Cryopreservation of threatened native Australian species - what have we learned and where to from here?

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Abstract

Cryogenic storage techniques have been developed and adopted for more than 100 (mainly agricultural) plant species worldwide, and within Australia at least 30 critically endangered plants have been stored long term using cryogenic approaches. Nevertheless, there are many species that are very difficult to store using current procedures, and organizations involved in plant germplasm conservation (such as botanic gardens, agricultural institutions, etc.) that utilise cryogenic storage techniques are in some respects at a crossroads in their endeavours to cheaply and effectively store a wide selection of species and genotypes for conservation and agricultural/horticultural purposes. For taxa that are not amenable to current cryogenic approaches, new ways of developing cryogenic storage techniques need to be investigated, including research into the ways in which cell membranes interact and change when cooled to cryogenic temperatures (-196^{o} C in liquid nitrogen) in the presence of various cryoprotective agents. This review highlights the current state of cryogenic research both within Australia and internationally, provides a case study on threatened plant species and also describes several new research initiatives that aim to provide answers to why some native species are quite amenable to widely utilised cryogenic approaches while others are currently non-responsive. New approaches aim to integrate laboratory and membrane modeling paradigms to provide guidelines for the development of new cryopreservation protocols and to assess the robustness of theoretical models in predicting optimum cryogenic conditions.

Key words

Cryobiology, cold acclimation, proteomics, plant conservation, membrane structure

Introduction

The flora of southwest Western Australia Floristic Region (SWAFR) is outstandingly rich in terms of species diversity and levels of endemism, making it one of the most distinctive floras worldwide, highlighted by the fact that the region is an internationally acknowledged biodiversity hotspot – one of only 34 currently recognised (Hopper et al. 1996; Brown et al. 1998; Myer et al. 2000; Hopper and Gioia 2004; Mittermeier et al. 2005). More than 8,000 plant species are estimated to occur in this region (Hopper and Gioia 2004). This high diversity is threatened by a multitude of largely anthropogenic processes, such as land clearing for agricultural, mining and urban development, the spread of plant diseases and pests and more recently climate change (Touchell et al. 2002; Malcolm et al. 2006). The number of rare and threatened plant species has increased markedly over the last few decades in Western Australia, with some 2,600 species currently classified as in need of some form of conservation. These are classified with a priority rating of P1-5, or declared endangered flora (DEF), or critically endangered flora (CR) (Atkins 2008). To preserve these species (especially CR species), efficient and cost-effective *ex situ* conservation methods are urgently required to compliment *in situ* conservation programs (Touchell et al. 2002; Bunn et al. 2007).

Ex situ methods for plant conservation

Ex situ methods for plant conservation include active growth collections (container and tissue culture collections) and seed banks that are usually maintained in botanic gardens or other national or international germplasm repositories (Guerrant et al. 2004). Most Western Australian species have orthodox (desiccation tolerant) seed, which can withstand drying to a low moisture content and storage at -18° C, making their long-term storage relatively straightforward (Touchell et al. 2002; Merritt and Dixon 2003; Merritt et al. 2003). Indeed, more than 200 rare and threatened taxa have been

accessioned in the Threatened Flora Seed Centre (Department of Environment and Conservation) and Western Australian Seed Technology Centre (Kings Park and Botanic Garden) (Cochrane 2004).

However, many threatened and endangered species produce little or no viable seeds, or have seeds that are deeply dormant. Often there are so few remaining individuals that the preservation of all remaining genotypes is considered of paramount importance (Bunn et al. 2007). Problematic species such as these can be maintained vegetatively through container collections, *in vitro* culture or in cryo-collections. Container collections are by far the cheapest and easiest way to maintain *ex situ* germplasm collections, though the space requirements for such material can be considerable – particularly if a large number of genotypes are to be maintained. Even the most carefully maintained *ex situ* collections are vulnerable to pests, diseases, climatic extremes (heat waves, frost or storms) and equipment failure (e.g., irrigation, cooling and/or heating); hence occasional (but often irreplaceable) losses can occur. In comparison, *in vitro* culture largely moderates many of these random abiotic and biotic factors by providing highly controlled temperature and light conditions within a sterile environment so that cultures are maintained in a pest and disease-free state. *In vitro* protocols for a significant number of rare native plant species have been developed and applied in Kings Park and Botanic Garden (Bunn et al. 2007), though as a general method for plant conservation there are some limitations, including relatively high maintenance costs, with large culture collections. A large proportion of this necessary maintenance involves sub-culturing of *in vitro* material that must occur regularly (3-4 wk) to maintain culture health. Other limitations include variable *in vitro* responses (not all plants respond equally well to the *in vitro* environment), and unwanted somaclonal variation may occur over long periods of continuous culture (Bunn et al. 2007). In addition, even though explant materials are surface sterilized during the initial establishment phase, secondary infections (bacteria, fungi) introduced by accident through operator error are commonly encountered and are an on-going management problem (Leifert et al. 1994; Kaczmarczyk 2008).

Although slow growth *in vitro* techniques (i.e., cool storage or incubation of cultures with growth-retarding media) can be used to reduce the frequency of

subculture, the need for regular subculture, accidental contamination risk and somaclonal variation are not completely removed but merely postponed. Growth retardants and extended subculture periods can still result in somaclonal variation (George and Sherrington 1993; Kumar 1994) and DNA methylation (Harding 1994). The requirement for specialised technical assistance for good culture maintenance also remains and invariably incurs higher costs. An additional significant issue is mislabeling of cultures that can readily occur with frequent subculture, particularly when technicians are constantly dealing with multiple genotypes from the one species. Once such mistakes are made, they are not readily identifiable and can cause significant problems later on during reintroduction programs (Schäfer-Menuhr 1996).

As an alternative to these other forms of germplasm storage, cryopreservation is a cost-effective, long-term option for the maintenance of important clonal material. Cryopreservation refers to the storage of biological material at liquid nitrogen (LN) temperatures (<-196° C) without the loss of viability, so when required, material can be readily retrieved and reinitiated, re-establishing desirable clonal lines. In cryopreservation, all cell division, metabolic and biochemical processes are arrested, and thus the plant material can be stored without deterioration or modification for a long period of time (Shibli et al. 2004; Zhu et al. 2006). Advantages are that material can be stored for a theoretically indefinite time with low maintenance costs and minimal space requirements. Input is needed mainly at the beginning when establishing clonal lines in tissue culture and when samples are prepared for cryogenic storage and cooled in LN (Kaczmarczyk, 2008). Once in cryostorage, the materials' only required maintenance is periodic (i.e., once a week) replenishment with LN to replace evaporated LN. Other advantages include halting genetic changes (due to somaclonal variation inherent in frequent subculture over long periods) and reducing the chances of introduced or accidental infections (bacterial, fungal or viral) due to frequent handling by tissue culture operators (Cole 1996). Critical for successful cryopreservation are the control and/or avoidance of intracellular ice nucleation, because this would destroy plant membranes and cause rapid cell death upon thawing (Benson 2008a).

Cryogenic collections for rare flora conservation should aim to maximise the level of genetic diversity in storage for the species in question to provide the greatest chance of re-introduction success through the re-establishment of genetically variable populations that have a high degree of inherent resilience (to pests, diseases, climatic extremes) which will most likely sustain their mid- to long-term future (Touchell 2000).

Cryopreservation has been important for preserving plant germplasm since the early 1970s (Reed 2001; Day et al. 2008) with more than 100 agricultural and economically important plant species (including many thousands of cultivars and genotypes) successfully preserved (and recovered) by this technology (Sakai and Engelmann 2007; Reed 2008; Panis et al. 2009). Aside from agriculture and economically valuable species, cryogenic storage is also highly applicable to the conservation of threatened flora. This is an increasingly important outcome, as more species become threatened, as technology matures and as the advantages of cryogenic storage become more widely acknowledged (Batty et al. 2002; Touchell et al. 2002; Flachsland et al. 2006; Sarasan et al. 2006; Bunn et al. 2007; Nikishina et al. 2007; Ray and Bhattacharya 2008; Mallón et al. 2008; Tanakaa et al. 2008; Hamilton et al. 2009a, 2009b; Trusty et al. 2009). Indeed, many conservation agencies, such as Kings Park and Botanic Garden in Perth, Western Australia, are currently applying cryogenic approaches to store threatened plant species (Table 1). Other institutions with active cryogenic programs for endangered taxa include: the National Seed Storage Laboratory in Fort Collins, Colorado (Touchell and Walters 2000), the Cincinnati Zoological and Botanic Gardens (Pence 2000) and the Royal Botanic Gardens, Kew (Sarasan et al. 2006).

Presently, different shoot tip cryopreservation protocols have been developed, including 2-step cooling, encapsulation/dehydration and vitrification, as well as combinations of these methods (Benson et al. 2007; Reed 2008). In recent years, the number of species cryopreserved using the combined droplet vitrification protocol with rapid cooling and rewarming has significantly increased (Leunufna and Keller 2005; Halmagyi and Pinker 2006; Sakai and Engelmann 2007; Sant et al. 2008; Senula et al. 2007; Yoon et al. 2007; Gonzalez-Arnao et al. 2008; Kim et al. 2009a; 2009b; Panis et al. 2009). On the other hand, 2-step cooling methods are used less often and

mostly for callus and suspension cultures (Gnanapragasam and Vasil 1992; Mikula et al. 2005). The reason for wider use of droplet vitrification lies in the fast and easy application, in which no expensive cooling apparatus (as for 2-step cooling) is necessary.

Successful results in the cryopreservation of Western Australian endangered plants have so far been obtained in more than 30 species through applying vitrification methods (Table 1) (Touchell et al. 2002; Hamilton et al. 2009a). However, not all species respond to the same protocol, so that some optimization (e.g., alteration to the constituents and timing of exposure to the preculture medium, and duration of incubation with cryoprotectants) has been necessary (Touchell 1995; Turner et al. 2001). Nevertheless, it has been demonstrated that through focusing on a model species, relatively rapid progress can be made in the development of more generic protocols for related species (Turner et al. 2001), as highlighted in the case study below.

Cryopreservation of threatened species of Haemodoraceae

The Haemodoraceae is a predominantly Gondwannan monocot family, well recognized for their spectacular flowers as seen in species such as *Anigozanthos manglesii, Dilatris pillansii, Macropidia fuliginosa* and *Wachendorfia thyrsiflora*. The family reaches the heights of diversity in the southwest of Western Australia, with at least 75 species belonging to 7 genera, 6 of which are endemic only to this region (Hopper 1993). However, among these diverse taxa, 28 are currently classified as threatened, including 5 subspecies of *Anigozanthos* and 15 species/subspecies of *Conostylis*, accounting for more than 35% of all extant Haemodoraceae (Western Australian Herbarium 1998-). With such a large number of taxa to conserve from the 1 family, cryopreservation is a critical part of an integrated conservation approach for ensuring that none of these species is lost forever.

Preliminary research on the common species *Anigozanthos viridis* ssp *viridis* found that post-LN survival (41%) was achievable using 1-2 mm-long shoot apices pre-cultured on a 0.4 M sorbitol medium for 2 d, before incubation in plant vitrification solution 2 (PVS2) for 25 min at 0° C, followed by direct immersion in LN (Turner et al. 2000). This protocol was then tested

on 7 other Haemodoraceae taxa, though the results for most of the species assessed were generally very low (Table 2) (Turner et al. 2000). A test species was then selected (the threatened *Anigozanthos viridis* ssp *terraspectans*), and a number of the originally used parameters investigated and modified (preculture medium, preculture duration, PVS2 formulation and the assessment of a loading phase), resulting in post-LN survival rising from 60% to 94% for this species. This new and improved cryogenic approach was then applied to another 5 threatened Haemodoraceae taxa; namely, *A. humilis* ssp *chrysanthus, A. kalbarriensis, Conostylis dielsii* ssp *teres*, *C. micrantha* and *C. Wonganensis,* resulting in higher survival in 3 out of the 5 taxa assessed. Regardless of the changes made to the original protocol, post-LN survival for the other 2 species (*C. dielsii* and *C. micrantha*) was still low - < 30% (Table 2)- (Turner et al. 2001).

Problems in cryopreservation

It is well known that intracellular ice formation needs to be avoided for successful cryopreservation, and this can be largely suppressed by using various cryoprotectants, either singly or in combinations (Benson 2008a). Nevertheless, many other biotic and abiotic factors influence the success of any cryopreservation method (Keller et al. 2008). On one side, the biological explant (shoot tip) and its genotypic characteristics, location within the *in vitro* plant, possible latent infections of endogenous (and/or exogenous) pathogens and physiological constitution play a significant role (Senula et al. 2007; Kaczmarczyk 2008, Keller et al. 2008). To provide the highest chance of cryogenic success, donor plants for shoot tips used in cryostorage need to be in optimum condition, free of contamination and precultured in high osmotic media. On the other side, physical, mechanical and oxidative stresses, which occur after the different steps in a cryopreservation protocol, have an impact on the regenerative ability of shoot tips after cryopreservation. Certain species and genotypes can be readily cryopreserved using established protocols while others are not able to survive cryopreservation, a response that can be highly variable even between different cultivars of the same species (Schäfer-Menuhr et al. 1997). The reasons for variation in results between different explants from the same species or genotype, or even the total absence of

survival after cryopreservation, are still not fully understood (Benson et al. 1996). To progress the science of cryopreservation, new studies and novel biotechnological tools are required to elucidate the mechanisms underlying cryogenic survival in plant tissues and to identify why cryosensitive species do not survive current cryogenic approaches.

New directions in cryopreservation of plant tissue

The future in cryobiology is closely tied in with recent advances in molecular biology and genomics over the last decade. The understanding of genetic adaptation to cold and stress tolerance is increasing, and the future of cryobiology is surely the dovetailing of formal biophysics with the study of life's adaptations to similar problems (Lane 2004).

Meryman (2004) suggested that we learn from nature's own laboratory. To put it simply, if a tissue or species is currently recalcitrant to cryopreservation protocols, the trick is not necessarily to change the protocol but to change (i.e., precondition) the tissues themselves (Lane 2004). Future research needs to find an answer to the question of which adaptations to freezing at high subzero temperatures can help precondition against the stresses of subsequent cryogenic storage. Part of the answer may have already been found with those plant species which possess the ability to cold acclimate. For example, it has been reported that cold acclimation of plants prior to cryopreservation leads to better regeneration results after rewarming from LN (Reed and Chang 1997). Niino and Sakai (1992), Chang and Reed (2000), Reed et al. (2003) and Towill and Ellis (2008) reported enhanced post-LN survival in several woody species; Seibert and Wetherbee (1977), Keller (2005), and Senula et al. (2007) reported similar results with a number of herbaceous species. Additionally, in other plants like yam and various potato cultivars, which generally do not show any obvious response to cold acclimation, cryopreservation results have been improved after preculture at low temperatures (Leunufna and Keller 2005; Kaczmarczyk et al. 2008).

Biochemical studies in relation to cryopreservation and cold acclimation of donor plants have shown positive correlation between increased sugar (sucrose, D-glucose, D-fructose) concentrations and linoleic acid content with shoot tip regeneration (Vandenbussche et al. 1999; Panta et al. 2009a,

2009b). Panta et al. (2009a) studied the responses of potato to abiotic stresses and compared these with the potato's cryo-ability, following the hypothesis that plant tissues preconditioned for cryopreservation exhibited similar biochemical responses as those shown by plants during drought or exposure to salinity and frost. As part of this study, a droplet vitrification protocol was applied to 6 cultivated frost sensitive and 2 wild frost-tolerant potato genotypes. Results revealed that frost resistant genotypes have significantly higher regeneration capacity after cryopreservation. Additionally, the linoleic acid content from each of these genotypes was also positively correlated with tolerance toward cryopreservation. The tolerance of cryosensitive genotypes with initially low recovery rates could be improved by the addition of the polyamine putrescine to the preculture medium, a growth factor known to influence cell division (Panta et al. 2009a).

Vandenbussche et al. (1999) also discovered that cold acclimation (21/5° C day/night temperature, 1 wk) of donor plants enhanced cryogenic tolerance in sugar beet shoot tips. The improved cryogenic tolerance was positively correlated with enhanced sugar concentration (sucrose, D-gluose, D-fructose) and a change in lipid composition (an increase in unsaturated membrane lipids [C18:2 / C18:1 ratio] and linolenic acid [C18:3] content).

Another approach to cold acclimation and enhancing tolerance to cryopreservation-induced stress involves applying additives in conjunction with cold treatment or as a substitute for chilling. Ramon et al. (2002) precultured banana meristems on medium with 0.4 M sucrose for 2 wk and found increases in polyamine levels, especially putrescine, and an increase in the ratio of unsaturated/saturated fatty acids, which was positively correlated to cryogenic survival. Zhu et al. (2006) also found that sucrose pre-treatment of banana meristems of several varieties increased the double bond index of the neutral lipid fraction; this was correlated with higher post-LN survival. Further examples include the abscisic acid-response proteins, bovine serum albumin, proline and sucrose-simulated cold acclimation as applied to *Ribes ssp*. (Luo and Reed 1997; Benson 2008b). Cross-talk between different types of plant stress responses, acclimation and development are now being confirmed using various "omics" approaches combined with molecular-physiological studies (Fujita et al. 2006; Beck et al. 2007; Zhu et al. 2007).

Molecular-physiological studies linked to cryopreservation

Biochemical characterization of explants can be assessed through determination of the plant protein expression. Proteomics approaches are used to analyze the complex functions of plants at different levels and are supported through the progress obtained in sensitive and rapid protein identification by mass spectrometry (Canovas et al. 2004).

The application of protein expression studies for cryopreservation is a new and rapidly developing research area. Capentier et al. (2007) used banana as a model to study protein expression in the meristem associated with sucrose-mediated osmotic stress. To facilitate this analysis, a protocol for 2-dimensional gels was developed for small amounts of tissue (30-50 mg). Polypeptide databases obtained by mass spectrometry were used for discriminative identification of proteins from other closely related plant species. This study concluded that homeostasis in shoot tips is perpetuated after sucrose-mediated osmotic stress through maintenance of an osmoprotective intracellular sucrose concentration, the enhanced expression of particular genes of glycolysis and the conservation of the cell wall integrity. All those factors are necessary for the explants to acclimate to and survive dehydration prior to cryopreservation (Carpentier et al. 2007; Samyn et al. 2007). The first proteome analyses in connection with cryopreservation have been made for potato shoot tips by Criel et al. (2008) and Kaczmarczyk (2008). Criel et al. (2008) analyzed proteins of *S. tuberosum* “Désirée” shoot tips and leaves after preculture of donor plants for 21 d on regular MS medium (Murashige and Skoog 1962) and MS complemented with 0.055 M, 0.110 M, and 0.220 M sorbitol. The regeneration rate after cryopreservation was increased from 50% to 80% with increased sorbitol concentration in the medium. Differences in 2D-difference-gel-electrophoresis (2D-DIGE) patterns were observed for samples pre-cultured on various sorbitol-enriched media. In total, 63 and 15 differently expressed proteins were determined in the shoot tips and leaves, respectively. Of them 43 and 3, respectively, were identified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The results indicated strong alteration of the primary metabolism and in carbon fixation. These results were additionally confirmed by parallel

biochemical analyses. Increase in carbohydrates was measured in plants treated with up to 0.11 M sorbitol, whereas the concentration of 0.22 M sorbitol showed decreased levels of carbohydrates. In conclusion, better cryopreservation response could be obtained due to high internal sugar concentration and alterations in primary metabolism as a result of the cryoprotectant function of sugars (Criel et al. 2008). Kaczmarczyk (2008) analyzed *S. tuberosum* “Désirée” and *S. demissum* after 2 different preculture conditions, followed by cryopreservation; 1-wk culture at 22/8 C day/night temperature in comparison to 1-wk culture at constant temperature of 22 C. The alternating temperature preculture of donor plants revealed higher survival and regeneration rates after cryopreservation. In protein expression, only minor changes were found between the preculture treatments, meaning that 2.7% or 0.4% of the proteins were changed in *S. demissum* and *S. tuberosum* “ésireé”, respectively. Proteins were mostly down-regulated in their expression level after preculture with alternating temperature. The identified proteins belonged to the functional groups of metabolism, signal transduction, defence, transcription, energy and secondary metabolism (Kaczmarczyk 2008). The first gene expression studies in response to cryoprotectant and LN exposure in *Arabidopsis thaliana* have also been recently completed (Volk et al. 2009). Volk et al. (2009) identified key gene families, which changed gene expression, encoding proteins involved in lipid transport, dehydration responses, seed maturation and detoxification. Some genes were upregulated in response to cryoprotectant treatment, indicating a critical step in the protocol; others had a more general response expressed during shoot tip recovery. This study showed that cryoprotectant treatments induced gene expression and critical biochemical pathways, including those involved in lipid transport and osmoregulation (Volk et al. 2009).

Nevertheless, to advance the science of cryopreservation the composition of shoot tip membranes and their interaction with sugars, and the function of cryoprotectants, need to be analysed and understood in more detail. Biophysical and molecular modeling studies of the effect of cryoprotectants such as DMSO, polyalcohols (e.g., glycerol, ethylene glycol and sorbitol) and sugars (e.g., sucrose) are providing insight at the molecular level of the interactions of these agents with phospholipid chains and headgroups and

their effect on the liquid crystallinity and stability of biological membranes (Pereira and Hünenberger 2008). In addition, investigation of the vitrification properties of these cryoprotectants on liquid water is shedding light on the molecular mechanism of solvent cryoprotection by explaining changes to the glass transition temperature of water following addition of cryoprotective agents (Mandumpal and Mancera 2010). Cross-disciplinary research between plant biology and biophysics is thus likely to provide new fundamental results to help rationalise and optimise cryopreservation protocols in the future.

Conclusion

Research in plant cryopreservation needs to move away from the empirical approaches that currently underpin the development of new cryogenic protocols for conservation of species of horticultural and agricultural value. Rising numbers of endangered plant species mean that there is less time to spend on each new species. In many cases the limited availability of *in vitro* shoot material means testing of large numbers of different treatment combinations using traditional multi-factorial experimental designs will simply be too slow to realistically serve the needs of *ex situ* conservation programs in the future. Therefore, to do more with less, the physiology of target plants in their natural habitats needs to be understood, as does the need for plants to be pre-conditioned to improve their tolerance to cryopreservation. New research possibilities are currently opening up with the rapid development of new scientific disciplines such as “omics.” Through understanding the fundamental parameters related to cold acclimation and dehydration tolerance and cultivating the means to connect this knowledge to formal biophysical processes, researchers should be able to develop new and innovative cryopreservation protocols. Theoretical models of membrane structure and their interaction with cryoprotectants are one promising way to provide new answers to fundamental questions. With the results of further research, improved cryogenic approaches will be developed, which will lead to better conservation outcomes and facilitate the custodians of threatened plants with an improved ability to secure the long-term future of these irreplaceable species.

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References

Atkins KJ (2008). Declared rare and priority flora list for Western Australia. Department of Environment and Conservation, Western Australi

Batty AL, Dixon KW, Brundrett M, Sivasithamparam K (2002) Long-term storage of mycorrhizal fungi and seed as a tool for the conservation of endangered Western Australian terrestrial orchids. Aust J Bot 49: 619-628

Beck EH, Fettig S, Knake C, Hartig K, Bhattarai T (2007) Specific and unspecific responses of plants to cold and drought stress. J Bioscience 32: 501-510

Benson EE (2008a) Cryopreservation theory. In: Reed BM (ed) Plant Cryopreservation. A Practical Guide. Springer, Berlin Heidelberg New York, pp 15-32

Benson EE (2008b) Cryopreservation of phytodiversity: a critical appraisal of theory & practice. Crit Rev Plant Sci 27: 141-219

Benson EE, Harding K, Johnston JW (2007). Cryopreservation of shoot tips and meristems. In: Stacey GN, Day JG (eds) Methods in Molecular Biology, vol. 368: Cryopreservation and Freeze-Drying Protocols, 2nd Ed, Totowa, NJ, USA, Humana Press Inc, pp 163-183

Benson EE, Wilkinson M, Todd A, Ekuere U, Lyon J (1996) Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot-tips. CryoLetters 17: 119-128

Brown A, Thomson-Dans C, Marchant N (1998). Western Australia's threatened flora. Perth, Western Australia, Department of Conservation and Land Management

Bunn E, Turner S, Panaia M, Dixon KW (2007) The contribution of in vitro technology and cryogenic storage to conservation of indigenous plants. Aust J Bot 55: 345-355

Canovas FM, Dumas-Gaudot E, Recorbet B, Jorrin J, Mock H-P, Rossignol M (2004) Plant proteome analysis. Proteomics 4: 285-298

Carpentier SC, Witters E, Laukens K, Van Onckelen H, Swennen R, Panis B (2007) Banana (Musa spp.) as a model to study the meristem proteome: Acclimation to osmotic stress. Proteomics 7: 92-105

Chang Y, Reed BM (2000) Extended alternating-temperature cold acclimation and culture duration improve pear shoot cryopreservation. Cryobiology 40: 311-322

Cochrane A (2004). Western Australia's *ex situ* program for threatened species: A model integrated strategy for conservation. In: Guerrant EO, Havens K, Maunder M (eds) *Ex Situ* Plant Conservation. Society for Ecological Restoration. Island Press, Washington DC USA pp 40-66

Cole M (1996) Microbial contaminants and aseptic techniques in plant tissue culture. In: Taji A, Williams R (eds)Tissue Culture of Australian Plants, University of New England, Armidale NSW, Australia, pp 204-239

Criel B, Panis B, Oufir M, Swennen R, Renault D, Hausman JF (2008). Protein and carbohydrate analyses of abiotic stress underlying cryopreservation in potato. Agrifood Res Working Papers 153: 37-38

Crowe M (1998) Survival of *Eucalyptus* species in cool storage and cryostorage: efficiency of choline chloride in cool storage media and glycerol and choline chloride in cryogenic preculture media. Honours Thesis, Murdoch University, Western Australia

Day JG, Harding KC, Nadarajan J, Benson EE (2008) Cryopreservation. Conservation of bioresources at ultra low temperatures. In: Walker JM, Rapley R (eds) Molecular Biomethods Handbook. 2nd Ed, Totowa, NJ,USA, Humana Press, pp 917-947

Flachsland E, Terada G, Scocchi A, Rey H, Mroginski L, Engelmann F (2006) Cryopreservation of seeds and in vitro-cultured protocorms of *Oncidium bifolium* sims. (Orchidaceae) by encapsulation-dehydration. CryoLetters 27: 235-242

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr Opin Plant Biol 9: 436-442

George EF, Sherrington PD (1993) Plant propagation by tissue culture, part 1. The technology. Edington, UK, Exegentics

Gnanapragasam S, Vasil IK (1992) Ultrastructural changes in suspension culture cells of *Panicum maximum* during cryopreservation. Plant Cell Rep 11: 169-174

Gonzalez-Arnao MT, Panta A, Roca WM, Escobar RH, Engelmann F (2008) Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell Tiss Org 92: 1-13

Guerrant EO, Havens K, Maunder M (2004) Ex Situ Plant Conservation. Society for Ecological Restoration. Island Press, Washington DC, USA

Halmagyi A, Pinker I (2006) Plant regeneration from Rosa shoot tips cryopreserved by a combined droplet vitrification method. Plant Cell Tiss Org 84: 100129-100137

Hamilton KN, Turner SR, Ashmore SE (2009a) Cryopreservation. In: Offord CA, Meagher PF (eds) Plant Germplasm Conservation in Australia: Strategies and Guidelines for Developing, Managing and Utilising ex situ Collections, Canberra, Australian Network for Plant Conservation Inc., pp 129-148

Hamilton KN, Ashmore SE, Pritchard HW (2009b) Thermal analysis and cryopreservation of seeds of Australian wild citrus species (Rutaceae): *Citrus australasica*, *C. inodora* and *C. garrawayi*. CryoLetters 30: 268-279

Harding K (1994) The methylation status of DNA derived from potato plants recovered from slow growth. Plant Cell Tiss Org 37: 31-38

Hopper SD (1993) Kanagaroo Paws and Cats Paws. Department of Conservation and Land Management, Perth, Western Australia, pp. 1-144

Hopper SD, Harvey MS, Chappill JA, Main AR, York Main B (1996) The Western Australian biota as Gondwanan heritage - a review. In: Hopper SD (ed) Gondwanan Heritage: Past Present and Future of the Western Australian Biota. Surrey Beatty, Chipping Norton, pp 1-16

Hopper SD, Gioia P (2004) The Southwest Australian Floristic Region: evolution and conservation of a global hot spot of biodiversity. Annu Rev Ecol Evol S 35: 623-650

Kaczmarczyk A. (2008). Physiological, biochemical, histological and ultrastructural aspects of cryopreservation in meristematic tissue of potato shoot tips. Verlag Dr. Köster, Berlin

Kaczmarczyk A, Shvachko N, Lupysheva Y, Hajirezaei MR, Keller ERJ (2008) Influence of alternating temperature preculture on cryopreservation results for potato shoot tips. Plant Cell Rep 27: 1551-1558

Keller ERJ, Senula A, Kaczmarczyk A (2008) Cryopreservation of herbaceous dicots. In: Reed BM (ed). Plant Cryopreservation: A Pratical Guide. Springer, Berlin Heidelberg New York, pp 281-332

Keller ERJ (2005) Improvement of cryopreservation results in garlic using low temperature preculture and high-quality *in vitro* plantlets. CryoLetters 26: 357-366

Kim HH, Lee JG, Park SU, Lee SC, Baek HJ, Cho EG, Engelmann F (2009a) Development of alternative loading solutions in droplet-vitrification procedures. CryoLetters 30: 291-299

Kim HH, Lee JG, Shin DJ, Ko HC, Gwag JG, Cho EG, Engelmann F (2009b) Development of alternative plant vitrification solutions in droplet-vitrification procedures. CryoLetters 30: 320-334

Kumar A (1994) Somaclonal variation. In: Bradshaw JE, Mackay GR (ed) Potato Genetics, CAB International, pp 197-212

Lane N (2004) The future of cryobiology. In: Fuller BJ, Lane N, Benson EE (eds) Life in the Frozen State. Florida, CRC Press LLC, pp 645-657

Leifert C, Morris CE, Waites EM (1994) Ecology of microbial saprophytes and pathogens in tissue-culture and field-grown plants - reasons for contamination problems in vitro. Crit Rev Plant Sci 13: 139-183

Leunufna S, Keller ERJ (2005) Cryopreservation of yams using vitrification modified by including droplet method: effects of cold acclimation and sucrose. CryoLetters 26: 93-102

Luo J, Reed BM (1997) Abscisic acid-responsive protein, bovine serum albumin, and proline pretreatments improve recovery of in vitro currant shoot-tip meristems and callus cryopreserved by vitrification. Cryobiology 34: 240-250

Malcolm JR, Liu C, Neilson RP, Hansen L, Hannah L (2006) Global warming and extinctions of endemic species from Biodiversity Hotspots. Conserv Biol 20: 538-548

Mallón R, Bunn E, Turner SR, González ML (2008) Cryopreservation of *Centaurea ultreiae* (Compositae) a critically endangered species from Galicia (Spain) CryoLetters 29: 363-370

Mandumpal JB, Mancera RL (2010) Molecular mechanism of solvent cryoprotection in aqueous DMSO solutions. Physl Chem Chem Phys, accepted for publication

Merritt DJ, Senaratna T, Touchell DH, Dixon KW, Sivasithamparam K (2003) Seed ageing of four Western Australian species in relation to storage environment and seed antioxidant activity. Seed Sci Res 13: 155-165

Merritt DJ, Dixon KW (2003) Seed storage characteristics and dormancy of Australian indigenous plant species: from the seed store to the field. In: Smith RD, Dickie JB, Linington SH, Pritchard HW, Probert RJ (eds) Seed Conservation, Turning Science into Practice. Royal Botanic Gardens Kew, Cromwell Press Pty Ltd. Great Britain, pp. 807-823

eryman H (2004) Foreword to Life in the Frozen State. Fuller BJ, Lane N, Benson EE (eds) Life in the Frozen State, CRC Press. Boca Raton, Florida, USA

Mittermeier RA, Gil PR, Hoffman M, Pilgrim J, Brooks T, Mittermeier CG, Lamoreux J, Da Fonseca GAB, Seligmann P (2005) Hotspots revisited. Conservation International,.University of Chicago Press, Chicago, IL, USA

Mikula A, Tykarska T, Mieczyslaw K (2005) Ultrastructure of *Gentiana tibetica* proembryogenic cells before and after cooling treatments. CryoLetters 26: 367-378

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Phys Plant 15: 473-497

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403: 803-808

Niino T, Sakai A (1992) Cryopreservation of alginate-coated in vitro-grown shoot tips of apple, pear, and mulberry. Plant Sci 87: 199-206

Nikishina TV, Popova EV, Vakhrameeva MG, Varlygina TI, Kolomeitseva GL, Burov AV, Popovich EA, Shirokov AI, Shumilov VY, Popov AS (2007) Cryopreservation of seeds and protocorms of rare temperate orchids. Russ J Plant Physiol 54: 121-127

Panis B, Piette B, Andre E, Van Den Houwe I, Swennen R (2009) Droplet vitrification: the first generic cryopreservation protocol for organized plant tissues? 1st International Symposium on Cryopreservation in Horticultural Species, Leuven, Belgium 55

Panta A, Panis B, Sanchez D, Canepa P, Ynouye C, Geuns J, Swennen R, Roca W, Tay D (2009a) Improved potato cryopreservation based on the identification of biochemical cell compounds linked to response to abiotic stress. 1st International Symposium on Cryopreservation in Horticultural Species, Leuven, Belgium, 5-8 April, Leuven, Belgium 16

Panta A, Panis B, Sanchez D, Ynouye C, Geuns J, Swennen R, Tay D, Roca W (2009b) Andean food tuber crops: cryopreservation and links with their response to abiotic stress. CryoLetters 30: 88-88

Pence VC (2000) Cryopreservation of in vitro grown fern gametophytes. Am Fern J 90: 16-23

Pereira CS, Hünenberger PH (2008) The influence of polyhydroxylated compounds on a hydrated phospholipid bilayer: a molecular dynamics study. Mol Simulat 34: 403-420

Ramon M, Geuns JMC, Swennen R, Panis B (2002) Polyamines and fatty acids in sucrose precultured banana meristems and correlation with survival rate after cryopreservation. CryoLetters 23: 345-352

Ray A, Bhattacharya S (2008) Cryopreservation of in vitro grown nodal segments of Rauvolfia serpentina by PVS2 vitrification. CryoLetters 29: 321-328

Reed BM (2008). Plant Cryopreservation. A Practical Guide. Springer, New York

Reed BM (2001) Implementing cryogenic storage of clonally propagated plants. CryoLetters 22: 97-104

Reed BM, Okut N, D'Achino J, Narver L, DeNoma J (2003) Cold storage and cryopreservation of hops (*Humulus* L.) shoot cultures through application of standard protocols. CryoLetters 24: 389-396

Reed BM, Chang Y (1997). Medium and long term storage of *in vitro* cultures of temperate and nut crops. In: Razdan MK, Cocking EC (eds.) Conservation of Plant Genetic Resources in vitro. Vol 1, General aspects. Science Publishers, Inc. Enfield, USA, pp 67-106

Sakai A, Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. CryoLetters 28: 151-172

Samyn B, Sergeant K, Carpentier S, Debyser G, Panis B, Swennen R, Van Beeumen J (2007) Functional proteome analysis of the banana plant (Musa spp.) using de novo sequence analysis of derivatized peptides. J Proteome Res 6: 70-80

Sant R, Panis B, Taylor M, Tyagi A (2008) Cryopreservation of shoot-tips by droplet vitrification applicable to all taro (Colocasia esculenta var. esculenta) accessions. Plant Cell Tiss Org 92: 107-111

Sarasan V, Cripps R, Ramsay MM, Atherton C, McMichen M, Prendergast G, Rowntree JK (2006) Conservation In vitro of threatened plants—Progress in the past decade. In Vitro Cell Dev-Pl 42: 206-214

Schäfer-Menuhr A (1996) Refinement of cryopreservation techniques for potato. Final report for the period September 1, 1991 - August 31, 1996. International Plant Genetic Resources Institute, Rome, Italy

Schäfer-Menuhr A, Schumacher HM, Mix-Wagner GM (1997) Long-term storage of old potato varieties by cryopreservation of shoot-tips in liquid nitrogen. Plant Genet Res Newslett 111: 19-24

Seibert M, Wetherbee PJ (1977) Increased survival and differentiation of frozen herbaceous plant organ cultures through cold treatment. Plant Physiol 59: 1043-1046

Senula A, Keller ERJ, Sanduijav T, Yohannes T (2007) Cryopreservation of cold-acclimated mint (Mentha spp) shoot tips using a simple vitrification protocol. CryoLetters 28: 1-12

Shibli RA, Al-Ababneh SS, Smith MAL (2004) Cryopreservation of plant germplasm: A review (Scientific Review). Dirasat, Agricultural Sci 31:60-73

Tan S (1998) Cryopreservation for conservation of *Grevillea* species. Honours thesis, Murdoch University, Western Australia

Tanakaa D, Niinoa T, Tsuchiyaa Y, Shirataa K, Uemuraa M (2008) Cryopreservation of shoot tips of endangered Hayachine-usuyukiso (*Leontopodium hayachinense* (Takeda) Hara et Kitam.) using a vitrification protocol Plant Genetic Resources: Characterization and Utilization 6 :164-166

Touchell DH (1995) Principles of Cryobiology for Conservation of Threatened Australian Plants. Department of Botany. Perth, University of Western Australia

Touchell DH (2000). Conservation of threatened flora by cryopreservation of shoot apices. In: Engelmann F, Takagi H (eds) Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Applikation. Tsukuba, Japan, Japan International Research Center for Agricultural Sciences, pp 269-272

Touchell DH, Walters C (2000). Recovery of embryos of *Zizania Palustris* following exposure to liquid nitrogen. CryoLetters 21: 261-270

Touchell DH, Turner SR, Bunn E, Dixon KW (2002) Cryostorage of somatic tissues of endangered Australian species. In: Towill LE, Bajaj YPS (eds) Cryopreservation of Plant Germplasm II. Springer, Berlin Heidelberg, pp 357-372

Towill LE, Ellis DD (2008) Cryopreservation of dormant buds. In: Reed BM (ed) Plant Cryopreservation. A Practical Guide. Springer, Berlin Heidelberg New York, pp 421-426

Trusty JL, Miller I, Boyd RS, Goertzen LR, Pence VC, Plair BL (2009) Ex situ conservation of the federally endangered plant species *Clematis socialis* Kral (Ranunculaceae). Nat Area J 29: 376-384

Turner SR, Touchell DH, Dixon KW, Tan B (2000) Cryopreservation of *Anigozanthos viridis* ssp *viridis* and related taxa from the south-west of Western Australia. Aust J Bot 48: 739-744

Turner SR, Senaratna T, Bunn E, Tan B, Dixon KW, Touchell DH (2001) Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. Ann Bot-London 87: 371-378

Vandenbussche B, Leuridan S, Verdoodt V, Gysemberg M, De Proft M (1999) Changes in sugar content and fatty acid composition of in vitro sugar beet shoots after cold acclimation: influence on survival after cryopreservation. Plant Growth Regul 28: 157-163

Volk GM, Henk A, Basu C (2009) Gene expression in response to cryoprotectant and liquid nitrogen exposure in *Arabidopsis* shoot tips. 1st International Symposium on Cryopreservation in Horticultural Species, Leuven, Belgium, 5-8 April, Leuven, Belgium 24

Western Australian Herbarium (1998-). FloraBase — The Western Australian Flora. Department of Environment and Conservation. http://florabase.dec.wa.gov.au/. Cited 08 April 2010

Yoon J-W, Kim H-H, Cho E-G, Ko H-C, Hwang H-S, Park Y-E (2007) Cryopreservation of cultivated and wild potato varieties by droplet vitrification procedure. Acta Hort 760: 203-208

Zhu GY, Geuns JMC, Dussert S, Swennen R, Panis B (2006) Change in sugar, sterol and fatty acid composition in banana meristems caused by sucrose-induced acclimation and its effects on cryopreservation. Physiol Plant 128: 80-94

Zhu JH, Dong CH, Zhu JK. (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Curr Opin Plant Biol 10: 290-295

Tables

Table 1. Percentage survival of excised shoot tips for Australian species cryopreserved using vitrification procedures (adapted from Touchell et al. 2002 and updated)

Family	Species	Survival	Reference
Dicotyledon			
Chloanthaceae	*Pityrodia scabra*	78.0%	Touchell (unpublished)
Goodeniaceae	*Lechenaultia formosa*	25.0%	Touchell (1995)
	Lechenaultia laricina	47.0%	Touchell (unpublished)
Lamiaceae	*Hemiandra gardneri*	8.3%	Touchell (1995)
Myoporaceae	*Eremophila caerulea ssp marellii*	27.0%	Touchell (1995)
	Eremophila resinosa	7.2%	Touchell (1995)
Myrtaceae	*Eucalyptus granticola*	60.0%	Crowe (1998)
	Ptychosema pusillum	12.1%	Touchell (1995)
Proteaceae	*Conospermum stoechadis*	5.4%	Touchell (1995)
	Grevillea cirsiifolia	46.0%	Tan (1998)
	Grevillea dryandroides spp. *dryandroides*	74.0%	Bunn (unpublished)
	Grevillea dryandroides spp. *hirsutus*	75.0%	Tan (1998)
	Grevillea flexuosa	68.0%	Tan (1998)
	Grevillea maccutcheonii	50.0%	Bunn (unpublished)
	Grevillea scapigera	64.1%	Touchell (1995)
	Grevillea scapigera	20.0%	Touchell et al. (1992)
	Hakea aculeata	62.0%	Bunn (unpublished)
	Lambertia orbifolia	62.0%	Bunn (unpublished)
	Leucopogon obtectus	17.4%	Touchell (1995)
Rutaceae	*Eriostemon wonganensis*	37.0%	Touchell (1995)
Sterculiaceae	*Rulingia sp. Trigwell Bridge*	17.9%	Touchell (1995)
Tremandraceae	*Tetratheca deltoidea*	9.6%	Touchell (1995)
Monocotyledon			
Haemodoraceae	*Anigozanthos kalbarriensis*	33.8%*	Turner et al. (2001)
	Anigozanthos humilis ssp. *chrysanthus*	80.4%*	Turner et al. (2001)
	Anigozanthos manglesii	31.9%	Turner et al. (2000)
	Anigozanthos pulcherrimus	17.1%	Turner (unpublished)
	Anigozanthos rufus	2.2%	Turner et al. (2000)
	Anigozanthos rufus x *Anigozanthos viridis ssp. Viridis*	17.6%	Turner et al. (2000)
	Anigozanthos viridis ssp. *viridis*	41.4%	Turner et al. (2000)
	Anigozanthos viridis ssp. *terraspectans*	94.4%*	Turner et al. (2001)
	Conostylis dielsia ssp. *teres*	11.6%	Turner et al. (2001)
	Conostylis micrantha	26.2%	Turner et al. (2001)
	Conostylis wonganensis	77.1%*	Turner et al. (2001)
	Macropidia fuliginosa	84.4%	Turner et al. (2000)
Liliaceae	*Sowerbaea multicaulis*	10.9%	Touchell (1995)
Restionaceae	*Hopkinsia anoectocolea*	46.4%	Touchell (unpublished)

Table 2. Post LN survival for six threatened species of Haemodoraceae using an original protocol developed for *A. viridis* ssp. *viridis* (Turner et al. 2000 i.e. 2 day pre-culture on 0.4 M sorbitol, incubation in PVS2 for 25 mins at 0 °C then direct immersion in LN) and a modified protocol (i.e. 3 day pre-culture on 0.8 M glycerol, 20 min incubation in a loading solution (2.0 M glycerol + 0.4 M sucrose) and then incubation in modified PVS2 for 25 mins at 0 °C followed by direct immersion in LN) developed using *A. vividis* ssp. *terraspectans* as a test species (Turner et al. 2001)

Species	Original cryogenic protocol	Modified cryogenic protocol
Anigozanthos humilis subsp. *chrysanthus*	24.4 ± 9.7	80.4 ± 3.9
Anigozanthos kalbarriensis	6.3 ± 3.6	33.8 ± 8.7
Anigozanthos viridis subsp. *terraspectans*	61.1 ± 3.2	94.4 ± 3.2
Conostylis dielsii subsp. *teres*	20.8 ± 8.4	11.6 ± 6.2*
Conostylis micrantha	14.6 ± 8.6	26.2 ± 3.8*
Conostylis wonganensis	17.6 ± 3.1	77.1 ± 12.4

*** Note: Due to a lack of shoot apices for experimentation the effectiveness of a loading phase in the cryogenic protocol was not assessed for these species.**