Department of Environmental Biology

Cryopreservation of Somatic Germplasm of Selected Australian Monocotyledonous Taxa (Haemodoraceae)

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This thesis is presented as part of the requirements for the award of the degree of Doctor of Philosophy of the Curtin University of Technology

August 2001

Candidates Declaration

Six of the eight chapters are presented as either published works (Chapters 2, 3, 4, 5 and 6) or as a paper in press (Chapter 7).

Dr Tissa Senaratna and Dr Darren Touchell provided overall guidance and advice on the physiological and cryobiological aspects of this study. Chapter 2 experiments were originally undertaken during the candidates honours year but written and submitted as a paper during PhD candidature. Additionally, Chapter 2 and the first experiment from Chapter 6 were originally suggested by Dr Touchell; while for Chapter 5 the original idea concerning the mode of action of sugars in preculture media was Dr Senaratna and Dr Touchell. However, for this chapter all experiments were designed and implemented by the candidatef. Additionally, Dr Senaratna provided advice on appropriate statistical methods, particularly for Chapter 5. Presentation of manuscripts was also improved with their valuable suggestions.

Overall supervision was provided by Dr Dixon, who also provided valuable information on conservation issues and other related matters.

Mr Eric Bunn provided further advice and discussions, notably on in vitro *methods of plant propagation of Australian native species, particularly in relation to Chapters 3, 5, 6 and 7. Mr Bunn also made many useful recommendations during manuscript revision.*

Dr Beng Tan provided help through idea development, experimental design, and kindly provided valuable plant cultures for the completion of Chapter 4. Dr Tan also helped in improving the presentation of manuscripts, particularly in relation to chapter 4.

Dr Siegy Krauss provided valuable advice on the experimental design and its implementation for chapter 7. He also helped in the interpretation of data, and the writing of the manuscript.

With these provisos the work presented in this thesis is entirely my own unless specifically stated otherwise. In addition, unless otherwise stated, the contents of this thesis have not been submitted previously, in whole or in part, in respect of any other academic award.

Shane Turner

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Abstract

The South West Botanical Province of Western Australia is one of the most floristically rich areas of the world with over, 8,000 species present, the majority of which (70%) are endemic to this region. Coupled with this high level of endemism, many taxa are threatened which makes them vulnerable to habitat alterations, modifications and destruction. Significant habitat alteration in many areas has resulted in 27 species becoming extinct in the South West Botanical Province, while an additional 327 species are classified as rare and endangered. In the context of stemming this loss of biodiversity, research in cryopreservation was undertaken to provide offsite protection and conservation of somatic germplasm.

Cryostorage techniques were evaluated in this study to determine the key factors which may affect the ability of somatic tissues of Haemodoraceae species to survive, recover and grow following liquid nitrogen (LN) immersion and storage. Using *Anigozanthos viridis* as a comparator in most experiments, the base vitrification protocol was established, which involved: (1) preculturing shoot apices on 0.4 M sorbitol for 48 h; (2) incubation in a vitrification cryoprotective solution (PVS2) for 25 min at 0°C; (3) LN immersion; (4) recovery to active growth through warming (immersion in a 40°C water bath). Using this procedure the highest post-LN survival of shoot apices for *A. viridis* was 41.4 ± 6.1%. Four additional taxa were successfully cryopreserved with this base protocol (*Anigozanthos manglesii, A. rufus, Conostylis wonganensis* and *A. rufus* x *A. pulcherrimus*); a fifth taxon, *Macropidia fuliginosa,* however, proved unresponsive.

To improve on post-LN survival, further research established that four of the six study taxa responded to the following amendments to the basic protocol: (1) longer preculture duration; (2) preculture on 0.8 M glycerol rather than sorbitol, (3) utilisation of PVS2 solutions with reduced DMSO content; and (4) incorporation of an additional loading phase (2 M glycerol plus 0.4 M sucrose for 20 mins at 0 °C).

Macropidia fuliginosa, a species with poor recovery after LN exposure, was successfully cryostored using somatic embryos. Treatments which resulted in

the highest survival (67.3% 5.7 ±%) included preculture with 0.4 M sorbitol, and incubation in PVS2 for 30 min. Further experimentation indicated that preculture for two days on 0.8 M glycerol (replacing 0.4 M sorbitol) was more beneficial for achieving high post-LN survival.

Post-LN survival was significantly correlated to the use of polyalcohols when the total number of hydroxyl (-OH) groups (regardless of molarity) present was the same as that found in 0.4 M sorbitol. It was hypothesised that hydroxyl number is more important than molarity in membrane stabilisation, during dehydration and cooling. Post-LN survival was also found to be significantly influenced by stereochemical arrangement of the -OH groups of polyalcohol molecules used in the preculture media. Finally, post-LN survival was also found to be significantly influenced by the size of the molecule, with smaller polyalcohols with more -OH groups on one flank of the carbon chain being superior as cryoprotective agents.

The influence of plant growth regulators on post-LN survival and recovery growth was also investigated. The survival of shoot apices was not correlated to cytokinin or auxin treatments administered in culture media prior to cryostorage. However, in the recovery medium, a combination of cytokinin and 0.5 μM GA_3 in the medium was found to be the most efficacious for obtaining healthy plantlets.

Genetic fidelity was then examined using Amplified Fragment Length Polymorphism (AFLP). Plantlets of one clone kept or maintained under the following conditions: (1) standard tissue culture conditions; (2) cold storage and (3) cryostorage, over a 12 month duration, showed no detectable genetic changes.

Further, shoot apex viability evaluated at regular intervals (after 0, 3, 6 and 12 months of LN storage) suggested that medium term storage of samplescryopreservation did not reduce shoot apex viability over this time span

This study has provided a better understanding of the factors influencing post-LN survival and recovery and, as a result, the cryopreservation protocols have been refined. Consequently, the prospects for conserving threatened

Haemodoraceae species from Western Australia through cryostorage is now significantly improved.

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Acknowledgments

I wish to offer my sincerest thanks to my supervisors, Dr Tissa Senaratna, Dr Darren Touchell, Dr Beng Tan and Dr Kingsley Dixon for their enthusiasm, patience and support during the planning, implementation and completion of this thesis. Not included in this list as an official supervisor, but who nevertheless, provided significant support and advice was Mr Eric Bunn, who was still a supervisor in ever sense of the word.

To present and former staff and students at Kings Park and Botanic Garden, in particular: David Merritt, Maggie Panaia, Dr Anle Tieu, Janet Anthony, Peter Hood, Robyn Taylor, Anthony Bishop, Keran Keys, Steve Easton and Michelle Crow. I am indebted to all of you in many ways for help, advice and friendship which has made the past four years such a rewarding experience.

I also wish to thank the staff at Environmental Biology, Curtin University, notably, Professor Jonathan Majer for his strong support, Lydia Kupsky, Gayle Monzu, Peter Mioduszewski, Jean Burgess, Enid Holt, Kathy Whiteman, Ian Abercrombie and Charles Lacoste for their support and professionalism. Even though I undertook my experimental work outside of Curtin I still feel honoured and indebted to Environmental Biology for allowing me the opportunity to continue my studies.

I also wish to thank to Curtin University of Technology for generously providing me with the financial support for the duration of this research program.

To my family and friends thanks for the support and friendship over the years and, finally, to Sarah and Talia, my inspiration and ultimate support, the last seven and a half years would not have been possible without your love and understanding.

Dedication

This thesis is dedicated to the memory of close family and friends, in particular my grandparents, Doris, and Cyril, and friends Paul, John, Jason and David; this achievement would not have been possible without their earlier guidance and friendship.

List of Publications

The list of publications presented below have arisen from research conducted during the Ph.D. candidature and are presented as individual chapters in this thesis.

Chapter 2: Turner, S. R., Touchell, D.H., Dixon, K. W and Tan B. (2000) Cryopreservation of *Anigozanthos viridis* ssp *viridis* and related taxa from the South West of Western Australia. Australian Journal of Botany. 48 (6): 739-744.

Chapter 3: Turner, S.R. Senaratna, T Bunn, E.. Tan, B. Dixon, K. W. Touchell, D.H (2001).Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. Annals of Botany 87: 371-378.

Chapter 4: Turner, S. R., Tan B., Senaratna, T. Bunn, E., Dixon, K. W and Touchell, D.H. (2000) Cryopreservation of the Australian species *Macropidia fuliginosa* (Haemodoraceae) by vitrification. Cryo-letters 21 (6): 21: 379-388.

Chapter 5: Turner, S, R., Senaratna, T., Touchell, D, H., Bunn, E., Dixon, K, W and Tan, B. (2001).Stereochemical arrangement of hydroxyl groups in sugar and polyalcohol molecules as an important factor in effective cryopreservation. Plant Science 160 (3): 489-497

Chapter 6: Turner , S, R., Senaratna, T,. Touchell D, H., Bunn, E., Tan, B and Dixon K. W. (2001). Effects of plant growth regulators on survival and recovery growth following cryopreservation. Cryo-Letters 22 (3): 163-174.

Chapter 7: Turner, S. R., Krauss, S. L., Bunn, E., Senaratna, T., Dixon, K, W., Tan, B. and Touchell, D. (in press). Genetic fidelity and viability of *Anigozanthos viridis* following tissue culture, cold storage and cryopreservation. Plant Science 161(6):1099-1106

Abbreviations and Definitions

ABA: abscisic acid

AFLP: amplified fragment length polymorphism

BAP: 6- benzylaminopurine

BM: basal medium

DMSO: dimethylsulfoxide

DNA: deoxyribose nucleic acid

EG: ethylene glycol

GA_3: gibberellic acid

IAA: indole-3 - acetic acid

IBA: 3 - indole butyric acid

kinetin: 6-furfurylaminopurine

LN: liquid nitrogen

MS: Murashige and Skoog

MES: 2-[N-morpholino]ethanesulfonic acid

NAA: 1-naphthalene acetic acid

-OH: hydroxyl

PEG: polyethylene glycol

PGR: plant growth regulator

Polyalcohols: polyhydritic alcohols

PPFD: photosynthetic photon flux density

PVS1: plant vitrification solution one

PVS2: plant vitrification solution two

PVS3: plant vitrification solution three

PVS4: plant vitrification solution four

RAPD: randomly amplified polymorphic DNA

RFLP: restriction fragment length polymerism

RM 1: recovery medium 1

RNA: ribose nucleic acid

2, 4-D: 2, 4-dichlorophenoxy acetic acid

2iP: N^6 - (2 -isopentyl) adenine

zeatin: 4-hydroxy-3-methyl-*trans*-2-butenylaminopurine

Chapter 1:

1.1. Introduction

Cryopreservation is the long-term storage of biological materials at ultra-low temperatures (below –139 °C) which is generally achieved through the use of cryogenic liquids, such as liquid nitrogen (LN) (Benson 1994; Morris 1981). By storing tissues at ultra low temperatures, all aging processes are negated and it is likely that genetic fidelity of material can be maintained indefinitely in a contamination free state (Bajaj 1995b). Cryostorage has been routinely applied for the conservation and preservation of animal tissues for the last 20 years; however, its utilisation in plants has so far been limited mostly to agriculturally important species (Bajaj 1995b). Nevertheless, cryopreservation is highly applicable for the conservation of endangered plant species, though specific techniques need to be elucidated in order for it to be fully utilised.

1.2 The need for Conservation and Cryopreservation

Worldwide, increasing pressures from expanding populations and other human activities is leading to a rapid decline in plant biodiversity with the possibility that 60,000 of the 250,000 vascular plant species in the world may become extinct within the next 50 years (Holsinger and Gottlieb 1991). In Australia a similar picture is emerging with 71 species of vascular plants so far extinct since European colonisation, with a further 2,245 taxa currently classified as threatened. (Walter and Gillett 1998).

Within Australia, the South-West Botanical Province of Western Australia is one of the most botanically-rich regions of the world, characterised by a high level of recent speciation coupled with extreme localisation and very high endemism (70 to 80%) (Hopper *et al.* 1990). As such, it is recognised as one of the most botanically diverse regions in the world (Brown *et al.* 1998), with more than 8000 described plant taxa (32% of the Australian flora) (Corrick and George 1996; Hopper *et al.*1996). In common with other biodiverse areas, many parts of this region have experienced severe habitat modification and disturbance through human activities such as land clearing for agricultural exploitation or

natural resources, and urbanisation. These activities, which have also increased the spread of exotic plant diseases and pests, have caused other ecological problems such as salination and erosion. In parts of the Western Australian Wheat Belt, extensive land clearing over the last 50 years has reduced natural vegetation in some parts by 98% (Hobbs and Saunders 1994).

Twenty-seven species have already been declared extinct in Western Australia; the majority are endemic to the south west region (Brown *et al.* 1998; Briggs and Leigh 1996). Further, the number of rare and threatened species has increased from 238 to 327 during the past ten years with 1,959 plant species currently classified as in need of conservation action (Hopper *et al.* 1990; Brown *et al.* 1998). Faced with this increasing number of endangered plant species, conservation agencies must turn towards *ex situ* methods such as seed banks, *in vitro* conservation and cryopreservation in order to conserve these threatened plant species.

1.2.1 Agricultural and Horticultural Species

For economically-important species similar problems of genetic erosion are recognised as having been exacerbated by the advent of modern farming techniques. Large-scale industrial farming practices have resulted in a genetic constriction of crop and horticultural species (Plucknett *et al* 1987). For example, between 1804 and 1904, over 85 % of American apple, asparagus, runner bean, carrot, peanut and sunflower varieties were lost, with similar rates of disappearance still occurring in many parts of the world (Maunder and Culham 1999) The narrow genetic base, which can make crops more vulnerable to pests, diseases and weather extremes (Plucknett *et al* 1987), has the potential to cause large -scale human famine.

Hidden within the genes of neglected crop varieties and their wild relatives, may be resistance to new pests and diseases, and tolerance to changing climatic conditions. Such germplasm may be utilised in breeding programs to facilitate the development of new crop varieties to feed an increasing human population and to also cope with rapidly changing weather patterns as a consequence of global warming (Gill 1989).

Cryopreservation as a tool for storage of agricultural and horticultural germplasm has been extensively reported for both somatic tissues (viz. shoot apices, somatic embryos and cell suspensions) and reproductive tissues, such as: seed, pollen and zygotic embryos (Bajaj 1995b, Engelmann 1997, Benson 1999, Engelmann and Takagi 2000a). However, the preferred tissue type for many species and cultivars are vegetative (somatic) tissues (e.g. single cells (cell suspensions), protoplasts, somatic embryos and meristems) which are genetically identical (clonal) to parental plants and, as such, grow true to type. In addition, differentiated explants such as shoot apices or somatic embryos are further preferred for conservation purposes as undifferentiated tissues such as cell suspensions and protoplasts may be more likely to be genetically unstable. Using somatic tissues, over 100 taxa from the world's major crop species have been evaluated using various cryopreservtion techniques however as yet most have not as yet been screened across a selection of cultivars or clones and techniques are still at the experimental stage (Appendix 1).

1.2.2 Rare and Endangered Plant Species

Research into cryostorage of endangered species is largely recent and somewhat limited. Using somatic tissues, such as shoot apices, protocols for the rare and endangered Western Australian native species *Grevillea scapigera*, were initially developed and later improved upon (Touchell *et al.* 1992; Touchell 1995; Touchell and Dixon 1999; and Touchell *et al.* in press (a)). Highest survival (64.1%) was achieved using a vitrification procedure involving: preculture of excised shoot apices on 0.6 M sorbitol; incubation for 30 min in Plant Vitrification Solution Two (PVS2) at 0 °C; direct immersion in LN; warming in a 40 °C water bath; then washing in 1 M sucrose washing solution and incubation on a recovery medium. This protocol (or modifications of) was then applied to 30 other rare and endangered native plant species with varying degrees of success.

More recently, Turner *et al.* (2001a), described the successful cryopreservation of six rare and endangered monocot species using a modified generic vitrification protocol, obtaining survival ranging across species from 14% to 94%.

Other endangered species in other parts of the world have also been successfully cryopreserved using shoot apices, such as: *Centaurium riguaii* (Gonzalez-Benito and Perez 1994), *Antirrhinum microphyllum* (Gonzalez-Benito *et al.* 1998b), *Holostemma annulare* (Decruse *et al.* 1999), *Cosmos atrosanguineus* (Wilkinson *et al.* 1998) and *Brunsfelsia densifolia* (Pence 1990). These species are facing extinction due to habitat modification and plant collecting, with most having a very limited distribution. The first two species are rare and endangered endemics from Spain, the third from India, the fourth from Mexico, while the last-named species is from Costa Rica.

Spores, seed and zygotic embryos from threatened species including tree ferns (Rogge *et al.* 2000), numerous Spanish endemics (Gonzalez-Benito *et al.* 1998a), and Texan wild rice (Touchell and Walters 2000) have also been successfully cryopreserved.

1.2.3 An Australian Perspective

Cryopreservation, as a means of conserving important germplasm, is a new conservation tool for Australian native species. Touchell (1995), Touchell and Dixon (1993, 1995, 1996, 1999) and Touchell *et al.* (1992), have successfully established procedures for the cryopreservation of seed and somatic tissues (shoot apices) from over 90 species in Australia.

Touchell and Dixon (1999) and Touchell *et al.* (in press (a)) have recently demonstrated successful cryostorage of somatic tissues for 30 species from the south west of Australia. Turner *et al.* (2000a, 2000b, 2001a, 2001b) have refined the techniques, and applied them to several native monocot species, one of which (*Conostylis wonganensis*) had previously proved recalcitrant to cryopreservation (Touchell and Dixon 1999).

Monod *et al.* (1992) and Blakesley and Kiernan (2001) have also successfully cryopreserved encapsulated shoot apices of several Australian eucalypt species, namely: *Eucalyptus gunnii, E. grandis* and a hybrid (*E. grandis* x *E. camaldulensis*).

Cryopreservation studies have also been undertaken on pollen from economically-important species such as: *Acacia* sp., (Sedgley and Harbard 1993) *Verticordia* sp. (Tyagi *et al.* 1992). and *Anigozanthos manglesii* (Mangles' kangaroo

paw) (Sukhvibul and Considine 1993). A summary of Australian species known to be successfully cryopreserved is presented in Appendix 2.

1.3 Advantages of Cryopreservation

In the context of germplasm conservation, cryopreservation has numerous advantages over conventional forms of germplasm storage. For example, it can be used for the storage of different tissues and organs, namely: orthodox seed, recalcitrant seed, shoot apices, protoplasts, somatic embryos, zygotic embryos and cell suspensions (Bajaj 1995b). Further, it is the only reliable method that allows for long-term storage of valuable germplasm; Touchell *et al.* (1997) categorised germplasm storage as follows:

- Short term storage: < 5 years and consists of active collections used for research, propagation and re-introductions. Material is maintained at 5 °C, or -18°C.
- Medium term storage: 5 - 25 years and consists of active collections maintained at -18 °C.
- Long term storage: > 25 years and is used to establish base collections where material is stored at -18 °C, or in LN (-196°C).

For clonal germplasm collections, cryostorage is inexpensive and space-saving while providing for long-term storage (Eberhart *et al.* 1991). Further, it provides a method for maintaining:

- cultivars which do not grow true to type when grown from seed
- species which take many years to germinate grow and mature from seed
- sterile hybrids
- species with complex dormancy mechanisms
- targeted clonal lines from genetically representative samples which are needed for maintaining maximum genetic diversity of critically endangered species (Touchell and Dixon 1999) (see Fig. 1.1.).

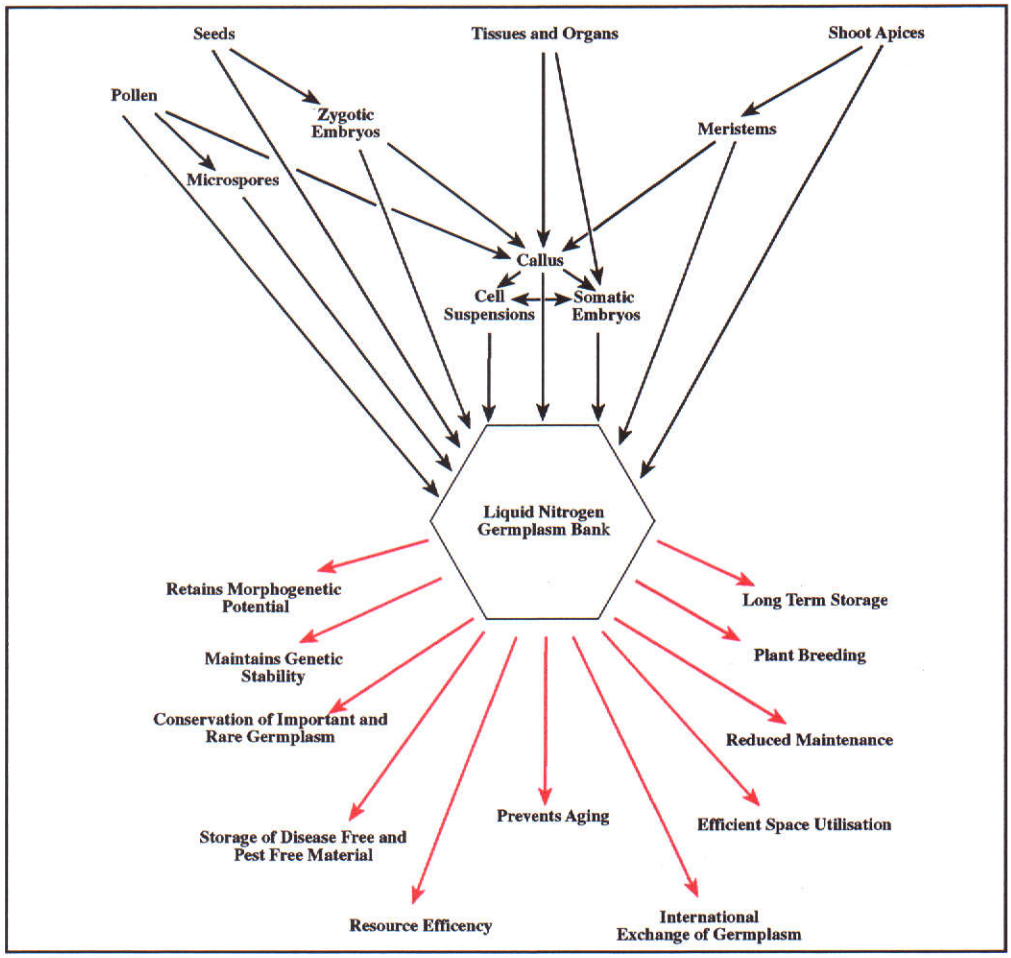

Figure 1.1. Advantages and integration of cryostorage with plant resource technology and improvement (after Bajaj 1995b).

1.4 Complimentary Germplasm Conservation Strategies

In order to risk manage critically endangered species, complimentary conservation actions may need to be considered, the choice of which will depend on many factors, such as germplasm availability and the degree of storage difficulty (Touchell *et al.* 1997). Such actions may include: the storage of different plant tissues (seed, whole plants (container collections), *in vitro* cultures); the utilisation of different storage techniques (room temperature, -5°C, -18°C, -196°C); and the creation of backup collections in other facilities (Touchell *et al.* 1997; Touchell *et al.* in press a; Bowes 1999; Reed 2001). Each different collection type will have advantages as well as disadvantages, which for seed, *in vitro* and container collections will include:

- Seed; advantages - can store a high level of genetic diversity; generally requires low technology to store and germinate. Disadvantages - seed may be recalcitrant; seed may be highly heterozygotic (and therefore will not grow true to type); if grown *ex situ*, unfit genotypes may be introduced into the population gene pool, creating a genetically unfit population; seed may be very difficult to germinate; produced in insufficient quantities or is very difficult to collect due to restricted seasonal availability, irregular flowering, and location on plant (Touchell *et al.* 1997; Iriondo and Perez 1999)
- Container collections; advantages - requires low technology; can be relatively inexpensive; seed can be regenerated for re-introduction programs. Disadvantages – collections can take up a large amount of space; may be losses due to pests and diseases; may die due to limited life span, or human error (Touchell *et al.* 1997, Touchell *et al.* in press a).
- *In vitro* collections; advantages - can be used to store genetically important clonal lines representing key genotypes; can be used to grow difficult to germinate species; can be used to propagate species which produce very little viable seed (or seed difficult to collect); can be used for mass propagation for re-introduction programs. Disadvantages - very expensive to set up and maintain a plant tissue culture laboratory; species without prior knowledge can be very difficult to initiate and maintain; requires specialised knowledge; collections can be lost due to equipment failure; somaclonal variation may occur randomly (Pence 1999; Fay *et al.* 1999; Touchell *et al.*1997).

Cryostorage (both of seed and somatic tissues) can be used to establish non-active base collections (in conjunction with other storage initiatives), which may be utilised to re-initiate active collections or lost clonal lines in cases of unforseen disaster such as: loss of seed viability (when kept at room temperature or 5°C), the loss of container collections due to pests and diseases or loss of valuable *in vitro* cultures due equipment failure (breakdown of air conditioners or incubators) (Touchell *et al.* 1997; Touchell *et al.* in press a). In addition, cryopreserved shoot apices may be used to re-initiate *in vitro* cultures which

may have undergone somaclonal variation, lost their morphogenic capacity or have become moribund (George 1993, Bajaj 1995b).

1.5 Integration of Cryopreservation into Biotechnology

The types of somatic tissues that can be cryopreserved include shoot apices, cell suspension cultures, protoplast suspensions, callus and somatic embryos. However, in the modern plant biotechnology laboratory there are reasons and distinct advantages for the storage of each of these tissue types (Figure 1.1). For example, shoot apices provide a useful method for conserving on a large scale a diverse array of elite germplasm, clonally. This material is easily accessed and can be rapidly grown to provide plants for plant breeding programs or plant tissues for genetic transformation or other biotechnology techniques (Benson 1999). Although shoot apices are difficult to handle under cryogenic conditions, they are the preferred tissues for long-term storage because of their inherent genetic stability Pluckett *et al* (1987). However, the successful use of shoot apices is highly dependent on the successful culture of parental plants *in vitro*, as the collection of viable shoot apices requires donor plants to be in optimal condition (Touchell *et al.* in press b). In addition, to successfully obtain plants following cryostorage of shoot apices requires both the application of successful cryostorage techniques, and optimisation of the post LN *in vitro* environment, to maximise recovery growth and to obtain healthy (genetically stable) robust plants in the shortest possible time (Turner *et al.* 2001c, Touchell *et al.* in press b).

In biotechnology, cell suspension cultures are being evaluated (and cryopreserved) for their potential use in supplying specific biological or pharmacological products such as pyrethrins (Hitmi *et al.* 1999a), ginsenosides (Mannonen *et al.* 1990) and cardenolides (Moran *et al.* 1999). However, after extended periods in culture, cell lines can be lost due to contamination, or they may begin synthesising reduced quantities of active compounds due to random genetic changes resulting from ploidy changes, random genetic mutation, translocations, and gene amplification (Evans *et al* 1981, Gamborg and Shyluk 1981; Bajaj 1991). Embryogenic cell cultures have also been utilised for the production of somatic embryos in species such as spruce (Moorehouse *et al.*

1996) and alfalfa (Binarova and Dolezel 1988). Again, these cultures may be easily lost due to contamination or genetic mutation. Several studies have shown that cell suspensions stored for various lengths of time in LN retain their morphogenetic potential and their capacity to regenerate complete plants following warming (Kartha 1981; Bajaj 1991).

Similar to cell suspension cultures are protoplast suspension cultures. Protoplasts are plant cells with their cell walls removed using various hydrolytic enzymes, typically pectinase, cellulase and hemicellulase, and resulting in an intact cell membrane, cytoplasm and nucleus (George 1993). Protoplasts can be used for genetic transformation involving DNA uptake and in somatic cell hybridisation via protoplast fusion (George 1993). Protoplasts of periwinkle (*Catharanthus roseus*), belladonna (*Atropa belladonna*), datura (*Datura innoxia*) and tobacco (*Nicotiana tabacum*) have been successfully cryopreserved (Gazeau *et al.* 1992; Bajaj 1988). Freezing of protoplasts is similar to that of cell suspensions (Grout 1995). However, as protoplasts differ from complete plant cells in their lack of a cell wall, these cells are highly sensitive and require critical concentrations of appropriate osmotica and cryoprotectants to prevent degradation of the plasma membrane, which results in cell collapse, damage and death (Withers 1985).

Callus cultures may be used to provide starting material for cell and protoplast suspensions, protoplast suspensions or somatic embryos (George 1993). The advantages and procedures of storing callus cultures are similar to those already described for cell suspensions and protoplast cultures. Species in which callus cultures have been successfully cryopreserved include citrus (Olivares-Fuster *et al.* 2000), rice (Moukadiri *et al.* 1999) and sugarcane (Martinez-Montero *et al.* 1998).

Somatic embryos are commonly derived from callus and are similar to zygotic embryos except that they develop from vegetative (somatic) cells (such as callus) rather that through the sexual process, as in zygotic embryos (Hartmann *et al* 1997). Somatic embryo's should be genetically identical to the parental plants from which the callus (or other somatic tissues) is derived. They can be produced from sterile hybrids, difficult-to-germinate species (Johnson

1996) and elite clonal lines. Like zygotic embryos, they will germinate and grow rapidly when given appropriate conditions. Somatic embryogenesis has been reported in more than 300 species from over 40 diverse plant families (George 1996). One of the more interesting developments in plant tissue technology is large-scale production of somatic embryo's in bioreactors (Paek *et al.* 2001). By utilising cryogenic storage techniques, it may be possible to store somatic embryo's at the appropriate stage of development can be stored until they are needed (Bajaj 1991).

Other tissues which may be of importance to biotechnology and which can be stored cryogenically include zygotic embryos derived from recalcitrant-seeded species, including economically important tropical plantation and timber species. Recalcitrant seeds normally possess high moisture contents (12-30%), germinate rapidly (some have precocious germination) and are normally very short-lived (from a few weeks to several months) (Iriondo and Perez 1999). Because of these characteristics these unorthodox taxa are difficult to preserve under standard storage conditions and in such cases cryopreservation of seed (zygotic) embryos is a logical alternative (Bajaj 1995b).

Another type of tissue which is of prime interest to plant breeders and horticulturists is pollen, which has been cryopreserved in a large number of species (Towill 1985). In biotechnology, the cryopreservation of pollen embryos may assist with the large scale production of haploid plants, maintenance of haploid culture stability and conservation of important genetic resources (Bajaj, 1991). Species that have had pollen embryos cryopreserved include *Arachis hypogaea, Brassica campestris, Nicotiana tabacum, Petunia hybrida, Primula obconica* and *Triticum aestivum* (Bajaj 1991).

1.6 Causes of Post LN damage

1.6.1 Physical and Biochemical

There are several ways in which cells exposed to LN temperatures can experience injury and death (Dumet and Benson 2000) (Figure 1.2). Generally, in the absence of appropriate pre-treatments, the type of injury is determined by the cooling rate, namely, gradual cooling or rapid cooling, with 95% of water

available within tissues being "free" and capable of converting to ice during freezing (Crowe *et al.* 1988). During gradual cooling cells may suffer from dehydration leading to various "solution effects". These solution effects are caused by osmotic dehydration of intracellular unfrozen water, moving from inside to outside the cell to compensate for the water vapour deficit as water freezes in the extracellular compartment (Steponkus *et al.* 1992). As a result cellular changes include: cell shrinkage and dehydration, and increased intra- and extra-cellular solute concentrations which may disrupt the cell's biochemical pathways and change in cellular pH, leading subsequently to injury (Morris 1981).

Rapid cooling may cause other types of cellular damage due to ice nucleation within cells (Finkle *et al* 1985). This may result in gross physical damage to the plasmalemma and cell organelles such as the tonoplast, mitochondria and chloroplasts, caused by large intracellular crystal formation leading to gross mechanical damage to these structures (Dumet and Benson 2000).

Additionally, cellular damage may be caused by stresses imposed on cells during cryopreservation such as metabolic uncoupling, leading to the production of highly toxic free radicals such as the hydroxyl radical ($^{\cdot}OH$) and the superoxide radical ($O_2^{\cdot-}$), and, less importantly, hydrogen peroxide (H_2O_2) and singlet oxygen (O_2^1) (Dumet and Benson 2000; Benson *et al.* 1992). These molecules degrade the lipid fraction of membranes, leading to the formation of lipid peroxides, which then break down to produce a wide range of secondary lipid peroxidation products. The aldehydic products of lipid peroxidation are highly cytotoxic and include: malondialdehyde (MDA) and hydroxyl-2-nonenal, (HNE) which can impair cell function as they cross link to macromolecules such as DNA and proteins (Dumet and Benson 2000). These may impair membrane properties and cell function, and interfere with DNA and protein synthesis (Benson *et al.* 1992).

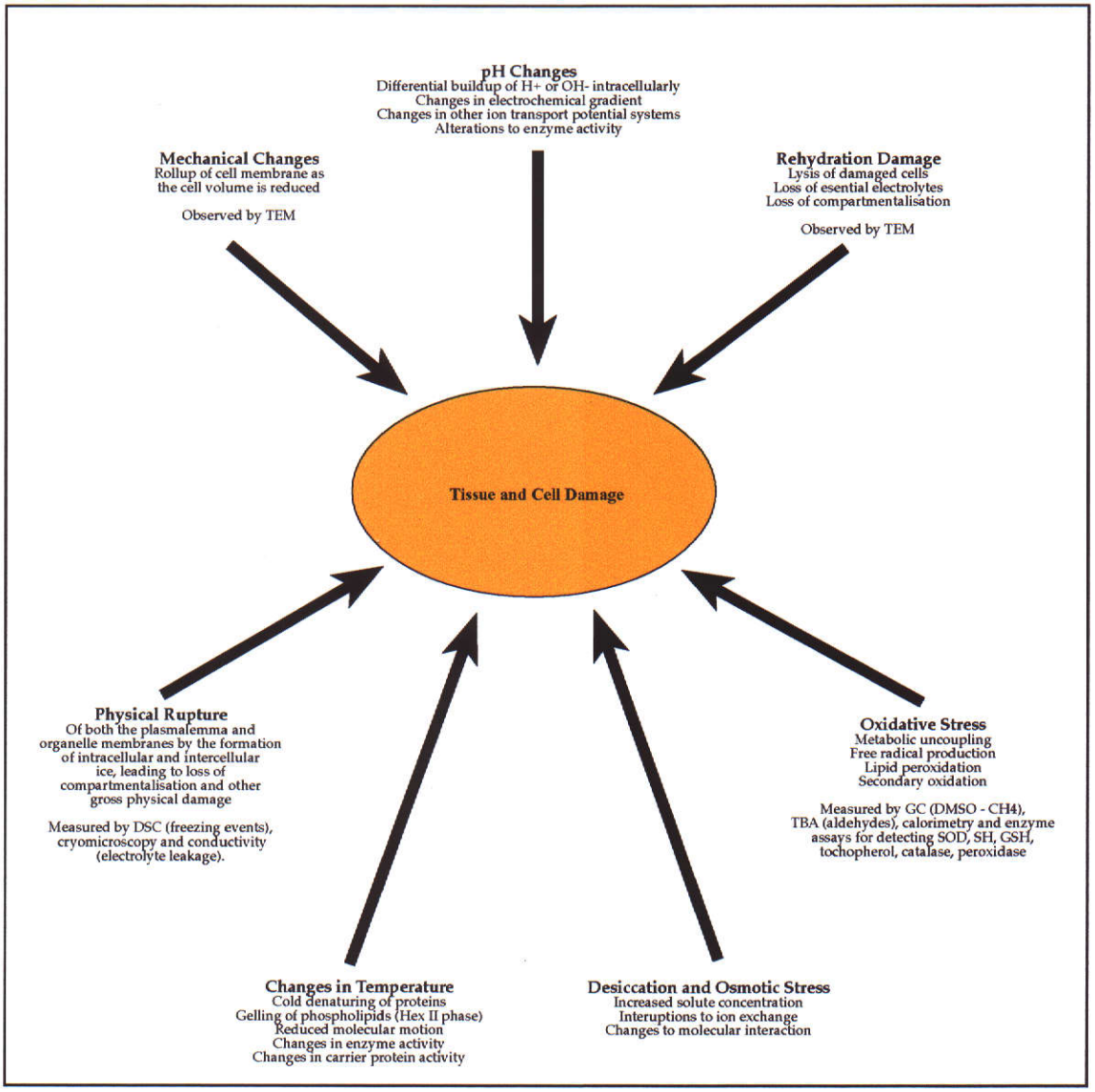

Figure 1.2. Main causes of damage to plant tissues during cooling and cryopreservation and ways in which this damage can be observed and quantified.

1.6.2 Genetic Alterations

Cryopreserved cells and tissues may also suffer from genetic changes caused by the cryoprotectants or by the cooling/warming cycle. Some studies suggest that genetic changes may be attributable to cryoprotectants like DMSO (Aronen *et al.* 1999). In animal cell lines, several studies have found that DMSO under some conditions (24 - 48 hour exposure at concentrations of about 1 - 2%) can

cause genetic aberrations such as DNA hypo-methylation, fragmentation, cell-cycle disturbance and gene activation (Ashwood Smith 1985). On the other hand, Finkle *et al.* (1985) has maintained that the probability of genetic mutations caused by DMSO is very low, when DMSO is applied for only short time periods at ice temperatures, and that being stored at -196 °C effectively stops all chemical and biological interactions.

Other types of genetic damage may also occur due to organelle disruptions (i.e. nucleus or nucleolus) and possibly chromosomes and DNA disruptions, when cells are cooled in LN, then warmed and recovered (Harding 1996). Results with potato have not found genetic changes caused by cryopreservation (or in associated treatments), although Harding (1997) has obtained some evidence of methylation in ribosomal DNA in one study. Most of these studies have utilised the less sensitive DNA techniques such as RAPDS or RFLP (Harding 1991; Harding and Benson 2000; Haggman *et al.* 1998; Aronen *et al.* 1999); therefore, the extent of genetic change attributable to cryo-procedures may not have been revealed.

1.7 Methods to Alleviate LN Damage

Many chemicals have been investigated as cryoprotectants including: DMSO (dimethyl sufoxide) glycerol, proline, sucrose, glucose, sorbitol, ethylene glycol, propylene glycol and polyethylene glycol (Finkle *et al.* 1985; Withers 1984; Mantell *et al.* 1985; Eberhart *et al.* 1991). Cryoprotectant solutions normally use combinations of these chemicals prepared in a base of standard culture medium. Only limited success has been achieved when using these compounds by themselves (Kartha 1981; Withers 1984).

Cryoprotectants work by depressing both the freezing point and the supercooling point of water (ie. the temperature at which the homogenous nucleation of ice occurs). They may also reduce the concentrations of other dissolved solutes and retard the growth of ice crystals (James 1983; Mantell *et al.* 1985).

James (1983) states that cryoprotectants can be broadly divided into two groups: penetrating and non-penetrating compounds. Penetrating compounds

include chemicals such as DMSO which act colligatively (mode of action depends on the concentration and not the nature of the permeating compounds), while glycerol, which is significantly less permeable across the cell membrane, is classified as non-penetrating (Kartha 1981). It is thought that non-penetrating cryoprotectants may interact specifically with the membrane, reducing freeze damage by binding to phospholipid head groups and membrane-bound proteins which help membrane stabilisation during cooling (James 1983; Crowe and Crowe 1986). Other non-penetrating compounds such as sorbitol are thought to act osmotically on the cell, thereby reducing intracellular water content and subsequent ice formation events during cooling (Merryman and Williams 1985). Penetrating compounds, (e.g. DMSO) on the other hand, cross the cell membrane and act through creating an environment that allows for the reduction in cell water content at temperatures sufficiently low to reduce the damaging effects of concentrated intra-cellular solutes and cryoprotectant toxicity (Finkle *et al.* 1985).

Finkle *et al.* (1985) summarised the characteristics of an ideal cryoprotectant as: (1) having a low molecular weight; (2) easily miscible with the solvent (usually water); (3) non-toxic at low concentrations; (4) ability to permeate rapidly into cells; (5) easily washable from cells. DMSO fulfils most of these characteristics and is a major component of most cryoprotectant solutions, although at higher concentrations it may reduce respiration and inhibit RNA and protein syntheses in isolated plant cells and tissues (Bajaj and Reinert 1977). DMSO is also thought to cause a redistribution of intra-membranous particles, which may increase membrane permeability.

Based on the above criteria, three main protocols have been developed to facilitate the cryopreservation of plant tissues, namely, slow cooling, encapsulation/dehydration and vitrification.

1.7.1 Slow Cooling

Slow freezing, the original procedure developed for cryostorage of somatic tissues, was first reported in the 1970's. The procedure involves the controlled cooling of plant tissues at rates of 0.1 to 5 °C per min to a terminal temperature of approximately - 40 °C before plunging into LN (Grout 1995). Factors that

affect the success of slow cooling include: cooling rate, cryoprotectant type, formulation and concentration, terminal freezing temperature, and the type and physiological state of the plant material. (Kartha 1985b). While it has proved widely applicable, its main disadvantages are that it is time consuming and the requirement for expensive equipment in order to undertake this procedure. For Australian species little work has been done with slow cooling but Touchell *et al.*(1992) have described the cryopreservation of *Grevillea scapigera, a* rare and endangered endemic plant species using this procedure, obtaining 20% post-LN survival.

1.7.2 Encapsulation/Dehydration

The encapsulation/dehydration technique was first described in 1990 by Fabre and Dereuddre for shoot apices from *Solanum phureja.* The procedure involves embedding somatic tissues such as shoot apices or somatic embryos, in calcium alginate beads, which are then precultured for one to seven days in liquid media containing high concentrations of sucrose (Decruse *et al.* 1999; Zhao *et al.* 1999; Engelmann 2000; Bachiri *et al.* 2001). The beads can then be further desiccated in a sterile air flow for several hours or over silica gel (Sakai *et al.* 2000; Engelmann 2000; Hornung *et al.* 2001). Desiccated encapsulated tissues are able to withstand direct immersion in LN without the need for expensive slow cooling equipment or protection from cryoprotectants (Benson 1994). However, the technique can be time consuming as each bead requires handling several times and, in addition, some plants may not tolerate the high sucrose concentrations required for dehydration (Reed 2001).

1.7.3 Vitrification

Vitrification is the process by which a concentrated aqueous solution solidifies into metastable glass (glassy solid) at ultra-low temperatures without crystallisation. This is achieved by the use of concentrated cryoprotective solutions (Yamada *et al* 1991), such as PVS1, PVS2 or PVS3. Most vitrification solutions are a mixture of various cryoprotectants, but core components usually include DMSO (5-15%), glycerol (19-50%), ethylene glycol (13-35%) in a sorbitol or sucrose (0.4-0.88 M) basal liquid medium (Table 1.1).

Table 1.1. Compositions of different plant vitrification solutions.

Vitrification Solution	Components	Reference
PVS1	19% w/v glycerol, 13%w/v ethylene glycol, 13% w/v propylene glycol, 6 % w/v DMSO in 1/2 MS liquid medium + 0.5 M sorbitol)	Uragami *et al.*, 1989)
PVS2	30% w/v glycerol, 15% w/v ethylene glycol and 15% w/v DMSO in 1/2 MS liquid medium + 0.4 M sucrose	Sakai *et al.* (1990)
Modified 1 PVS2	30% w/v glycerol, 15% w/v ethylene glycol, 7.5% w/v DMSO, 7.5% propylene glycol in 1/2 MS liquid + 0.4 M sucrose	Turner *et al.* (2001a)
Modified 2 PVS2	30% w/v glycerol, 15% w/v ethylene glycol, 15% propylene glycol in 1/2 MS liquid medium + 0.4 M sucrose	Turner *et al.* (2001a)
PVS3	50% w/v glycerol, 50% w/v sucrose in water	Nishizwa *et al.* (1993)
Modified PVS3	50% w/v glycerol, 50% w/v sucrose, 5% DMSO in water	Nishizwa *et al.* (1993)
PVS4	35 % w/v glycerol, 20 % w/v ethylene glycol in 1/2 MS liquid medium + 0.6 M sucrose	Sakai (2000)
A	20 % glycerin, 15 % sucrose	Maruyama and Ishii (2000)
B	30 % glycerin, 15 % sucrose, 15 % ethylene glycol, 15 % DMSO	Maruyama and Ishii (2000)
C	25 % glycerin, 15 % sucrose, 15 % ehtylene glycol, 13 % DMSO, 2% poly ethylene glycol 4000 (PEG)	Maruyama and Ishii (2000)
D	35 % ethylebne glycol, 10 % DMSO, 5 % PEG	Maruyama and Ishii (2000)
unnamed	0.7 M sucrose, 15 % ethylene glycol, 15 % DMSO, 30 % glycerol	Ng and Ng (2000)
unnamed	38 % 1, 2-propandiol,, 20 % sucrose	De Boucaud *et al.* (1996)
unnamed	15 % w/v ethylene glycol, 25 % glycerol, 3 % polyethylene glycol (PEG 8000) and 13.6 % sucrose	De Boucaud *et al.* (1996)
Mix B	25 % glycerol, 15 % sucrose, 15 % ethylene glycol, 13 % DMSO, 2 % PEG in WPM liquid medium	Maruyama *et al.* (1997)
Mix C	35 % ethylene glycol, 10 % DMSO, 5 % PEG in WPM liquid medium	Maruyama *et al.* (1997)
unnamed	7 M ethylene glycol, 0.88 M sorbitol, 6 % (w/v) BSA	Langis and Steponkus (1990)

Vitrification of plant tissues was first described by Uragami *et al.* (1989) when they successfully cryopreserved somatic embryos of *Aspapragus officinalis* (48% survival). Since then, over 30 species have been successfully cryopreserved using various cryoprotectant solutions such as PVS1, PVS2, PVS3 (Table 1.2), or with other vitrification solutions as listed in Table 1.1. However, the most commonly used vitrification solution is PVS2 (Table 1.2).

Table 1.2. Plant species in which vitrification procedures utilising PVS1, PVS2 or PVS3 have been reported

Species	Vitrification Solution	Tissue Type	Reference
Actinidia chinensis (kiwi fruit)	PVS2	callus	Hakozaki *et al.* (1996)
Allium sativum	PVS2, PVS3	shoot apices	Niwata (1995), Makowska *et al.* (1999),
A. wakegi	PVS2	shoot apices	Kohmura *et al.* (1994)
Ananas comosus	PVS2	shoot apices	Gonzalez-Arnao *et al.* (1998)
Anigozanthos kalbarriensis	Modified PVS2	shoot apices	Turner *et al.* (2001a)
A. humilis spp *chrysanthus*	PVS2	shoot apices	Turner *et al.* (2001a)
A. manglesii	PVS2	shoot apices	Turner *et al.* (2000a)
A. pulcherrimus	PVS2	shoot apices	Turner *et al.* (2000a)
A. rufus	PVS2	shoot apices	Turner *et al.* (2000a)
A. viridis ssp *terraspectans*	Modified PVS2	shoot apices	Turner *et al.* (2001a)
A. viridis ssp *viridis*	PVS2	shoot apices	Turner *et al.* (2000a)
Armoracia rusticana	PVS2	shoot primordia	Phunchindawan *et al.* (1997)
Artocarpus heterophyllus (jackfruit)	PVS2	embryonic axes	Thammasiri (1999)
Asparagus officinalis	PVS1, PVS2, PVS3	cell suspension, somatic embryos ,Multiple bud clusters, cell suspension	Uragami *et al.* (1989), Kohmura *et al.* (1992), Nishizawa *et al.* (1993)
Beta vulgaris	PVS2	shoot apices	Vandenbussche *et al.* (2000)
Bletilla striata	PVS2	zygotic embryos	Ishikawa *et al.* (1997)
Bromus inermis	PVS2	suspension cultures	Ishikawa *et al.* (1996)
Camellia sinensis	PVS2	shoot apices	Kuranuki and Sakai (1995)
Carica papaya	PVS2	Shoot apices	Ashmore *et al.* (2000)
Citrus sinensis	PVS2	cell suspensions	Seijo (2000), Kobayashi *et al.* (1994), Sakai *et al.* (1991), Sakai *et al.* (1990)
Colocasia esculenta	PVS2	shoot apices	Takagi *et al.* (1997)
Conospermum stoechadis	PVS2	shoot apices	Touchell and Dixon (1999)
Conostylis dielsii spp *teres*	Modified PVS2	shoot apices	Turner *et al.* (2001a)
C. micrantha	Modified PVS2	shoot apices	Turner *et al.* (2001a)
C. wonganensis	Modified PVS2	shoot apices	Turner *et al.* (2001a)
Daucus carota (carrot)	PVS2	cell suspension	Seijo (2000)
Dendranthema grandiflorum	PVS2	shoot apices	Ahn and Sakai (1994)
Dendrobium candidum	PVS2	seeds and protocorms	Wang *et al.* (1998)
Doritaenopsis cv. New Toyohashi	PVS2	cell suspension	Tsukazaki *et al.* (2000)
Doritis pulcherrima	PVS2	seeds	Thammasiri (2000)
Eremophila caerulea ssp *marellii*	PVS2	shoot apices	Touchell and Dixon (1999)
E. resinosa	PVS2	shoot apices	Touchell and Dixon (1999)
Eriostemon wonganensis	PVS2	shoot apices	Touchell and Dixon (1999)
Eucalyptus dolorosa	PVS2	shoot apices	Wilson (1999)
E. granticola	PVS2	shoot apices	Crowe (1998)
E. todtiana	PVS2	shoot apices	Wilson (1999)
Fragaria x ananassa	PVS2, PVS3	meristems, cell suspension	Hirai *et al.* (1998), Yongjie *et al.* (1997)
Grevillea cirsiifolia	PVS2	shoot apices	Tan (1998)
G. dryandroides spp *dryandroides*	PVS2	shoot apices	Bunn (unpublished)
G. dryandroides spp *hirsutus*	PVS2	shoot apices	Tan (1998)
G. flexuosa	PVS2	shoot apices	Tan (1998)
G. maccutcheonii	PVS2	shoot apices	Bunn (unpublished)
G. scapigera	PVS2	shoot apices	Touchell and Dixon (1999)
Hakea aculeata	PVS2	shoot apices	Bunn (unpublished)
Hemiandra gardneri	PVS2	shoot apices	Touchell and Dixon (1999)
Hopkinsia anoectocolea	PVS2	shoot apices	Touchell (unpublished)
Hordeum vulgare	PVS2	immature inflorences	Wang *et al.* (1996)
Ipomoea batatas	PVS2	shoot apices	Pennycooke and Towill (2000)
Lambertia orbifolia	PVS2	shoot apices	Bunn (unpublished)
Leucopogon obtectus	PVS2	shoot apices	Touchell and Dixon (1999)
Lechenaultia formosa	PVS2	shoot apices	Touchell and Dixon (1999)
L. laricina	PVS2	shoot apices	Touchell and Dixon (1999)

Lilium sp.	PVS2	shoot apices	Matsumoto *et al.* (1995)
Macropidia fuliginosa	PVS2	somatic embryos, shoot apices	Turner *et al.* (2000b)
Malus sp. (apple)	PVS2	shoot apices	Niino *et al.* (1992a)
Manihot esculenta	PVS2	shoot apices	Charoensub *et al.* (1999)
Mentha spicata	PVS2	shoot tip meristems	Hirai and Sakai (1999a)
Morus alba (mulberry)	PVS2	shoot apices	Niino *et al.* (1992b)
Musa spp.	PVS2	shoot apices	Thinh *et al.* (1999)
Olea europea	PVS2	somatic embryos	Shibli *et* and Al-Juboory. (2000)
Oryza sativa	PVS2	cell suspension	Huang *et al.* (1995)
Panax ginseng	PVS2	hairy root cultures	Yoshimatsu *et al.* (1996)
Pityrodia scabra	PVS2	shoot apices	Touchell and Dixon (1999)
Populus alba	PVS2	shoot apices	Lambardi *et al.* (2000)
Prunus cerasus (sour cherry)	PVS2	shoot apices	Niino *et al.* (1997)
P. domestica	PVS2	shoot apices	De Carlo *et al.* (2000)
P. dulcis	PVS2	shoot apices	Shatnawi *et al.* (1999)
P. jamasakura (cherry)	PVS2	shoot apices	Niino *et al.* (1997)
P. perscia (peach)	PVS2	embryonic axes, shoot apices	De Boucaud *et al.* (1996), Paulus *et al.* (1993)
Ptychosema pusillum	PVS2	shoot apices	Touchell and Dixon (1999)
Pyrus communis (pear)	PVS2	shoot apices	Niino *et al.* (1992a)
Rulingia sp. Trigwell Bridge	PVS2	shoot apices	Touchell and Dixon (1999)
Ribes sp. (currants)	PVS2	meristems	Reed and Yu (1995)
Solanum tuberosum	PVS2	meristems	Hirai and Sakai (1999b)
Solemoostemon rotundifolius	PVS2	lateral buds	Niino *et al.* (2000)
Sowerbaea multicaulis	PVS2	shoot apices	Touchell and Dixon (1999)
Limonium altaica x *L. caspium*	PVS2	meristematic clumps	Matsumoto *et al.* (1997)
Tetratheca deltoidea	PVS2	shoot apices	Touchell and Dixon (1999)
Trifolium repens	PVS2	callus	Yamada *et al.* (1993), Yamada *et al.* (1991)
Vitis vinifera	PVS2	shoot apices	Matsumoto *et al.* (1998b)
Wasabia japonica	PVS2, PVS3	meristems	Matsumoto *et al.* (1998a), Matsumoto *et al.* (1995), Matsumoto *et al.* (1994)

When compared to other protocols, namely, slow cooling and encapsulation/dehydration, vitrification facilitates the direct transfer of tissues into LN, circumventing the need for expensive and sophisticated equipment or time consuming protocols (Nishizawa *et al* 1993). Critical factors influencing success may include: cold acclimation of tissues before vitrification, appropriate pre-treatments or preculture on media with high concentrations of sugars or polyalcohols, the temperature of the PVS2 and duration of PVS2 exposure (Reinhoud *et al* 1995).

Nevertheless, the vitrification procedure has several inherent problems including: inadvertent crystallisation due to the transfer of vitrified materials between different temperatures (warming), potential toxic effects of cryoprotectant components, and potential damaging osmotic effects that may occur during cryoprotectant removal (Benson 1994).

Critical to any successful use of vitrification solutions is that components of the vitrification solution should penetrate the cell and provide internal cryoprotection and promote vitrification. Toxicity effects are avoided by altering the duration and/or the temperature at which the vitrification solution is applied. In some protocols the cells are allowed to equilibrate with a lower concentration of cryoprotectants (called loading) and the final amount is added prior to rapid cooling. In other protocols the mixture may be added in one step with the cells being cooled before they have absorbed lethal amounts of cryoprotectant, or have become excessively dehydrated. Upon warming, the cryoprotective solution should be rapidly diluted with non-toxic substances to prevent cellular damage (Reinhoud *et al.* 1995). A generalised vitrification protocol for somatic tissues is presented in Figure 1.3. (Sakai 1995).

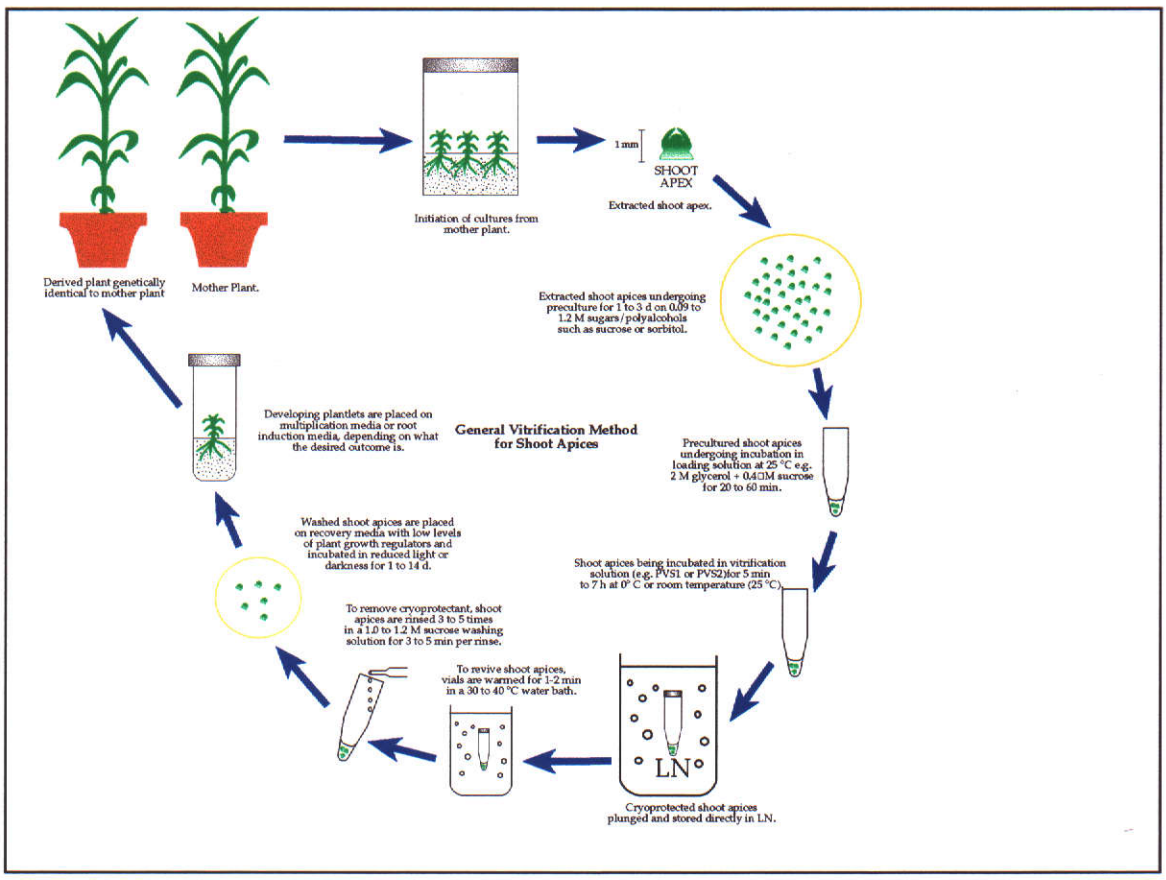

Figure 1.3. Key steps in a generalised vitrification protocol (After Sakai 1995).

1.8 Key Aims of the Thesis

Cryopreservation offers an effective and economical way of conserving the germplasm of agricultural, horticultural and threatened plant species. Cryostorage of somatic tissues using the vitrification procedure is not only more efficient than earlier procedures, but results so far have indicated that it will prove highly effective in minimising LN damage in Australian species (Touchell *et al.* in press (a)).

In Chapter 2, using *Anigozanthos viridis* as the reference species, the aims were to develop a base cryostorage protocol, which would: determine the optimum concentration of sorbitol for preculture, and the optimum duration for PVS2 exposure. The second part of this chapter investigated the application of the developed protocol to other members of the Haemodoraceae. The final objective of this chapter was to determine whether concentration and types of cytokinin used in culture media can influence post-LN survival.

Using the protocol developed in Chapter 2, the objectives in Chapter 3 were to examine post-LN survival of six rare and endangered Haemodoraceae species in response to modifications in the original vitrification protocol. These include: variation to preculture duration, the effects of different sugars/polyalcohols in preculture medium, the use of different vitrification solutions and the addition of a loading step.

Further investigations into the cryopreservation of *Macropidia fuliginosa* were made in Chapter 4; as it was discovered in Chapter 2, excised shoot apices of this species had no survival following LN immersion. Somatic tissues consisting of somatic embryos and shoot apices were used for further investigation. The aims were to:

- evaluate post treatment survival following incubation on different concentrations of sorbitol
- investigate the interaction of sorbitol and PVS2 on post-treatment and post LN immersion survival
- examine the effect on post LN survival after incubation on glycerol or sorbitol for different times (two or three days)

- compare the responses of two tissue types, namely somatic embryos and shoot apices, to the optimised cryopreservation protocol

The mode of action of sugars/polyalcohols in the preculture medium was investigated in Chapter 5 using the principal study species (*A. viridis*). The hypothesis was that molecules with more OH groups arranged along one side of the polyalcohol carbon chain interact more efficiently with the membrane bilayer, and improve preservation of the membrane's integrity at low temperatures. This hypothesis was tested by evaluating a range of sugars and polyalcohols of varying molecular size and with different numbers of, and orientation of OH groups, for their cryoprotective ability.

The effect of plant growth regulators (PGR's) (applied both before and after cryostorage) on successful cryopreservation was investigated in Chapter 6. One aim was to determine the effects of different types and concentrations of plant growth regulators in the culture medium (prior to extraction, cryogenic treatments and LN immersion) on post LN survival of shoot apices derived from these different treatments. The second aim was to determine the most effective plant growth regulator(s) for recovering shoot apices following cryopreservation, and the most appropriate time for their application to recovery medium to maximise quality recovery growth. Parameters such as survival, length and number of leaves were measured and evaluated for this part of the study.

Chapter 7 assessed, firstly, the genetic fidelity of plantlets under standard culture conditions, and under cold storage conditions; and shoot apices under cryostorage conditions after a 12-month storage period. Genetic fidelity was assessed using the powerful PCR-based multi-locus DNA fingerprinting technique of AFLP. A second aspect of this chapter evaluated shoot apex viability of cryogenically stored shoot apices over the same duration (12 months) to determine if storage in LN has lowered viability over time.

The final chapter integrates preceding chapters with a discussion of the results, as well as their implications and significance to both Australian and international plant conservation efforts, horticulture and agricultural science.

Chapters 2 to 7

The list of publications presented below have arisen from research conducted during the Ph.D. candidature and are presented as individual chapters in this thesis

Chapter 2: Turner, S. R., Touchell, D.H., Dixon, K. W and Tan B. (2000) Cryopreservation of *Anigozanthos viridis* ssp *viridis* and related taxa from the South West of Western Australia. Australian Journal of Botany. 48 (6): 739-744.

Chapter 3: Turner, S.R. Senaratna, T Bunn, E.. Tan, B. Dixon, K. W. Touchell, D.H (2001).Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. Annals of Botany 87: 371-378.

Chapter 4: Turner, S. R., Tan B., Senaratna, T. Bunn, E., Dixon, K. W and Touchell, D.H. (2000) Cryopreservation of the Australian species *Macropidia fuliginosa* (Haemodoraceae) by vitrification. Cryo-letters 21 (6): 21: 379-388.

Chapter 5: Turner, S, R., Senaratna, T., Touchell, D, H., Bunn, E., Dixon, K, W and Tan, B. (2001).Stereochemical arrangement of hydroxyl groups in sugar and polyalcohol molecules as an important factor in effective cryopreservation. Plant Science 160 (3): 489-497

Chapter 6: Turner , S, R., Senaratna, T,. Touchell D, H., Bunn, E., Tan, B and Dixon K. W. (2001). Effects of plant growth regulators on survival and recovery growth following cryopreservation. Cryo-Letters 22 (3): 163-174.

Chapter 7: Turner, S. R., Krauss, S. L., Bunn, E., Senaratna, T., Dixon, K, W., Tan, B. and Touchell, D. (in press). Genetic fidelity and viability of *Anigozanthos viridis* following tissue culture, cold storage and cryopreservation. Plant Science 161(6):1099-1106

Chapter 8:

8.1 Introduction

The results described in this study have shown the wide applicability of cryopreservation techniques for the conservation of Western Australian species of Haemodoraceae. The strategy of using one species (*Anigozanthos viridis*) as an indicator facilitated the rapid development of the cryogenic procedures. Such a strategy may allow for the rapid implementation of cryo-conservation procedures for routine germplasm storage of a diverse range of native taxa. In an international context the results obtained are highly relevant as there have been few studies on cryopreservation of somatic tissues of Australian native species. A particularly significant finding is an explanation why certain cryoprotectants are more effective than others; the understanding establishes broad principles which should be applicable to many other species.

The significant achievements of the investigation can be summarised as follows:

1) Establishment of a simple but effective protocol which successfully facilitated the cryopreservation of *Anigozanthos viridis* and taxa from congeneric and intra-familial plant groups.
2) Application of the original protocol to six rare and endangered Haemodoraceae species; modifications and the addition of a loading phase significantly increased survival for four of the six species examined.
3) Successful establishment of a new protocol for cryopreservation of somatic embryos of *Macropidia fuliginosa,* a species which previously proved recalcitrant to cryopreservation.
4) Experimental evidence that the cryoprotective properties of polyalcohols are imparted by the orientation of their hydroxyl (OH) groups and the size of their carbon backbone.
5) Optimised recovery environment with the appropriate plant growth regulators (PGR) added immediately after post-warming treatments.
6) Demonstration that genetic fidelity is maintained during 12 months of LN immersion, and that LN exposure for the same duration does not reduce shoot apex viability.

8.2 Cryostorage of Monocot Species

For *A. viridis* (Chapter 2) and *M. fuliginosa* (Chapter 4) optimal post-LN immersion survival was independently found to require incubation on basal medium supplemented with 0.4 M sorbitol (or 0.8 M glycerol - Chapters 2, 3 and 4), 25 min PVS2 exposure (*A. viridis*) or 30 min PVS2 exposure (*M. fuliginosa*). Further, for the other species examined in Chapter 3 (*C. wonganensis, C. micrantha, C. dielsii, A. kalbarriensis* and *A. humilis*) these exhibited some post-LN survival and for four of these species survival was significantly increased. Therefore It would appear that the pre-treatment requirements for this group of species are generally similar. Given that these related species occur in the same geographical region (i.e. the south west of WA) and are adapted to more or less similar ecological environment (i.e. nutrient deficient sandy soils, extremes of summer heat and drought, followed by cool wet winters) it could be suggested that their general physiological adaptations are not dissimilar.

Nevertheless, there are some significant differences in the niche requirements among these species (Hopper 1993), as well as differences in their life histories. *Conostylis* species, for example, can be classed as sprouter species persisting for many years, through establishment of a large rhizomatous base which re-sprouts each growing season (Elliot and Jones 1984), while *A. kalbarriensis*, and *A. viridis* could be classified as an extended seeder species (Hopper 1993), which are characteristically short-lived (1-2 yrs), quick growing and germinate only when the appropriate conditions prevail (post-fire). *A. humilis* is longer lived than the other two *Anigozanthos* species and is probably more similar to sprouter species (Hopper 1993).

In comparison, the two species with the lowest post-LN survival were *C. dielsii* and *C. micrantha*, both of which are typical sprouter species, (i.e. slow growing, long-lived). When cultured *in vitro*, both were also observed to have the slowest growth and multiplication rates, as well as being prone to hyperhydricity. All or some of these characteristics may be attributed to their different life cycles (compared with the other species). It is hypothesisied that alterations to their culture conditions, to increase both the quality and quantity of shoots, as well as their tolerance to dehydration stress (possibly through ABA treatments), would result in higher post-LN survival, or at least in those stages leading up to LN immersion.

In comparison, vitrification studies on other monocot species such as banana (Thin *et al.* 1999) and rice (Huang *et al.* 1995), have found that preculture on low concentrations of sugars (0.3 M sucrose for banana) and polyalcohols (0.4 M sorbitol for rice) was normally much more effective than at higher concentrations, which are more commonly applied for eudicot species, such as cherry (0.7 M sucrose) and olive (0.75 M sucrose) (Niino *et al.* 1997; Martinez *et al.* 1999).

However, for optimal PVS2 exposure, the species examined in this study required only 25 – 30 min exposure to the vitrification solution, which is comparatively brief, although there are also species such as rice which require only 7.5 mins exposure to PVS2 (at 0° C) for optimal cryoprotection (Huang *et al.* 1995). At the other extreme, several species such as asparagus (Kohmura *et al.* 1992) and pineapple (Gonzalez-Arnao *et al.* 1998) require 2 - 7 hrs exposure (at 0 ° C), which would be supra-optimal or event toxic for Haemodoraceae species.

There are fewer published reports on cryostorage of monocot species compared with those for eudicot taxa. Less than 20 % of cryopreservation studies involve monocot species, (Appendix 1), the more significant of which include the study by Thin (1997) who investigated cryostorage procedures for some 20 tropical monocot species including taro, banana, pineapple and several orchids species, while lily (Matsumoto *et al.* 1995) and asparagus (Uragami *et al.* 1989) have also been successfully cryopreserved.

8.3 Parameters of Effective Cryoprotection

Apart from *M. fuliginosa*, all the species investigated in this study appeared to benefit from preculture for three days rather than the originally adopted duration of two days. Survival in the non-cooled treatments suggest that the extra day had beneficial effects by increasing the osmo-tolerance of shoot apices prior to subsequent treatments, and this has resulted in a significant increase in survival after cryoprotection and ultimately post-LN survival. For *M. fuliginosa*, however, three days' preculture was supra-optimal; it is likely that after two days preculture, tissues were already in optimal condition to survive the subsequent stages in the protocol such as vitrification and cooling. Hence, an additional day of preculture may result in excessive stress and, later, irreversible tissue damage following LN immersion

Post-LN survival was also shown not to depend on the presence of DMSO in the cryoprotectant solution, with similar levels of survival being achieved when none (0 %) or reduced (7.5 %) concentrations of DMSO were present in the vitrification solution. The DMSO concentration in PVS2 is 15 % and this concentration was considered optimal prior to this study. Previous to this finding DMSO was thought to be a key component of this commonly used vitrification solution. However, the results presented here suggest that higher concentrations of DMSO may not be as important as first thought. The results also suggest that other less toxic compounds such as 1,2 – propandiol (as used in this study), which have similar permeating qualities to DMSO, may be used successfully in vitrification solutions. In comparison, there are other successful vitrification solutions such as PVS3 (Nishizawa *et al.* 1993) or PVS4 (Sakai 2000) which contain zero to five percent DMSO. These solutions have been reported to have excellent cryoprotectant qualities for species such as asparagus and strawberries (Nishizawa *et al.* 1993; Yongjie *et al.* 1997), although in this present study PVS3 was found to be unsuitable under the conditions it was administered (Chapter 3).

A significant finding in this study is that both molecular size and stereochemical arrangement of OH groups of polyalcohol molecules have a significant effect on post-LN survival. It had previously been assumed that sugars and polyalcohols used in preculture media acted in several ways during the conditioning process leading up to cryoprotection and cryopreservation. Firstly, sugars and polyalcohols were thought to desiccate plant tissues (Panis *et al.* 1996), and it had also been postulated that sugars and polyalcohols may increase membrane stability under conditions of severe dehydration by replacing membrane-bound water molecules (Crowe *et al.* 1989). Finally, sugars and polyalcohols were also thought to induce a stress response through increasing the intracellular concentration of sugars (Grospietsch *et al.* 1999), lowering the freezing point (freezing point depression) (Panis *et al.* 1996), and through the biochemical response to osmotic stress. The latter will induce the production of compounds such as ABA or proline (Hitmi *et al.* 1999a; Reinhoud 1996), and prime the tissues for cryoprotection and tolerance to LN.

The results presented here suggest that there are significant interactions between sugar/polyalcohol molecules and cellular membranes, assisting in

stabilisation during subsequent stress and cryopreservation. This is indicated in a number of ways: firstly, by the significant difference in tissue survival with similar-sized molecules but with different OH orientations – specifically, sugars/polyalcohols with aligned OH molecules were shown to be superior in imparting cryoprotection; secondly, smaller molecules such as glycerol and erithitol were found to impart higher survival (possibly due to better packing qualities around cell membranes); thirdly, molecules with higher numbers of OH groups (such as polyalcohols) were found to have superior cryoprotection qualities (more hydrogen bonding sites); and finally, similar survival can be achieved when polyalcohol preculture media were formulated with equimolar concentrations of OH groups present. This survival was achieved regardless of molarity of the preculture media. Based on these properties, glycerol was shown to be very effective at twice the molarity (0.8 M) (Chapters 3, 4, 5) compared with optimal molarity for sorbitol (0.4 M) (Chapters 2 and 4). These concentrations were found to be deleterious with other sugars and polyalcohols (Chapter 5).

Glyerol was shown to be effective for cryostorage of *A. viridis* (Chapter 3) and *M. fuliginosa* (Chapter 4) and appeared to be equally effective for the other Haemodoraceae species (Chapter 3). While the most commonly used sugar in preculture media is sucrose (Kohmura *et al.* 1994, Niino *et al.* 1997), glycerol has been reported to be necessary for preculture conditioning prior to cryostorage for several species, including horseradish hairy root cultures (at 1.0 M) (Phunchindawan *et al.* 1996) and embryonic axes of jackfruit (at 0.5 M) (Thammasiri 1999). However, both these studies reported the use of glycerol in conjunction with sucrose. The results presented in this study are the first reported usage of glycerol, singularly, as the sugar/polyalcohol source in the preculture medium.

8.4 Optimised Culture and Recovery Conditions

One of the most important factors in successful cryopreservation is the condition of the plantlets prior to shoot apex excision, pre-treatment(s) and cryopreservation. Plantlets (and subsequently shoot apices) need to be in optimal condition to obtain maximum survival following cryopreservation. Different species require different concentrations' of and types of PGR's, to grow optimally. Yet the effects of these hormonal regimes were hitherto

unknown. The results for *A. viridis* (Chapter 6) indicate that both of these factors (concentration and type) do not appear to influence post-LN survival in this species. Previous observations for *Grevillea scapigera* (Touchell *et al.* in press b) and *A. viridis* (Chapter 2) had indicated that both, type and concentration of plant growth regulator, may significantly influence post-LN survival. For *A. viridis* these earlier differences (Chapter 2) may be due to sub-optimal pre-treatments, as indicated by the low survival of post-LN treatments. Such results may help in the formulation of more appropriate culture conditions (prior to cryopreservation), as there appears to be no PGR-or concentration-specific effect on post-LN survival. This finding may be applicable for species such as *C. dielsii* and *C. micrantha,* which have been observed to grow very poorly on the standard culture medium (1/2 MS supplemented with 0.5 μ M BAP) (Chapter 3) and which may require different growth regulators and factors and/or media formulation to obtain high-quality, actively-growing shoots.

The importance of PGR's in the recovery media has been demonstrated in this study. For *A. viridis,* a combination of GA_3 and cytokinin from day 0 facilitated the development of rapidly growing plantlets following cryopreservation. This combination did not induce callusing (a possible source of somaclonal variation) but resulted in vigorously growing plantlets. It is recommended that warmed and washed shoot apices be placed as quickly as possible on the appropriate medium to stimulate active growth. However, PGR levels need to be reasonably low to avoid callusing which is usually induced by interactions of PGR's with injured cells, the latter being sensitive to callusing and possibly prone to mutations.

As no callusing of recovering shoot apices, or phenotypic changes, were observable in regenerated plantlets, the type and concentrations of PGR's used in this study appear to be physiologically acceptable.

Following cryopreservation, it is recommended that if callusing is observed during recovery, the addition of PGR's should be postponed to reduce the risk of somaclonal variation occurring (Touchell 1995).

An optimised recovery medium is of particular importance for species such as *C. wonganensis,* which exhibits a high initial survival following cryostorage (Chapter 3) but a very high mortality rate in the subsequent 2-4 weeks, when non-optimised recovery media were utilised. This species in

particular would be expected be respond to the new recovery medium regime, although this hypothesis needs to be tested.

8.5 Maintenance of Genetic Integrity

Under the experimental conditions reported in this study it appears that no genetic changes were induced in any of the treatments on shoot tissues of *A. viridis* (Chapter 7). The AFLP technique, which is a very powerful DNA profiling tool, facilitated the detailed examination of generated fragments to a degree which had previously not been possible. Of a total of 95 fragments generated with three different primers, no differences were detected between the different treatment samples examined. These had been maintained under different storage conditions over a 12 month period. Nevertheless, as stated previously, this study examined only one clone, with five replicates from each treatment; this restricted sample size may have been insufficient for detecting small changes occurring in only a small percent of the population. As a preliminary investigation, however, these results do suggest that genetic fidelity can be maintained under cryogenic conditions. Given the small number of studies that have investigated the maintenance of genetic integrity after LN immersion, none or only minor aberrations have so far been reported (Harding 1991; Harding and Benson 2000; Haggman *et al.* 1998). The results presented here add further confidence for the cryogenic procedures which are now being developed and utilised.

The other key issue is the maintenance of tissue viability over extended periods of LN storage. The results presented in Chapter 7 suggest that tissue viability can be maintained over a period of 12 months, but this is considered short term in the context of germplasm storage. Although, tissue viability can be expected to be maintained for a much longer duration, further studies involving cryostorage for much longer periods (such as 5 - 10 years) are needed, and with a wider range of species. In this context, Gonzales-Arnao *et al.* (1999) Haskins and Kartha (1980), and Kartha *et al.* (1980) have shown that sugarcane, pea and strawberry shoot apices stored cryogenically have maintained viability for up to one, two, and ten years respectively.

8.6 Establishment of Cryogenic Germplasm Collections

The study by Touchell (1995) highlighted the importance of undertaking detailed cryogenic studies on one species (*G. scapigera*), and extending the application to other species. Given the numerous species and even clones which are currently in need of being conserved, it is critically important that generic techniques with high applicability are developed. If detailed studies were required for all taxa requiring cryogenic (germplasm) storage, the time, money and resources needed for this undertaking would be enormous and in most cases will simply not be available.

The technique developed by Touchell (1995) for *G. scapigera* has so far led to the successful cryopreservation of more than 20 West Australian species, while the techniques developed for *A. viridis* during this study has facilitated the successful cryopreservation of a further nine Haemodoraceae taxa. Similar successes have been reported overseas for kiwifruit (Bachiri *et al.* 2001), banana (Thin *et al.* 1999) and carnations (Fukai *et al.* 1991).

For species that appear to be recalcitrant to these techniques, new approaches need to be developed. Consideration may be given to: ABA pre-conditioning and cold hardening (Reed 1990), ASA (acetyl salicylic acid) pre-conditioning, (Senaratna *et al.* 2000), or the addition of anti-oxidants (e.g. desferrioxamine) (Benson *et al.* 1995) to culture, preculture or recovery media; additionally other loading and/or vitrification solutions may also be examined (Table 8.1). Finally new procedures such as encapsulation/dehydration (Sakai *et al.* 2000) may also be evaluated.

Table 8.1. Factors that have been found to be beneficial for increasing post-LN survival.

Stage	Factor	Reference
Culture	Addition of ABA, cold acclimation, applying osmotic agents, preculturing in media which contain anti-stress agents such as proline, incubation for extended durations	Reed (1990); Reed (1996); Wu *et al.* (1999)
Preculture	Different sugars or polyols (sucrose, glycerol, sorbitol), different concentrations, different incubation times	Turner *et al.* (2001b)
Loading	Different loading solutions, different exposure times	Langis and Steponkus (1990); Chang *et al.* (2000)
Vitrification	Different solutions (PVS1, PVS2, PVS3, PVS4), different exposure times, different exposure temperatures, stepwise additions	Uragami *et al.* (1989); Sakai *et al.* (1990); Makowska *et al.* (1999); Sakai (2000)
Recovery and Post Recovery	Culture on choline chloride, on increased osmoticiums (sucrose), on appropriate PGR's	Touchell *et al.* (in press a); Makowska *et al.* (1999).

8.7 Future Implications of Cryopreservation Principles

With increasing encroachment, exploitation and ultimately destruction of natural ecosystems, radical measures are needed to avert or at least reduce the loss of biodiversity. Increasingly, integrated conservation programs are being formulated and implemented covering not only countries, but entire global regions as well. International programs such as the Millennium Seed Bank Project which, within 10 years, aims to store seed of 10 % of all the world's plant species are presently taking shape. This particular program aims to store orthodox seeded species at –18° C for up to 200 years at the Kew Royal Botanic Gardens. Similar but less ambitious programs are needed around the world, particularly in countries such as Australia with its highly endangered and unique flora.

In Australia there are encouraging signs with more communication and integration of conservation efforts. Botanic gardens of several states are already beginning to help conserve and store rare and endangered germplasm including those from outside their immediate jurisdictions (Dixon pers. com). For example, Kings Park and Botanic Garden (KPBG), in Western Australia, is actively coordinating, collecting and storing seed and symbiotic fungi of critically endangered orchids from Victoria, New South Wales and South Australia. A part of this collection is now maintained under cryogenic conditions.

Further advances in cryostorage and integration of programs may see the establishment of large germplasm base collections of seed and somatic tissues from native Australian species. KPBG is currently maintaining shoot apices of over ten critically endangered Western Australian species in cryogenic storage, with other rare species being constantly evaluated for addition to the storage list (Bunn pers. com).

Once equipment, blueprint and cryo-procedures are in place, the implementation of a cryogenic germplasm bank becomes a matter of routine. With increasing scientific and technical knowledge on cryopreservation, cryoconservation of valuable germplasm such as Haemodoraceae taxa will become an integral part of a larger modern conservation and biotechnology program encompassing many other plant species.

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Appendix 1.

Summary of successful cryopreservation studies on non Australian species, using various cryogenic procedures such as slow cooling, encapsulation/dehydration and vitrification, on somatic tissues like protoplasts, cell suspensions, shoot apices, and tissue and organ cultures (* modified from Bajaj 1995b and Sakai 2000 (see thesis reference list))

Species	Plant Tissues Used	*Reference
Abies cephalonica	embryogenic cultures	Aronen *et al.* (1999)
Abies nordmanniana	cell cultures	Norg *et al.* (1993),
Acacia mangium	shoot apices	Sudarmonowati (2000)
Acer pseudoplatanus (sycamore)	cell suspension	Sugawara and Sakai (1974)
Aesculus hippocastanum	embryonic axes	Pence (1992)
Actinidia chinensis (kiwi fruit)	stem segment	Jian and Sun (1989)
Aesculus hippocastanum (horse chestnut)	somatic embryos	Jekkel *et al.* (1992),
Allium sativa (garlic)	shoot apices, apical meristems	Niwata (1992) Niwata (1995)
A. wakegi (shallot)	apical meristems	Kohmura *et al.* (1994)
Ananas comosus (pineapple)	shoot apices	Gonzalez - Arnao (1998)
Angelica acutiloba	hairy root cultures	Yoshimatsu *et al.* (2000)
Anisodus acutangulus	cell suspension	Zheng Guang-zhi *et al.* (1983)
Antirrhinnum microphyllum	nodal explants	Gonzales - Benito *et al.* (1998)
Arachis hypogaea (peanut)	meristems, embryonic axes	Bajaj (1979), Runthala *et al.* (1993)
Armoracia rusticana (horseradish)	hairy root culture, shoot primordia	Hirata *et al.* (1995), Phunchindawan *et al.* (1997),
Anisodus acutangulus	callus	Zheng *et al.* (1983)
Artemisia annua	hairy root cultures	Teoh *et al.* (1996)
Artocarpus heterophyllus (jackfruit)	embryonic axes	Fu *et al.* (1990)
Asparagus officinalis	somatic embryos, cell suspensions, nodal segments, shoot apices, multiple bud clusters	Uragami *et al.* (1989), Suzuki *et al.* (1998), Suzuki *et al.* (1997), Kohmura *et al.* (1992)
Atropa belladonna (belladonna)	cell suspension, protoplasts,	Nag and Street (1975), Bajaj (1988)
Azadirachta indica	embryonic axes	Berjak and Dumet (1996)
Beta vulgaris (sugarbeet)	meristems, root cultures, shoot apices	Braun (1988), Benson and Hamill (1991), Vandenbussche and De Proft (1996)
Betula pendula	shoot apices, dormant buds	Ryynanen (1996), Ryynanen (1996)
Brassica campestris (mustard)	cell suspension	Langis *et al.* (1990)
B. napus	shoot tips, somatic embryos	Withers *et al.* (1988), Li *et al.* (1999)
Bromus inermis (bromegrass)	protoplasts, cell suspension	Mazur and Hartmann (1979), Ishikawa *et al.* (1996)
Brunsfelsia densifolia	cell suspension	Pence (1990)
Camellia japonica	somatic embryos, embryonic axes	Janeiro *et al.* (1996)
Camellia sinesis (tea)	embryonic axis, shoot apices	Chaudhury *et al.* (1991), Kuranuki and Sakai (1995)
Cannabis sativa (hemp)	cell suspension	Jekkel *et al.* (1989)
Carica papaya	embryogenic cultures	Ashmoore *et al.* (2000)
Castanea sativa	embryonic axes	Pence (1992)
Catharanthus roseus (periwinkle)	cell suspension, protoplasts	Chen *et al.* (1984), Gazeau *et al.* (1992)

Cedrela odorata	shoot apices	Maruyama and Ishii (2000)
Centaurium rigualii	nodal explants	Gonzalez *et al.* (1997)
Chamomilla recutita	cell suspension	Cellarova *et al.* (1992)
Chrysanthemum cinerariaefolium	shoot apices, cell cultures	Hitmi *et al.* (1999), Hitmi *et al.* (1997)
C. morifolium	shoot apices	Fukai (1990)
Cicer arietinum (chickpea)	meristems	Bajaj (1983)
Cichorium intybus (chicory)	shoot apices	Demeulemeester *et al.* (1993),
Cinchona ledgeriana	callus	Hunter (1986)
Citrus sp.	somatic embryos, cell suspensions	Marin and Duran-Vila (1988), Aguilar *et al.* (1993)
Coffea arabica (coffee)	somatic embryos	Bertrand-Desbrunais *et al.* (1988)
C. canephora	cell suspension, somatic embryos	Tessereau *et al.* (1991),
C. racemosa	shoot apices	Mari *et al.* (1995)
C. sessiliflora	shoot apices	Mari *et al.* (1995)
Colocasia esculenta	shoot apices	Takagi *et al.* (1997)
Corylus avellana (hazelnut)	embryonic axes	Reed *et al.* (1994
Cucumis melo (melon)	somatic embryo, shoot primordia	Shimonishi *et al.* (1991), Ogawa *et al.* (1997)
Datura innoxia	protoplasts	Bajaj (1988)
D. Stramonium	cell suspension	Bajaj 1976)
Daucus carota (carrot)	cell suspension, somatic embryos, protoplasts	Nag and Street (1975), Withers (1979), Takeuchi *et al.* (1980)
Dendranthema grandiflorum (chrysanthemum)	shoot apices	Fukai *et al.* (1994)
Dendranthema sp.	shoot apices	Fukai *et al.* (1991)
Dendrobium candidum	protocorms	Wang *et al.* (1998)
Dianthus caryophyllus (carnation)	shoot apices	Fukai (1989)
Dianthus sp.	shoot apices	Fukai *et al.* (1991)
Digitalis lanata	cell suspension	Dietterich *et al.* (1982
D. thapsi	cell suspension	Vloran *et al.* (1999)
Dioscorea alata	shoot apices	Malaurie *et al.* (1998)
D. bulbifera	shoot apices	Malaurie *et al.* (1998)
D. deltoidea (yam)	cell suspension, shoot apices	Butenko *et al.* (1984), Manda *et al.* (1996)
Elaeis guineesis (oil palm)	somatic embryos, polyembryonic cultures	Engelmann *et al.* (1985), Dumet *et al.* (1994)
Euphorbia longan (longan)	embryonic axes	Fu *et al.* (1990)
Festuca sp.	cell suspension	Wang *et al.* (1994
Fragaria x ananassa (strawberry)	runner apex, meristems, cell suspension	Sakai *et al.* (1978), Hirai *et al.* (1998), Yongjie *et al.* (1997)
Gentiana scabra (gentian)	axillary buds	Suzuki *et al.* (1998)
Gladiolus grandavansis	callus	Li and Wang (1989)
Glycine max (soybean)	cell suspension protoplasts	Bajaj (1976), Takeuchi *et al.* (1982)
Gossypium arboreum (cotton)	callus, cell suspension	Bajaj (1982), Rajasekaran (1996)
G hirsutum	callus	Bajaj (1982)
Guazuma crinita	shoot apices, multiple bud clusters	Maruyama *et al.* (1998), Maruyama *et al.* (1997),
Gypsophila paniculata	shoot apices	Fukai *et al.* (1991)
G.elegans	shoot apices	Fukai *et al.* (1991)
G.repense	shoot apices	Fukai *et al.* (1991)
Haplopappus gracilis	shoot primordia	Taniguchi *et al.* (1988)
Helianthus tuberosus (Jerusalem artichoke)	cell suspension	Swan *et al.* (1999)
Hevea brasiliensis (rubber)	embryonic axes, embryogenic callus	Normah *et al.* (1986), Veiesseire *et al.* (1993)
Holostemma annulare	shoot apices	Decruse *et al.* (1999)

Hordeum vulgare (barley)	protoplasts, callus, cell suspension,	Takeuchi *et al.* (1982), Hahne and Loerz (1987), Fretz and Loerz (1995)
Humulus lupulus (hops)	shoot apices	Martinez *et al.* (1998)
Iris nigricans (black iris)	somatic embryos	Shibli Rida (2000)
Ipomoea sp. (sweet potato)	cell suspension, shoot apices, embryogenic callus	Latta (1971), Towill and Jarret (1992), Blakesley *et al.* (1995)
Juglans regia (walnut)	embryonic axis, somatic embryos	de Boucaud *et al.* (1991), de Boucaud *et al.* (1994)
Landolphia kirkii	embryonic axis	Vertucci and Berjak (1991)
Lavandula vera (lavender)	cell suspension	Watanabe *et al.* (1983),
Larix x eurolepis (larch)	cell cultures	Klimaszewska *et al.* (1992)
Lilium sp. (lily)	meristems	Matsumoto and Sakai (1995),
Limonium sp. (statice)	apical meristems, shoot primordia	Matsumoto *et al.* (1998), Matsumoto *et al.* (1997)
Linum usitatissimum (flax)	cell suspension	Quatrano (1968)
Litchi chinensis (lychee)	embryonic axes	Fu *et al.* (1993)
Lolium sp.	meristems, cell suspension	Yongjiang *et al.* (2000), Wang *et al.* (1993)
Lychnis coronaria	shoot apices	Fukai *et al.* (1991)
chalcedonica	shoot apices	Fukai *et al.* (1991)
coelirosa	shoot apices	Fukai *et al.* (1991)
Lycopersicon esculentum (tomato)	meristems	Grout *et al.* (1978))
Malus sp. (apple)	shoot apices, callus, buds	Niino and Sakai (1992), Kurola and Nishiyama (1983), Tyler and Stushnoff (1988)
Manihot esculenta (cassava)	shoot apices, somatic embryos,	Bajaj (1977), Sudarmonowati and Henshaw (1990)
Marchantia polymorpha	protoplasts	Takeuchi *et al.* (1980)
Medicago sativa (alfalfa)	callus	Finkle *et al.* (1980)
Mentha sp. (mint)	shoot apices	Towill (1990)
Moricandia arvensis	cell suspension	Craig *et al.* (1997)
Morus alba (mulberry)	shoot apices, winter buds	Niino and Sakai (1992), Niino *et al.* (1993)
M. bombysis	meristems, shoot apices	Yakuwa and Oka (1988)
Musa sp. (banana),	cell suspension, shoot apices	Panis *et al.* (1990), Panis *et al.* (1996)
Nephelium lappaceum	shoot apices	Sudarmonowati (2000)
Nicotiana tabacum (tobacco)	cell suspension, protoplasts	Bajaj (1976), Bajaj (1988)
N. plumbaginifolia	cell suspension	Maddox *et al.* (1983)
N. rustica	root cultures	Benson and Hamill (1991)
Olea europaea (olive)	shoot apices	Martinez *et al.* (1999)
Onobrychis viciaefolia (sainfoin)	callus	Sun and Jian (1990)
Oryza meyeriana	cell cultures	He *et al.* (1998)
O. sativa (rice)	cell suspension, callus	Sala *et al.* (1979), Watanabe *et al.* (1990)
O. sativa + *Pisum sativum*	fused protoplasts	Bajaj (1983)
O. sativa + Sorghum bicolor	fused protoplasts	Bajaj (1983)
Panax ginseng (ginseng)	cell suspension, root cultures	Butenko *et al.* (1984), ,Yoshimatsu *et al.* (1995)
Panicum maximum (guinea grass)	cell suspension	Gnanapragasam and Vasil (1992)
P. miliaceum	callus	Sun *et al.* (1988)

Papaver somniferum (opium poppy)	cell cultures	Elleuch *et al.* (1998)
Paraserianthes falcataria	embryogenic callus	Sudarmonowati (2000)
Pennisetum glaucum (pearl millet)	cell suspension	Gnanapragasam and Vasil (1992)
Phoenix dactylifera (date palm)	meristems, callus, somatic embryos	Finkle *et al.* (1980), Mycock *et al.* (1995), Bagniol and Engelmann (1991)
Picea abies (Norway spruce)	cell cultures	Gupta *et al.* (1987)
P. glauca (white spruce)	cell cultures, somatic embryos	Kartha *et al.* (1988), Cyr *et al.* (1994)
P. glauca - engelmanni	cell cultures	Cyr *et al.* (1994)
P. mariana (black spruce)	cell cultures	Klimaszewska *et al.* (1992)
P . sitchensis (sitka spruce)	cell cultures	Find *et al.* (1993)
Pinus patula	somatic embryos	Ford *et al.* (2000)
P. sylverstris (Scots pine)	buds	Kuoksa and Hohtola (1991),
P. Caribaea (Caribean pine)	cell suspensions	Laine *et al.* (1992)
P. taeda (loblolly pine)	cell suspensions	Gupta *et al.* (1987)
Pisum sativum (pea)	protoplasts, embryonic axis, excised seedlings, meristems, somatic embryos	Mycock *et al.* (1991), Sun (1958), Haskins and Kartha (1980), Wesley-Smith (1995), Mycock *et al.* (1995)
Polygonium aviculare	cell suspension	Swan *et al.* (1998)
Pometia pinnata	embryogenic callus	Sudarmonowati (2000)
Poncirus trifolata (citrus)	embryonic axes, shoot apices	Radhamani and Chandel (1992), Gonzalez - Arnao *et al.* (1998)
Populus alba (white popular)	shoot apices	Lambardi *et al.* (1999)
P. euramericana (poplar)	cell suspension	Sakai and Sugawara (1973)
Primula x *pubescens*	shoot apices	Hornung *et al.* (2001)
Prunus cerasus (sour cherry)	dormant buds	Towill and Forsline (1999)
P. dulcis (almond)	shoot apices, embryonic axes	Channuntapipat *et al.* (2000), Chaudhury and Chandel (1995)
P. ferlenain	shoot apices	Helliot and De boucaud (1997)
P. jamasakura	shoot apices	Niino *et al.* (1997)
P. perscia (peach)	embryogenic axes	De boucaud *et al.* (1996)
P. cv. Fereley - Jaspi	shoot apices	Brison *et al.* (1995)
P. cv. Ferlenain - Plumina	shoot apices	Brison et al. (1995)
Pterocarpus indicus	embryogenic axes	Krishnapillay *et al.* (1995)
Puccinellia sp. (halophyte grasses)	cell suspension	Heszky *et al.* (1990)
Pyrus communis (pear)	shoot apices, winter buds	Moriguchi *et al.* (1985), Oka *et al.* (1991)
P. pyrifolia	shoot apices	Niino and Sakai (1992)
P. koehnei	shoot apices	Reed (1990)
P. cossonii	shoot apices	Reed (1990)
Quercus faginea	embryonic axes	Gonzalez - Benito and Perez - Ruiz (1992)
Q. ilex	embryonic axes	Gonzalez - Benito *et al.* (1999)
Q. suber	embryonic axes	Gonzalez - Benito *et al.* (1999)
Ribes sp. (currants)	apical meristems	Reed (1992)
Rosa multiflora	shoot apices	Lynch *et al.* (1996)

Rubus sp. (brambles)	apical meristems, shoot apices, cell suspension	Reed and Lagerstedt (1987), Chang and Reed (1999), Lett and Schmitt (1992)
Saccharum officinarum (sugarcane)	callus, cell suspension, meristems, shoot apices	Bajaj *et al.* (1987), Ulrich *et al.* (1979), Paulet *et al.* (1993)
Secale cereale (rye)	protoplasts	Langis and Steponkus (1990)
Setaria italica (foxtail millet)	callus, cell suspension	Lu and Sun (1992)
Silene armeria	shoot apices	Fukai *et al.* (1991)
S. pendula	shoot apices	Fukai *et al.* (1991)
Solanum tuberosum (potato)	meristems, axillary buds, shoot apices	Bajaj (1977, 1978), Towill (1984)
S. goniocalix	meristems	Grout and Henshaw (1978)
S. etuberosum	meristems	Towill (1981)
S. phureja	encapsulated shoot apices	Fabre and Dereuddre (1990)
Swietenia macrophylla (mahogany)	shoot apices	Aguilar *et al.* (2000)
Taxus chinensis	cell suspension	Kim *et al.* (2001)
Theobroma cacoa (coco)	immature zygotic embryos	Pence (1991)
Trichilia dregeana	embryonic axes	Kioko *et al.* (1998)
Trifolium repens (clover)	apical meristems, callus	Yamada *et al.* (1991), Yamada *et al.* (1993)
Triticum aestivum (wheat)	callus cultures, cell suspension	Bajaj (1980), Kendall *et al.* (1990)
T. aestivum + *Pisum sativum*	fused protoplasts	Bajaj (1983)
Vaccinium pahalae	cell suspension	Rida *et al.* (1999)
V. sp.	apical meristems	Reed (1989)
Vanda pumila	shoot primordia	Na *et al.* 91996)
Vigna mungo x *V. radiata*	hybrid embryos	Bajaj (1990)
Vitis vinifera (grapes)	cell suspension, shoot apices	Dussert *et al.* (1992), Plessis *et al.* (1993)
Wasabia japonica (wasabi)	apical meristems	Matsumoto *et al.* (1994),
Xanthosoma brasiliense (tanier spinach)	somatic embryos	Jayos *et al.* (1993)
Zeas mays (maize)	callus cultures, cell suspension, root meristems,	Withers and King (1979), Sun and Jian (1989), Issacs and Mycock (1999)
Zoysia sp.	meristems	Yongjiang *et al.* (2000)

Appendix 2.

List of all Australian species which have successfully survived cryopreservation. This list includes seed, pollen, somatic embryos and shoot apices.

Family	Species	Tissues Used	Protocol	Reference
	Eudicots			
Aizoaceae	*Trianthema oxycalyptra*	seeds	direct immersion	Touchell and Dixon (1993)
Amarantaceae	*Gomphrena canescens*	seeds	direct immersion	Touchell and Dixon (1993)
Asteraceae	*Brachycome latisquamea*	seeds	direct immersion	Touchell and Dixon (1993)
	Helichrysum davenportii	seeds	direct immersion	Touchell and Dixon (1993)
	Helipteru roseum	seeds	direct immersion	Touchell and Dixon (1993)
	Schoenia cassiniana	seeds	direct immersion	Touchell and Dixon (1993)
Caesalpiniaceae	*Cassia venusta*	seeds	direct immersion	Touchell and Dixon (1993)
	Labichea teretifolia	seeds	direct immersion	Touchell and Dixon (1993)
	Petalostylis millefolium	seeds	direct immersion	Touchell and Dixon (1993)
Casuarinaceae	*Allocasuarina fraseriana*	seeds	direct immersion	Touchell and Dixon (1993)
Chenopodiaceae	*Atriplex nummularia*	seeds	direct immersion	Touchell and Dixon (1993)
Chloanthaceae	*Pityrodia scabra*	shoot apices	vitrification	Touchell and Dixon (1999)
Crassulaceae	*Crassula colorata*	seeds	direct immersion	Touchell and Dixon (1993)
Epacridaceae	*Lysinema ciliatum*	seeds	direct immersion	Touchell and Dixon (1993)
Goodeniaceae	*Goodenia beardiana*	seeds	direct immersion	Touchell and Dixon (1993)
	Lechenaultia formosa	shoot apices	vitrification	Touchell and Dixon (1999)
	Lechenaultia laricina	shoot apices	vitrification	Touchell and Dixon (1999)
Lamiaceae	*Coleus scutellarioides*	seeds	direct immersion	Touchell and Dixon (1993)
	Hemiandra gardneri	shoot apices	vitrification	Touchell and Dixon (1999)
Menyanthaceae	*Villarsia capitata*	seeds	direct immersion	Touchell and Dixon (1993)
Mimoaceae	*Acacia acuminata*	seeds	direct immersion	Touchell and Dixon (1993)
	Acacia auriculiformis	pollen	equilibration and direct immersion	Sedgley and Harbard (1993)
	Acacia iteaphylla	pollen	equilibration and direct immersion	*Sedgley and Harbard (1993).
	Acacia mangium	pollen	equilibration and direct immersion	*Sedgley and Harbard (1993).
Myoporaceae	*Eremophila caerulea* ssp *marellii*	shoot apices	vitrification	Touchell and Dixon (1999)
	Eremophila resinosa	shoot apices	vitrification	Touchell and Dixon (1999)
Myrtaceae	*Agonis flexuosa*	seeds	direct immersion	Touchell and Dixon (1993)
	Beaufortia incana	seeds	direct immersion	Touchell and Dixon (1993)
	Callistemon phoeniceus	seeds	direct immersion	Touchell and Dixon (1993)
	Calothamnus tuberosus	seeds	direct immersion	Touchell and Dixon (1993)
	Eremaea beaufortioides	seeds	direct immersion	Touchell and Dixon (1993)
	Eucalyptus burracoppinensis	seeds	equilibration and direct immersion	Touchell and Dixon (1993)
	Eucalyptus dolorosa	shoot apices	vitrification	Wilson (1999)
	Eucalyptus grandis	axillary buds	encapsulation/ dehydration	Blakesley and Kiernan (2001)
	Eucalyptus grandis x *Eucalyptus camaldulensis*	axillary buds	encapsulation/ dehydration	Blakesley and Kiernan (2001)
	Eucalyptus granticola	shoot apices	vitrification	Crowe (1998)
	Eucalyptus gunnii	shoot apices	encapsulation/ dehydration	Monod *et al.* (1992)
	Eucalyptus lane-poolei	seeds	direct immersion	Touchell and Dixon (1993)
	Eucalyptus loxophleba var. *gratiae*	seeds	direct immersion	Touchell and Dixon (1993)
	Eucalyptus todtiana	shoot apices	vitrification	Wilson (1999)
	Hypocalymma angustifolium	seeds	direct immersion	Touchell and Dixon (1993)
	Kunzea baxteri	seeds	direct immersion	Touchell and Dixon (1993)
	Leptospermum spinescens	seeds	direct immersion	Touchell and Dixon (1993)
	Melaleuca cuticularis	seeds	direct immersion	Touchell and Dixon (1993)
	Melaleuca cuticularis	seeds	direct immersion	Touchell and Dixon (1993)
	Melaleuca radula	seeds	direct immersion	Touchell and Dixon (1993)
	Verticordia etheliana	pollen	equilibration and direct immersion	Tyagi *et al.* (1992).
	Verticordia helichrysantha	pollen	equilibration and direct immersion	Tyagi *et al.* (1992).
	Verticordia monadelpha	pollen	equilibration and direct immersion	Tyagi *et al.* (1992).

	Verticordia picta	pollen	equilibration and direct immersion	Tyagi *et al.* (1992).
	Verticordia staminosa	pollen	equilibration and direct immersion	Tyagi *et al.* (1992).
Papillionaceae	*Bossiaea ornata*	seeds	direct immersion	Touchell and Dixon (1993)
	Brachysema aphyllum	seeds	direct immersion	Touchell and Dixon (1993)
	Burtonia scabra	seeds	direct immersion	Touchell and Dixon (1993)
	Chorizema dicksonii	seeds	direct immersion	Touchell and Dixon (1993)
	Clianthus formosus	pollen	equilibration and direct immersion	*Hughes *et al.* (1991)
	Daviesia cordata	seeds	direct immersion	Touchell and Dixon (1993)
	Gastrolobium bilobum	seeds	direct immersion	Touchell and Dixon (1993)
	Gompholobium marginatum	seeds	direct immersion	Touchell and Dixon (1993)
	Hardenbergia comptoniana	seeds	direct immersion	Touchell and Dixon (1993)
	Hovea elliptica	seeds	direct immersion	Touchell and Dixon (1993)
	Indigofera australis	seeds	direct immersion	Touchell and Dixon (1993)
	Isotropis atropurpurea	seeds	direct immersion	Touchell and Dixon (1993)
	Jacksonia floribunda	seeds	direct immersion	Touchell and Dixon (1993)
	Kennedia stirlingii	seeds	direct immersion	Touchell and Dixon (1993)
	Mirbelia dilata	seeds	direct immersion	Touchell and Dixon (1993)
	Oxylobium atropurpureum	seeds	direct immersion	Touchell and Dixon (1993)
	Pultenaea capitata	seeds	direct immersion	Touchell and Dixon (1993)
	Sphaerolobium fornicatum	seeds	direct immersion	Touchell and Dixon (1993)
	Swainsona formosa	seeds	direct immersion	Touchell and Dixon (1993)
	Temletonia retusa	seeds	direct immersion	Touchell and Dixon (1993)
	Viminaria juncea	seeds	direct immersion	Touchell and Dixon (1993)
	Ptychosema pusillum	shoot apices	vitrification	Touchell and Dixon (1999)
Proteaceae	*Banksia ashbyi*	seeds	equilibration and direct immersion	*Merritt *et al.* (2000)
	Banksia cuneata	seeds	direct immersion	Touchell and Dixon (1993)
	Banksia menziesii	pollen	equilibration and direct immersion	*Maguire and Sedgley (1997)
	Banksia occidentalis	seeds	direct immersion	Touchell and Dixon (1993)
	Conospermum stoechadis	shoot apices	vitrification	Touchell and Dixon (1999)
	Grevillea bipinnatifida	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea cirsiifolia	shoot apices	vitrification	Tan (1998)
	Grevillea dryandroides spp *dryandroides*	shoot apices	vitrification	Bunn (unpublished)
	Grevillea dryandroides spp *hirsutus*	shoot apices	vitrification	Tan (1998)
	Grevillea fistulosa	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea flexuosa	shoot apices	vitrification	Tan (1998)
	Grevillea maccutcheonii	shoot apices	vitrification	Bunn (unpublished)
	Grevillea macrostylis	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea nana	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea nudiflora	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea obtusifolia	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea olivaceae	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea ripicola	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea saccata	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea scapigera	pollen, shoot apices	equilibration and direct immersion	Touchell (1995) Touchell *et al.* (1992), Touchell and Dixon (1999)
	Grevillea thelemanniana	pollen	equilibration and direct immersion	Touchell (1995)
	Grevillea thyrsoides	pollen	equilibration and direct immersion	Touchell (1995)
	Hakea aculeata	shoot apices	vitrification	Bunn (unpublished)
	Hakea costata	seeds	direct immersion	Touchell and Dixon (1993)
	Isopogon cuneatus	seeds	direct immersion	Touchell and Dixon (1993)
	Lambertia orbifolia	shoot apices	vitrification	Bunn (unpublished)
	Leucopogon obtectus	shoot apices	vitrification	Touchell and Dixon (1999)

Ranunculaceae	*Clematis pubescens*	seeds	direct immersion	Touchell and Dixon (1993)
Rhamnaceae	*Guichenotia ledifolia*	seeds	direct immersion	Touchell and Dixon (1993)
	Thomasia glutinosa	seeds	direct immersion	Touchell and Dixon (1993)
	Keraudrenia hermanniifolia	seeds	direct immersion	Touchell and Dixon (1993)
Rutaceae	*Boronia crenulata*	pollen	equilibration and direct immersion	*Astarini *et al.* (1999)
	Boronia heterophylla	pollen	equilibration and direct immersion	*Astarini *et al.* (1999)
	Boronia megastigma	pollen	equilibration and direct immersion	*Astarini *et al.* (1999)
	Boronia molloyae	pollen	equilibration and direct immersion	*Astarini *et al.* (1999)
	Boronia purdienana	pollen	equilibration and direct immersion	*Astarini *et al.* (1999)
	Boronia ramosa	pollen	equilibration and direct immersion	*Astarini *et al.* (1999)
	Eriostemon wonganensis	shoot apices	vitrification	Touchell and Dixon (1999)
Sapindaceae	*Diplopeltis huegelii*	seeds	direct immersion	Touchell and Dixon (1993)
	Dodonaea hackettiana	seeds	direct immersion	Touchell and Dixon (1993)
Sterculiaceae	*Rulingia* spTrigwell Bridge	shoot apices	vitrification	Touchell and Dixon (1999)
Stylidaceae	*Stylidium adnatum*	seeds	direct immersion	Touchell and Dixon (1993)
Surianaceae	*Stylobasium australe*	seeds	direct immersion	Touchell and Dixon (1993)
Tremandraceae	*Tetratheca deltoidea*	shoot apices	vitrification	Touchell and Dixon (1999)
Zygophyllaceae	*Zygophyllum aurantiacum*	seeds	direct immersion	Touchell and Dixon (1993)
	Monocots			
Haemodoraceae	*Anigozanthos kalbarriensis*	shoot apices	vitrification	Turner *et al.* (2001a)
	Anigozanthos humilis spp *chrysanthus*	shoot apices	vitrification	Turner *et al.* (2001a)
	Anigozanthos manglesii	pollen, seeds, shoot apices	equilibration and direct immersion in LN (pollen and seeds), vitrification	Sukhvibul and Considine (1993) Touchell and Dixon (1993) Turner *et al.* (2000a)
	Anigozanthos pulcherrimus	shoot apices	vitrification	Turner *et al.* (2000a)
	Anigozanthos rufus	shoot apices	vitrification	Turner *et al.* (2000a)
	Anigozanthos viridis ssp *terraspectans*	shoot apices	vitrification	Turner et al. (2001a)
	Anigozanthos viridis ssp *viridis*	shoot apices	vitrification	Turner *et al.* (2000a)
	Conostylis dielsii spp *teres*	shoot apices	vitrification	Turner *et al.* (2001a)
	Conostylis micrantha	shoot apices	vitrification	Turner *et al.* (2001a)
	Conostylis wonganensis	shoot apices	vitrification	Turner *et al.* (2001a)
	Macropidia fuliginosa	shoot apices, somatic embryos	vitrification	Turner *et al.* (2000b)
Liliaceae	*Burchardia umbellata*	seeds	direct immersion	Touchell and Dixon (1993)
	Sowerbaea multicaulis	shoot apices	vitrification	Touchell and Dixon (1999)
Restionaceae	*Hopkinsia anoectocolea*	shoot apices	vitrification	Touchell (unpublished)

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Appendix 3.

Publications (listed below) presented in Appendix 3 have arisen from research conducted during Ph.D. candidature.

Turner, S. R., Touchell, D.H., Dixon, K. W and Tan B. (2000) Cryopreservation of *Anigozanthos viridis* ssp *viridis* and related taxa from the South West of Western Australia. Australian Journal of Botany. 48 (6): 739-744.

Turner, S.R. Senaratna, T Bunn, E.. Tan, B. Dixon, K. W. Touchell, D.H (2001).Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. Annals of Botany 87: 371-378.

Turner, S. R., Tan B., Senaratna, T. Bunn, E., Dixon, K. W and Touchell, D.H. (2000) Cryopreservation of the Australian species *Macropidia fuliginosa* (Haemodoraceae) by vitrification. Cryo-letters 21 (6): 21: 379-388.

Turner, S, R., Senaratna, T., Touchell, D, H., Bunn, E., Dixon, K, W and Tan, B. (2001).Stereochemical arrangement of hydroxyl groups in sugar and polyalcohol molecules as an important factor in effective cryopreservation. Plant Science 160 (3): 489-497

Turner , S, R., Senaratna, T,. Touchell D, H., Bunn, E., Tan, B and Dixon K. W. (2001). Effects of plant growth regulators on survival and recovery growth following cryopreservation. Cryo-Letters 22 (3): 163-174.

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(Co-ordinator, ADT Project (Retrospective), Curtin University of Technology, 12.5.03)