** shoots cultured on a reduced nutrient medium consisting of 0.5 MS, 0.5 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite. Treatments B, C and D were under a 16 h light/8 h dark photoperiod at 200 μE. m⁻².s⁻¹ for 10 months [**bar = 17.4 mm**].

Table 2.3 The effect of shoot storage under low light intensity ($4 \mu\text{E} \cdot \text{m}^2 \cdot \text{s}^{-1}$) and temperature ($10 \text{ }^\circ\text{C}$) on survival and multiplication rates. Storage medium consisted of MS, $3.5 \text{ g} \cdot \text{l}^{-1}$ Gelrite and $30 \text{ g} \cdot \text{l}^{-1}$ sucrose for a period of 8 months. Samples were subcultured from the storage medium to a multiplication medium consisting of MS nutrients, $0.1 \text{ mg} \cdot \text{l}^{-1}$ biotin, $0.1 \text{ mg} \cdot \text{l}^{-1}$ calcium pantothenate, $0.2 \text{ mg} \cdot \text{l}^{-1}$ BAP, $30 \text{ g} \cdot \text{l}^{-1}$ sucrose and $3.5 \text{ g} \cdot \text{l}^{-1}$ Gelrite at $28 \text{ }^\circ\text{C}$ under a 16 h light and 8 h dark photoperiod at $200 \mu\text{E} \cdot \text{m}^2 \cdot \text{s}^{-1}$ PPFD for four weeks. Levels of significant difference (ANOVA) were represented by letters a-b. Multiplication rates with similar letters were not significantly different ($f = 0.05$). $n = 50$ shoots (5/bottle) \pm SE

Time in storage (months)	% survival	multiplication (shoots/explant)	% contamination
0	100	6.20 ± 1.12 a	0
2	80	5.35 ± 1.92 a	20
4	70	3.00 ± 2.82 a	30
6	70	3.88 ± 2.90 a	30
8	0	0 b	0

In an earlier study of *in vitro* storage of *Eucalyptus* spp. shoots in our laboratory, shoots were stored on a medium containing quarter-strength MS, $5 \text{ g} \cdot \text{l}^{-1}$ sucrose and $3.5 \text{ g} \cdot \text{l}^{-1}$ Gelrite at $4 \text{ }^\circ\text{C}$ under a constant, low light intensity ($50 \mu \text{E} \cdot \text{m}^2 \cdot \text{s}^{-1}$) (Berjak, Mycock, Wesley-Smith, Dumet and Watt, 1996). Results obtained by those authors showed a decline in multiplication rates from 18.3 to 10.8 shoots/explant after a maximum storage period of 6 months. In this study, the rates of multiplication were not significantly affected by storage at low temperature and light intensity for the first 6 months. The differences in the response of shoots to the investigated storage conditions in both studies could have been influenced by the different sources of seedlings used. In this work, seedlings were obtained from *E. grandis* M5684 while Berjak *et al.* (1996) used *E.*

grandis M5. These observed differences correlated with observations made in other studies. For example, a broad variability in response to cold storage of a core collection of *Coffea* spp. (Dussert *et al.*, 1997) was observed and different *Solanum* spp. (Wescott, 1981a) showed varying responses to cold storage. However, in both this study and that of Berjak *et al.* (1996), it was observed that storage for periods longer than 6 months under low-light and temperature conditions had deleterious effects on cultures. In contrast, Mascarenhas and Agrawal (1991) reported storage of *Eucalyptus citriodora* at 10 °C for 10 months.

Temperature reduction has been shown to be one of the most effective methods of suppressing growth of cultures *in vitro* and thus increasing culture storage length. Similar results were obtained for example, with storage of *Colocasia esculenta* for more than 8 years with transfer intervals of 3 years (Bessembinder *et al.*, 1993), *Populus alba* x *P. grandidentata* shoots stored for 5 years (Son *et al.*, 1991) and shoot-tips of *Actinidia chinensis* maintained for 1 year in the dark at 8 °C (Monette, 1986). However, other workers have reported cold storage treatment to be ineffective or resulting in a short longevity for some species, for example, banana (Benerjee and De Langhe, 1985) and coffee (Bertrand-Desbrunais *et al.*, 1992). According to Blakesley *et al.* (1996), one of the most important factors that affect success of cold storage is the susceptibility to chilling- injury of the species being stored. Species that are of tropical and sub-tropical origin seem to suffer physical dysfunction as a result of storage at temperatures below 16 °C.

Tropical and sub-tropical species or those that are chilling sensitive such as cassava (Roca, Rodriguez, Beltran, Roa and Mafla, 1982), banana (Van de houwe *et al.*, 1995) and coffee (Kantha *et al.*, 1981b) are good examples where cold storage at below 16 °C was unsuccessful. With such species, exposure to reduced temperatures resulted in chilling-injury and concomitant physiological damage, thus reducing post storage survival (Lyons, 1973; Lyons, Raison and Steponkus, 1979). *Eucalyptus grandis*, which is widely planted in warmer and moist areas, is also likely to be chilling sensitive thus storage at 10 °C may have resulted in injury hence the rapid decline leading to death in 6

months (Table 2.3).

Another factor that contributes to the success of cold storage of shoot cultures is the size of the explant. According to George (1996) small shoot clumps did not survive as well as those four times their size. Small shoots are also prone to hyperhydricity, a situation where shoots become abnormally glassy or water soaked. Hyperhydricity has been recorded to occur in stored cultures and is prevalent in woody species or occasionally herbaceous plant groups. It is often triggered by conditions that are less than optimal for *in vitro* plant growth such as those that were being investigated in this study. According to George (1996), hyperhydricity can be promoted by high temperatures, low irradiance or storing cultures in the dark. Due to the fact that shoots were cultured in less than optimal conditions, it is possible that hyperhydricity may have prevailed causing low survival rates during storage. Moreover, in a review by Le Roux and van Staden (1991), it was argued that *Eucalyptus* shoots can become hyperhydric even when stored under normal culture conditions.

In conclusion, medium-term storage of *Eucalyptus* shoot cultures was maintained for 6 months at low temperature and light intensity with high survival rates (70%) but lower rates of explant multiplication ($3.88 \pm 2.90\%$) compared with initial multiplication rates of $6.20 \pm 1.12\%$. However, because *E. grandis* is chilling-sensitive, storage at 4 °C could have resulted in the poor shoot multiplication. In future, attempts to increase storage periods, survival and multiplication rates could be by storing eucalypt cultures at 16 °C.

2.3.1.3 Storage of shoots on a mannitol-containing medium

The use of sugar alcohols such as mannitol or sorbitol is often recommended for minimal-growth storage on the basis of their ability to impose a level of osmotic stress on shoot cultures (Grout, 1995). Under *in vivo* growth conditions and at low concentrations, those sugars can be taken up by plants and used as an alternative source of carbohydrate thus stimulating growth. Uptake of those sugars has also been recorded in *in vitro* storage studies. For instance, Taylor *et al.* (1996) reported increased growth rates of sweet potato when stored on a medium that contained of less than 3% (w/v) concentrations of sorbitol

or mannitol. However, above a certain level, the use of such sugars may result in a negative water potential of the medium thus inhibiting growth. For example, growth rates of *Vitis vinifera* were retarded by incorporation of peodin 3-glucoside in the culture medium (Do and Cormier, 1991). It is this negative effect on growth, which often makes mannitol a successful growth retardant. In this study, therefore, the use of osmotic regulating sugars as growth retardants was tested. Shoots were stored on a medium consisting of 60 g.l⁻¹ mannitol, 0.5 MS nutrients, 0.5 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite. During storage, shoots exhibited stunted growth and no root development at all. On the second month of storage, they gradually started yellowing and by the end of four months they had all turned brown (Fig 2.4B). However, browning did not seem to have any significant effect on the rates of explant multiplication (Table 2.4). Although by the end of 6 months overall survival was still high (70%), multiplication rates had declined significantly from 6.20 ± 1.12 to 1.62 ± 1.51 and shoots had lost the ability to multiply by the end of 8 months in storage (Table 2.4). Percentage contamination that occurred as a result of subculturing ranged from 0 - 30 per transfer.

Sugar alcohols such as mannitol and sorbitol do not only act as a source of carbohydrate but are responsible for regulating osmotic potential of different media. Such sugars are usually not metabolised by plants although a few instances have been reported (Thompson and Thorpe, 1981). The approximate osmotic potential of a medium due to dissolved substances can be estimated from the total potential of macronutrients and that of sugars (George, 1993). Addition of sugars to a growth medium clearly plays a greater role in changing the osmotic potential of that medium. Thorpe (1978) showed that addition of 1%, 3%, 6% and 10% (w/v) mannitol to MS medium produced osmotic pressures of -4.4, -7.0, -10.9 and -15.2 bar, respectively. Concentrations that effectively inhibit growth seem to occur between 3 and 6% (w/v) (-7.0 and -10.9 bar). In this study mannitol was used at 6% (w/v) which would have reduced the osmotic potential of the medium to -10.9 bar.

Osmotic potential seems to have an influence on cellular processes during culture although how this occurs is still not very clear (Thorpe, 1978). Cells maintained in an

environment with low (highly negative) osmotic potential lose water and as a consequence the water potential of the cell decreases (Amador and Steward, 1987). This

Table 2.4 The effect of shoot storage on a medium containing mannitol on survival and multiplication. Shoots were stored on a medium consisting of 0.5 MS, 3.5 g.l⁻¹ Gelrite, 60 g.l⁻¹ mannitol and 0.5 g.l⁻¹ sucrose for 8 months. Samples were then subcultured to a multiplication medium consisting of MS nutrients, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.2 mg.l⁻¹ BAP, 30 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite at 28 °C under a 16 h light and 8 h dark photoperiod at 200 μE. m⁻².s⁻¹ PPFD for four weeks. Levels of significant difference (ANOVA) were represented by letters a-c. Multiplication rates with similar letters were not significantly different (f = 0.05). n = 50 shoots (5/bottle) ± SE

Time in storage (months)	% survival	multiplication (shoots/explant)	% contamination
0	100	6.20 ± 1.12 a	0
2	80	7.33 ± 2.22 a	20
4	90	4.13 ± 2.31 a	10
6	70	1.62 ± 1.51 b	30
8	80	0 c	20

brings about changes in metabolism such as accumulation of high levels of proline in cells and conditions of osmotic stress further induce an alternative oxidative system (George, 1993). These changes result in senescence and inhibition of cell division. The stunted growth that was observed in *Eucalyptus grandis* shoots stored in this study was probably due to inhibition of cell division.

It must be noted though that when osmotic regulators are used to retard growth, it is under the assumption that they are not taken up by plants as their uptake may be toxic but rather to alter the osmotic potential of the medium (Amador and Steward, 1987). However, with the long-term use of mannitol, this substance has been shown to occasionally penetrate into the cell thus causing abnormal growth characterised by small

leaves, short internodes and loss of vigour (Wescott, 1981b; Lipavska and Vreugdenhil, 1996). Our results clearly indicated a case of slow growth and loss of vigour, which could be due to the long-term use of mannitol or the possibility that it was taken up by shoots thus becoming toxic. The reduced growth metabolism resulted in the ability to store shoots for as long as 6 months.

2.3.1.4 The effect of ABA on stored shoot cultures

The effect of ABA as a growth retardant has been studied at various concentrations by several researchers (Wescott, 1981b; Grout, 1995; Taylor *et al.*, 1996). The most commonly used concentrations of ABA for growth and morphogenesis are those between 0 and 50 mg.l⁻¹ (Grout, 1995). In this study, shoots were cultured on a medium containing either 10 mg.l⁻¹ (Table 2.5) or 25 mg.l⁻¹ ABA (data not shown). It was observed that shoots stored on the medium that contained 25 mg.l⁻¹ ABA showed stunted growth, yellowing with subsequent browning and senescence in the first month of storage. Upon subculturing at the end of two months, shoots could not be regenerated indicating complete loss of viability. Therefore, all material stored in 25 mg.l⁻¹ ABA was discarded and no further tests were undertaken from that treatment.

Shoots cultured on a medium that contained 10 mg.l⁻¹ ABA (Table 2.5) continued to grow in storage such that by the end of 6 months an extensive root system had developed (Fig 2.4C). However, at this point, plantlets started showing signs of stress indicated by browning on the leaf margins and some of the leaves started falling off. Nevertheless, when subcultured, shoots still showed high survival and multiplication rates (e.g. 9.54 ± 2.04 after 6 months storage). Data shown on Table 2.5 also indicated that there was a significant increase in the rates of shoot multiplication after 4 and 6 months in storage compared with that prior to treatment. However, multiplication and survival rates after 8 months of storage declined significantly. Analysis of average multiplication rates (ANOVA) indicated that rates at 8 months had declined to similar rates as those at the beginning of the treatment.

Table 2.5 The effect of shoot storage on a medium containing 10 mg.l⁻¹ ABA on survival and multiplication. Shoot storage was on MS, 3.5 g.l⁻¹ Gelrite, 10 mg.l⁻¹ ABA and 30 g.l⁻¹ sucrose for 10 months. Samples were subcultured from the treatment medium to a multiplication medium consisting MS nutrients, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.2 mg.l⁻¹ BAP, 30 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite at 28 °C under a 16 h photoperiod at 200 μE.m⁻².s⁻¹ PPFD for four weeks. Levels of significant difference (ANOVA) were represented by letters a-b. Multiplication rates with similar letters were not significantly different (f = 0.05). n = 50 shoots (5/bottle) ± SE

Time in storage	% survival	Multiplication (shoots/explant)	% contamination
0	100	6.20 ± 1.12 a	0
2	80	4.26 ± 2.13 a	20
4	90	8.81 ± 3.58 b	10
6	80	9.54 ± 2.04 b	20
8	60	3.52 ± 3.17 a	40
10	80	3.94 ± 3.21 a	20

Abscisic acid, which occurs naturally in plants, has numerous regulatory effects on plants (George, 1996). It is often regarded as an inhibitor because it can control seed and bud dormancy and also due to the fact that it often inhibits auxins, which are responsible for cell elongation. Other regulatory effects include regulation of stomatal closure and control of water and ion uptake by roots, leaf abscission and senescence. It appears that depending on the concentration, ABA can either inhibit or promote growth, with lower concentrations promoting growth while higher concentrations are inhibitory. This would therefore explain why shoots stored on a medium containing 25 mg.l⁻¹ ABA (results not included) withered suddenly whereas those on 10 mg.l⁻¹ ABA (Table 2.5) showed increasing rates of growth and morphogenesis. Similar results were observed in studies

with potato cultures (Wescott, 1981b). That author noticed that the use of 15 mg.l⁻¹ ABA for *in vitro* storage of potato cultures resulted in better survival rates compared with storage using concentrations higher than 30 mg.l⁻¹. Several other instances have also been cited where ABA has been inhibitory to culture growth at higher concentrations for example adventitious shoots (Shepard, 1980), callus (Torizo and Zapata, 1986) and somatic embryos (Ammirato, 1974).

Storage of shoot cultures in a medium that contained relatively low concentrations of ABA (10 mg. l⁻¹) allowed continued growth of shoots for the first 6 months of storage (Table 2.5), after which multiplication rates started to decline. In total, shoot cultures were maintained in storage, using ABA, for 10 months with relatively high rates of survival and multiplication.

2.3.1.5 Storage of shoots under reduced nutrient concentration

Storage under reduced nutrients consisted of culturing shoots on a medium with half strength MS nutrients, 0.5 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite. Data showing contamination and survival rates, growth and development during storage and after subculture were shown in Table 2.6.

During the first 6 months of storage, shoots showed normal growth and extensive root development (Fig 2.4D). At the end of the seventh month, plantlets had grown very long reaching the lids of the storage jars. At this stage signs of stress were noted by the development of callus on the leaf margins, leaves curling and eventually abscission (Fig 2.4D). However, when subcultured, shoots still showed high survival and multiplication rates (Table 2.6). After removal from storage and transfer to appropriate medium, multiplication rates of shoots increased consistently until 6 months of storage, after which a decline was observed. The reduced rates of multiplication and survival after 8 months coincided with observed dehydration of media (Fig 2.4D). It was deduced that long-term storage of cultures on the same medium led to dehydration and lack of nutrients imposing a physiological stress on the plants. In this study, shoots were stored in tightly sealed culture bottles filled to about 1/3 capacity with medium to prevent this problem, although

this was obviously inadequate for such long storage periods. For future studies, alternative methods of preventing dehydration such as applying a layer of vaseline on the lids of the culture bottles could be applied.

Table 2.6 The effect of shoot storage on a medium with reduced nutrients on subsequent survival and multiplication. Storage period was 10 months on 0.5 MS, 3.5 g.l⁻¹ Gelrite and 30 g.l⁻¹ sucrose. Samples were subcultured from the treatment medium to a multiplication medium consisting of MS nutrients, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.2 mg.l⁻¹ BAP, 30 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite at 28 °C under a 16 h photoperiod at 200 µE.m⁻².s⁻¹ PPFD for four weeks. Levels of significant difference (ANOVA) were represented by letters a-b. Multiplication rates with similar letters were not significantly different (f = 0.05). n = 50 ± SE

Time in storage	% survival	Multiplication (shoots/explant)	% contamination
0	100	6.20 ± 1.12 a	0
2	60	8.13 ± 3.58 a	40
4	100	11.69 ± 4.83 b	0
6	70	11.46 ± 3.69 b	30
8	80	7.46 ± 1.05 a	20
10	90	13.65 ± 7.05 a	10

Alteration of media components has been used in various studies as a means of medium-term storage. Such alterations include reduction of carbohydrate supply (Wescott, 1981a; Gunning and Langerstedt, 1986), reduction of MS media components or concentration (Schnapp and Preece, 1986; Moriguchi and Yamada, 1989; Bonnier *et al.*, 1997) and/or reduction of inorganic nutrients (Kantha *et al.*, 1981a; Vysotskaya, 1994). The approach that was taken in this study was that of reducing MS concentration. Contrary to results

obtained in other studies (Kantha *et al.*, 1976; Schnapp and Preece, 1986; Bonnier *et al.*, 1997), reducing MS concentration from full to half did not reduce growth *in vitro*. The observed effects of reduced nutrient on shoot storage were rather similar to those characteristic of 'laissez-faire' where cultures under normal growth conditions are left untouched during storage and because of the depletion of media nutrients, shoot growth becomes retarded (George, 1993). *Coffea* sp. for example was stored for two years (Kantha *et al.*, 1981b) and *Mentha* sp. for 6-13 months (Gunning and Langerstedt, 1986) by leaving the media untouched. However, the use of reduced nutrient treatment was very effective in increasing periods of shoots storage of up to 10 months while maintaining very high rates of multiplication and survival.

2.3.1.6 Summary remarks on the effect of tested storage treatments

One of the advantages to be derived from using *in vitro* culture techniques for conserving germplasm is the ability to maintain cultures 'clean' (Grout, 1995; George, 1996; Blakesley *et al.*, 1996; Watt, Thokoane, Mycock and Blakeway, 1998b). In all treatments tested, shoot storage was generally associated with very little contamination. Most of the contamination recorded (Table 2.3; 2.4; 2.5 and 2.6) was due to subculturing of shoots from the different storage media to the multiplication medium. Contamination might present problems with regard to meeting targets for commercial production. However, this problem can be avoided by storing about 20 - 30% more explants than estimated.

A comparison of all four storage treatments (Fig 2.5) indicated that mannitol and reduced light and temperature conditions were more effective as a method of retarding growth. Using these protocols, shoot cultures were maintained for 6 months (multiplication rates of 3.88 ± 2.90 and 1.62 ± 1.51 reduced temperature and mannitol respectively) without subculturing. Storage on a medium containing mannitol and at 10 °C could be used as short-term storage of germplasm for material required on a seasonal basis. It may also benefit as a temporary means of storing cold sensitive species such as *E. grandis* to avoid putting them in the greenhouse thereby obviating heating costs. These temporary means of storage may be useful in times of limited staff availability such as long holidays.

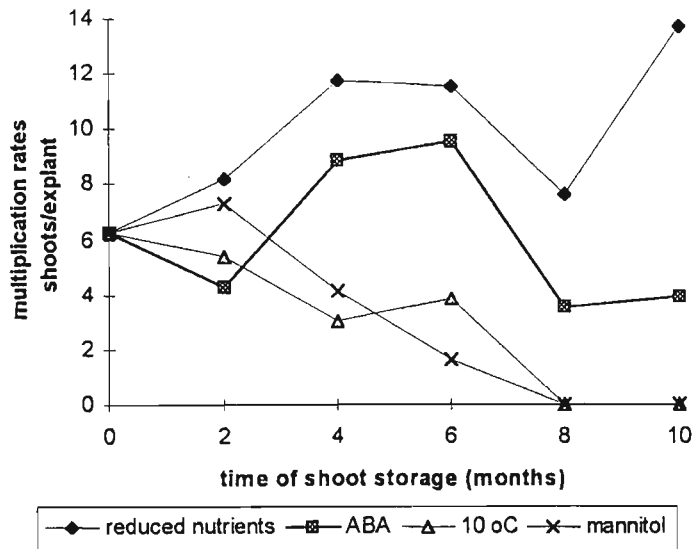


Fig 2.5 Comparison of multiplication rates of shoots after retrieval from four different storage treatments. Shoots were stored as follows: **A) reduced nutrient** :- consisted of MS nutrients, 0.5 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite. **B) ABA** :- consisted of MS nutrients with 30 g.l⁻¹ sucrose, 10 mg.l⁻¹ ABA and 3.5 g.l⁻¹ Gelrite. **C) 10 °C** :- cultures were stored low light and temperatures conditions on a medium which consisted of 0.5 MS, 0.5 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite. **D) Mannitol** :- consisted of storage on a medium with 0.5 MS, 60 g.l⁻¹ mannitol, 0.5 g.l⁻¹ sucrose and 3.5 Gelrite. Treatments were at 28 °C under a 16 h photoperiod at 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD, except for storage at 10 °C where light was continuous at 4 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Every two months, shoots were subcultured a multiplication medium (MS, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.2 mg.l⁻¹ BAP, 30 g.l⁻¹ sucrose and 3.5 g.l⁻¹ Gelrite) at 28 °C at 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD.

Material can be kept in storage until needed. Shoots can also be held over temporarily during peak production and heavy greenhouse utilisation until space is available in the greenhouse. In contrast, the use of ABA or reduction of nutrients during storage may not have successfully reduced growth but the two methods enabled storage for 10 months without subculture while relatively high explant multiplication rates of 3.94 ± 3.21 and 13.65 ± 7.05 respectively were retained. These two forms of storage can be used as a medium-term storage facility for materials required annually. For instance, during breeding procedures it is sometimes necessary to maintain all potential parents vegetatively, it may also be desirable to propagate the chosen parents so that sufficient plants are available to produce a large amount of seed. The ability to store and retrieve material with high multiplication potential would therefore lessen time and labour requirements. This is a particular advantage in tree breeding where it is necessary to keep ramets from a clone while others are field- or progeny-tested. For this purpose, cultures would have to be maintained for years so that they could be available for propagation on a large scale once promising genotypes have been identified (Aitken-Christie and Singh, 1987).

Both *in vitro* storage protocols developed in this study meet one of the essential requirements of such conservation protocols, that of security against loss by pests, pathogens and climatic hazards. Moreover, maintaining hundreds of cultures on a few shelves rather than the many hectares that would have been utilised as field provenances saves vast amounts of space.

2.3.1.7 Effect of temperature on viability of stored alginate encapsulated buds

Synthetic seeds (also known as artificial seeds) can be considered as encapsulated somatic embryos and/or axillary buds engineered to be of use in commercial plant propagation (Piccioni, 1997). The most widely used method of producing synthetic seeds is hydrogel encapsulation with the use of 3 % (w/v) sodium alginate and calcium chloride (e.g. Gill, Senaratna and Saxena, 1994; Piccioni and Standardi, 1995; Pliegoalfaro *et al.*, 1996; Maruyama *et al.*, 1997; Castillo *et al.*, 1998). The mechanism by which alginate capsules are formed is a function of the cations and the complexing time (Gill *et al.*,

1994). No heat is required for the production of gels as sodium alginate complexes when mixed with a di- and trivalent metal cation to form calcium alginate via formation of ionic bonds between carboxylic acid groups on the guluronic acid molecules (Redenbaugh, 1993). Minimum complexing time has been found to be 15 minutes otherwise the resultant beads might still be liquid inside (Redenbaugh *et al.*, 1991; Piccioni, 1997). Based on the reviewed literature, *Eucalyptus* sp. buds were encapsulated in this study using 3 % (w/v) sodium alginate complexed with calcium chloride for 25 minutes. The resultant synthetic seeds were stored at different temperatures for 6 months (Table 2.7).

Table 2.7 Survival of encapsulated buds after storage at 10 °C and 28 °C for 6 months. Buds were stored in jars on a water based non-nutritive medium at 10 or 28 °C for 6 months. Every month, samples were retrieved and cultured on multiplication medium consisting of MS, 30 g.l⁻¹ sucrose, 3.5 g.l⁻¹ Gelrite, 0.01 g.l⁻¹ ascorbic and 0.01 g.l⁻¹ citric acid, in the growth room at 16 h/8 h photoperiod at temperatures between 24 - 28 °C for four weeks. Survival was scored when shoots had emerged from within the capsule and were about 10 mm long.

Time in storage (months)	10 °C	28 °C
0.5	100	100
1	90	100
2	87	60
3	93	0
4	59	0
6	47	0

Several methods have been successfully used for storage of encapsulated explants. Such methods include pretreating explants with ABA to enhance desiccation tolerance (Senaratna *et al.*, 1989; Anandarajah and McKersie, 1990; Lecouteux *et al.*, 1993; Pliegoalfaro *et al.*, 1996) followed by desiccation of capsules in the laminar flow

(McKersie *et al.*, 1989; Anandarajah *et al.*, 1991), reduction of light and temperature (Pliegoalfaro *et al.*, 1996), minimum-nutrient supply (Maruyama *et al.*, 1997) and/or a combination of treatments (Bapat, 1993; Piccioni, 1997; Castillo *et al.*, 1998). Pretreatment with ABA and desiccation is more often used for somatic embryos while the combination of light, temperature and nutrients has been attempted for encapsulated buds of many woody species (Bapat, 1993). For example, Maruyama and co-workers (1997) in their work with *J. mimosaeifolia*, *C. odorata* and *Guazuma crinita* found that storage of encapsulated buds of those species in a water-based, non-nutritive medium at reduced light and temperature effectively suppressed growth. In this study, alginate beads were therefore stored in jars (Fig 2.6), and maintained on a non-nutritive water-based medium at either 10 or 28 °C. Buds were observed carefully during the first few months of storage and it was noted that those stored at 28 °C continued to grow steadily such that at the end of one month they had grown slightly out of their capsules. By the end of two months, water in the storage jars had evaporated completely causing buds to be entirely dehydrated and this resulted in total loss of viability within three months.

Several factors could have contributed towards the short storage period of buds at 28 °C. One of them was that although shoots were stored on a non-nutritive medium consisting of water alone, the fact that the sodium alginate was prepared with 0.3M sucrose and this might have presented enough carbohydrate for the continued growth of buds during storage. Moreover, storage of shoots at 28 °C was theoretically under optimal temperature conditions which would have contributed towards the continued growth and development of stored buds. Another factor that could have caused failure of this form of storage is the fact that buds were stored in a relatively large container which allowed rapid evaporation of water resulting in total dehydration of buds leading to senescence.

Storage at 10 °C was more successful with buds retaining viability for up to 6 months (Table 2.7). However, due to the limited material available, the effect of storage time on survival was not studied beyond six months. Buds stored at 10 °C behaved similarly to shoots stored under slow-growth conditions whereby, low temperatures reduce metabolism of stored explants increasing longevity (Bapat, 1993; Redenbaugh, 1993).

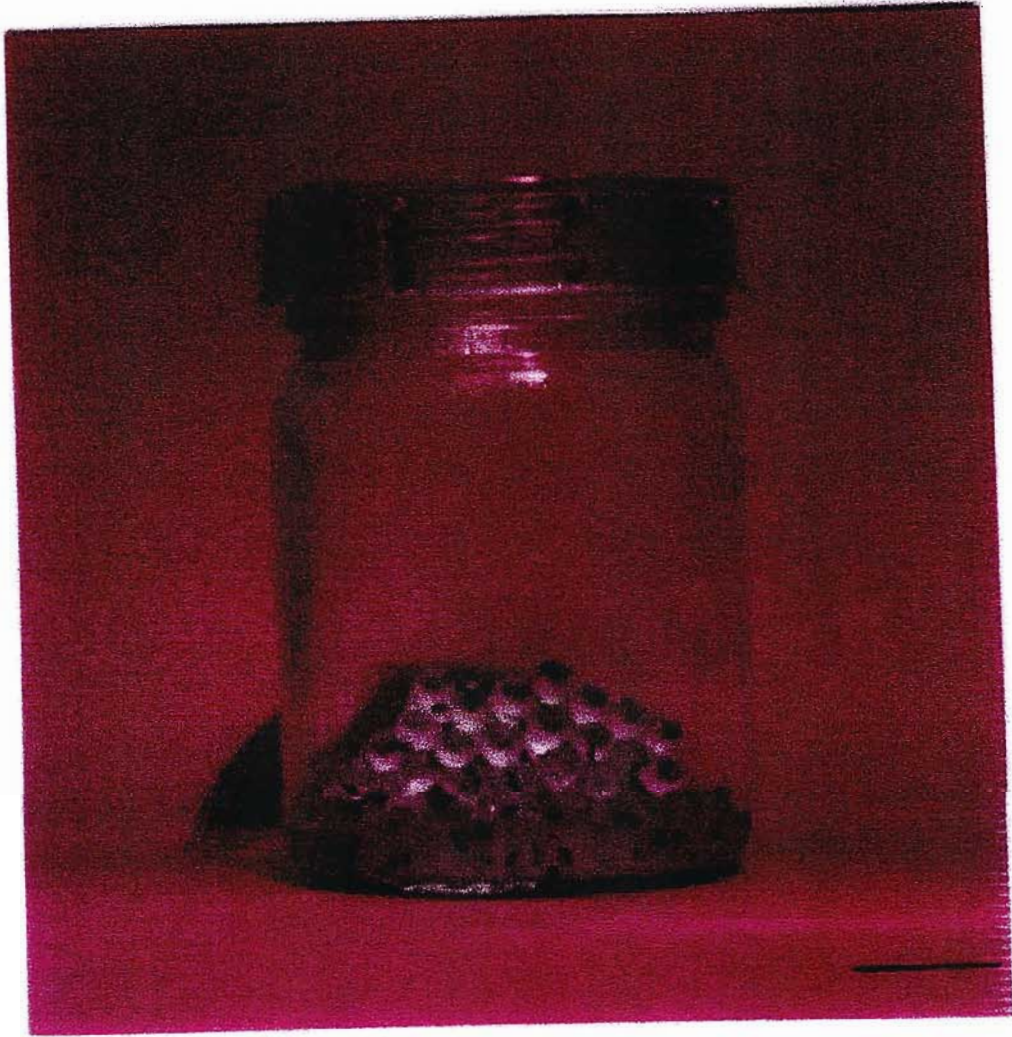


Fig 2.6 Storage of encapsulated buds in 60 ml jars at 10 °C [bar = 16.9 mm].

Synthetic seeds have many diverse applications in agriculture particularly in the forestry industry (Redenbaugh *et al.*, 1991; 1993; Maruyama *et al.*, 1997; Piccioni, 1997). In tree breeding programmes, where vegetative propagation and clonal multiplication play such an important role (1.1.2), the ability to store buds as synthetic seeds for 6 months as shown (Table 2.7) with high survival rates (47 %) would be a great benefit. This advantage would allow for buds to be retrieved and multiplied for supply on demand. Moreover, heterozygous plants that cannot be stored by conventional seed storage methods due to genetic recombination can be stored using buds (Fujii, Slade and Redenbaugh, 1989; Lai, Lecouteux and McKersie, 1995). As discussed earlier (1.1.2), hybrid production is another important aspect of tree breeding. However, most hybrids are sterile and/or do not set seed, storage of encapsulated buds obtained from those hybrids would therefore be an alternative method of conserving such germplasm. Furthermore the use of encapsulated buds could be an alternative method of storing hybrid material created by cross-pollination, since at times it may not be possible to repeat crosses for seed production because of the associated expenses.

Synthetic seed technology may also be applied in short-term storage of propagules to enable synchronised planting commodities in a greenhouse (Grout, 1995). One of the limitations of current micropropagation techniques is that the tissue culture facility and the greenhouse production facility must be physically linked, that is, there must be immediate transfer of tissue culture products into the greenhouse so that production could be synchronised in order to meet peak market demand (Grout, 1995; Gill *et al.*, 1994). Synthetic seeds could be used to uncouple this linkage enabling micropropagation to be done all year round at a site distant from the production facility.

2.3.2 Medium-term storage of pollen

2.3.2.1 Determination of pollen viability

As discussed in section 2.1.3.3, pollen viability can be assessed using staining, germination, seed or fruit-set techniques. In most studies involving testing viability of *Eucalyptus* pollen, germination (Boden, 1958) or seed-set techniques (Sedgley and Smith, 1989) have been used. No information was found regarding the use of staining

techniques for viability assessment of *Eucalyptus* spp pollen. Thus, in this study, in addition to germination, staining techniques were investigated.

Different pollen species require varying periods of incubation in stains before the necessary colour changes can be observed (Vietz, 1952). It is therefore, essential to establish optimal incubation time in staining solutions for each pollen species prior to establishment of staining protocols. This section was therefore aimed towards the establishment of suitable times of incubation for pollen of *Eucalyptus* sp in MTT and TTC assays as well as determining the reliability of each assay. Towards this, samples of pollen incubated at 28 °C in either TTC or MTT assays were retrieved and viewed with an Olympus light microscope at 6 h or 15 min intervals respectively. Observations were recorded in accordance with pollen colour changes: light red to red (Fig 2.7A) and red to black indicated viability with TTC and MTT assays respectively. In both cases non-viable pollen remained yellow (Fig 2.7B).

Staining in the cytoplasm by tetrazolium salts occurs as a result of reduction of the salts by the dehydrogenase enzymes within the cell to produce an insoluble red formazan complex (Cook and Stanley, 1960). As shown in Fig 2.8A, at 6 h, insufficient stain had penetrated the cell to initiate the reduction process, hence fewer pollen grains stained positive. At 12 h, maximum penetration had occurred and the highest number of stained grains was scored (1.5%, 11.4% and 15.8% for pollen harvested in February, May and June, respectively). After 12 h, percentage viability started to decline significantly, and the initially colourless staining solution changed to red implying that the stain leaked out of pollen tissue when kept for longer than 12 h. Incubation of cells overnight (approximately 12 h) using TTC was also found to be effective for *Vitis vinifera* (Iborra *et al.*, 1992), while incubation times of 4 h or less have also been recorded (e.g. Norton, 1966; Enikeev *et al.*, 1995; Khatun and Flowers, 1995; Popov and Vysotskaya, 1996). All subsequent TTC assays were incubated for 12 hours.

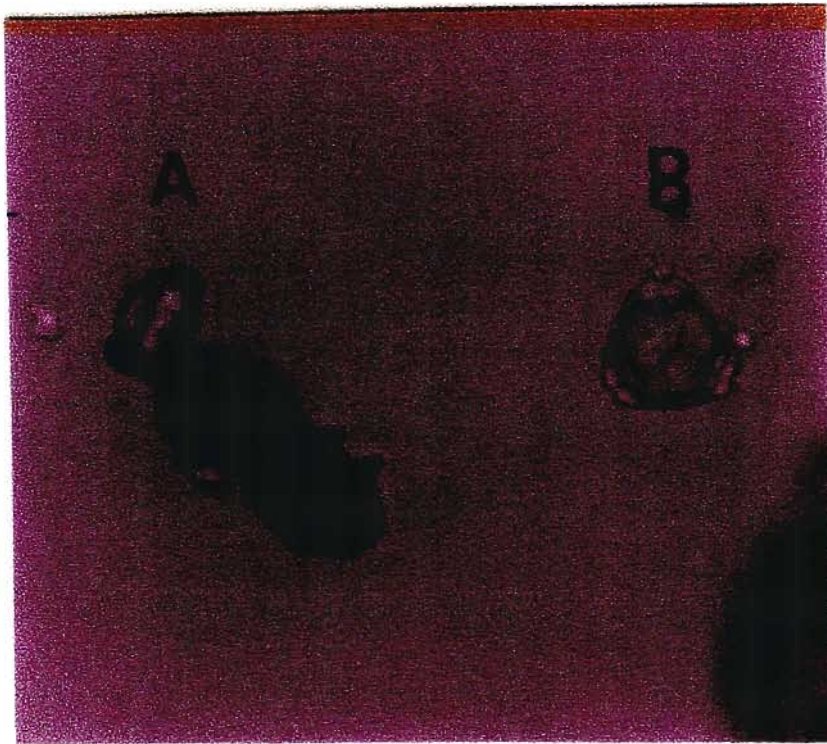


Fig 2.7 Pollen assayed with 2,3,5-triphenyl tetrazolium chloride salt (TTC) A) Positively stained pollen assayed with tetrazolium chloride salt showing red colour staining [**bar = 25 μm**]. **B)** unstained non-viable pollen [**bar = 25 μm**]. Dry pollen (0.02 g) was placed in Eppendorf tubes, mixed with 1% (w/v) TTC staining solution and incubated at 28 °C for 12 h after which a drop of the polle/TTC mixture was mounted on a haemocytometer slide and viewed using an Olympus light microscope.

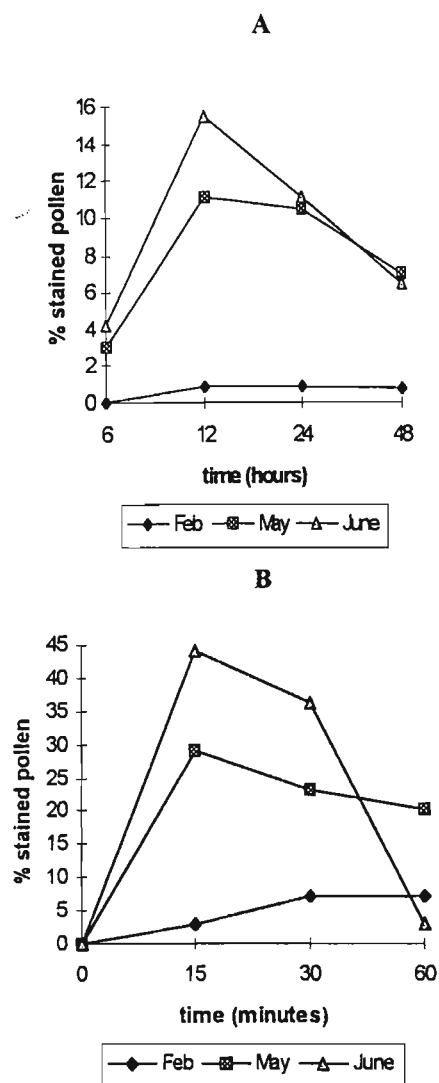


Fig 2.8 Percentage of stained pollen grains after various incubation times in two tetrazolium salts. A) 1% (w/v) 2,3,5-triphenyl tetrazolium chloride (TTC) B) 0.9% (w/v) 3-{4,5-dimethyl-thiazolyl 1-2-}-2,5-diphenyl monotetrazolium bromide (MTT). In both tests, three lots of pollen were tested (pollen harvested in February, May and June 1996). Pollen (0.02 g) was mixed with either 1% (w/v) TTC or 0.9% (w/v) MTT stains then incubated at 28 °C for 60 h and 60 min.

Results shown in Figure 2.8B indicated that optimum incubation time for MTT to be at 15 min, where maximum percentage staining of pollen was observed (3.5%, 30% and 44% for pollen harvested in February, May and June respectively). At longer incubation times, a decline in the number of stained pollen grains accompanied by changes in the colour of the staining solution from originally yellow to black was observed. Therefore it was concluded that incubation periods of 15 min for MTT assays would be used for all subsequent investigations. The incubation periods obtained here were similar to those obtained in other studies for example, rice pollen (Khatun and Flowers, 1995), mammalian cells (Rexan and Emborg, 1992) and plum pollen (Norton, 1966).

It should be noted that the three pollen lots tested were harvested at different times, kept in storage in the presence of silica gel at 4 °C for varying periods of time then assessed for viability. There was a significant difference in viability with regard to harvest times as tested using both TTC and MTT assays. With both assays, pollen harvested in June had the highest viability followed by that of May while that which was harvested in February had the least viability. These results could be due to the fact that at the time of viability testing, lots obtained in February and May had been stored in silica gel for 3 and 1 month respectively. It was assumed, therefore, that pollen harvested in May and June was still relatively fresher than that which was harvested in February. Moreover, storage in the presence of silica gel, as would be shown later (Section 2.3.2.3. Fig. 2.10) had an adverse effect on *E. grandis* pollen.

In order to test the reliability of each tetrazolium assay, pollen samples of 0.02 g were killed by heating in a water bath at 80 °C for 24 h then tested with MTT or TTC stains (Table 2.8). This approach was taken as according to Oberle and Watson (1953), tetrazolium salts tend to give inconsistent results when used to assay pollen viability. Other reports where pollen was killed by increased temperature include rice (Khatun and Flowers, 1995), *Cannabis sativa* (Zottini, Mandolino and Ranalli, 1996) *Banksia menziesii* (Maguire and Sedgley, 1997).

Table 2.8 Percentage viability of pollen after heating (at 80 °C) for 24 h.

Viability was tested using either MTT and TTC assays.

Stain	Treatment	Approximate % Viability
TTC	Heated	0
TTC	not heated	64.6±1.6
MTT	Heated	72.5 ± 1.3
MTT	not heated	89.6 ± 2.8

It was expected that very few or no pollen grains would be viable after heating when tested with either of the salts, but these results were observed only with the TTC assay. On the other hand, MTT assay showed no significant difference between the percentage of dead and viable pollen (Table 2.8). The positive staining of dead pollen with MTT could have been brought about by a heat stable component of the pollen. A similar hypothesis was put forward by Khatun and Flowers (1995) when they observed that some of the rice pollen they had killed by exposure to high temperatures tested positive with MTT. From the results discussed above, it was concluded that MTT assay was an inaccurate method of testing viability in *Eucalyptus* sp. as results could not accurately reflect the state of pollen cells. Therefore all subsequent viability assays were conducted using TTC assays.

2.3.2.2 Assessment of pollen germination *in vitro*

In contrast to the staining methods, *in vitro* pollen germination has been found to correlate well with *in vivo* pollen germination (Stanley and Linskens, 1974) and is regarded as an accurate measure of viability (Karthi, 1985). Therefore, a successful system of *in vitro* pollen germination is a pre-requisite for pollen research, male gametophyte selection in eucalypt breeding (Potts and Marsden-Smedley, 1989) and is important for testing the quality and viability of pollen that is to be used for controlled crosses (Griffin, Ching, Johnson, Hand and Burgess, 1982; Heslop-Harrison and Heslop-Harrison, 1970). Although there is sufficient literature on the handling and storage of eucalypt pollen (Boden, 1958; Eldridge, 1978; Griffin *et al.*, 1982), very little

information is available on pollen germination in the species. From the reviewed literature on *in vitro* pollen germination of eucalypts, success has been achieved mainly with the use of liquid medium (Griffin *et al.*, 1982) or semi-solid media (Cauvin, 1984; Marien, 1988) on a BK (Brewbaker and Kwack, 1963) pollen germinating medium with 10 - 30% sucrose.

Investigations of *in vitro* pollen germination in this study were hampered by both bacterial and fungal contamination (results not shown). To establish a sterilisation protocol, varying concentrations of sodium hypochlorite were tested on dry or imbibed pollen. After each step of sterilisation, a preliminary viability test was conducted with TTC assay and pollen that stained positive was germinated *in vitro*. Regarding TTC staining, when no or a low concentration (0-0.05 % [v/v]) of sodium hypochlorite was used, viability determined using TTC assay remained fairly high (22.3 - 54.5%). However, due to high contamination rates, no distinction could be made between fungal hyphae and pollen tubes during subsequent *in vitro* germination, and it was impossible to record percentages of germination (Table 2.9). In an attempt to reduce contamination, sodium hypochlorite concentration was increased and viability tests conducted. Viability (TTC assay) declined steadily with increasing concentrations of the sterilant. After treatment with 0.05 % with sodium hypochlorite viable pollen (as determined using TTC assay) was assessed for germination ability. Pollen germination was attempted on BK medium, pollen was viewed with a microscope and results recorded. Although viewing was no longer hampered by fungi as before, the pollen had been affected by the high concentrations of sodium hypochlorite such that no germinating pollen tubes were scored. At concentrations above 0.1 % (v/v) sodium hypochlorite, viability was 0 with TTC, thus samples were not tested for germination.

Data shown on Table 2.9 also indicated that imbibing pollen before sterilisation generally resulted in higher viability compared with non-imbibed pollen. For example, 22.3% compared with 6.8 % positively stained pollen was observed after sterilising with 0.05 % (v/v) sodium hypochlorite imbibed and non-imbibed pollen, respectively. Mature pollen

Table 2.9 The effect of pollen surface sterilisation on viability as tested using TTC and *in vitro* germination techniques. Pollen was sterilised using various concentrations of sodium hypochlorite, centrifuged and the sterilant decanted. It was then centrifuged three times in sterile water to remove all traces of the sterilant. After sterilisation, pollen was assayed with TTC as described on section 2.2.3.2.a and percentage viability recorded for the various concentrations. I refers to imbibition of pollen for 1 hr prior to sterilisation, NI was used in cases where pollen was not imbibed. n = 3 ± SE

<u>% (v/v) sodium hypochlorite</u>	<u>treatment</u>	<u>av. % viability</u>	<u>% germination <i>in vitro</i></u>
control	NI	54.7 ± 1.6	*
0.01	I	49.0 ± 1.3	*
0.01	NI	46.5 ± 1.8	*
0.05	I	22.3 ± 2.5	*
0.05	NI	6.8 ± 1.6	0
0.1	I	5.0 ± 1.2	0
0.1	NI	0	-
0.2	I	0	-
0.2	NI	0	-
1	I	0	-
1	NI	0	-

* was used to denote cases where *in vitro* germination was attempted but due to the high fungal contamination, accurate determination of pollen tube growth was not possible.

A dash (-) was used in cases where *in vitro* germination was not attempted because viability as tested using TTC assays was 0.

grains are enclosed in a double wall structure, the inner wall is called the intine, and the outer one is referred to as the exine. The role of these walls is to protect the sensitive nuclei. When pollen is imbibed, water is conducted throughout the wall softening the hard protective double layer (Stanley and Linskens, 1974; Brogalia and Brunori, 1994). This would therefore have facilitated easier penetration of the staining salts into the cells, hence the relatively higher indication of percentage viability whenever pollen was imbibed prior to assay.

In addition to testing different concentrations of sodium hypochlorite, another way of eliminating fungal contamination attempted was germination on a BK medium containing of 100 mg.l⁻¹ Benlate. This medium successfully eliminated growth of contaminants while retaining pollen germination to about 26 ± 3.0 % (Fig 2.9). According to Kartha (1985), the major components for germinating pollen of most woody species are sucrose (10-40 % [w/v]) and boron (50 - 200 mg.l⁻¹) calcium and magnesium (Visser, 1955). Sucrose is believed to provide an energy source as well as being a suitable osmoticum and current evidence suggests that the external sucrose is metabolised by the germinating pollen. Boron is also an essential component for germinating pollen. Lack of boron was shown to inhibit pollen tube formation of Douglas fir (Fernando, Owens, Vonaderkas and Takaso, 1997). Kartha (1985) also suggested that if pollen germination is assessed in the absence of boron, results would be highly inaccurate. The BK medium that was tested had an adequate composition of all the required elements, hence optimum germination should have been obtained. Moreover, the same medium was successfully used for other *Eucalyptus* species by other authors (e.g. Boden, 1958; Potts and Marsden-Smedley, 1989).

In vitro pollen germination has the advantage of accuracy and better correlation with *in vivo* germination compared with staining techniques. However, the application of staining techniques has the advantage of being rapid and thus saves a lot of valuable time for day-to-day breeding activities. Moreover, stains are very useful in field conditions as unlike *in vitro* germination, they do not require special equipment (Norton, 1966). Optimisation of pollen germination conditions may also be difficult to obtain therefore staining can be



Fig 2.9 *In vitro* germinating pollen grain after sterilisation [bar = 25 μm]. Pollen was germinated on a BK (Brewbaker and Kwack, 1963) medium consisting of 10 mg.l^{-1} H_3BO_3 , 30 mg.l^{-1} $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 20 mg.l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg.l^{-1} KNO_3 and 200 g.l^{-1} sucrose (Boden, 1958). A drop of the medium was placed on a sterile coverlip in a sterile Petri dish and dry pollen was slowly tapped on top of the drop. Pollen was incubated for 48 h at 28 °C under a 16 h photoperiod at 200 $\mu\text{E.m}^{-2}.\text{s}^{-1}$ PPFD. Germinated pollen was placed on haemocytometer and viewed under a light microscope.

used as an alternative viability assessment method (Stanley and Linskens, 1974; Kartha, 1985). Lorenzo and de Almeida (1997) showed that because of optimisation problems, the use of acetic carmine staining solution for pollen viability assessment of onion hybrids correlated well with field germination in contrast to germination obtained on various culture media. Nevertheless, such cases where staining techniques correlate with *in vivo* germination better than *in vitro* germination are quite rare.

In order to combine and effectively use the accuracy of germination and rapidity of staining techniques, it would be ideal to establish both assays and estimate correlation between the two could be made (Khatun and Flowers, 1995). In such cases, whenever rapid viability estimates are required, staining would be applied and germination rates could be calculated. Thus, in this study, TTC and germination viability results were compared and a correlation between the two was estimated. Results obtained indicated that for pollen that had $56 \pm 2.5\%$ viability after assaying with TTC, germination *in vitro* was about $26 \pm 3.0\%$ (Table not shown). It should be noted that different sub-samples were used for TTC and germination assays. This therefore indicated that approximately half the pollen that stained positive would to be expected to germinate *in vitro*.

2.3.2.3 Storage tests

Any attempts in plant breeding programmes that involve production of controlled pollinated seed require development of an efficient pollen storage system (Hawtin, Iwanaga and Hodgkin, 1996). Some of the desirable features of such a system are a) that highly viable pollen must be available whenever required by the breeder and b) that storage space requirements of the system must be as few as possible. Most research regarding storage of *Eucalyptus* pollen (e.g. Boden, 1958; van Wyk, 1977; Eldridge, 1978) fall short of the above mentioned requirements (Griffin *et al.*, 1982). The method recommended by Boden (1958), which involved storage of flowers at anthesis stage in a deep freeze ($-16\text{ }^{\circ}\text{C}$), was at least able to meet the availability requirement. However, it involved the use of unnecessarily large amounts of space. Griffin *et al.* (1982) were able to design a method enabling constant availability whilst utilising minimum amount of space for *Eucalyptus regnans*. Our storage methods for *E. grandis* were thus based on an

approach similar to that of Griffin *et al.* (1982). Those authors investigated storage at room temperature (10 - 35 °C), refrigerator (5 °C) and deep freezer (-16 °C). In our investigation, the following treatments were analysed: room temperature (28 °C), refrigerator (4 °C), and cryogenic storage (below -70 °C). This chapter reports only on medium-term storage, that is, storage at 4 and 28 °C. Both treatments included storage in the presence or absence of two types of drying agents, silica gel and talc (Johnson and Johnson Baby powder). The use of talc as a desiccating agent was described by Kindiger and Dewald (1993). Those authors reported the use of talc for pollen storage of gamagrass to have increased longevity as well as prevented pollen from becoming sticky or clumpy. Silica gel has been customarily used as a drying agents in experiments investigating storage of pollen, examples including *Eucalyptus regnans* (Griffin *et al.*, 1982) and pine (Matthews and Kraus, 1981).

Storage in the presence of talc presented problems with regard to viability analysis. Talc could not be separated from pollen prior to evaluation and therefore, TTC assay was used on the mixture. Talc, due to its finer particles formed a cloud or suspension that did not allow visibility of viable pollen when viewing with a microscope. All treatments with talc were therefore discarded.

With regard to storage in the presence of silica gel, results obtained indicated that pollen lost viability after 6 months when stored at room temperature, whereas the absence of silica gel during storage increased longevity by 2 months (Table 2.10). Storing pollen under reduced temperature (4 °C) without silica gel increased pollen longevity as tested using TTC to ten months with viability of 6.73%. In both cases pollen had longer storage life if it was maintained without silica gel. These results therefore led to the conclusion that generally, pollen favoured storage at low temperatures without silica gel. These results were similar to those of Griffin *et al.* (1982). That author observed that storage of *E. regnans* pollen for less than 6 months was not significantly affected by temperature, whereas if pollen was stored for more than 6 months, lower temperatures were more effective than room temperature. Several other researchers, for example, Kindiger and Dewald (1993) and Snyder (1961) using Mexican gamagrass and southern pine

respectively, made similar observations. Those authors observed that pollen longevity increased with decreasing temperature. Our results, however, contradicted those of other researchers regarding desiccation of pollen during storage. While most species seem to favour storage in the presence of a desiccating agent (e.g. Barnabas *et al.*, 1988; Hughes, Lee and Towill, 1991; Kindiger and Dewald, 1993; Kovacs and Barnabas, 1993), results obtained in this study (Table 2.10) showed a consistent decline in pollen when pollen was stored in the presence of silica gel (Table 2.10). It was therefore essential to investigate further the effect of silica gel on pollen viability.

Pollen was placed in a weighing boat in a jar half-filled with silica gel to dry over 24 hours (section 2.2.3.1a). Fresh weight and pollen viability (TTC assay) were monitored through out the period of desiccation. Initial weight of 0.445 g declined to 0.418 g in the first half of an hour of desiccation (Fig 2.10). This was accompanied by viability loss of 23%. Thereafter, fresh weight stabilised with a final weight of 0.415 g after 24 h.

Table 2.10 Percentage stained pollen grains stored for 10 months as determined using the TTC assay. + SG = with and - SG = without silica gel. Levels of significant difference (ANOVA) were represented by letters a-g (f = 0.05). n = 3 ± SE

Time (months)	storage at 4 °C		storage at 28 °C	
	+ SG	- SG	+ SG	- SG
0	100	100	100	100
2	38.07 ± 1.02 d	80.76 ± 3.45 g	53.84 ± 0.10 e	22.61 ± 1.22 c
4	39.61 ± 2.10 d	62.30 ± 2.21 f	11.53 ± 2.21 b	25.50 ± 1.91 c
6	32.69 ± 3.37 d	32.49 ± 5.53 cd	0	6.61 ± 1.10 a
8	10.11 ± 1.12 b	12.76 ± 3.21 b	0	0
10	0	6.73 ± 1.21 a	0	0

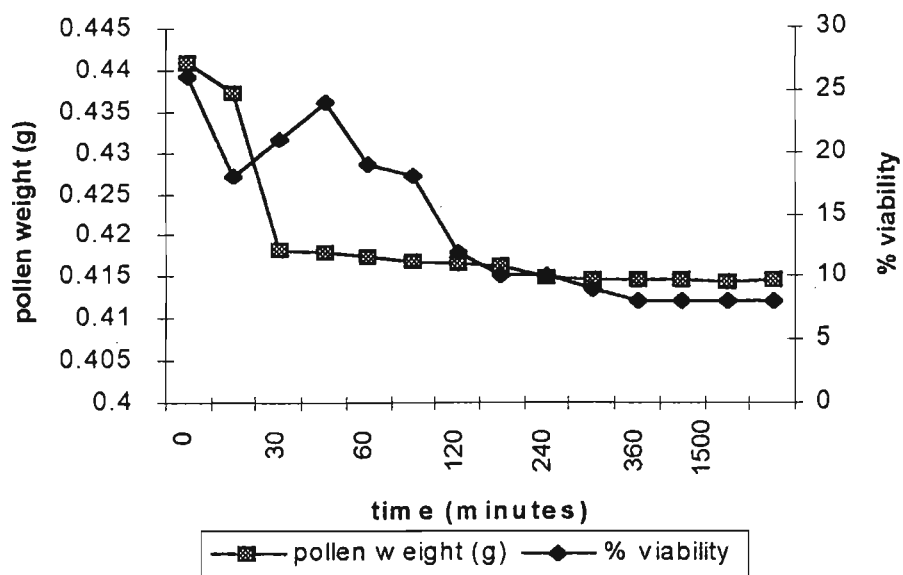


Fig 2.10 The effect of drying with silica gel on pollen viability and fresh weight. Two aluminium-weighing boats were each placed in a 100 ml jar with 60 g silica gel. Samples were taken from one boat and viability assessment using TTC assay conducted after every 15 min while the other fresh weight was recorded at similar time intervals.

However, by the end of 24 h viability had declined to 7%, indicating a total viability loss of 73% (Fig 2.10). These results confirmed the previous observation that silica gel had adverse effects on the pollen of *E. grandis*. However, it should be mentioned that results obtained in Fig 2.10 were of pollen that had been stored for 3 months in silica gel. This was depicted by the low initial survival rates observed (Fig 2.10) thus further confirming pollen sensitivity to desiccation. From work presented here on pollen, it can be concluded that the best methods of storage are those that do not require desiccation, and storage should be at low temperatures (4 °C).

The success of *Eucalyptus* sp. pollen storage obtained in this study is of considerable value to breeding activities practised at Mondi. Maintenance of pollen with acceptable germination competence will allow for hybridisation of species that flower at different times and those that are at distant places. It will also allow for international and national germplasm exchange. The ability to determine pollen viability rapidly using staining and germination techniques is an added advantage as time requirements of cross pollination are reduced and the costs of carrying out crosses using non-viable pollen will not be incurred. Pollen once tested and found to be viable but not necessarily fertile, may still be used for other purposes. Viable but infertile pollen of many woody perennials e.g. poplar (Kexian and Nagarajan, 1990) and apple (Zhang, Lespinasse and Chevreau, 1990b) has been used for the production of haploids by pollination-induced egg cell development.

Viable infertile pollen is also useful as mentor or pioneer pollen for intra-specific and other incompatible hybridizations (Shintaku, Yamamoto, and Nakajima, 1988; Andreichenko and Grodzinskii, 1991). The ability to store pollen temporarily, allows geneticists and/or breeders to accomplish crosses which hereto were not possible due to the occurrence of varied flowering periods. It also offers breeders the opportunity to extend their germplasm resources and expand their research capabilities.

2.4 Conclusions

Medium-term storage of shoot cultures, axillary buds and pollen is important in the forestry industry to facilitate production of genotypes with superior qualities. Of the various minimal-growth conditions investigated, reduced nutrient was found to be the best storage method and shoots were maintained for 10 months with high multiplication levels of 13.75 ± 7.05 shoots/explant. Axillary buds encapsulated in 3% (w/v) sodium alginate and in 0.3M sucrose were stored in jars at 10 °C or 28 °C. Of these two treatments, viability was sustained for longer (6 months) with the former method, compared with 4 months with storage at 28 °C.

Before establishing pollen storage, viability assessment methods were determined and these consisted of *in vitro* germination on a BK (Brewbaker and Kwack, 1963) medium consisting of 10 mg.l⁻¹ H₃BO₃, 30 mg.l⁻¹ Ca(NO₃)₂.4H₂O, 20 mg.l⁻¹ MgSO₄.7H₂O, 10 mg.l⁻¹ KNO₃, 200 g.l⁻¹ sucrose and 100 mg.l⁻¹ Benlate for 24 hours ($26 \pm 3.0\%$), and staining with 1 % (w/v) 2,3,5-triphenyl tetrazolium chloride (TTC). Medium-term storage of pollen was best achieved by maintenance in the fridge (4 °C) without desiccation, resulting in 8 months longevity and a percentage viability of 6.73 ± 1.21 .

CHAPTER 3: ESTABLISHMENT OF CRYOPRESERVATION PROTOCOLS FOR THE LONG-TERM STORAGE OF *Eucalyptus* spp. POLLEN AND AXILLARY BUDS

3.1 Introduction and Literature review

3.1.1 Cryopreservation

In view of the imminent danger of losing rare germplasm and the prevention of endangered ones from extinction, it is highly desirable to strengthen the existing germplasm banks and incorporate new and innovative methods of storage (Bajaj, 1995; Blakesley *et al.*, 1995). Presently, *in vitro* culture storage has been used for purposes of short- to medium-term storage, but germplasm banks can only be supplemented by the use of long-term conservation practices such as cryopreservation (Withers and Engelmann, 1996). Cryopreservation is based on the reduction and subsequent arrest of all cellular divisions and metabolic processes of the plant material by storage at ultra low temperatures, usually in liquid nitrogen (-196 °C). At this temperature, plant material can be stored without alteration or modification for a theoretically unlimited period of time (Kantha, 1985; Towill, 1985; Grout, 1991; Blakesley *et al.*, 1996; Engelmann, 1997). However, only few biological materials can be frozen to subzero temperatures without adversely affecting cell viability. As a result, much research is currently focused on the development of optimum conditions for successful cryopreservation protocols of various species. However, it should be mentioned that cryopreservation of plant cells and organs is still in its infancy and only limited information is available concerning the various aspects pertaining to its success (Grout, 1991; 1995; Ashmore, 1997; Engelmann, 1997).

The development of cryopreservation for plant cells and organs has followed advances made with mammalian species, albeit several decades later. The first report on survival of plant tissue exposure to ultra-low temperatures was made by Sakai in 1960, when he demonstrated that hardy mulberry twigs could withstand freezing in liquid nitrogen after dehydration mediated by extra-organ freezing (Engelmann, 1996). Although there is a general awareness of the potential application of cryogenic storage to the long-term preservation of plant genetic resources, in reality, the actual number of species to which this technique is successfully applied is rather small. However, in the last few years, the

number of plant species successfully cryopreserved has increased rapidly (Table 3.1).

3.1.2 Factors affecting cryogenic storage

3.1.2.1 Choice of explant

The need to conserve plant cells that are true-to-type limits the range of plant material that can be considered suitable for storage (Kartha, 1982; Withers, 1986; George, 1993). Cell suspension cultures or any other cultures obtained via a callus-mediated organogenetic pathway have proved likely to include induced genetic abnormalities in the population of the regenerants (Warren, 1991). There is also an added difficulty of many suspension cultures not being morphogenic (Dodds and Roberts, 1985; Collin and Dix, 1990; Grout, 1995; George, 1996). The use of callus mediated cultures, therefore, depends on the ability to select genetically stable and morphogenically competent cell lines. In *Eucalyptus grandis*, embryogenic suspensions have been successfully developed (Blakeway, Herman and Watt, 1993). However, for the reasons discussed above, they are not ideal germplasm material for storage.

An immediate aim of long-term conservation is to ensure representatives of all genotypes are maintained for future research purposes in breeding. It is thus essential to conserve explants that represent the same genotype as the parent plant. Because of their genetic stability and therefore 'true' clonal progeny, apical meristems or meristems within larger buds and possibly somatic embryos from a genetically stable regeneration system, are the most suitable explants for storage (Kartha, 1985; Withers, 1986; Withers and Engelmann, 1996). In cryopreservation, the terms 'meristem' and 'shoot tip' are often used interchangeably (Grout, 1995). Shoot meristem is anatomically defined as a structure that contains the apical dome, and the youngest, unexpanded leaf primordia directly associated with the dome meristem (Towill, 1995). Excised meristems are usually of the order of 0.4 - 1.0 mm from the apex base (Kartha, 1985; Towill, 1995). In practice, it is the larger shoot tip that is used in 'meristematic cryopreservation', for example potato

Table 3.1 Examples of plant species in which cryopreservation studies have been conducted from 1990 until 1998 (date in which the current work was submitted). csc = cell suspension cultures; m = meristems; se = somatic embryos; st = shoot tips; ea = embryonic axes; ze = zygotic embryos; c = callus; p = protoplasts

Plant genera and species	Material frozen	Reference
<i>Asparagus officinalis</i>	csc	Nishizwa, Sakai, Amano and Matsuzawa (1992)
<i>Brassica campestris</i> (mustard)	csc	Langis, Schnabel, Earle and Steponkus (1990a)
<i>Abies nordmanniana</i>	csc	Norg, Baldursson and Krogstrup (1993)
<i>Aesculus hippocastanum</i> (chestnut)	se	Jekkel, Kiss, Kiss, Gyulai and Heszky (1992)
<i>Allium sativum</i>	ms	Niwata (1992)
<i>Beta napus</i>	ms	Chen and Beversdorf (1992)
<i>Brufelsia densifolia</i>	csc	Pence (1990)
<i>Camellia sinensis</i> (tea)	ea	Wesley-Smith, Vertucci, Berjak, Pammenter and Crane (1992);
<i>Carya illinoensis</i> (pecan)	ze	Abou Taleb, Yates, Wood and Fouad (1992)
<i>Chamomilla recutita</i>	st	Diettrich, Popov, Pfeiffer, Neumann, Butenko and Luckner (1990)
	csc	Cellarova, Cernicka, Vranova, Brutovska and Lapar (1992)
<i>Chrysanthmum morifolium</i>	st	Fukai (1990); Fukai, Goi and Tanaka (1991)
<i>Cinchona intybus</i> (chicory)	st	Demeulemeester, Vandenbussche and de Proft (1993)
<i>Citrus</i> sp.	se	Normah, Hamidah and Ghani, (1997) Perez, Navarro and Duran-Vila (1997)
	ze	Kobayashi, Sakai and Oiyama (1990); Sakai, Kobayashi and Oiyama (1991)
<i>Cocos nucifer</i> (coconut)	ze	Englemann <i>et al.</i> (1995)
<i>Coffea arabica</i> (coffee)	ze	Abdelnour-Esquivel, Villabos and Engelmann (1992 a)
<i>C. canephora</i>	csc	Tessereau, Lecouteux, Florin, Schlienger and Petiard (1991)
<i>Cucumis melo</i> (melon)	se	Shimonishi, Ishikawa, Suzuki and Cosawa (1991)
<i>Daucus carota</i> (carrot)	se	Dereudde, Blandin and Hassen (1991); Lecouteux, Florin, Tessereau, Bollon and Pitiard (1991)
<i>Dianthus caryophyllus</i> (carnations)	st	Langis Schnabel, Priekstas, Earle and Steponkus (1990b); Tannoury, Tanaka, Ashitani, Kaminski and Dereudde (1991)
<i>Dioscorea deltoidea</i> (yam)	csc	Popov and Fedorovskii (1992)
	st	Mandal, Chandel and Dwevedi (1996)
<i>Elaeis guineensis</i> (oil palm)	se	Dumet, Engelmann, Chabrilange and Duval (1993a)
<i>Euphorbia longan</i>	ze	Fu, Zhang, Wang, Qiao and Haung (1990)
<i>Grevillea scapigera</i>	st	Touchell, Dixon and Tan (1992)
<i>Ipomea</i> sp.	st	Towill and Jarret (1992)
<i>Juglans regia</i>	ze	de Boucaud, Brison, Ledoux, Germain and Lutz (1991)
	se	de Boucaud, Brison and Negrier (1994)
<i>Landolphia kirkii</i>	ze	Vertucci and Berjak (1991)
<i>Larix x eurolepis</i> (larch)	csc	Klimaszewska, Ward and Cheliak (1992)
<i>Malus</i> (apple)	m	Niino and Sakai (1992)
	st	Niino, Sakai, Yakuwa and Nojiri (1992a)
	st	Stushnoff (1991); Stushnoff, Remmele and Esensee (1992)
<i>Mamhot</i> (cassava)	se	Sudarminiwati and Henshaw (1990)
	ze	Marin, Mafla, Roca and Withers (1990)
	st	Chabrilange, Engelmann and Benson (1996) Escobar <i>et al.</i> (1997)
<i>Medicago sativa</i> (alfalfa)		

	st	Niino, Sakai, Yakuwa and Nojiri (1992a)
	st	Stushnoff (1991); Stushnoff, Remmele and Esensee (1992)
<i>Manihot</i> (cassava)	se	Sudarminiwati and Henshaw (1990)
	ze	Marin, Mafla, Roca and Withers (1990)
	st	Chabrillange, Engelmann and Benson (1996) Escobar <i>et al.</i> (1997)
<i>Medicago sativa</i> (alfalfa)	c	Cachita, Zapirtan, Craciun, Rakosky, Cristea, Varga and Craciun. (1991)
<i>Mentha</i> (mint)	st	Towill (1990)
<i>Morus alba</i> (mulberry)	st	Niino, Sakai, Enomoto, Mogoshi and Kaot (1992b)
<i>Musa</i> sp. (banana)	csc	Panis, Withers and De Langhe (1991)
	ms	Panis, Totte, Van Nimmen, Withers and Swennen (1996)
<i>Onobrychis viciaefolia</i> (sainfoin)	c	Sun and Jian (1990)
<i>Oryza sativum</i> (rice)	c	Watanabe, Ueno and Matsuda (1990) Kuriyama, Watanabe, Ueno and Matsuda (1990)
<i>Phoenix dactylifera</i> (date palm)	m	Bagniol and Engelmann (1992)
<i>Picea glauca-engelmanni</i>	csc	Cyr, Lazaroff, Grimes, Quian, Bentune, Dunstan and Roberts (1994)
<i>Picea mariana</i> (black spruce)	csc	Klimaszewska <i>et al.</i> (1992)
<i>Pinus sylvestris</i> (Scots pine)	st	Kuoska and Hohtola (1991)
<i>P. nigra</i>	ea	Pita, Barrero and Escudero (1997)
<i>P. caribea</i> (Caribbean pine)	csc	Laine, Bade and David (1992)
<i>Pisum sativum</i> (pea)	ze	Mycock, Watt and Berjak (1991)
<i>Poncirus trifoliata</i>	ze	Radhamani and Chandel (1992)
<i>Prunus jamasakura</i>	st	Niino, Tashiro, Suzuki, Ohuchi, Magoshi and Akihama (1997)
<i>Puccenellia</i> sp. (halophyte grasses)	csc	Henszky, Jekkel and Ali (1990)
<i>Pyrus communis</i> (pear)	st	Reed (1990) Dereuddre, Scottez, Arnaud and Duron (1990); Niino <i>et al.</i> (1992a)
<i>Quercus petraea</i> (oak)	ze	Jorgensen (1990); Gonzales-Benito and Perez-Ruiz (1992)
<i>Ribes</i> sp. (currants)	ms	Reed (1992)
<i>Saccharum officinarum</i> (sugarcane)	c/csc	Gnanapragasam and Vasil (1990) Vandenbussche and De Proft (1998)
<i>Secale cereale</i> (rye)	p	Langis and Steponkus (1990)
<i>Setaria italica</i> (foxtail millet)	c/csc	Lu and Sun (1992)
<i>Solanum tuberosum</i> (potato)	m	Fabre and Dereuddre (1990), Harding, Benson and Smith (1991) Schafer-Manuhr, Schumacher and Mix-Wagner (1997)
<i>Trifolium repens</i> (clover)	m	Yamada, Sakai, Matsuru and Haguchi (1991)
<i>Triticum aestivum</i> (wheat)	c/csc	Kendell, Qureshi, Kartha, Leung, Chevrier, Caswell and Chen (1990)
<i>Vigna mungo x Vigna radiata</i> hybrid	ze	Bajaj (1990)
<i>Vitis vitifera</i>	csc	Dussert, Mauro and Engelmann (1992)
<i>Wasabia japonica</i> (wasabi)	m	Matsumoto, Sakai and Yamada (1994)
<i>Xanthosoma brasiliense</i> (tanier spinach)	se	Jayos, Hoekstra and Staritsky (1993)
<i>Zea mays</i> (maize)	p	Zhang, Jian, Kuo, Qu and Qian (1990a)

shoot tips (Grout and Henshaw, 1978; Schafer-Menuhr *et al.*, 1997), freeze-preservation of *Ribes* shoot tips (Reed, 1992) and storage of mint shoot tips (Towill, 1990). This structure comprises of the meristematic apex, sub-adjacent tissue, and several larger often expanded leaf primordia. The meristematic zone of apices, from which organized growth originates, is composed of a relatively homogeneous population of small, actively dividing cells, with small vacuoles and high nucleo-cytoplasmic ratio. It is those characteristics that make them less susceptible to desiccation-injury compared to highly vacuolated and differentiated cells. These organs are also suitable subjects for cryopreservation on account of their small size, high cytoplasmic density and low water content (Towill, 1985; Grout and Roberts, 1995).

Pollen as a sexual organ is an ideal storage explant especially in view of its uses for controlled breeding. Traditionally, pollen has been stored at reduced temperatures of zero and slightly below. However, it has become apparent that those temperatures can only prolong storage for a few months and definitely not on a long-term basis (2.2.3, 2.3.2.3). Cryopreservation provides an additional dimension for pollen storage, offering the possibility of significantly extended storage periods and ensuring maximum genetic stability. One of the earliest reports that pollen could survive very low temperatures was by Knowlton (1922) who observed that *Antirrhinum* pollen germinate after exposure to -180 °C. Since then, a considerable amount of research has been undertaken in pollen cryopreservation. Table 3.2 shows a few examples of papers on cryopreserved pollen available in the literature. Although some pollen has appeared to lose germination ability with time in cryostorage, it is often the contrary in most species. Some researchers have found that *in vitro* pollen stored at ultra-low temperatures for short periods often have a higher germinability than that of fresh pollen (Visser, 1955; Stanley and Linskens, 1974). Although no clear explanation has been found for this behaviour, this effect may be attributed to the after ripening processes occurring after shedding or the release of some needed nutrients triggered by the freezing process (Stanley and Linskens, 1974).

Table 3.2 A few examples of some of the species where pollen was successfully cryopreserved

Species	Reference
<i>Allium victorialis</i>	Kanazawa, Kobayashi and Yakuwa (1992)
<i>Arachis villosa</i>	Bajaj (1984)
<i>Artocarpus heterophyllus</i>	Chin, Krishnapillay and Hor (1989)
<i>Banksia menziesii</i>	Maguire and Sedgley (1997)
<i>Brassica olearacea</i>	Crisp and Grout (1984)
<i>Camellia sinensis</i>	Chaudhuray, Radhamani and Chandel (1991)
<i>Citrus limon</i>	Ganeshan and Alexander (1991)
<i>Clanthus formusas</i>	Hughes <i>et al.</i> (1991)
<i>Glycine max</i>	Collins <i>et al.</i> , 1973
<i>Helianthus annus</i>	Andreica and Sparchez (1990)
<i>Lycopersicon esculentum</i>	Sacks and Stclair (1996)
<i>Manihot esculenta</i>	Marin <i>et al.</i> (1990)
<i>Narcissus sp.</i>	Bowers (1990)
<i>Nepheium lappaceum</i>	Chin <i>et al.</i> (1989)
<i>Oryza sativa</i>	Chin <i>et al.</i> (1989)
<i>Pennisetum glaucum</i>	Hanna (1994)
	Inagaki and Mujeebkazi (1996)
<i>Phytolacca dodecandra</i>	Demeke and Hughes (1991)
<i>Secale cereale</i>	Demeke and Hughes (1991)
<i>Solanum tuberosum</i>	Wheatherhead, Grout and Henshaw (1978)
<i>Trifolium praetense</i>	Engelke and Smith (1974)
<i>Triticum aestivum</i>	Bajaj (1984)
	Inagaki <i>et al.</i> (1997)
<i>Zea mays</i>	Barnabas and Rajki (1976)
	Georgieva and Kruleva (1993)

3.1.2.2 Pretreatments

Preconditioning of plant material prior to cryopreservation is beneficial for increasing the survival of plant tissue (Reed, 1996). Pretreatment of plant cells and tissues can be divided into three categories: a) culture pretreatments, which involve culturing explants in a growth medium with osmotica or growth regulators and cold acclimation (e.g. Reed and Chang, 1997), b) chemical treatments, which include osmotic or penetrating cryoprotectants (e.g. Mycock, Wesley-Smith and Berjak, 1995) and c) desiccation using laminar air flow (e.g. Niino, 1995) or the use of desiccants such as silica gel (e.g. Dumet *et al.*, 1993a). Often, individual pretreatments cannot provide adequate protection to cells or tissues, rather a combination of pretreatment techniques is needed in order to condition the cells to withstand the stresses imposed by freezing and thawing (Withers, 1982; Grout and Morris, 1987; Benson and Hamill, 1991; Grout, 1991; Potter and Jones, 1991).

a) Culture

Culture pretreatments include growing mother plants on a suitable medium with preconditioning chemicals for several days or weeks before subjecting explants to liquid nitrogen treatments. Osmotically active chemicals like dimethylsulfoxide (DMSO) and growth regulators such as abscisic acid are often used in the growth for preconditioning cells, tissues or mother plants (Ashwood-Smith and Farrant, 1980; Withers, 1986; Grout, 1991; Benson, 1995). The effectiveness of an osmoticum is often attributed to the fact that negative water potential in the cells is induced thus enhancing sugar and overall solute levels within the cell (Dereuddre, Fabre and Bassaglia, 1988). According to Dumet, Engelmann, Chabrillange, Duval, and Dereuddre (1993b), increased sugars and solutes within the cell lead to increased resistance to freezing and consequently less freezing injury. This type of behaviour has been reported in several papers, a few examples include work with carnation shoot tips (Dereuddre *et al.* 1988; Langis *et al.*, 1990b), *Eucalyptus* apices (Poissonier, Monod, Paques and Dereuddre, 1992), sugar cane apices (Paulet, Engelmann and Glaszmann, 1993; Gonzalez-Arno, Moreira and Urra, 1995) and horse radish shoot primordia (Phunchindawan, Hirata, Sakai and Miyamoto, 1997).

Cold acclimation is another form of culture pretreatment. This method is used to trigger a plant's natural resistance mechanisms to cold weather (Steponkus and Bannier, 1971; Reed, 1993). It causes an increase in starch grains, lipid bodies, sugar content and dry weight (Tanino, Weiser, Fuchigami and Chen, 1990). Cold acclimation was found to increase the capacity of canola tissue to synthesize phosphatidylcholine and induced other changes such as an increase in the ratio of unsaturated to saturated fatty acids and an increase in phospholipids and linoleic acid (Tanino *et al.*, 1990; Reed, 1993). Changes in the amount and composition of the membrane lipids following acclimation are thought to protect cells against freeze-induced injury (George, 1993). In other studies acclimation was achieved by growing mother plants at low temperatures for one to several weeks prior to cryopreservation (Seibert and Whetherbee, 1977; Matsumoto and Sakai, 1995). Alternatively, growth regulators such as ABA, have been used as their effect on the plant mimic a short winter day (Reed, 1988; 1990; Timbert, Barbotin and Thomas, 1996; Perez de Juan, Irigoyen and Sanchez-Diaz, 1997). Johnson-Flanagan, Huiwen, Thiagarajah and Saini (1991) showed that the freezing tolerance of *Brassica napus* cell suspension cultures was enhanced after hardening for six days with 50 μM ABA. However, not all plants respond positively to cold acclimation, generally, the treatment is most effective on temperate plants but totally ineffective or detrimental to tropical plants (Grout, 1995; Henshaw and Blakesley, 1996).

b) Chemical

Chemical treatments can be used as a part of culture pretreatments by adding the chemical to the growth medium for various periods of time or they may be used immediately before the freezing process. Chemical and culture pretreatments are often used together for the best results (Ashwood-Smith and Farrant, 1980; Kartha, 1985). Various chemicals exhibit cryoprotective properties on biological samples. Such cryoprotectants have little in common except a high solubility in water (George, 1993). Their mode of action is not entirely known, it may depend on their ability both to reduce the concentration of salts in the cell and to restrict the growth of damaging ice crystals (Grout, 1995). According to Bajaj and Reinert (1977), an efficient cryoprotectant should have the following properties: a) non-toxicity to plants, b) low molecular weight, c) be

readily miscible with water, d) have the ability to permeate cells rapidly and e) be easy to wash away from cells. Some of the more commonly used cryoprotectants include DMSO, glycerol, mannitol, sorbitol and polyethylene glycerol, either alone or in combination (Towill, 1995). A mixture of 10% (v/v) DMSO and 5% (w/v) sucrose were successfully used in freezing of immature embryos of *Fragus* sp. (Jorgensen, 1990), immature embryos of *Cocos* sp. were cryoprotected with a mixture of DMSO and glycerol at 10% (v/v) each and successfully frozen (Assy-Bah and Engelman, 1992a) and a standard preculture of explants in 0.5-0.75M sucrose, mannitol or sorbitol has been recommended by Benson (1995).

c) Desiccation

Dehydration is another pretreatment mechanism for cryopreservation. According to Stanwood (1985), many seeds can be safely stored in liquid nitrogen when they have low moisture contents. However, some seeds and most actively growing plant parts cannot tolerate drying (Reed, 1996). Dehydration pretreatments, therefore, provide ways to overcome the lack of natural tolerance to drying in such species or plant material. Three methods of drying plant material have been used: a) drying under the laminar hood (Niino, 1995), b) flash drying using a direct stream of air (Berjak, Farrant, Mycock and Pammenter, 1990) and c) using dehydrants such as silica gel (Dumet *et al.*, 1993a). It is often recommended that explants be dried to a moisture content of 20% (wet weight basis) (Towill, 1995). Reviewed literature showed that partial desiccation of chemically cryoprotected embryos of *Cocos mucifera* followed by rapid desiccation increased from 33 to 63% survival rates (Assy-Bah and Englemann, 1992b) and seeds of coffee were air dried to 17% resulting in 53% post-freezing survival rates (Becwar, Stanwood and Leonhardt, 1983).

3.1.2.3 Freezing methods

Nearly all the damage that may occur to cells during transfer to very low temperatures or subsequent thawing is a consequence of their water content (Engelmann, 1996; Bajaj, 1995; Kartha, 1985). A possible exception would be the damage caused by interference with biochemical or biophysical pathways during freezing. Living cells are damaged

mostly by the internal formation of ice crystals as a result of exposure to freezing. Hence, for successful cryopreservation, the physical state of water within a cell must be changed to a frozen glass-like condition without crystallization. This 'transformation' of water from a liquid to a solid glass-like state is known as vitrification (Fahy, MacFarlane, Angell and Meryman, 1984; Kartha, 1985). There are two methods of freezing which have to be applied with caution to prevent the appearance of intra-cellular ice crystals namely, slow and rapid freezing.

a) Slow freezing

Methods of freezing plant meristems slowly are based on the physico-chemical events that occur during freezing (Kartha, 1985). Slow freezing may be achieved by cooling material at slow and controlled rates to temperatures between -30 and -60 °C before directly plunging into liquid nitrogen (Grout and Morris, 1987). With progressive temperature reduction, the cell and its external medium initially supercool, followed by ice formation in the external milieu (Fig 2.1). In the case of plant cells, the cell wall and the plasma membrane act as barriers to prevent ice from seeding the cell interior at temperatures above -10 °C and thus the cell remains unfrozen. As the temperature is further lowered, an increasing fraction of extra-cellular solution is converted to ice, resulting in concentration of extra-cellular solutes. Since the interior of the cell remains supercooled, its aqueous vapour pressure exceeds that of the frozen exterior. The cell then equilibrates by loss of water to external ice (dehydration) (Fig 3.1). Slowly cooled cells reach an equilibrium with the external ice by efflux of water and the remaining water within the cell is vitrified without ice formation (Kartha, 1985). Slow freezing has been successfully attempted for several recalcitrant species including zygotic axes of *Landolphia kirkii* (Vertucci, Berjak, Pammenter and Crane, 1991) and meristematic tissues of woody plant species such as *Citrus sinensis* (Kobayashi *et al.*, 1990), *Pyrus* sp. (Reed, 1990) and *Picea abies* (Kartha, Fowke, Leung, Caswell and Hawkman, 1988).

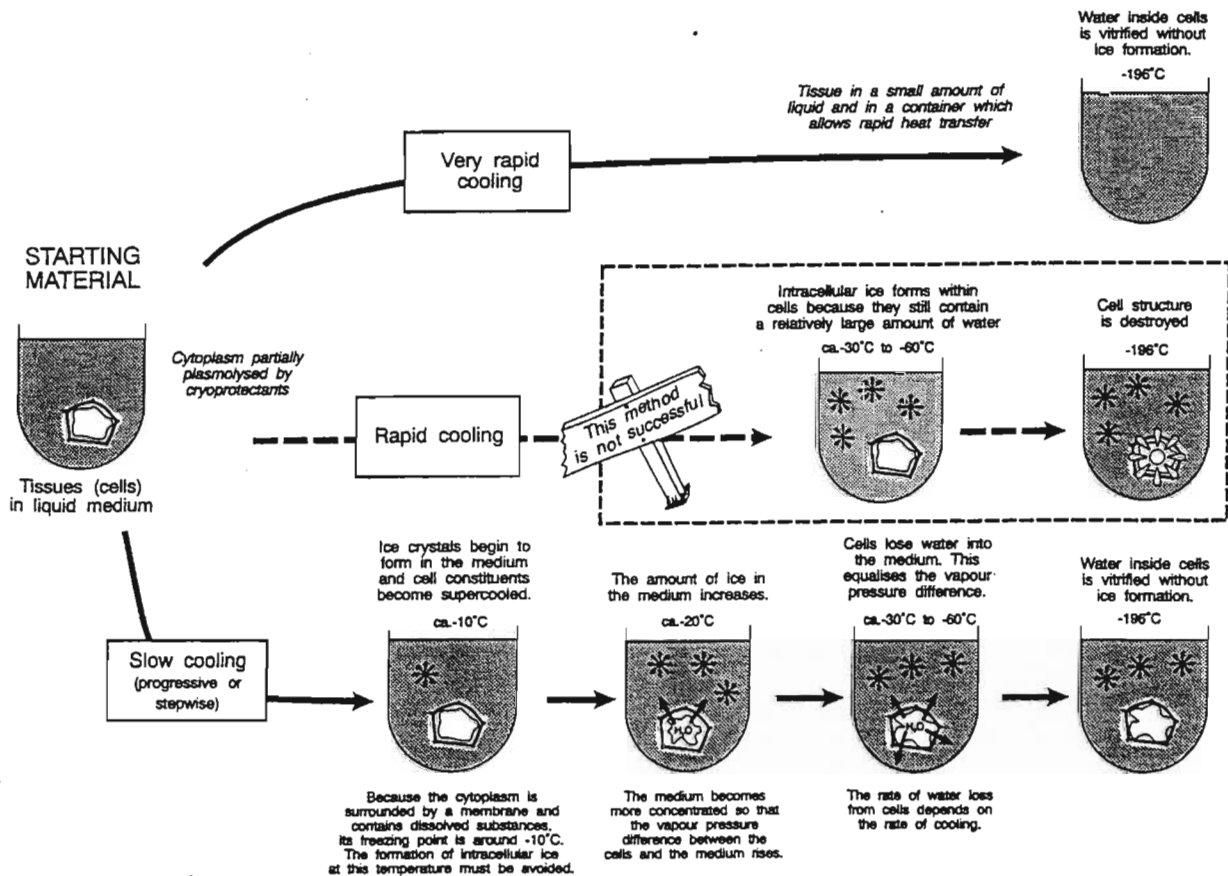


Fig 3.1 Physico-chemical events during different methods of cryogenic cooling. For successful cryopreservation, material must either be cooled slowly to about -30°C to -60°C before transfer to liquid nitrogen, or else cooled very quickly in a small volume or liquid (George, 1993).

b) Rapid freezing

The high costs of apparatus needed to achieve various experimental rates of controlled slow cooling has limited the application of cryopreservation research to a few specialised laboratories (George, 1993). However those cells with dense cytoplasm and a relatively low water content, can be preserved if they are cooled sufficiently rapidly such that internal water directly becomes vitrified without prior slow cooling (Luyet, 1937). Cooling rates imposed during rapid freezing of plant meristems are of the order of several hundred degrees per minute and often there is no control on the rate at which cells are frozen (Henshaw, 1975; Kartha, 1985). In contrast to regulated freezing, during rapid freezing, cells do not have sufficient time to equilibrate with external ice or vapour-pressure deficit by efflux of cellular water (Benson, 1995; Grout, 1995). Therefore, they attain equilibrium by intra-cellular freezing which is often lethal to biological specimens (Fig 3.1). However, cells which have dense cytoplasm and a relatively low water content can be preserved if they are cooled sufficiently rapidly for internal ice to become directly vitrified. It has also been shown that small plant samples can be transferred immediately to $-196\text{ }^{\circ}\text{C}$ without damage, providing that the tissue to be frozen has been induced to take up cryoprotectant chemicals and they have been placed in a thin walled container which allows rapid heat transfer (Grout, 1991).

The first few reports of successful cryopreservation by rapid freezing concerned the cryopreservation of shoot tips of potato (Bajaj, 1977; Grout and Henshaw, 1978; Bajaj, 1979; Towill, 1981), tomato (Grout, Wescott and Henshaw, 1978), chickpea (Bajaj, 1977) and cassava (Kantha *et al.*, 1981a). However, more recently, a significant increase in successful cryopreservation via rapid freezing has been reported. Examples include cell suspension cultures of *Brassica compestris* (Langis *et al.*, 1990a) by vitrification with 1.5M ethyl glycol solution followed by rapid freezing, nucellar cells of *Citrus* sp. (Sakai *et al.*, 1991), cultures of *Beccaurea motleyana* by Normah and Marzalina (1996), *Musa* spp. Panis *et al.* (1996), *Solanum* spp. (Schafer-Menuhr, 1996) and sugar cane (Vandenbussche and De Proft, 1998).

3.1.2.4 Storage, thawing and viability

At the temperature of liquid nitrogen all metabolic activities of meristematic cells are at a standstill and theoretically, plant meristems can be stored indefinitely. For example pea, and strawberry meristems have been stored for over two years in liquid nitrogen with no serious consequences with regard to retention of viability (Kantha, 1985). More examples where liquid nitrogen has been used for successful long-term storage of germplasm can be found on Table 3.1.

Thawing, which is the reversal of freezing, has attendant hazards (Bowers, 1990). At around $-130\text{ }^{\circ}\text{C}$, ice structures can change from vitreous to crystalline. As in freezing, whenever ice crystals result within the cell they become damaging (George, 1993). If ice formation occurs at $-70\text{ }^{\circ}\text{C}$ and above, ice crystals may form and grow on the surface of smaller pre-existing crystals by a process of recrystallization. Rapid thawing is therefore usually most successful in preserving viability, as in that way the amount of ice crystal formation which occurs before water in cells melts is reduced to a minimum. Rapid thawing can be carried out in a water bath at $25 - 30\text{ }^{\circ}\text{C}$ (e.g. Reed, 1991) or $35 - 40\text{ }^{\circ}\text{C}$ (e.g. Grout, 1995) when using cryoprotected material. However, occasionally, very high thawing temperatures have been quoted in the literature as being suitable for thawing. For instance, *Coleus blumei* suspensions and callus were thawed at $60\text{ }^{\circ}\text{C}$ with no apparent negative effect (Reuff, Seitz, Ulrich and Reinhard, 1988).

One of the methods of assessing viability is a qualitative assessment of structural damage detected by direct microscopic examinations (Kantha, 1985; George, 1993). Ultrastructural examination with electron microscopes has also provided information on cellular damage and the pattern of recovery (e.g. Berjak *et al.*, 1996). Histological viability tests are also available and these are often objective, providing information about physiological conditions of frozen and thawed cells. One of the basic requirements prior to cryopreservation of plant meristem, is the availability and development of an efficient and reliable protocol for regeneration of plants (Kantha and Engelmann, 1994). Once established, regeneration is one of the best methods of determining viability after

cryogenic storage. However, the true physiological condition still remains best assessed with the combination of results from a wide range of tests and, ultimately, the ability of the cells or organs to grow and regenerate.

3.1.3 Approaches to cryopreservation

In recent reviews (Blakesley *et al.*, 1995; Ashmore, 1997; Engelmann, 1997), it has been suggested that the available cryopreservation methodologies may be categorised into two groups, namely, classical procedures developed in the 1970s and the more recent techniques. The development of classical techniques was based on the following sequence of treatments: chemical cryoprotection, slow dehydrative cooling followed by rapid immersion and storage in liquid nitrogen, rapid thawing, washing and recovery. For instance, date palm was slow frozen at 0.33 °C/min at -30 °C (Dumet *et al.*, 1993a), somatic embryos of coffee at 0.5 °C/min (Bertrand-Desbrunais *et al.*, 1992) while Klimaszewska *et al.* (1992) used classical procedures to freeze *Larix* meristematic tissues.

In recent years, several new cryopreservation techniques have been developed and such methods allow application of cryopreservation to a larger range of tissues and organs (Kantha and Engelmann, 1994; Blakesley *et al.*, 1996; Withers and Engelmann, 1996; Ashmore, 1997; Engelmann, 1997). *In vitro* culture systems to be cryopreserved generally contain high amounts of cellular water and are thus extremely sensitive to freezing injury since most of them are not inherently freezing-tolerant (Withers and Engelmann, 1996). Samples thus have to be dehydrated artificially to protect them from damages caused by crystallization (Meryman, 1960; Mazur, 1969). The main difference between classical and new cryopreservation methods is based on the method of dehydration employed; classical techniques apply freeze-induced dehydration whereas new techniques are based on vitrification (Henshaw and Blakesley, 1996). Vitrification, has been defined by Fahy *et al.* (1984), as the transition of water directly from liquid phase into an amorphous phase or glass, whilst avoiding the formation of crystalline ice.

Vitrification based procedures offer practical advantages in comparison to classical techniques (Steponkus, 1992; Phunchindawan, *et al.*, 1997) and are consequently more appropriate for complex organs such as shoot tips and embryos. Because they preclude ice formation in the system, vitrification based procedures are less complex than classical ones and do not require expensive equipment (controlled freezers). Pretreatment is followed by rapid cooling, instead of slow cooling, as is the case in classical methods and in this way, all factors which affect intra-cellular ice formation are avoided. Four different procedures facilitating vitrification have been identified: encapsulation-dehydration, encapsulation-vitrification, encapsulation-desiccation and pre-growth-desiccation (Engelmann, 1997).

Encapsulation-dehydration is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pre-grown in liquid medium enriched with sucrose for several days, partially desiccated to a water content of about 20% (fresh water basis) then rapidly frozen (Dereuddre *et al.*, 1990). Survival rates are often high and growth recovery of cryopreserved samples is generally rapid and direct (without callus formation). For instance, survival of carrot and coffee somatic embryos was observed to be 100% when high concentrations of sucrose (0.75 - 1M) were used as a sole source of cryoprotection (Tessereau *et al.*, 1991; Thierry, Tessereau, Florin, Meschine and Petiard, 1997). This technique has been successfully developed for apices of many woody plant species, for example, apple (Stushnoff and Seufferheld, 1995), pear (Moriguchi, 1995), eucalypts (Poissonier *et al.*, 1992), species of tropical origin such as sugarcane (Paulet *et al.*, 1993) and cassava (Chabrillange *et al.*, 1996).

Encapsulation vitrification combines encapsulation of explants in alginate beads with subsequent treatment with vitrification solutions before freezing (Sakai, Niino, Yamada and Kohmura, 1992). Vitrification solutions are often highly concentrated cryoprotectants and at low temperatures, they become so viscous that they solidify into a metastable glass. These solutions have been applied with success to apices of lily (Matsumoto, Sakai and Yamada, 1995a) and wasabi (Matsumoto and Sakai, 1995). Sakai *et al.* (1990) successfully cryopreserved nucellar cells of *Citrus sinensis* using a concentrated PVS2

solution (containing 30% (v/v) glycerol, 15% (v/v) ethylene glycol, and 15% (v/v) DMSO together with sucrose).

Encapsulation-desiccation has mainly been used for freezing zygotic embryos of recalcitrant and intermediate species (Pence, 1991, Withers and Engelmann, 1996; Dumet, Berjak and Engelmann, 1996). It involves desiccation of explants in a stream of air, laminar air-flow or over silica gel. Pregrowth involves the preculturing of plant material on a medium containing cryoprotectant, then rapid freezing. Pregrowth-desiccation therefore combines the use of both pregrowth in the presence of a cryoprotectant and desiccation using silica gel or laminar air flow.

3.1.4 Applications and limitations

Long-term preservation plays a crucial part in the establishment of germplasm banks for those species that are rare, recalcitrant or vegetatively propagated (Fig. 3.2). Banks of this kind can assist in the international exchange of genetic resources between countries. The risk of importing diseases in culture is clearly far less than from the import of whole plants or plant parts (Ashmore, 1997). Furthermore, cryogenic storage saves time, space and money, as there is no need to subculture. Freeze-preserved specimens have an advantage over *in vitro* stored accessions as they retain their morphogenic potential and should maintain genetic stability (Engelmann, 1997).

Besides the many advantages associated with cryopreservation (Fig 3.2), it should be emphasised that the process of 'freezing-storage-thawing-culture' can be complicated. An error at any stage or alteration of factors can result in lethal injury to the material. Also, it should be noted that different cryopreservation protocols have to be established for each species, hybrid, cultivar variety, clone and/or line (George, 1993; Bajaj, 1995; Grout, 1995).

Another problem with cryopreservation has been found to be the lack of a universal method; the cryostorability of cells or tissues depends greatly on the genotype, age and physiological state of the plant and/or cultures. Thus, it is difficult to repeat an

established protocol if any of those factors change (Benson, 1990; Grout, 1991; George, 1993; Benson, 1995). Seasonal variations can also change the soluble protein and increase sugar levels and alcohols (Heber, 1958; Imelda, 1996) which are generally believed to increase the cold resistance of plants (Sakai, 1960). Thus, material taken from a plant in winter may behave differently from plants grown under extreme summer conditions. A further difficulty is that *in vitro* cultures have been found to differ from field samples hence a protocol that is successful with *in vitro* material might not necessarily apply to greenhouse material of the same species (Harding *et al.*, 1991; Kozai *et al.*, 1997).

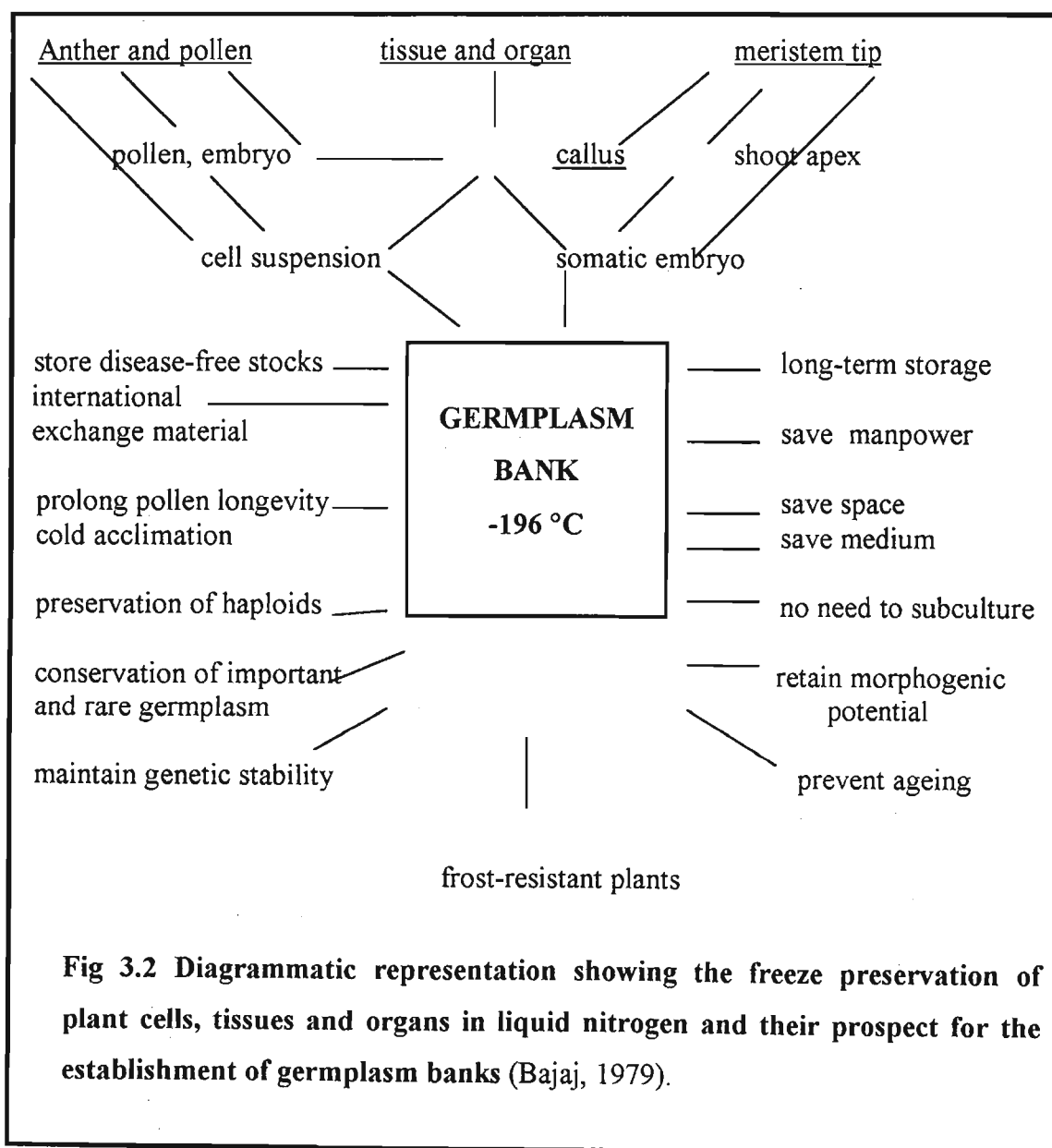


Fig 3.2 Diagrammatic representation showing the freeze preservation of plant cells, tissues and organs in liquid nitrogen and their prospect for the establishment of germplasm banks (Bajaj, 1979).

Despite problems associated with cryopreservation, the quest of the tree breeder to obtain hybrids with superior wood quality, and the rapid disappearance of already existing genotypes makes utilisation of freeze preservation methods for the storage of desired germplasm a must. In view of the obvious dangers associated with loss of genetic variability, cryopreservation methods must further be refined and simplified for routine use in tree breeding programmes.

3.1.5 Aims of the investigation

The aims of this study were to establish cryopreservation protocols for the long-term conservation of *Eucalyptus* clone GC 559 germplasm. The explants selected were axillary buds or pollen. Axillary buds were deemed ideal material for long-term storage as they are known to result in true-to-type genotypes once regenerated in culture. Pollen, on the other hand, offered an additional dimension of germplasm conservation for *Eucalyptus* spp. because of its value in breeding. However, cryopreservation is a complex technique consisting of many intrinsic steps. Hence in this study, the size of explant, its pretreatment, freezing and thawing procedures were investigated for both pollen and axillary buds.

3.2 Materials and Methods

3.2.1 Cryopreservation of axillary buds

3.2.1.1 Explant preparation and culture

Mature *Eucalyptus* clone GC 559 greenhouse plants (6 months old) were obtained from Mondi Nursery, Hilton. Shoots were harvested from those plants, trimmed, then placed in a 500 ml Schott bottle for sterilisation. Two sterilisation procedures were investigated. In protocol A, shoots were washed in a solution that consisted of 1 g.l⁻¹ benomyl (Benlate®), 0.5 ml.l⁻¹ chlorothalonin (Bravo®) fungicide and 1 g.l⁻¹ boric acid. A drop (0.02 ml) of Tween 20 was added to the solution and left for 30 min on an orbital shaker (110 rpm). The bottle containing the shoots was then transferred to the laminar flow and the shoots were rinsed three times in sterile water then soaked in 1 % (w/v) calcium hypochlorite. After 2 min, shoots were rinsed with sterile water three times. Thereafter, buds were excised from shoots then cultured in tubes on a medium consisting of MS (Murashige and Skoog, 1962), 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.01 mg.l⁻¹ NAA, 0.2 mg.l⁻¹ BAP, 3.5 g.l⁻¹ Gelrite and 30 g.l⁻¹ sucrose (multiplication medium) pH 5.6 – 5.8.

Protocol B was basically similar to protocol A except that 0.01 g.l⁻¹ ascorbic and 0.01 g.l⁻¹ citric acid were added to both the washing solution and the culture medium. For both protocols, buds were divided into three size categories: small (2 mm), medium (4 - 5 mm) and large (10 mm) and cultured in the growth room under 16 h light/8 h dark photoperiod at 200 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ photosynthetic photon flux density. Each sample consisted of 35 buds with 2 replicates per treatment. The rate of survival was compared between sterilisation protocols A and B and between the different size classes in each protocol.

Buds were also encapsulated using sodium alginate (section 2.2.1.3.), divided into small (0-5 mm) and large (5-10 mm) sizes then cultured on the multiplication medium consisting of MS, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.01 mg.l⁻¹ NAA, 0.2 mg.l⁻¹ BAP, 3.5 g.l⁻¹ Gelrite, 30 g.l⁻¹ and sucrose 0.01 g.l⁻¹ supplemented with ascorbic and 0.01 g.l⁻¹ citric acid (protocol A) pH 5.6 – 5.8.

3.2.1.2 Encapsulation-desiccation

A sample of 120 encapsulated buds per treatment was placed on sterile filter papers in Petri dishes without lids. Each Petri dish contained 20 alginate beads, and they were left to dry using the laminar flow for 0 - 6 h and sampled every 1 h. At each interval, a sample of 10 buds was taken for moisture content analysis and another 10 for viability determination. Buds were thereafter dried in an oven at 80 °C for 98 h, and moisture content was determined using the formula given below:

$$\frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}} \times 100$$

3.2.1.3 Encapsulation-dehydration

Sucrose-induced dehydration was attempted following the method of Poissonier *et al.* (1992). Buds were immersed in a liquid medium which consisted of MS and 0.1M sucrose for 48 h, sucrose concentrations were thereafter increased successfully every 48 h from 0.1, 0.3, 0.5, 0.75, 1.0, 1.25 and 1.5M. At each concentration, a sample of 10 buds was transferred into culture on a solid multiplication medium consisting of MS, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.2 mg.l⁻¹ BAP, 3.5 g.l⁻¹ Gelrite and 30 g.l⁻¹ sucrose (pH 5.6 – 5.8) for four weeks. Buds, after 0.1 and 1.0M sucrose dehydration treatments were desiccated using the laminar air flow for 0-5 h (3.2.1.2) then moisture content and viability were assessed.

3.2.1.4 Cryoprotection

Encapsulated buds (size 5 mm) were initially immersed for 30 min in one of the following: a) 5% (v/v) DMSO, b) 5% (v/v) DMSO with 0.5M sucrose, c) 5% (v/v) DMSO with 5% (v/v) glycerol or d) 5% (v/v) glycerol. After 30 min buds were transferred into either a) 10% (v/v) DMSO, b) 10% (v/v) DMSO with 1.0M sucrose, c) 10% (v/v) DMSO with 10% (v/v) glycerol or d) 10% (v/v) glycerol respectively. Cryoprotectants were prepared in MS basal medium and all treatments were carried out on an orbital shaker at 110 rpm.

3.2.1.5 Freezing and thawing

After the various tested pretreatments, encapsulated buds were placed in disposable polypropylene 2 ml cryovials (Corning Costar Corporation) (3 buds per vial) and mounted on aluminium canes. Samples were then rapidly immersed in a liquid nitrogen bath and the time taken for liquid nitrogen to stop bubbling was noted. After an hour, canes were removed and rapidly immersed in a water bath (37 °C) for 30 min to thaw. Alternatively, buds were thawed slowly by leaving on the laminar flow bench for 30 min. To wash off cryoprotectants, buds in alginate beads were placed in MS basal medium with 4 g.l⁻¹ sucrose in a 100 ml jar at 37 °C for 30 min. Viability after freezing was determined by culturing encapsulated buds on the multiplication medium consisting of 30 g.l⁻¹ sucrose, 3.5 g.l⁻¹ Gelrite, 1 g.l⁻¹ Benlate, 0.1 g.l⁻¹ citric and 0.1 g.l⁻¹ ascorbic acid, pH 5.6 – 5.8 (Protocol B in 3.2.1.1). Each sample consisted of 15 tubes. Replicates or sometimes triplicates of each treatment were made. Results were recorded after four weeks.

3.2.2 Cryopreservation of pollen

Samples of pollen weighing approximately 0.02 g were placed in Eppendorfs (1.5 ml), sealed and mounted on aluminium canes. Samples were then rapidly immersed in liquid nitrogen and after an hour, they were immediately transferred into cryo-boxes and stored in shelves in an ultra-low freezer at -78 °C. Pollen viability in storage was tested every second month for 12 months. To test viability, a sample of pollen in an Eppendorf was retrieved and allowed to thaw slowly on a laminar flow bench for 30 min. After 30 min, pollen viability was assessed using TTC assay described in section 2.2.3.2. a.

3.2.3 Photography and Microscopy

Growth and development of encapsulated buds was recorded using a Nikon FM2 camera with a 60 mm Mikro Nikon macro lens.

3.2.4 Data Analysis

Average survival values were recorded for the different pretreatments and the percentage moisture contents with standard error were calculated after desiccation. Where

appropriate, One Way Analysis of Variance (ANOVA) (SAS, 1982) was used to assess differences in the recorded mean values of the variables investigated. Alphabetical values were assigned to the mean values recorded per treatment. Mean values that did not share the same letter were recognised as being significantly different.

3.3 Results and Discussions

3.3.1 Studies using axillary buds

In this study germplasm conservation strategies for *Eucalyptus* spp. were divided into two categories, a) active collections for medium-term storage and/or utilisation and b) base collections for long-term conservation and as a 'back-up' for medium-term storage. The previous chapter (Chapter 2), reported on short and medium-term storage protocols of *Eucalyptus grandis*. However, maintaining active collections at minimal-growth conditions is merely aimed towards supplying material for day-to-day use in breeding and it is not suitable for long-term conservation of germplasm. Moreover, because of the routine subculturing that is associated with *in vitro* culture storage, there are possible hazards such as losses that may occur due to *in vitro* contamination (Wilkins *et al.*, 1982; Kartha, 1985; Withers, 1988). Another problem encountered with the use of *in vitro* storage techniques for conservation of germplasm is that those techniques only reduce rather than stop metabolic activity, hence the possibility of genetic instability in culture cannot be eliminated (Wilkins *et al.*, 1982; Wilkins and Dodds, 1983; Ashmore, 1997). Additionally, such growth retardants as mannitol and sorbitol used for minimal-growth have been found to cause genetic alterations to shoot cultures if stored for very long periods (section 2.3.1.3.). Medium-term storage, therefore, does not present a solution for the long-term conservation of germplasm. So far, long-term base collection can only be maintained through the use of cryopreservation techniques, so, it is essential to establish such protocols in order to have a comprehensive conservation system.

An ideal method of truly conserving genotypes is one which maintains tissues in their original state as far as possible (Ashwood-Smith and Farrant, 1980; Wilkins and Dodds, 1983). Cryostorage is such a method as, theoretically, all metabolic activities of tissues stored in this way are arrested. However, most biological material cannot tolerate freezing. The key to successful cryopreservation of any tissue lies in the prevention or minimization of damage caused by ice crystal formation within individual cells (Blakesley *et al.*, 1995). Two main variables determine the response of cells to the stresses of freezing and subsequent injury. Firstly, these are intrinsic or cellular factors

which include stages in the cell cycle, age and the size of tissues to be frozen and secondly, extrinsic or physical determinants such as the type and concentration of cryoprotectant, rate of cooling and the warming or thawing of tissue after freezing (Ashwood-Smith and Farrant, 1980; Towill, 1985; George, 1993; Engelmann, 1997). Cryopreservation thus becomes a complex process with intricate steps starting with the choice of explant, use of pretreatments, freezing and finally thawing methods. This investigation therefore focused on the establishment of optimal conditions for each of the steps and thus establishing a cryopreservation protocol for long-term conservation of *Eucalyptus* germplasm.

3.3.1.1 Choice of freezing material

The choice of explant for preservation is determined with regard to four main factors: a) the usefulness of material to the research or commercial application, b) the genetic stability of the explant, c) the size and d) the age of tissue to be cryopreserved (Withers, 1980; 1982; Kartha, 1985). Of these factors, perhaps the first two are of utmost importance with regard to germplasm conservation. Because of their stability and the uniformity of progeny to which they give rise and the fact that methods exist for their multiplication *in vitro*, axillary buds were chosen as an ideal explant for cryopreservation in this investigation. Moreover, buds have within them meristematic tissue, which is composed of homogenous populations of small, actively dividing cells with small vacuoles (Benson, 1995; Grout, 1995). Those characters are highly desirable with regard to size and age of the tissue to be cryopreserved. Ideally, the smaller the tissue the less vulnerable it is to freezing injury.

Before establishment of any pretreatment or freezing protocols, it was essential to determine methods of testing viability in order to assess the effects of investigated pretreatments on tissues. The first stage of this study was, therefore, devoted towards confirming *in vitro* bud proliferation protocols. Buds were obtained from 6 months old greenhouse plants. However, it is well known that explants from greenhouse *Eucalyptus* plants are constantly affected by both bacterial and fungal contaminations (McComb and Bennett, 1986; Warrag *et al.*, 1990; Le Roux and Van Staden, 1991; Watt *et al.*, 1996).

Stringent methods of sterilisation are therefore necessary. Several types of sterilants have been used to remove surface contamination of *in vivo* eucalypt explants. Some of those include soaking buds in sodium hypochlorite (De Fossard, 1974; Watt *et al.*, 1995; 1996) or mercuric chlorite (Blakeway *et al.*, 1993) and the use of fungicides such as Benlate[®], Bravo[®] and Previcur N[®] (Le Roux and Van Staden, 1991; Blakeway *et al.*, 1993; Watt *et al.*, 1996). Work done earlier in our laboratory indicated that the use of Bravo and Benlate fungicides effectively eliminated fungal contamination from greenhouse *Eucalyptus* spp. (Makwarela, 1996). Based on those results, two sterilisation protocols (A and B) using axillary buds of various sizes (3.2.1.1) were designed. Rates of bud survival after sterilisation were recorded after four weeks on a multiplication medium. Results indicated significantly low levels of contamination for both methods: (0 – 6% for method A and 3 – 4% for method B) (Table 3.3). It was therefore concluded that the chosen fungicides were successful in eliminating contaminants.

Even though both protocols effectively eliminated contaminants, shoots treated according to protocol A, showed significantly low survival rates due the high rates of browning (Table 3.3). Browning is often a result of phenolic exudates in culture (Herman, 1995). Death of cultures due to browning is caused by oxidation of phenolics produced from cut or damaged ends of explants and the resultant phytotoxicity. This problem is often intensified by explant sterilisation in some species of *Eucalyptus* (Cresswell and De Fossard, 1974; Cresswell and Nitsch, 1975). It was, therefore, assumed that injury was inflicted on when buds were harvested and the intensive sterilization procedure that followed could have further aggravated phenolic production. Antioxidants such as polyvinylpyrrolidone (Tulecke, 1987; Warren, 1991), ascorbic (Grewal *et al.*, 1980; George, 1993), citric and boric acid (Creemers-Molenaar and van Oort, 1990) have successfully been used to prevent phenolic production in culture. From this background information, abscisic and citric acid (both antioxidants) were added to the washing solution as well as the culture medium in protocol B and this resulted in reduction of browning by approximately 98-100% (Table 3.3B). Protocol B was therefore adopted as the best method of sterilising explants, and all subsequent sterilisation procedures were carried out using that protocol.

Table 3.3 Percentage survival of different size axillary buds obtained from greenhouse plants after sterilisation protocols A or B n = 35 buds per size class.

A) Buds were sterilised by washing with 1 g.l⁻¹ Benlate[®], 1 g.l⁻¹ boric acid and 0.5 ml.l⁻¹ Bravo[®] fungicide, then cultured on the multiplication medium consisting of MS, 0.1 mg.l⁻¹ biotin, 0.1 mg.l⁻¹ calcium pantothenate, 0.2 mg.l⁻¹ BAP, 3.5 g.l⁻¹ Gelrite and 30 g.l⁻¹ sucrose. **B)** Buds sterilised with 1 g.l⁻¹ Benlate, 1 g.l⁻¹ boric acid and 0.5 ml.l⁻¹ Bravo fungicide, 0.01 g.l⁻¹ ascorbic and 0.01 g.l⁻¹ citric acid were cultured on the multiplication medium supplemented with 0.01 g.l⁻¹ ascorbic and 0.01 g.l⁻¹ citric acid. Buds from both treatments were cultured at 28 °C, under a 16 h light and 8 h dark photoperiod at 200 μE.m.⁻².s⁻¹ for four weeks. n = 15 (replicated). sur - survival, br - browning, cont - contamination. Levels of significant difference (ANOVA) were represented by letters a-c (f = 0.05).

Size class (mm)	Protocol A			Protocol B		
	% sur	% br	% cont	% sur	% br	% cont
< 2	61 b	33	6	97 c	0	3
4-5	32 a	65	3	100 c	0	0
> 10	38 a	62	0	95 c	1	4

Aside from the question of contamination, the other problem was establishing an explant size that would be as small as possible to survive freezing but still multiply in culture. Results obtained (Table 3.3A) indicated that there was no significant difference between medium (4-5 mm) and large buds (> 10 mm), but that both had significantly lower survival rates than smaller buds (< 2 mm). The apparent reason for the low rates of survival was the high phenolic production in those size categories. However, once the problem of browning had been removed, there was no significant difference in the rates of survival of the different sizes (Table 3.3 protocol B). It was therefore decided to use the smallest sizes (approximately 2 mm) of buds for all cryopreservation experiments. The use of smaller explants was an advantage as they have been shown to freeze more

easily than larger ones and their ability to withstand desiccation is much higher. Escobar *et al.* (1997) studied the effect of shoot-tip size on freeze preservation of cassava. In their work, those authors found that viability and shoot growth of smaller shoots (1-2 mm) after freezing and desiccation increased by up to 80 and 64% respectively when compared with larger shoots (> 4 mm).

3.3.1.2 Survival of buds after encapsulation in sodium alginate

Having established relatively small explants with high survival rates, the next step was to subject explants to various pretreatments and subsequently freezing. The size of buds chosen (approximately 2 mm) was most appropriate in terms of reducing freezing injury and thus increasing survival after freezing but it presented practical problems with regard to handling during treatments. Other workers, through the use of encapsulation techniques during cryopreservation have avoided this problem. Explants from many species, for example, Japanese lily (Matsumoto and Sakai, 1995), horse-radish (Phunchindawan *et al.*, 1997), *Eucalyptus* (Monod, Poissonier, Paques and Dereuddre, 1992) and *Hevea* sp. (Engelmann, Lartaud, Chabrilange, Carron and Etienne, 1997) were encapsulated to enable handling during freezing. Additionally, classical cryopreservation techniques, which were used in this study, are based on encapsulation of explants, for example encapsulation-dehydration and encapsulation-desiccation. Encapsulation of explants prior to freezing is also an advantage as it has been shown to enhance vitrification thus reducing the possibility of freezing injury (Dereuddre *et al.*, 1990).

In view of the deemed value of encapsulation of buds for cryopreservation purposes, it was essential to establish whether encapsulation had any adverse effect on buds. Two size classes of buds, small (2 mm) and large (5-10 mm) were encapsulated using sodium alginate and the resultant alginate beads (Fig 3.3 A) were placed in culture. After four weeks on the multiplication medium, survival rates of buds were recorded and results were shown on Table 3.4 and Fig 3.3 B. Size did not seem to have any significant effect on survival of encapsulated buds (Table 3.4). These results concur with those obtained by other workers (Bapat and Rao, 1990; Redenbaugh, 1993; Piccioni and Standardi, 1995; Anon, 1997). It was interesting to note that survival after encapsulation remained

significantly high with very little contamination occurring during culture (Table 3.4). Retaining high survival rates prior to pretreatment of explant is very important as during the various manipulations and preparation of explant for freezing, the rates of survival are likely to decrease.

Table 3.4 Percentage survival of encapsulated buds. Buds were immersed in 3 % (w/v) sodium alginate dissolved in 3M sucrose for 30 min. Using a sterile 3 ml pipette, buds were dispensed into a complexing solution that consisted of 11 g.l⁻¹ CaCl₂ for 25-30 min then rinsed three times in sterile water. Survival rates were recorded after four weeks on the multiplication medium. n = 10

Size	% survival	% contamination	% browning
small (2-5 mm)	90	2	8
large (10 mm)	92	2	6

Encapsulation can be considered an important application of micropropagation, in that it can improve the success of *in vitro*-derived plant delivery to greenhouse and/or field, and contribute towards synthetic seed technology. Successful encapsulation of small sized axillary buds with very little loss of viability achieved in this study is highly significant for the forestry industry, not only with regard to the possible ease of cryopreservation but because of the ease of exchange of sterile germplasm material between laboratories (Bapat, 1993). Because of their small size and relative ease of handling, these structures, if properly preserved, can also be planted like true seed in the greenhouse or field making them extremely useful where tissue culture facilities are not available. This is possible as Bapat and Rao (1990) demonstrated by germinating encapsulated axillary buds of mulberry directly in soil.

3.3.1.3 Effects of pretreatments on axillary buds.

Living cells are seriously harmed by the internal formation of ice crystals if frozen with high moisture contents (Kantha, 1985; 1987; Withers, 1987; Grout and Morris, 1987; Grout, 1995). For a successful protocol to be achieved, it is necessary to change the physical state of water within the cells to a frozen glass-like state without crystallization. This transformation of water from liquid to a solid phase is known as vitrification (Luyet, 1937; Fahy *et al.*, 1984). Various forms of pretreatments have been devised to prevent intra-cellular ice formation and subsequent freezing injury. Three types of pretreatments were investigated in this study. They were cryoprotection using cryoprotective chemicals, desiccation and sucrose-induced osmotic dehydration. However, because it has often been found that a combination of one or two pretreatments is more effective than the use of one individually (Ashwood-Smith and Farrant, 1980), some of the treatments were also combined.

From prior discussions, it is clear that one of the main causes of freezing injury is intra-cellular water (Grout, 1995; Ashmore, 1997). During freezing, intra-cellular ice may form within cells due to the high water levels and this may cause membranes to rupture resulting in damage of the cellular structure (Kraak, 1993). Tissue desiccation is perhaps the simplest method of reducing tissue moisture content prior to freezing (Berjak *et al.*, 1989; Abdelnour-Esquivel, *et al.*, 1992a; Mycock *et al.*, 1995). This technique has been used mainly for freezing embryonic axes of some recalcitrant (Mycock *et al.*, 1995) and some orthodox seed species (Mycock *et al.*, 1991; Berjak, Farrant and Pammenter, 1990). Optimal survival rates are generally obtained when samples are frozen at moisture contents between 10 and 20 % fresh weight basis (Grout, 1995; Ashmore, 1997; Engelmann, 1997). Some of the methods of desiccation cited in the literature include rapid drying or flash-drying (e.g. Berjak *et al.*, 1989), laminar flow air drying (e.g. Grout, 1995) and drying using silica gel (e.g. Dumet *et al.*, 1993a). Of the three methods of drying, laminar flow air drying in combination with sucrose-induced dehydration is more often used for cryostorage of buds of most woody species (Stushnoff and Seufferheld, 1995; Engelmann *et al.*, 1997). Desiccation using the laminar flow hood was therefore chosen as a method of drying for cryostorage of eucalypt buds.

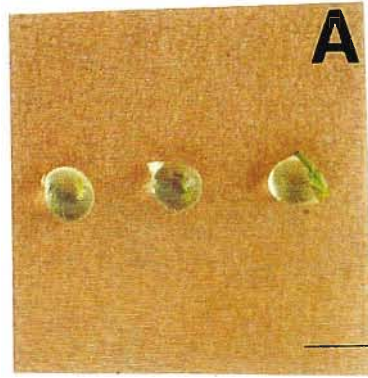


Fig 3.3 Bud encapsulation, growth and development A) Encapsulated buds [bar = 7.1 mm]. B) Encapsulated buds on a multiplication medium showing different stages of growth [bar = 6.28 mm].

In this study, the effects of laminar flow desiccation on axillary buds were noted at 1 hour intervals for 6 h (Table 3.5). Within the first 3 h, buds lost approximately 60% (from 95 - 34.1%) water by which time survival declined to 60% (Table 3.5A). Optimal pre-freezing moisture content of approximately 20% was attained only after 4 h desiccation and at this moisture, survival had declined to 40% (Table, 3.5B). Several hypothesis have been put forward in attempt to explain the effects of desiccation on tissues. One states that during drying, membrane deterioration occurs in desiccation-sensitive tissue leading to leakage of electrolytes (Tetterroo, Hoekstra and Karssen, 1995; Tetterroo, Henselmans, de Bruijn, Wolkers, van Aelst and Hoekstra, 1996). Another, that drying might interfere with the normal cellular processes by increasing production of a reactive oxygen species such as hydrogen peroxide and superoxide (Leprince, Artherton, Thorpe, Deltour and Hendry, 1990; Leprince, Artherton, Deltour and Hendry, 1994). These reactive free radicals then damage cellular constituents such as proteins, DNA and membranes leading to increased levels of fatty acids and loss of phospholipids (Leprince *et al.*, 1990, 1994). In this study, a steady decline in survival rates was observed indicating that buds were desiccation-sensitive (Table 3.5). This may have resulted in membrane deterioration that could have led to leakage hence reducing post-desiccation survival (Tetterroo *et al.*, 1995; 1996). Cellular damage could have also occurred as a result of free radicals (Leprince *et al.*, 1994). However, it was difficult to determine accurately the causes of the damage as no in-depth analysis was performed on desiccated tissues.

Buds desiccated for 0, 1 and 2 hours were not subjected to freezing due to their high moisture contents (Table 3.5). Nevertheless, buds desiccated for 3, 4 and 5 h (30, 22 and 12% moisture contents respectively) (Table 3.5), were rapidly frozen in liquid nitrogen and plated on the multiplication medium, to determine survival after four weeks. None of the desiccated buds survived freezing (Table 3.5C). It is important to understand the dynamics of desiccation together with freezing as this might help explain the inability to regenerate frozen material successfully. According to Pence (1992) tissue damage can

Table 3.5 Survival rates of encapsulated buds after drying to various moisture contents, before and after freezing in liquid nitrogen. **A)** Percentage moisture content (fwb) of buds desiccated under the laminar flow for up to 5 h. Buds were weighed before and after drying in an oven at 78 °C for 48 h. **B)** Percentage survival of buds after desiccation on a multiplication medium for 4 weeks. **C)** Percentage survival of buds after freezing in a liquid nitrogen bath for 1 h then cultured on the same medium for 4 weeks. NA refers to cases where freezing was not attempted.

Time (hours)	A	B	C
0	95.0 ± 0.02	90	NA
1	89.7 ± 0.01	90	NA
2	68.7 ± 0.11	60	NA
3	34.1 ± 0.07	60	0
4	21.5 ± 0.09	40	0
5	11.8 ± 0.08	30	0

occur when moisture levels drop below a ‘critical’ point. However, if levels are higher than the ‘critical’ level, ice formation may occur during liquid nitrogen exposure resulting in tissue damage. Thus there is only a narrow ‘window’ at which survival through liquid nitrogen is obtained (Grout, Shelton and Pritchard, 1983; Normah *et al.*, 1986; Pritchard and Pendergast, 1986). When tissues are dried, freezable water is lost first, followed by non-freezable water, which is associated with macromolecules. If freezing is carried out while freezable water is present in cells, potentially damaging phase transitions can be observed (Vertucci, 1989). At the threshold moisture level (estimated to be between 10 and 20%), all freezable water is removed and non-freezable water is present. Ideally, this is the point of successful freezing. However, this becomes very complex as threshold moisture levels differ with different tissues in relation to the lipid content (Vertucci and Leopold, 1987). This is further complicated by the fact that exposure to liquid nitrogen also results in tissue desiccation (Vertucci, 1989). Therefore,

depending on the species and the procedures used, some tissues may survive liquid nitrogen exposure with low levels of freezable water present while some tissues with no freezable water may still be damaged by liquid nitrogen (Vertucci, 1989). The ability to survive desiccation therefore might not guarantee that cellular damage may be avoided during cryopreservation (Pence, 1992). This has been the case in this study (Table 3.5). Even though theoretically non-freezable water would have been removed at 20% moisture, the relatively narrow range of moisture at which liquid nitrogen exposure can be tolerated made cryostorage difficult to achieve.

Although desiccation on its own was not successful in the prevention of freezing injury, information that was of future relevance was obtained from this exercise. Namely, it was determined that alginate encapsulated buds can be dehydrated to the required moisture content of between 10 - 20 % within 4 to 5 h and that the expected rates of survival on average were between 30 and 40 % respectively. This information was used in subsequent studies.

Desiccation alone as a freezing pretreatment has been most popularly used when freezing explants such as embryonic axis (Pritchard and Pendergast, 1986; Vertucci *et al.*, 1991; Wesley-Smith *et al.*, 1992) and seedlings (Pita *et al.*, 1997). This technique has also been successfully used for somatic embryos (Dumet *et al.*, 1993a; 1993b; Berjak *et al.*, 1996; Mycock *et al.*, 1995; Pence, 1995). However, no reports were found where desiccation alone resulted in successful cryopreservation of encapsulated buds. Rather, desiccation in combination with osmotic-induced dehydration has been successfully used for the cryopreservation of encapsulated buds and it is often referred to as encapsulation-dehydration (Matsumoto *et al.*, 1995b; Puchindawan *et al.*, 1997).

Encapsulation-dehydration is one of the recently developed methods of cryopreservation procedures and it entails pregrowth of explants in high concentrations of sucrose. In this technique, progressive osmotic dehydration causes extraction of water from cells which is replaced by sucrose (Dereuddre *et al.*, 1990). Additional water is removed by subsequent evaporation during desiccation which further increases the concentration of sucrose in the

beads (Dumet *et al.*, 1993b). It is well known that at high concentrations, sucrose like other sugars, plays an important role in the mechanism of resistance of plant cells and organs to desiccation (Leopold, Sun and Benal-Lugo, 1994; Dumet *et al.*, 1993a; Hoekstra, Wolkers, Buitink, Golovina, Crowe and Crowe, 1997). Results obtained after desiccation indicated a rapid decline in survival as a result of desiccation (Table 3.5), establishing that the chosen explant was intolerant to desiccation below 20% moisture level. Hence as incorporation of desiccation with sucrose-induced dehydration has been shown to increase tissue tolerance to desiccation, the two treatments were combined (Dereuddre *et al.*, 1990; Paulet *et al.*, 1993; Gonzalez-Arno *et al.*, 1995).

The use of osmotic dehydrants such as sucrose has an advantage over the more commonly used cryoprotectants like DMSO, which are often toxic and sometimes less efficient with regard to cryoprotection of most woody species (Matsumoto and Sakai, 1995). Significantly high post-freezing survival rates have been reported using encapsulation-dehydration techniques on explants from woody trees such as walnut somatic embryos (de Boucaud *et al.*, 1991), shoot tips of apples, pears and mulberry (Niino and Sakai, 1992), meristem of banana (Panis *et al.*, 1996) and shoot tips of grapevine (Plessis, Ledder, Collas and Dereuddre, 1993). Monod *et al.* (1992) has also successfully used treatment with high concentrations of sucrose in the genus *Eucalyptus* on encapsulated buds of *E. gunnii*. Those authors noted that during preculture, the higher the sucrose concentration the higher the tissue tolerance to desiccation and subsequent freezing. However, at very high concentrations, sucrose was detrimental to tissue survival. This was also observed by authors such as Matsumoto and Sakai (1995), Monod *et al.* (1992), Scottez, Chevreau, Godard, Arnaud, Duron and Dereuddre (1992) and Bachiri, Gazeau, Hansz, Morisset and Dereuddre (1995). It was therefore decided to initiate a preliminary study in order to determine the effects of different sucrose concentrations on encapsulated buds.

Preculturing of buds in sucrose generally caused a slight decline in survival (Fig 3.4) however, the highest decline was observed at 1.5M preculture. This was to be expected because very high concentrations of sucrose have often been found to be detrimental to

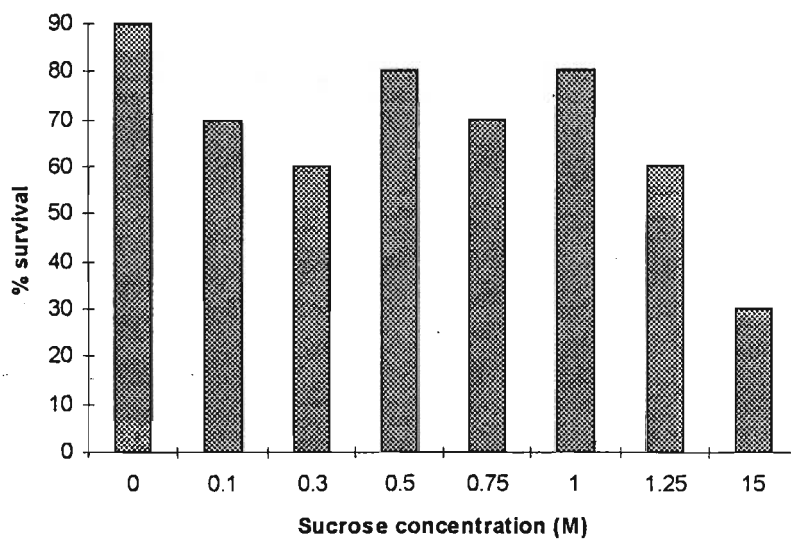


Fig 3.4 Effect of sucrose concentration on survival of encapsulated buds. Buds were precultured in sucrose from 0.1 - 1.5M progressively increased at 48 h intervals and then cultured for four weeks on the multiplication medium.

the survival of most tissues (Blakesley *et al.*, 1996). This is a consequence of osmotic gradient that is created because of the difference in cellular water potential and that of the surrounding high concentration of sugar solution (Koster and Leopold, 1988). As a result, cellular water leaves the cell too rapidly to be replaced by sucrose causing the cell to shrink and hence irreversible damage results (Crowe and Crowe, 1988; Koster and Leopold, 1988; Sun, Irving and Leopold, 1990; Hoekstra *et al.*, 1997). It was for this reason that in this study high concentrations were not introduced rapidly. Rather, buds were initially precultured at low concentrations, which were increased progressively to higher concentrations at 48 hour intervals. However, as has been observed in this study and other studies (Monod *et al.*, 1992; Scottez *et al.*, 1992) at very high levels of sucrose, damage is inevitable and in our study, damage occurred at 1.5M (Fig 3.4).

From this investigation (Fig 3.4), it was concluded that buds were sensitive to sucrose concentrations above 1.25M, hence further work was restricted to this and lower concentrations. Consequently, the next stage was to test if sucrose would increase tolerance to desiccation as has been reported (Sun and Leopold, 1997). The choice of explants to be desiccated following sucrose-induced dehydration was between the highest (1.0M) and the lowest (0.1M) concentrations. Buds precultured at 0.1 and 1M sucrose were thus desiccated using the laminar flow hood for 5 h and comparative observations on the appearance, the rate of survival and loss of moisture were made.

The general appearance of freshly encapsulated alginate beads was that of transparent spherical balls which could be handled easily without sticking (Fig 3.3A). With regard to appearance during desiccation, 0.1M precultured buds were initially transparent and the buds inside the capsule clearly visible. After the first 3 h of desiccation, beads had shrunk considerably losing their spherical shape, becoming sticky and difficult to handle. Shoots precultured in 1M sucrose, on the other hand, had an opaque glassy appearance. This appearance together with the shape was not affected even after desiccation for 5 h. Samples of buds from 0.1 or 1M preculture treatments were analysed for moisture content while others were cultured on the multiplication medium, after hourly intervals of desiccation.

Initially buds, precultured at 1M sucrose had considerably low levels of moisture (approximately 63%) compared with buds precultured on 0.1M (above 90%) (Fig 3.5 B and A respectively). Presumably, this was due to the fact sucrose had penetrated and replaced most of the cellular water by osmotic gradient. In their work with embryogenic potato callus, Blakesley *et al.* (1996) also found that moisture content after preculture at high concentrations of sucrose, but before desiccation, were significantly lower than when buds were not precultured. Other researchers made similar observations, for instance Paulet *et al.* (1993) working with apices of plantlets of sugar cane and Matsumoto and Sakai (1995) with *Wasabi japonica*. In this study, because the initial moisture content of high sucrose precultured buds was so low, the time necessary to reach 20% moisture content was reduced to 3 h as opposed to 4-5 h (Fig 3.5A) needed in cases of buds precultured at 0.1M. In terms of survival, buds precultured in high concentrations of sucrose had consistently higher survival rates through out drying (between 65% and 85%) while a steady decline was observed with buds cultured at lower concentrations (from 88% to 24%). From these results, it was concluded that buds precultured at 1.0M could withstand desiccation to 20% moisture levels while maintaining high rates of survival. Theoretically, once tissue is saturated with sucrose and desiccated to the given levels of moisture, freezing must occur without ice damage (Koster, 1991). In support of this theory, Gonzalez-Arno and co-workers (1995) performed a differential thermal analysis on sucrose dehydrated buds and found that increasing sucrose molarity had a marked effect on the toleration-ability of buds to drying and freezing. The use of sucrose build-up in tissues as a cryoprotectant has been extensively applied in cryopreservation of buds (e.g Niino and Sakai, 1992; Scottez *et al.*, 1992; Reed, 1996; Schafer-Manuhr, 1996) with much success, however no reports showing actual tissue analysis to ascertain the role of sucrose as a cryoprotectant were found.

Having ascertained the role that sucrose has in the ability of buds to withstand desiccation, the next step was to determine whether a combination of sucrose-induced dehydration and desiccation had any effect on post-freezing survival. To determine the

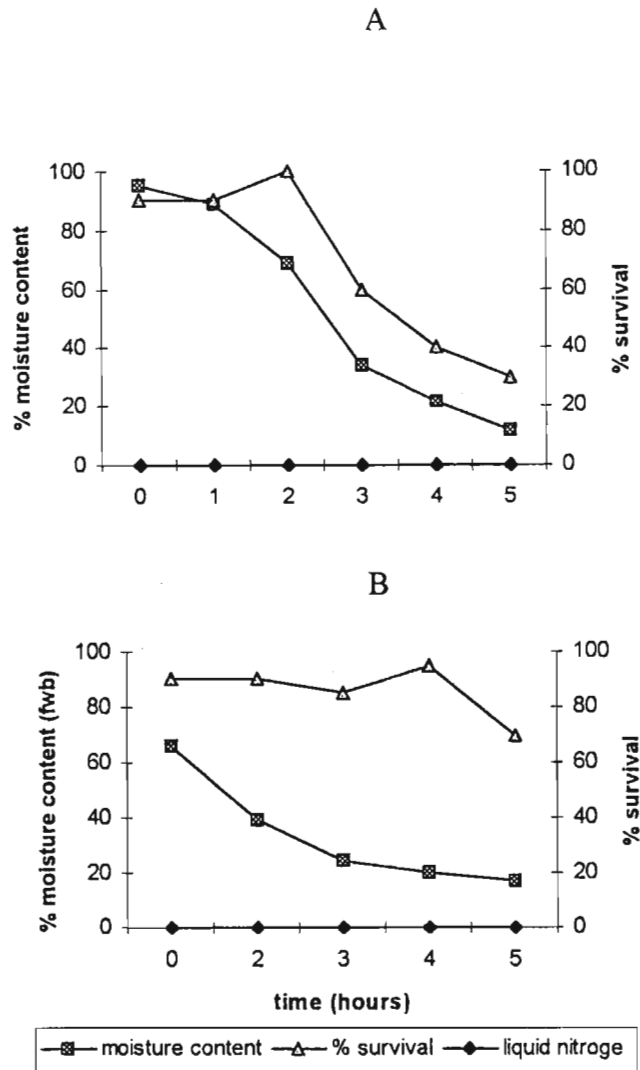


Fig 3.5 Effect of sucrose preculture and laminar flow desiccation on survival of encapsulated buds A) 0.1 M sucrose and B) 1.0 M sucrose pretreatment, followed by drying using the laminar flow hood for 1 to 5 h and rapid freezing in a liquid nitrogen bath for 1 hour prior to culture on multiplication medium for four weeks.

freeze-tolerance of precultured buds to liquid nitrogen treatment, buds that were precultured in sucrose (0.1M and 1.0M) and desiccated for 0-5 h, were frozen then thawed in a 37 °C water bath for 30 minutes and placed on a multiplication medium. None of the frozen material survived after four weeks on the multiplication medium. This was contrary to most of other cited studies. Preculturing with high concentrations of sugars such as sorbitol and sucrose was reported to be very important in improving survival of cryopreserved cells, meristems and somatic embryos (Kantha *et al.*, 1988; Uragami, Sakai and Yamada, 1990; Dereuddre, Hassen, Blandin and Kamanski, 1991; Niino and Sakai, 1992; Matsumoto *et al.*, 1994). The mechanism by which the accumulation of sugars reduces injury is based on the removal of crystallizable water to the point of reaching a glassy state (Williams and Leopold, 1989; Koster, 1991). Their presence in cells thus ensures that crystallization during freezing is avoided. Sugars such as sucrose are also believed to have a stabilizing effect on the lipid bilayer membranes by forming hydrogen bonds between free sugars and lipid headgroups (Dumet *et al.*, 1993b; Thierry *et al.*, 1997), thus allowing membranes to withstand dehydration stress. However, the lack of survival of desiccated sucrose-dehydrated buds indicated that intracellular ice formation had occurred resulting in cellular damage. Due to the fact that buds were exposed to very high sucrose concentrations, they were theoretically saturated with high concentrations of sucrose and presumably all crystallizable water was successfully removed without a significant loss of survival (Fig 3.5B). It would seem, therefore, that the damage was more likely to have occurred as a result of the freeze/thaw process rather than poor sucrose protection (a detailed discussion can be found in section 3.3.1.4). Consequently, another pretreatment involving chemical cryoprotection was attempted since the possibility of poor sucrose protection however remote could not be totally ignored.

Under natural circumstances, very few plant cells can survive freezing to and thawing from -196 °C as has been shown through the various exercises attempted to freeze eucalypt buds in this study. Thus the effect of a large number of potential cryoprotectants on cellular recovery from freezing has been extensively studied in both mammals and

plants. Some of the commonly used cryoprotectants are dimethylsulphoxide (DMSO), glycerol, mannitol, sorbitol and (poly)ethylene glycol (PEG). The mechanism of such protective chemicals is based mainly on their ability or lack of ability to penetrate the cell thus they are broadly grouped into penetrating and non-penetrating. Cryoprotectants are believed to reduce chilling-injury by depressing the freezing/melting and supercooling point (the temperature at which homogenous nucleation of ice occurs) (Ashwood-Smith and Farrant, 1980; Kartha, Leung and Gamborg, 1979; Grout, 1995; George, 1996). By penetrating through the cell membranes, they replace cellular water, reduce the concentration of other dissolved solutes and reduce the possibility of ice formation. They also increase viscosity of cellular solutions thus further retarding ice nucleation and making vitrification easier to achieve.

The use of cryoprotectants has successfully eliminated problems of freezing injury and resultant death of frozen tissue where encapsulation-dehydration has failed in such species as *Hevea brasiliensis* (Engelmann *et al.*, 1997). Since attempts to freeze axillary buds of *Eucalyptus* sp. using encapsulation-dehydration techniques had proved unsuccessful (Table 3.5; Fig 3.5A and B), an alternative route using cryoprotective chemicals was investigated, viz. the effectiveness of numerous cryoprotectants on the ability to reduce chilling-injury during freezing. However, the use of cryoprotective chemicals is often associated with toxicity that has often prompted the need for washing after cryoprotection (Rowe and Lenny, 1980; Langis and Steponkus, 1989; 1990; Steponkus, 1992). The first step of this study was, therefore, to establish whether the selected cryoprotectants had any deleterious effects on plant cells and if washing could reverse the toxic effects of such chemicals. All cryoprotectants were tested at room temperature, with concentrations between 5 and 10% (v/v), using a two step increment of concentration. The chosen concentrations were based on several studies, which had successfully used a similar group of cryoprotectants at those concentrations. Examples include 10% (v/v) DMSO (*H. brasiliensis*) (Engelmann *et al.*, 1997) and *C. sinensis* (Oerez, Navarro and Duran-Vila, 1997), 15% (v/v) DMSO (*Quercus faginea*) (Gonzalez-Benito and Perez-Ruiz, 1992), 10% (v/v) DMSO combined with 10% (v/v) glycerol (*Cocos nucifera*) (Bajaj, 1984) and 0.5M sucrose and 10% (v/v) DMSO (*Theobroma*

cacao) (Pence, 1991).

All cryoprotectants were found to have an adverse effect on encapsulated buds *Eucalyptus* sp. although their effects varied in gravity (Table 3.6A). Compared with the control, survival after pretreatment with DMSO alone decreased by 70% (from 95 - 25%)

Table 3.6 The effect of various cryoprotectants on encapsulated buds. Bud size = 5 mm. Cryoprotection was achieved in two stages, stage 1: alginate beads were immersed in a medium that consisted of 5% (v/v) DMSO, 5% (v/v) DMSO with 0.5 M sucrose or with 5% (v/v) glycerol, and 5% (v/v) glycerol alone for 30 min on an orbital shaker. Stage 2: beads were transferred to 10% (v/v) DMSO, 10% (v/v) DMSO with 1.0M sucrose, 10% (v/v) DMSO with 10% (v/v) with glycerol and 10% (v/v) glycerol for another 30 min. All cryoprotectants were prepared on MS basal medium. After cryoprotection, buds were transferred on to a multiplication medium and results were recorded after four weeks. **A)** Survival of axillary buds after pretreatment with various cryoprotectants. **B)** Survival after pretreatment followed by washing in MS and 4 g.l⁻¹ sucrose. **C)** Survival after pretreatment with cryoprotectants, laminar flow air drying for 4 h (20% moisture content), rapid freezing in liquid nitrogen (LN) for 1 hour and rapid thawing in MS and 4 g.l⁻¹ sucrose at 37 °C for 30 min. n = 15 (3 replicates) NA refers to cases where freezing was not attempted. Levels of significant difference (ANOVA) were represented by letters a-d (f = 0.05).

Cryoprotectant	% survival after treatment		
	A (cryo)	B (cryo + wash)	C (cryo + LN + dry + wash)
control	90 a	85 a	0
DMSO	25 d	50 c	0
DMSO + sucrose	33 d	50c	NA
DMSO + glycerol	63 b	63 b	0
glycerol	50 c	30 d	NA

but after washing with a solution of MS and 4 g.l⁻¹ sucrose percentage survival increased two fold (Table 3.6A and B). This was expected as, even though DMSO is highly effective as a cryoprotectant of plant cells, it is also extremely poisonous to cells particularly after long-term use (Ashwood-Smith and Farrant, 1980; Morris, 1980; Withers, 1987; 1988). There is also evidence for interference by DMSO with microfibrils, microtubules and cell division processes, which consequently curtail the cells' ability to grow normally (Fahy, 1986; Withers, 1987). By introducing a washing step, a negative gradient was introduced so that the sucrose washing solution replaced the toxic DMSO within the cell. Similar observations were made when a mixture of DMSO and sucrose was used (Table 3.6). A significant increase in survival as compared with unwashed buds was obtained after the washing stage was introduced. However, the inclusion of a washing step had a negative effect on buds pretreated with glycerol (survival decreased from 50 to 30% without and with washing respectively). Glycerol is a high molecular weight additive that penetrates plant cells very slowly (Kantha, 1985; Grout and Morris, 1987). Thus damage is highly likely to occur during its introduction and removal. If cells are transferred abruptly from a glycerol-containing medium to one without, water may rapidly permeate the cell in response to the osmotic gradient before glycerol can leave the cell and this will cause shrinking of the of the cell membrane away from the cell wall (Ashwood-Smith and Farrant, 1980). This was possibly the reason why lower survival rates were observed after introducing the washing stage in glycerol protected buds.

Combinations of additives have been often used and this reportedly has greater effect than individual cryoprotective agents (Grout and Morris, 1987; Withers, 1987). The combination of glycerol and DMSO used in this study proved to be more effective than each cryoprotectant on its own (Table 3.6). In contrast, sucrose combined with DMSO was not as effective. Glycerol and DMSO are believed to make a good combination based on the fact that the relatively low molecular weight DMSO rapidly penetrates cells while glycerol, which has a larger molecular weight, is slower to penetrate (Ashwood-Smith and Farrant, 1980; Kantha, 1985). Since DMSO permeates cells easily and rapidly,

it modifies the permeability characteristics of the cell, thus making it possible for the slower penetrating glycerol to be taken up more rapidly (Grout, 1995).

Due to the fact that buds pretreated with a mixture of DMSO and glycerol showed the best survival rates, buds obtained from this treatment were chosen and partially desiccated to 20% moisture content. Partial desiccation of cryoprotected cells is important during freezing because it reduces cellular water levels and thus solutes within the cell become more concentrated (Ashwood-smith and Farrant, 1980). It was essential to partially desiccate tissues in this study as this has been reported to increase post-freezing survival of embryonic axes. Cryoprotected pea embryonic axes, for example, were partially dehydrated and rapidly immersed into liquid nitrogen and relatively high survival rates were obtained (Mycock *et al.*, 1991).

Partial desiccation of explants, pretreated with DMSO and glycerol for 4 h, using the laminar flow to approximately 20% moisture resulted in survival rates of $78.3 \pm 9.5\%$ (Table not included). From those results it was concluded that desiccation of cryoprotected buds had no significant effect on survival. Since desiccation did not have a significant effect on survival of cryoprotected encapsulated buds, buds pretreated with DMSO, glycerol or a combination of the two were thus rapidly frozen in liquid nitrogen without desiccation. After 1 hour, buds were thawed by plunging into a washing solution consisting of MS and 4 g.l^{-1} sucrose at 37°C and rotated on an orbital shaker at 110 rpm for 30 min. Survival of beads cultured on the multiplication medium for four weeks remained at 0% (Table 3.6C).

Treatment with cryoprotectants is a complex procedure that involves many factors. In order to ensure effective cryoprotection, several parameters can be manipulated (Wilkins and Dodds, 1983). For example, Morris (1980) showed that the effectiveness of penetrating additives is a function of temperature, time of exposure and concentration of cryoprotectant. That author demonstrated that survival of *Euglena gracilis* cells after exposure to 1.5M DMSO decreased with increasing time, while exposure at lower temperatures (0°C) resulted in better survival rates than at higher temperatures (20°C). It

is these varying responses of individual species that require extensive variations of conditions to suit each particular species. In this study, the limited time available made it impossible to explore all possible variations in order to increase the effectiveness of cryoprotectants.

3.3.1.4 Post-freezing effects

Successful cryopreservation can be carried out by using either slow cooling or vitrification procedures (Benson, 1990; Potter and Jones, 1991; Blakesley *et al.*, 1996). Besides the use of various pretreatments to enable vitrification, the rate of cooling and thawing is very important in prevention of intracellular ice formation (Henshaw, 1975; Withers, 1980; Grout and Morris, 1987; Grout, 1991; 1995). Freezing rates can thus be categorised into three groups, step-wise cooling, slow and/or rapid freezing. Slow freezing may be achieved through controlled cooling rates of -30 to -60 °C/h followed by rapid immersion in liquid nitrogen. Slow cooling gives rise to cellular dehydration, which progressively concentrates cellular constituents and depresses freezing point and thus 'ice damage' is prevented. Once cellular solutions have been concentrated by slow cooling, remaining water can be easily vitrified by rapid freezing (Grout, 1995). An alternative method is one in which samples may be progressively subjected to a series of sub-zero temperatures (step-wise cooling) before plunging into liquid nitrogen. For example, cell suspension cultures of *Acer psuedoplatanus* pretreated with 5% glycerol were held for 3 min at -10, -15, -20, -30 and -40 °C before plunging in liquid nitrogen (Niino, 1995).

The high costs associated with slow and step-wise cooling have limited their application in most cryopreservation studies to a few specialized laboratories. Moreover, slow and step-wise cooling have been found to be unsuccessful for cryopreservation of most organized plant structures such as shoot apices and buds (Nitzsche, 1983; Yamada *et al.*, 1991). Due to the lack of specialized equipment, those two methods were ruled out as inappropriate in this study. Moreover, the use of organised plant explants in this work further rendered those methods undesirable. Rapid freezing was therefore the only alternative method of freezing that could be applied in this case. Using that method, cells that are at relatively low moisture contents (approximately 20%) can be cooled

sufficiently for rapid internal water to become directly vitrified without any need for prior slow cooling (Grout and Roberts, 1995). Rapid cooling has proved highly successful for freezing encapsulated shoot apices of several species such as *Dioscorea* spp. (Mandal *et al.*, 1996), alginate coated meristematic cells of *Musa* spp. (Abdelnour-Esquivel, Mora and Villalobos, 1992b; Panis *et al.*, 1996), encapsulated sugar cane apices (Paulet *et al.*, 1993) and apical meristem of wasabi (Matsumoto *et al.*, 1995) thus it was a highly advantageous route for freezing encapsulated axillary buds.

After various pretreatments, buds were frozen in cryo-vials mounted on aluminium canes. The time taken from the moment cryo-vials were immersed in liquid nitrogen up to complete freezing (estimated as the time taken for the initially boiling liquid nitrogen to settle) was found to be approximately 15 seconds, giving estimated freezing rates of approximately 800 °C/min. Those rates of freezing were acceptably high as according to George (1993) rates of between 300 and 1000°C are rapid enough to prevent injury. However, no frozen material was successfully retrieved from storage as no growth was observed after a four week culture period on the multiplication medium (Table 3.5; 3.6 and Fig 3.4; 3.5).

It has been found that the type of container used for freezing can also play a significant role in the rates of explant freezing that may be achieved (Grout and Morris, 1987). While direct contact of liquid nitrogen with explant can be detrimental, it is essential to maintain the distance between the two as minimal as possible (Kantha, 1985). Often, enclosing large samples in thick-walled containers can reduce rates of freezing and hence freezing injury may occur with the consequent decrease in rates of survival (Grout, 1995). The use of cryo-vials, in this study, was attributed as one of the factors that could have caused injury during freezing or thawing during the course of this investigation. For the purpose of this study, in order to increase contact between explant and liquid nitrogen, the use of slightly more thin-walled containers such as aluminium envelopes was attempted (Grout *et al.*, 1978). Although freezing rates obtained as a result of using aluminium envelopes would have slightly increased, this did not have any effects on viability (results not included). Moreover, high rates of contamination (approximately

60%) were associated with the use of aluminium envelopes hence this method was abandoned. The use of thin-walled containers to increase freezing rates has been attempted in several other studies (Mycock *et al.*, 1991; Grout *et al.*, 1978). Mycock *et al.* (1991) attempted cooling explants using cryo-vials, aluminium envelopes or wire meshes, and they found that the latter were most suitable. Their results therefore further established the fact that allowing as much explant/liquid nitrogen contact as possible can increase survival. From the results obtained in this study, it was obvious that freezing injury had occurred during either freezing or thawing resulting in lethal injury thus the consequent lack of survival (Table 3.5; 3.6; Fig. 3.4; 3.5).

The manner in which tissues are thawed after freezing is equally as important as the rate of freezing with regard to intracellular ice formation (Ashwood-Smith and Farrant, 1980; Kartha, 1985; George, 1993) and resultant freezing injury. During re-warming from -196 °C to room temperature, it has been shown that the vitreous ice structure achieved during freezing can become crystalline at about -130 °C. If crystals result, they will cause irreversible damage to cellular membranes causing a decrease in survival. In this way, even if lethal ice formation was successfully avoided during freezing, its occurrence during thawing can be equally as dangerous. Thus the importance of thawing could not be ignored in this study as it can easily destroy tissue that has successfully undergone various stages of preparation.

Frozen tissue can be thawed in one of two ways, rapid or slow re-warming. If rapidly thawed, re-crystallization temperatures can be bypassed quickly enough to prevent ice formation. For these reasons, rapid thawing is usually more successful in preserving viability of frozen material. Rapid re-warming has often been achieved by agitating sample vials in a 37 °C water bath (Withers and Engelmann, 1996). Results presented in this study (Table 3.5; 3.6 and Fig 3.5) were from explants that were rapidly thawed. In this investigation, rapid re-warming was achieved by either plunging vials containing explants in a 37 °C water bath and subsequently washing out cryoprotectants, where appropriate, in a solution of MS nutrients and 4 g.l⁻¹ sucrose (Table 3.6). Alternatively, explants were immersed directly in the washing solution, which was at 37 °C and agitated

at 110 rpm for 30 minutes. However, neither approach was effective as no frozen tissue survived. The alternative slow thawing procedures were therefore attempted.

The process of thawing samples slowly is often linked with recrystallization and intracellular ice formation (Grout, 1995). However, there are few instances where slow thawing gave higher survival rates than rapid thawing methods. Examples include work of Mandal *et al.* (1996) who thawed encapsulated yam buds by leaving them on the laminar flow for 15 to 30 min. Slow thawing by leaving frozen samples in cryo-vials on a laminar flow bench for 30 min was thus investigated and in this work results of bud survival on the multiplication medium were noted after four weeks. No survival was obtained for tissues thawed in this way either (results not presented).

3.3.2 Freeze-preservation of pollen

The preservation of pollen is very important for various purposes such as international exchange of germplasm, haploid cell production, biochemical and physiological studies. Moreover, pollen conservation is especially important for breeding and tree improvement programs as it can be applied in hybridization (Towill, 1985; Bajaj, 1987; Grout and Roberts, 1995). Hybridization of trees that flower at different times or places and also cross pollination of species that mature at different times can be easily attained by maintaining a steady supply of viable pollen. As mentioned previously, storage methods at room temperature (28 °C) or in the fridge (4 °C) are for short- to medium-term use and pollen viability is reduced or lost with the passage of time (Table 2.10). Thus, for meaningful long-term conservation of germplasm, cryopreservation in liquid nitrogen seems to be the most viable possibility (Alexander and Ganeshan, 1988; Grout, 1995). Cryopreservation of pollen is rather simple compared with that of other explants such as axillary buds, shoot tips and embryonic axes (Stanley and Linskens, 1974; Kartha, 1985; Towill, 1985). This perhaps is due to the low water content, highly packed storage material such as sugars, starches, and oils, the non-vacuolated nature and resistant exine characteristic of pollen (Stanley and Linskens, 1974).

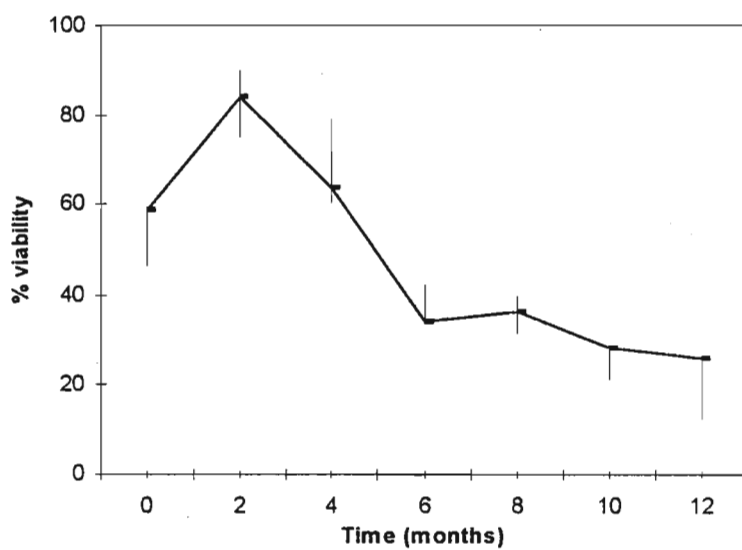


Fig 3.6 Percentage germination of pollen rapidly frozen in liquid nitrogen for 1 h followed by storage at -78 °C for 12 months as determined using TTC assay

A simple procedure of cryopreserving pollen was adopted in this study. It involved rapid freezing of pollen in liquid nitrogen followed by storage at $-78\text{ }^{\circ}\text{C}$ for 1 year. Because of logistic problems of collection and delivery, pollen used for this work had been in storage for one month at $4\text{ }^{\circ}\text{C}$ in silica gel. Viability of pollen during the 12 months of storage at $-78\text{ }^{\circ}\text{C}$ was periodically tested using TTC assay on pollen slowly thawed in the laminar flow for 30 min (Fig 3.6).

At the time of storage, pollen germination was relatively low followed by a significant increase after two months of storage (from $53\% \pm 6.6$ to $87\% \pm 7.5$). By the sixth month, germination had deteriorated significantly from $67.7\% \pm 9.9$ obtained after 4 months to $38.8\% \pm 4.2$. The type of increase in germination recorded here after the first two months of storage has been observed by several authors who studied pollen storage at very low temperatures (Visser, 1955; Heslop-Harrison, 1971). For example, exposure of pine pollen to $-20\text{ }^{\circ}\text{C}$ for a few days increased seed yields over those obtained with non-frozen pollen (Heslop-Harrison, 1971; Farmer and Barnett, 1974). Germination of tomato pollen after 3 years of storage also increased from 47 to 63%, followed by normal seed set (Grout *et al.*, 1978). Although not experimentally proven, these effects appear to be due to after-ripening processes that may occur after shedding or, possibly, freezing procedures may have produced some needed nutrients resulting in increased viability after storage (Kantha, 1985; Towill, 1985). According to Stanley and Linskens (1974) very little is known about the factors that cause decreased pollen viability during storage. However, those authors attributed such changes to the possible response of pollen cells to inactivation of enzymatic activities and the resultant reduction of metabolic substrates that are essential for pollen germination. Inactivation of enzymatic activities occurs as a result of storage at ultra low temperatures. Even though this is an advantage in terms of genetic conservation, it may be a disadvantage as it has been shown to be the ultimate cause of death of pollen cells during storage (Stanley and Linskens, 1974).

Pollen cryopreservation procedures are often achieved through vacuum- or freeze-drying and this has significantly extended storage periods of mature pollen (Kantha, 1985; Towill, 1985; Grout and Roberts, 1995). This technique has provided storage periods of

up to 4 years for a wide variety of fruit, forest and other agricultural species (Table 3.1) (King 1965; Akihama, Omura and Kozai, 1978; Towill, 1985). However, for this particular study, drying samples prior to cryopreservation was not attempted for several reasons, the main being that results presented in section 2.3.2.3 in the previous chapter led to the conclusion that desiccation using silica gel was detrimental to pollen survival. The other reason was that samples had been in storage in the presence of silica gel for almost a month before cryopreservation was attempted and their moisture content was presumably relatively low (it must be noted that due to limited availability of samples, moisture content was not determined prior to freezing).

Pollen exposure to cryogenic temperatures is usually by direct immersion into liquid nitrogen bath with the use of small vessels. For example, rose pollen was frozen rapidly by Marchant, Power, Davey, Chartier-Hollis and Lynch (1993), while Hughes *et al.* (1991) found that direct exposure of pollen over vapours of liquid nitrogen for 1 h resulted in 54 - 74% germination and maize pollen was successfully cryopreserved in small ampoules (Barnabas *et al.*, 1988). Thawing methods more prevalent in the reviewed literature are rapid rather than slow. Rates of up to 2000 °C/min were successfully used for thawing pollen of several species. For example, *Clanthus formosus* pollen, which was rapidly thawed in a 40 °C water bath at rates close to 2000 °C/min (Hughes *et al.*, 1991). In this study, *Eucalyptus* pollen was thus rapidly frozen in liquid nitrogen and thawed on the laminar flow bench for 30 minutes. The high rates of survival obtained here correspond with those of other workers who had successfully cryopreserved pollen without any prior cryoprotection (Grout *et al.*, 1978; Weatherhead *et al.*, 1978; Hughes *et al.*, 1991; Inagaki and Mujeebkazi, 1996; Sacks and Stclair, 1996; Pooler and Scorza, 1997).

Cryostorage of pollen was first reported in 1922 by Knowlton while working with *Antirrhinum* pollen. In the last few decades, pollen of many crops and woody species has been successfully preserved e.g. *Glycine max* (Collins *et al.*, 1973), *Citrus limon* (Ganeshan and Alexander, 1991), *Triticum* (Inagaki *et al.*, 1997), *Manihot esculenta* (Marin *et al.*, 1990), *Rosa* sp. (Marchant *et al.*, 1993), sub-tropical peaches and nectarine

species (Barbosa, Campo-Dall'Orto, Ojima, Martins and Boaventura, 1991) and some deciduous trees (Akihama *et al.*, 1978; Bueno, Gomez, Boscaiu, Manzanera and Vicente, 1997). Cryopreservation of pollen provides an additional dimension for germplasm storage and it offers the possibility of significantly extended storage periods for the generally difficult to maintain pollen (Barnabas and Rajki, 1976; Grout, 1995; Grout and Roberts, 1995). *Eucalyptus* pollen stored at cryogenic temperatures retained viability for up to 12 months and this enabled longer storage periods compared with storage at 28 and 4 °C (2.3.2.3). The relatively short storage period at -80 °C could have been due to the fact that pollen used for freezing was not fresh, that is it had been stored in the presence of silica gel for a few months before freezing. Theoretically, pollen should survive infinitely during cryostorage for example, cryopreservation of *Zea mays* resulted in extended storage duration of up to 10 years storage (Barnabas and Kovacs, 1997).

Controlled breeding is of essence especially where parentlines for hybridisation flower at different times or are at different locations. Maintenance of viable pollen at all times can therefore cut down on the time, expenses and space required to keep parents with genes of interest. Moreover, pollen stored at ultra-low temperatures can be of use in national and international germplasm exchange. Since medium-term storage of *Eucalyptus* sp. resulted in longevity of less than a year (Table 2.10), cryopreservation can be a useful long-term alternative. For example, in cases where one species does not flower well the one year, frozen pollen from the previous year could be used for pollination. Moreover, because the ability to cryopreserve pollen presents a long-term solution for pollen preservation, it can become applicable in cases where for instance a particular cross has to be repeated 5 or more years later. In this way, the space, time and expenses required to maintain parentlines could be highly reduced. Storage pollen frozen can also be used as a means of reducing dissemination associated with inter-regional transportation of plants for breeding.

Cryopreservation theoretically arrests all metabolic activities of tissues during storage (Withers and Street, 1977). Using cryopreservation, pollen could be stored at different stages of development. In addition, the ability to store and successfully retrieve pollen at

various stages of growth could be used as a tool for understanding the physiology and biochemistry of *Eucalyptus* pollen. According to Potts and Potts (1986), despite the popular traits exhibited by eucalypts, very little work has been done with regard to understanding pollen behaviour in that species.

3.3 Conclusions

In this chapter, the possibility of long-term storage of *Eucalyptus* germplasm was investigated using pollen and axillary buds. Cryopreservation technique, which is currently the only available method of long-term conservation *in vitro*, was attempted for both explant types. Pollen was successfully stored at $-78\text{ }^{\circ}\text{C}$ for 12 months with final survival rates of 23 %. Cryopreservation of axillary buds was, on the other hand, a complex procedure that involved encapsulation, of the appropriate sized explant to be exposure to specific pretreatments, freezing and thawing. Explants that were 2 mm were successfully encapsulated in 3% (w/v) sodium alginate. Pretreatment with various chemical cryoprotectants indicated the use of a mixture of DMSO and glycerol facilitated high survival rates (63%) after washing with MS and 4 g.l^{-1} sucrose solution. Desiccation of buds in the laminar flow for 4 h to a moisture content of approximately 20% (fwb) resulted in poor survival rates (40%). Preculturing buds in 1.0M sucrose (progressively from 0.1 - 1.0M at 48 h intervals) increased desiccation-tolerance (pretreated buds desiccated for 4 h in the laminar flow had high survival rates of 85%). Although pretreatments were successfully established, none of the pretreated material survived after frozen storage.

CHAPTER 4: CONCLUDING REMARKS AND FUTURE RESEARCH STRATEGIES

4.1 Towards *in vitro* germplasm storage of *Eucalyptus* spp.

Breeders are faced with the task of meeting the high demand on *Eucalyptus* products through the development of superior quality hybrids. However, without a wide genepool to select from, genetic improvements of that genus will be hindered. Conservation of existing genotypes and any that might be created through breeding efforts is therefore of essence. The aim of this investigation was to establish short-to-medium storage methods for conservation of *Eucalyptus* germplasm. Storage strategies were thus devised into two categories with regard to their significance in tree improvement. These were, short-to-medium term storage for day-to-day breeding practices and long-term storage as a means of 'back up'.

With regard to short and medium-term storage, three types of explants were put in storage, namely, shoot cultures, axillary buds and pollen. Shoot cultures and axillary buds both being vegetative material play an important role in clonal and micropropagation of parent lines for selection or multiplication of genetically modified material, while pollen conservation is essential in view of controlled pollination (section 1.1.1). The investigation into storage of shoot cultures achieved considerable success. Shoots were stored and maintained for 10 months in a medium that consisted of low nutrients (section 2.3.2.4) while retaining high multiplication rates of $(13.75 \pm 7.05 \text{ shoots/explant})$. Storage of encapsulated buds was achieved for 6 months at 4 °C (Table 4.1). Before establishing pollen storage protocols, it was important to establish methods of determining viability. Pollen viability was assayed using 1 % (w/v) 2,3,5-triphenyl tetrazolium chloride (TTC) and *in vitro* germination on a BK (Brewbaker and Kwack, 1963) medium for 24 hours ($26 \pm 3.0\%$). Longevity was found to be highest if pollen was stored at 4 °C without desiccation.

With regard to long-term conservation, pollen was successfully stored at -78 °C for 10 months (Table 4.1). Axillary bud cryopreservation consisted of establishing explant size suitable for freezing, pretreatments, freezing and thawing methods. Explants encapsulated in

sodium alginate approximately 2 mm in size were successfully pretreated and the best pretreatments were a mixture of DMSO and glycerol, or sucrose induced dehydration combined with laminar flow desiccation. However, after freezing and thawing, no regeneration was obtained (Table 4.1).

Table 4.1 A summary of success achieved during this investigation and areas of future research.

	PROTOCOL	RESULTS
1. Short-to-medium term storage		
1.1 SHOOT CULTURES	Storage of shoot cultures for short and medium term use	obtained
1.2 AXILLARY BUDS	Storage of encapsulated buds	obtained
1.3 POLLEN	Methods of pollen viability assessment	obtained
	Medium-term storage of pollen	obtained
2. Long-term storage		
2.1 POLLEN	Storage of pollen under cryogenic temperatures	obtained
2.2 AXILLARY BUDS	Establishment of sterilisation and regeneration procedures	obtained
	Establishment of explant size	obtained
	Establishment of pretreatments	obtained
	Establishment of freezing and thawing methods	unsuccessful

4.2 Future research strategies

A crucial point of germplasm storage is maintaining genetic composition of stored material as a true representative of existing germplasm (Withers, 1986). However, it has been shown that medium-term storage of plants under growth limiting factors may alter

the genetic composition of that plant (Ashmore, 1997). This would be a major disadvantage of *in vitro* germplasm storage and a danger for breeding stations. A study on the genetic stability of *in vitro* maintained material would be of importance.

With cryopreservation, one has to appreciate the fact that such techniques are still relatively new in plant studies and a great deal of empirical work is necessary done when attempting to cryopreserve a new species. Successful execution of such work requires full understanding of the mechanisms underlying all stages of pre-freezing preparation. Moreover, all stages are intrinsically interrelated thus making this type of work complex. As has been shown from prior discussions, the choice of explant, its preparation through to the rate at which it is cooled and thawed has a strong bearing on the type of results that can be obtained. At each stage, several parameters can be varied in attempts to optimize conditions for survival.

With regard to pretreatments, the use of chemical cryoprotectants such as DMSO and glycerol could be optimised by varying temperature, time and the concentration of individual cryoprotectants as previously discussed (Section 3.3.1.3). Material pretreated with the best combination of the above variables could then be subjected to freezing in order to determine the level of cryoprotection attained. Regarding desiccation and sucrose-induced dehydration, it would be suggested that a study of the cellular structural changes be attempted in order for better understanding and determination of 'critical' moisture levels discussed in section 3.3.1.3. Once optimisation of pretreatments has been achieved, freezing and thawing could be attempted. The use of pollen for cryogenic storage proved highly successful hence very little of further studies can be suggested in that respect.

In vitro conservation is poised to make an impact in all areas of agricultural research that depend upon the availability of defined genetic material (Ashmore, 1997). It is likely to make a significant effect in the forestry industry particularly for genetically modified and clonally propagated germplasm. However, despite this optimistic claim, several other technical difficulties regarding cryopreservation have to be realized. In cryopreservation,

procedures must be improved for organized structures and those of macroscopical size. In both medium and long-term approaches to storage, the methodology must be adequately tested on a wide range of genotypes and the limits of efficacy must be determined in order that intelligent choices may be made with minimum empirical investigation.

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