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Current issues in plant cryopreservation and importance for *ex situ* conservation of threatened Australian native species

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Abstract. An alarming proportion of Australia's unique plant biodiversity is under siege from a variety of environmental threats. Options for *in situ* conservation are becoming increasingly compromised as encroaching land use, climate change and introduced diseases are highly likely to erode sanctuaries regardless of best intentions. *Ex situ* conservation is currently limited to botanic garden living collections and seed banking, with *in vitro* and cryopreservation technologies still being developed to address *ex situ* conservation of species not amenable to conventional storage. Cryopreservation (storage in liquid nitrogen) has been used successfully for long-term biosecure storage of shoot tips of several species of threatened Australian plants. We present a case for building on this research and fostering further development and utilisation of cryopreservation as the best means of capturing critical germplasm collections of Australian species with special storage requirements (e.g. recalcitrant-seeded taxa and species with short-lived seeds) that currently cannot be preserved effectively by other means. This review highlights the major issues in cryopreservation that can limit survival including ice crystal damage and desiccation, toxicity of cryoprotective agents, membrane damage, oxidative stress and mitochondrial function. Progress in understanding and mitigating these stresses is vital for advancing cryopreservation for conservation purposes.

Additional keywords: cryobank, cryobiology, cryobiotechnology, cryostorage, freezing, in vitro conservation, plant tissue culture, vitrification.

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Australian unique plant biodiversity under threat

Threats to Australian ecosystems and plant diversity include land clearing for agriculture and urban development, habitat fragmentation, changed water use, pollution (water, soil and air), natural disasters, illegal collection, loss of seed dispersal agents and the introduction of alien species (Engelmann 2004; Maunder *et al*. 2004; Li and Pritchard 2009; Williams *et al*. 2011; Berjak and Pammenter 2014; Merritt *et al*. 2014*a*; Millennium Seed Bank Partnership 2015; Broadhurst and Coates 2017; O'Donnell and Sharrock 2017; Sommerville *et al*. 2017). Introduced diseases, such as 'root rot' or 'dieback', caused by the soil-borne water mould *Phytophthora cinnamomi*, are linked to the decline of many rainforest and other species in the wet tropics (Williams *et al*. 2011; Sommerville *et al*. 2017) and south-west Western Australian eucalypt and banksia woodlands (Anderson *et al*. 2010; Duque-Lazo *et al*. 2016). A more recent invasive pathogen, myrtle rust (*Austropuccinia psidii*) (Beenken 2017), originating in South America and first observed in Australia in 2010, is of particular concern given the dominance of Myrtaceae in Australian ecosystems. A study by Carnegie *et al*. (2016) reported 232 potential host species for *A. psidii* in Australia, including a keystone wetland species (*Melaleuca quinquenervia*), with a further 115 species susceptible after artificial inoculation. Berthon *et al*. (2018) showed 1285 species to be at risk of exposure if the fungus continues its spread across Australia. Two major Australian plant biodiversity hotspots that contain a high percentage of rare or threatened plant (and animal) species most at risk include the Forests of East Australia, which also contain significant areas of rainforest that have persisted since Gondwanan times and

sheltered species such as the endangered 'living fossil' *Wollemia nobilis* (Ashmore *et al*. 2011; Williams *et al*. 2011), and the south-west Australia hotspot with 5570 species of mainly endemic vascular plants including four endemic monospecific families: Ecdeiocoleaceae, Emblingiaceae, Eremosynaceae and Cephalotaceae (Conservation International 2018).

Conservation options for the Australian flora

Although *in situ* conservation of species and ecosystems is considered the simplest and most effective method of conservation, where such areas can be adequately protected (Prance 1997), *in situ* conservation alone cannot save all threatened species from the various threats outlined in the preceding paragraph. *Ex situ* conservation methods are necessary to provide support for *in situ* actions by preventing the extinction of species, providing a source of material for reintroduction or population enhancement of individual species, and providing a source of material for research to better understand the biological factors contributing to a species' decline (Offord and Makinson 2009). A low cost, long-term method for *ex situ* conservation applicable to seed-bearing species is seedbanking (i.e. storage of seeds, after proper moisture equilibration, at –20°C). This option is applicable to a high proportion of species that possess orthodox (desiccation and low temperature tolerant) seed (Tweddle *et al*. 2003; Guerrant *et al*. 2014). Orthodox seeds generally have stable storage behaviour that allows drying, freezing, and storage at low temperatures over long periods of time (at least decades), after which germination and growth into healthy, reproductive adult plants is possible (Dickie and Pritchard 2002; Guerrant *et al*. 2014; O'Donnell and Sharrock 2017). However, not all seeds can be stored under conventional seed banking conditions. Seeds with special storage requirements are generally chilling sensitive, short lived, desiccation sensitive, or have a combination of those characteristics (Hay *et al*. 2010; Merritt *et al*. 2014*b*). Many of these seeds can be classified as recalcitrant, i.e. seeds that are unable to survive drying to low moisture contents (MC) and are sensitive to chilling (Ellis *et al*. 1990; Engelmann 2012). Most recalcitrant seeds studied to date are produced by evergreen rainforest tree or shrub species in moist tropical and subtropical zones. These seeds tend to be larger and have a higher water content than orthodox seeds, and are metabolically active and temperature sensitive; these characteristics make them unsuitable for storage by conventional seed banking methods (Tweddle *et al*. 2003; Li and Pritchard 2009; Hamilton *et al*. 2013; Funnekotter *et al*. 2017*c*). Many threatened species fall under the recalcitrant category and are thus much more suited to other *ex situ* storage methods such as *in vitro* (tissue) culture and/or cryopreservation (Ashmore *et al*. 2011).

Tissue culture is a technique used to grow plant cells, tissues and organs on synthetic media in an aseptic environment with controlled light, temperature and humidity conditions (Dagla 2012). Tissue culture allows a large amount of plant material to be produced from a small amount of starting material while maintaining key genotypic features of elite genotypes (or simply a limited number of existing plants in the case of threatened taxa), which may be lost in storage of genetically varying seed accessions (Ashmore *et al*. 2011; Bunn *et al*. 2011; Funnekotter *et al*. 2017*c*). However, tissue cultures require regular maintenance, may be accidentally contaminated via operator error, may accumulate somaclonal (or epigenetic) variation over many generations of continuous culture cycles, and may decline in viability over time (Larkin and Scowcroft 1981; Miguel and Marum 2011), and the presence of endophytes may hasten the death of the explants by availing more nutrients from either the plant culture medium or damaged tissues (Cassells 1991; Fouda *et al*. 2015). Hence other technologies are needed and cryo-storage or cryopreservation presents as a more attractive option for the long-term, biosecure storage of key germplasm collections (Benson 2008*b*).

Cryopreservation

Cryopreservation offers a superior long-term method of plant germplasm conservation that, in principle, overcomes the issues listed above for *in vitro* material, and also offers the only current alternative long-term storage option for recalcitrant-seeded species. Cryopreservation, in theory, allows indefinite storage of plant tissues in liquid nitrogen (LN), thereby maintaining the tissues at –196°C, with cellular metabolism effectively halted (Benson 2008*b*). Cryopreservation has been increasingly favoured as a plant conservation method in recent years as it requires very little storage space, minimal upkeep (i.e. periodic replenishing of LN), all but eliminates the risk of contamination, and is applicable to a wide range of plant tissues (Kaczmarczyk *et al*. 2012). It also mitigates the risk of any epigenetic variation in the stored tissues over time (Harding 2004). A wide range of plant tissues and organs can be cryopreserved including pollen, seeds, shoot tips, dormant buds, cell suspensions, embryonic cultures, somatic and zygotic embryos and callus tissue (Benelli *et al*. 2013; Engelmann 2012). The choice of propagule to use for cryo-storage is dependent on the conservation goal, with seeds and embryos capturing species diversity, whereas propagules like shoot tips and dormant buds capture specific genotypes (Reed 2008). Initial research on plant cryopreservation began in 1965, with a series of experiments investigating the use of the cryoprotective agent (CPA) dimethyl sulfoxide (DMSO) on mulberry twigs (Sakai 1965). Cryopreservation techniques have been steadily evolving and improving since these early beginnings.

Biophysics of cryo-storage

The development of cryopreservation protocols requires an understanding of water behaviour, cryo-injury and cryo-protection (Day *et al*. 2008). Temperature greatly influences the formation of the four most common states of water: liquid, glass, ice and vapour (Pukacki and Juszczyk 2015). The main goal that cryo-protocols aim to achieve for successful cryopreservation is the manipulation of water in its liquid, glassy and ice states, in order to avoid the formation of lethal intracellular ice crystals (Pukacki and Juszczyk 2015). Sufficient dehydration and cooling during cryo-storage ensures that the temperature drops rapidly below the glass transition temperature (T_g), resulting in the formation of an amorphous 'glassy' state (Shamblin *et al*. 1999; Walters 2015). The stability of this glassy state is related to the strength of the hydrogen-bonding network in

water (Kreck and Mancera 2014). The advantages of the formation of this glassy state are that it (i) prevents cellular collapse; (ii) precludes chemical reactions requiring diffusion, ensuring stability during the storage period; and (iii) allows chaotropic solutes to be trapped by an amorphous glass which prevents them from becoming concentrated (Burke 1986; Moelbert *et al*. 2004).

Formation of ice crystals can occur during cryo-storage because the vitrified state is metastable. The behaviour of glasses in biological tissue is highly complex, and they can easily devitrify to form ice and/or revert back into a liquid (Benson 2008*b*; Day *et al*. 2008). When water molecules form intermolecular hydrogen bonds, the size of any ice crystals increases, so they become lethal to the cell (Walters 2015). Crystallisation can occur extracellularly or intracellularly depending on the rate of cooling. Rapid cooling results in intracellular ice formation, whereas slow cooling results in extracellular ice formation (Gonzalez-Arnao *et al*. 2007). Extracellular ice formation is responsible for osmotic shock injury (Benson 2008*a*). The formation of intracellular ice damages cell membranes directly as the growing ice crystals push apart and rupture membranes (Burke *et al*. 1976).

Desiccation of cells minimises the formation of ice crystals as the removal of water causes the cells to shrink and increases their solute concentration, in turn, increasing viscosity of the cytoplasm and improving the stability of the glassy state (Walters *et al*. 2010; Verhoeven *et al*. 2018). Successful cryopreservation protocols all centre on the controlled reduction of tissue water content to sufficiently low levels that the cell contents become concentrated and highly viscous, promoting the likelihood of vitrification taking place over ice crystal formation (Benson 2008*a*). Several measures can be incorporated into cryopreservation protocols to achieve this, including desiccation and pretreatment with CPAs (O'Donnell and Sharrock 2017).

Cryopreservation protocols

Cryopreservation is a multi-step process, involving (i) the initial excision of the germplasm such as seed embryos or shoot tips to obtain sufficiently small material; (ii) desiccation or preculture on osmotic media to reduce water content; (iii) cryoprotection through exposure to CPAs to promote vitrification; (iv) cryopreservation in LN; (v) re-warming; and finally (vi) the washing (unloading of CPA solutions) and recovery of the germplasm after cryopreservation (Fig. 1; Funnekotter *et al*. 2017*b*; Kaczmarczyk *et al*. 2012). There is wide variation in cryo-capability among species, and also among different clonal lines of the same species, therefore each species requires its own cryopreservation protocol to achieve the maximum possible post-cryogenic survival (Reed 2008). Different protocols need to be developed and optimised depending on the tissue and species to be cryopreserved, by experimentally altering factors such as desiccation conditions (preculture), mixtures of CPAs and recovery environments (Hamilton *et al*. 2009*b*).

CPAs can be classified into two main types that are often used in combination: non-penetrating, such as sucrose and polyethylene glycol; and penetrating (also known as 'colligative'), such as DMSO and glycerol (Day *et al*. 2008; Berjak and Pammenter 2014). Non-penetrating CPAs do not cross cell membranes and remain external to the cells, causing potentially freezable water to be osmotically removed from the cells, thus increasing intracellular solute concentration (Pammenter and Berjak 2014). Penetrating CPAs cross cell membranes and act in the following various ways: (i) as agents that inhibit the formation and growth of intracellular ice by disrupting crystalline structure; (ii) as solutes that increase the number of particles dissolved in the intracellular water and, therefore, lower the freezing point; (iii) as diluents for intracellular electrolytes, which counteract the potentially toxic effects of increased concentrations of solute as water is removed; and (iv) as promoters of the glassy state (Berjak and Pammenter 2014). Molecular simulation studies have revealed the mechanism by which addition of CPAs to water promotes the formation of the glassy state of water (Kreck *et al*. 2011; Mandumpal *et al*. 2011).

The most commonly used combination of CPAs, Plant Vitrification Solution 2 (PVS2), was developed in 1990 by Sakai *et al*. (1990) and contains (in w/v) 30% glycerol, 15% DMSO and 15% ethylene glycol in Murashige-Tucker medium (however, Murashige-Skoog medium is now more commonly used) containing 0.15 M sucrose. One of the most widely used techniques incorporating CPAs today is droplet vitrification. This method was developed in 2004 by Panis *et al*. (2005) and involves treatment with CPAs before rapidly freezing each shoot tip in a droplet of vitrification solution and subsequently storing in LN. Using droplets rather than large volumes of CPAs contributes to higher cooling and re-warming rates, resulting in improved survival rates after cryopreservation (Sakai and Engelmann 2007). An alternative cryo-protocol, developed in the 1990s by Fabre and Dereuddre (1990) and known as encapsulation-dehydration, involves encapsulation of plant material within alginate beads which provide protection from mechanical damage. The beads are osmotically dehydrated in a concentrated sugar solution, then further dehydrated by silica gel or airflow to achieve sufficiently low water content for cellular vitrification, thereby avoiding the use of toxic CPAs (Fig. 1), before immersion in LN. A variation on this technique known as encapsulation-vitrification replaces the osmotic and air dehydration steps with cryoprotection using CPAs for more desiccation sensitive species (Sakai and Engelmann 2007).

Progress in the cryopreservation of Australian species

Most of the cryopreservation research for native species in Australia to date has focussed on Western Australian species due to the location of appropriate research facilities. There is, however, a range of herbaceous and woody species from different ecosystems represented in the literature (Table 1). Cryopreservation of other Australian species has previously been reported by Bunn *et al*. (2007), Kaczmarczyk *et al*. (2011) and Funnekotter *et al*. (2017*b*), while Merritt *et al*. (2014*a*) has reviewed cryopreservation success with seeds of various orchid species. Due to the high diversity of endemic species in Australia, little is known about the physiology of each species, resulting in slow progress in the development of new cryopreservation protocols. Further progress in understanding the fundamental requirements for successful cryopreservation and

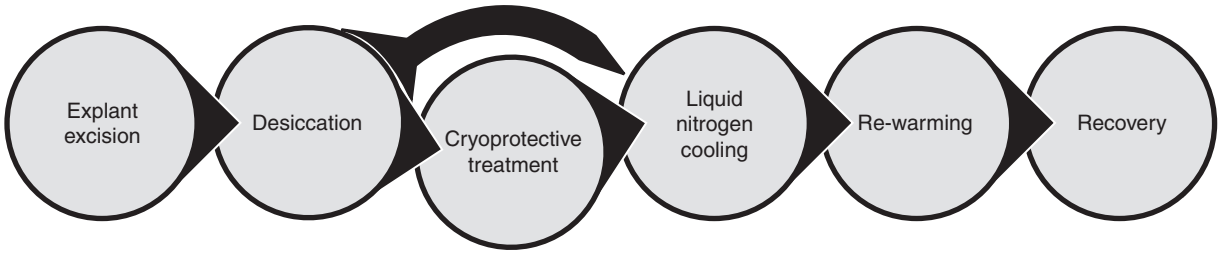

Fig. 1. Common stages for cryopreserving plant material: (i) the initial excision of the germplasm such as seed embryos or shoot tips to obtain sufficiently small material; (ii) desiccation or preculture on osmotic media to reduce water content; (iii) cryoprotection through exposure to CPAs to promote vitrification; (iv) cryopreservation in LN; (v) re-warming; and finally (vi) the washing (unloading of CPA solutions) and recovery of the germplasm after cryopreservation. With sufficient desiccation, the cryoprotective treatment can be circumvented in the cryopreservation process.

Table 1. Summary of published protocols for cryopreservation of Australian plant taxa undertaken in Australia. Maximum (mean) survival rate found after cryopreservation

Propagule types: C, callus; EA, embryonic axis; G, gametophyte; P, protocorm; S, spore; ST, shoot tip; WS, whole seed

Family	Species	Propagule	Cryopreservation method	Survival rate	Reference
Asparagaceae	*Lomandra sonderi*	ST	Droplet-vitrification	32.0%	(Menon *et al*. 2012)
	Sowerbaea multicaulis	ST	Vitrification	10.9%	(Touchell 1995)
Cunoniaceae	*Ceratopetalum gummiferum*	ST	Encapsulation-dehydration	61.4%	(Shatnawi and Johnson 2004)
Cyatheaceae	*Cyathea australis*	S, G	Encapsulation-dehydration	>80%	(Mikuła *et al*. 2009)
Haemodoraceae	*Anigozanthos humilis* subsp. *chrysanthus*	ST	Vitrification	71.8%	(Turner *et al*. 2000, 2001;
	Anigozanthos kalbarriensis	ST	Vitrification	18.4%	Funnekotter *et al*. 2017*a*)
	Anigozanthos manglesii	ST	Vitrification	31.9%	
	Anigozanthos rufus	ST	Vitrification	2.2%	
	Anigozanthos rufus × *A. pulcherrimus*	ST	Vitrification	17.6%	
	Anigozanthos viridis subsp. *terraspectans*	ST	Vitrification	80.3%	
	Anigozanthos viridis	ST	Cryo-mesh	83%	
	Anigozanthos viridis subsp. *viridis*	ST	Vitrification	34.9%	
	Conostylis dielsia subsp. *teres*	ST	Vitrification	11.3%	
	Conostylis micrantha	ST	Vitrification	27.5%	
	Conostylis wonganensis	ST	Vitrification	67.3%	
	Macropidia fuliginosa	ST	Vitrification	0%	
Malvaceae	*Androcalva perlaria*	ST	Droplet-vitrification	82.0%	(Whiteley *et al*. 2016)
	Rulingia sp. 'Trigwell Bridge'	ST	Vitrification	67.9%	(Bunn *et al*. 2007)
Myrtaceae	*Eucalyptus graniticola*	ST	Vitrification	48.3%	(Bunn *et al*. 2007)
	Syzygium francisii	ST	Encapsulation-dehydration	58.3%	(Shatnawi *et al*. 2004)
Orchidaceae	*Caladenia huegelii*	P	Droplet-vitrification	79.0%	(Bustam *et al*. 2016*a*)
	C. latifolia	P	Droplet-vitrification	85.0%	(Bustam *et al*. 2016*b*)
Proteaceae	*Banksia ashbyi*	WS	Direct immersion	>80%	(Merritt *et al*. 2000)
	Grevillea althoferorum	ST	Droplet-vitrification	77.5%	(E. Bunn, unpubl. data)
	Grevillea dryandroides subsp. *dryandroides*	ST	Vitrification	74%	(Kaczmarczyk *et al*. 2011)
	Grevillea dryandroides hirsutifolia	ST	Vitrification	75%	(Touchell *et al*. 1992)
	Grevillea scapigera	ST	Droplet-vitrification	52%	(Funnekotter *et al*. 2013)
	Lambertia orbifolia	ST	Vitrification	95.0%	(Bunn *et al*. 2007)
Restionaceae	*Loxocarya cinerea*	ST, C	Droplet-vitrification	<1%, 90%	(Kaczmarczyk *et al*. 2013)
Rutaceae	*Citrus australasica*	WS	Direct immersion	94.0%	(Hamilton and Ashmore 2008;
	Citrus garrawayi	WS	Direct immersion	>75%	Hamilton *et al*. 2009*a*)
	Citrus inodora	WS	Direct immersion	>75%	
Scrophulariaceae	*Eremophila resinosa*	ST	Vitrification	60.6%	(Bunn *et al*. 2007)
Stylidiaceae	*Stylidium coroniforme*	ST	Vitrification	100%	(Bunn *et al*. 2007)
	Stylidium expeditionis	ST	Vitrification	92.7%	(Bunn *et al*. 2007)

the development of novel protocols is vital to accelerate successful conservation of Australian species through cryopreservation.

Current issues in cryopreservation

Differential explant response

The materials most frequently used for cryopreservation of wild plants in Australia to date include tissue cultured shoot tips and whole seeds (Table 1). The use of excised seed embryos or embryonic axes (the root and shoot axis of the embryo with cotyledons removed) has, for the most part, been limited to commercially important recalcitrant and intermediate species such as almond, chestnut, coffee, hazelnut and rubber (Normah and Makeen 2008). Although both shoot tips and embryos are composed chiefly of meristematic cells, they can respond very differently to cryopreservation pretreatments and the processes of freezing and thawing. Shoot tips consist largely of apical meristem tissues surrounded by leaf primordia and subtended by partially differentiated cells forming the primary meristems, i.e. ground meristem (that will produce the stem pith), procambium (that will produce the vascular system) and protoderm (that will produce the epidermis) (Evert and Eichhorn 2013). Shoot tips – at least those produced in tissue culture – are generally intolerant of desiccation unless pre-treated with suitable cryoprotective agents (Volk and Walters 2006). Embryonic axes are composed largely of primary meristems with cell characteristics and tissue arrangement related to the mature tissues they will eventually form (Esau 1977). In addition to an apical meristem, the embryonic axis has a root meristem, which in some embryos may have developed further into a radicle (a primordial root), while the apical meristem may have developed further into a plumule (a stem-like axis with one or more leaves surrounding the apical meristem) (Evert and Eichhorn 2013). In some taxa, the embryonic axis has the capacity to survive rapid desiccation even though the whole seed is sensitive to drying (Normah and Makeen 2008). In other taxa, the axis may be just as sensitive to drying as a shoot tip (Pammenter and Berjak 2014; Sommerville *et al*. 2017).

There have not, as yet, been any published studies comparing desiccation-tolerant and desiccation-sensitive embryonic axes in terms of ultrastructure, membrane composition or other cellular components. Likewise, there have been no studies comparing the effect of cryopreservation on the ultrastructure of embryonic axes with shoot tips of the same species. There have, however, been several ultrastructural studies focussed on a single tissue type for individual species and these may provide some clues to the mechanisms at work. Wesley-Smith *et al*. (2015), for example, observed that cryopreservation of fresh, untreated, embryonic axes of *Acer saccharinum* resulted in far greater damage to the highly vacuolated cells of the plumule than to the cells of the radicle. The apical and root meristems were both lethally damaged and regrowth occurred only from the primary meristems – from ground meristem and procambium in the radicle, and from pockets of ground meristem and protoderm in the plumule. Similarly, in a study on the effects of cryopreservation on potato (*Solanum tuberosum*) shoot tips pre-treated with DMSO, Kaczmarczyk *et al*. (2008) observed extensive post-cryopreservation damage in the meristematic dome and part of the epidermis. Survival and regeneration occurred mainly in the leaf primordial tissues. These results suggest that material with a higher proportion of partially differentiated tissues may be more tolerant of cryopreservation than undifferentiated meristem. However, in a study of cryopreservation of yam (*Dioscorea alata*) shoot tips by encapsulation-dehydration, Barraco *et al*. (2014), found that only cells in the meristematic zone survived. Similarly, Volk and Caspersen (2007) found that recovery of cryopreserved *Mentha* × *piperita* shoot tips pre-treated with PVS2 occurred from surviving clumps of cells in the outer layers of the original meristem or in the axil of the shoot and leaf primordia, whereas Helliot *et al*. (2003) observed that recovery of banana meristems pre-treated with PVS2 occurred only from small areas of surviving cells in the leaf primordial tissues and in the tunica of the meristematic dome. These results may indicate that different cryoprotective treatments favour different tissues, or may be an indication of species-dependent differences in cell structure and composition.

Dormant buds are a possible alternative source of material for cryopreservation not yet tested in Australia. Dormant buds or scions holding dormant buds have been successfully used for preservation of genetic material in forest, fruit, nut and ornamental species (Towill and Ellis 2008; Benelli *et al*. 2013). The method used for cryopreservation is dependent on the species as well as on the level of cold-hardiness of the collected material (Towill and Ellis 2008). For successful cryopreservation of dormant buds, the most important factor identified to date is the extent of cold acclimation that the species can attain, and the extent of acclimation that the material possesses at the time of collection (Towill and Ellis 2008). Regrowth after cryopreservation can be achieved by direct grafting of the bud or micrografting of the excised shoot tip from the dormant bud (e.g. apple), by culture of the shoot tip on suitable medium (e.g. persimmon), or by direct rooting of the cryopreserved scion (e.g. willow) (Towill and Ellis 2008; Benelli *et al*. 2013). However, only 0.15% of the Australian continent experiences winter snow-cover (Pickering *et al*. 2008), and thus there are unlikely to be many species that are sufficiently cold tolerant to be suitable for this method.

Cryoprotective agent toxicity

The successful use of CPAs requires exposure to these agents in an adequate concentration and for a sufficiently long time to achieve dehydration and ultimately, vitrification. Volk and Walters (2006) showed that the extent of PVS2 penetration into garlic and mint shoot tips was directly proportional to the exposure time, and a significant decrease in water content was seen with increased exposure time to PVS2. The effect of CPAs on cellular ice formation can be measured through the use of thermal analysis via differential scanning calorimetry (DSC), with extended exposure times significantly reducing the formation of ice crystals (Benson 2008*a*; Menon *et al*. 2012). CPAs, however, can be toxic to cells, such that limiting the concentration of CPAs to reduce toxicity is a major barrier to cryopreservation by vitrification (Best 2015). CPAs are deemed toxic if they cause breaches or damage in the cell membrane, impair the function of enzymes, diminish cell development, and damage DNA, proteins or macromolecules (Best 2015). The high

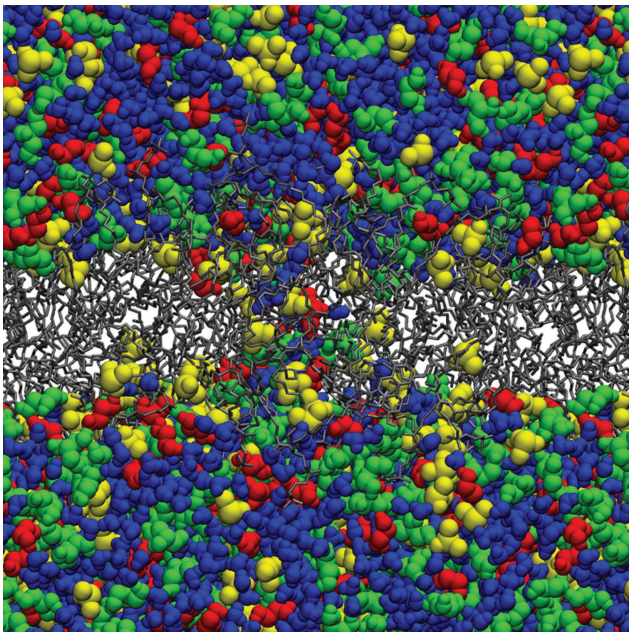

Fig. 2. Pore formation in a model cell membrane composed of DOPC upon the action of a mixture of cryosolvents representative of PVS2. Each cryosolvent molecule is shown with a different colour and the lipid bilayer is shown with grey sticks (Hughes and Mancera 2014).

concentration of CPAs causes an exchange of intracellular water with the external environment, creating acute osmotic stress (Lai *et al*. 2015), with the resulting rapid efflux of water from cells causing them to collapse (Fuller 2004). Molecular simulation of model cell membranes has shown that addition of CPAs at high concentrations can damage membrane integrity, ultimately causing the formation of water pores (Fig. 2) and the subsequent destruction of the cell membrane (Hughes *et al*. 2012; Hughes *et al*. 2013; Hughes and Mancera 2013; Malajczuk *et al*. 2013). However, this damaging effect is reduced when CPAs are used in mixtures, such as in PVS2 (Hughes and Mancera 2014).

Various approaches have been developed to reduce CPA toxicity, including their sequential 'loading' and 'unloading' to prevent osmotic shock (Day *et al*. 2008). The temperature and timing of CPA treatments are critical in achieving sufficient cryoprotection, while avoiding toxicity and damage to cells (Kaczmarczyk *et al*. 2012; Pence 2014). In the development of cryopreservation protocols for Musacae (Panis *et al*. 2005) and *Anigozanthos viridis* (Turner *et al*. 2000), a 5 or 10 min difference in CPA incubation times was observed to translate to a change in survival rate of around 20%. The consequence of the damaging effects of CPAs for cryopreservation protocols is that optimising CPA incubation time is essential for post cryopreservation survival. If plant tissues are incubated insufficiently, ice crystals will damage cells, but just as much damage can occur with longer incubation times due to osmotic stress and CPA toxicity (Turner *et al*. 2000; Panis *et al*. 2005).

The role of cell membranes in cryopreservation

The fundamental purpose of the cell membrane is to form a stable but fluid barrier that is semi-permeable between two aqueous compartments: the intra- and extracellular environments. Cell membranes are considered one of the primary sites of cryo-injury. During cryopreservation, water stress damage occurs due to changes in intracellular and extracellular water concentrations caused by desiccation or freezing. This results in external osmotic pressure on cells, forcing the cells to shrink in volume (Moussa *et al*. 2008). The maintenance of membrane integrity and stability under water stress is considered a major factor in drought tolerance (Bajji *et al*. 2002). In cryopreservation protocols, plant material is desiccated on culture media containing a high concentration of sugars. This removal of water can mimic the effects of water stress on the cellular membrane. There are two widely accepted hypotheses around the effect of water stress on membranes: (i) that when electrolyte concentration reaches a critical level, the cell membrane is damaged, and the cells die from osmotic shock (Lovelock 1953); and (ii) that membrane damage occurs because cells are unable to shrink enough to maintain osmotic equilibrium (Meryman 1968). Additionally, the process of cryopreservation can result in physical membrane damages, such as (i) expansion-induced lysis, where overexpansion of cells occurs due to increased extracellular osmotic pressure during thawing/warming; (ii) loss of osmotic responsiveness, where due to a slow cooling rate there is no osmotic change during warming (i.e. cells stay dehydrated); (iii) altered osmotic behaviour, where water and solutes are released into the surroundings because cell membranes become 'leaky'; and (iv) intracellular ice crystal formation due to rapid cooling that causes membrane disruption. (Steponkus 1984).

Minimising cryo-injury depends on membrane stabilisation, typically achieved through preconditioning, causing changes in lipid composition in cell membranes, accumulation of sugars and production of membrane-protecting polypeptides (Thomashow 1999). Preconditioning is the method of exposing plants to an abiotic stress before cryopreservation to alter its cellular properties (Kaczmarczyk *et al*. 2012). Temperature preconditioning is commonly used as a method to increase cell membrane stability at low temperatures through increased levels of unsaturated acyl chains (Menon *et al*. 2014). Studies on two Australian species (*Grevillea scapigera* and *Loxocarya cinerea*) exposed to 20/10°C alternating temperature preconditioning resulted in a decrease in common acyl chains (16 : 0, 18 : 0, 18 : 1 and 18 : 2) with an increase in α-linoleyl tails (18 : 3) (Funnekotter *et al*. 2013). Larger concentrations of the more unsaturated α-linoleyl tails induce a greater level of membrane molecular disorder, leading to increased membrane fluidity, stabilisation of the lamellar phase and maintenance of diffusion across membranes when subjected to reduced temperatures and desiccation. An increase in double bonds within phospholipids (PLs) was also seen in both species; however, these changes only correlated with increased cryopreservation survival in one (*G. scapigera*). Studies on cryopreservation of banana meristems have also seen positive correlation between unsaturation and cryopreservation survival (Zhu *et al*. 2006). Higher concentrations of double bonds are generally thought to be beneficial in dehydration and cooling by reducing the liquid crystal-to-gel transition temperature through increased membrane fluidity (Kong and von Aderkas 2011). However, too high a concentration of double bonds may not be

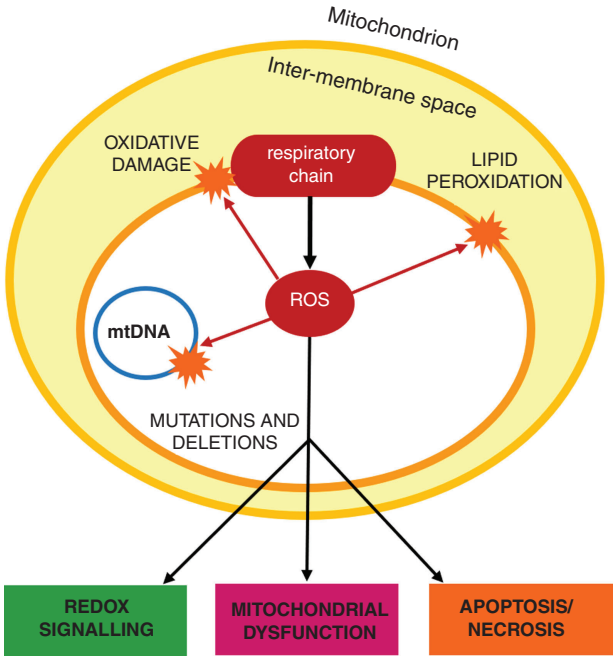

Fig. 3. Production and impact of reactive oxygen species on mitochondria. Adapted from Murphy (2009).

beneficial as the membrane becomes more susceptible to free radicals acting on electron rich double bonds and causing lipid peroxidation (Benson 1990).

Oxidative stress damage in cryopreservation

Plant tissues are susceptible to oxidative stress, caused primarily by reactive oxygen species (ROS) (Benson and Bremner 2004; Kaczmarczyk *et al*. 2012). ROS are oxygen-based molecules with an unstable number of electrons, making them highly reactive (Murphy 2009). Mitochondria are generally accepted as the major producers of ROS in the cell (Murphy 2009; Andreyev *et al*. 2015), although chloroplasts and peroxisomes also produce ROS in plants (Taylor *et al*. 2004; Suzuki *et al*. 2012). The accumulation of these ROS results in an imbalance of redox reactions within cells, resulting in oxidative stress and several other deleterious effects, potentially resulting in cell apoptosis (Fig. 3) (Berjak and Pammenter 2014).

Oxidative stress constitutes a major component of cryo-injury and is a major obstacle for successful cryopreservation (Benson and Bremner 2004; Chetverikova 2012; Funnekotter *et al*. 2017*c*). Each of the steps in the vitrification technique of cryopreservation (excision, pre-culture, loading solution, CPA treatment, cooling, rewarming, washing and regrowth) presents the possibility of oxidative stress, due to physical damage and osmotic stress involved in the process, and many studies have shown this (Uchendu *et al*. 2010). Hydrogen peroxide (H_2O_2) and superoxide production has been observed during excision, desiccation, exposure to LN, and rewarming and recovery (Johnston *et al*. 2007; Fang *et al*. 2008; Whitaker *et al*. 2010; Berjak *et al*. 2011; Skyba *et al*. 2012). During the desiccation stage, Johnston *et al*. (2007) found *Ribes ciliatum* produced more than three times the amount of ethylene (an indicator of oxidative membrane damage) as *Ribes nigrum*. Whitaker *et al*. (2010) showed that the tropical tree species *Trichilia dregeana* produced twice as much superoxide as *Castanea sativa*, and for a greater duration. One study found that H_2O_2 production in *Hypericum perforatum* persisted for several weeks after cryopreservation (Skyba *et al*. 2012). Roach *et al*. (2008) studied ROS formation during the first steps of the cryopreservation protocol, specifically wounding and desiccation, and showed that excision of embryonic axes from fresh seeds of recalcitrant sweet chestnut (*C. sativa*) induced a burst of extracellular superoxide in the first 5 min following excision. The isolated axes were subjected to varying levels of desiccation stress, and the combined effects of wounding and desiccation from 60 to 30% MC, were found to double the initial rates of extracellular superoxide production (Roach *et al*. 2008). These results indicated that there is a complex interaction between excision and subsequent drying of the isolated axes, which showed a reduction in viability and vigour and increase in an electrolyte leakage, which suggests impaired membrane integrity (Roach *et al*. 2008). Similar results were found by Whitaker *et al*. (2010) using recalcitrant seeds from the tropical tree species *Trichilia dregeana*.

Lipid peroxidation

ROS interact with cellular biomolecules, and can cause serious oxidative damage to proteins, nucleic acids and lipids, with lipid peroxidation in particular leading to disruption of membranes that are critical to cell survival (Sies and Cadenas 1985; Uchendu *et al*. 2010; Halliwell and Gutteridge 2015). Lipid peroxidation results from the oxidation of PLs and other unsaturated lipids when the production of ROS overwhelms the ability of scavenging antioxidant defence systems to protect cells (Rachmilevitch *et al*. 2006). Lipid peroxidation is concerning as it affects the integrity of membrane structure, and alters its functions, which leads to cell death (Uchendu *et al*. 2010).

Some of the by-products of lipid peroxidation, such as the aldehydes, are highly reactive and may be regarded as secondary toxic messengers, which spread and cause an increase in initial free radical events (Catalá 2006). The aldehydes that have been most intensively studied so far are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), with the identification of their formation commonly used as an indicator of oxidative stress (Kaczmarczyk *et al*. 2012). Due to its carbonyl functional groups, MDA is chemically reactive; however, the toxicity of MDA is debatable, as its effects were previously overestimated and it has been found to be less reactive than HNE (Halliwell and Gutteridge 2015; Tsikas 2017). MDA can polymerise easily and undergoes various reactions with proteins (leading to loss of function) and DNA (leading to mutations) (Halliwell and Gutteridge 2015; Tsikas 2017). However, HNE formation has greater toxicity to cells than MDA, as it can cause mitochondrial damage, inhibit DNA and protein synthesis, and inhibit the action of chaperone repair proteins (Halliwell and Gutteridge 2015).

Ren *et al*. (2013) studied how peroxidation due to CPA treatment affects *Arabidopsis thaliana* seedling survival and found that MDA content increased after desiccation and cryoprotection (with PVS2) treatment in both 48 and 72 h (cultured) seedlings. Their data showed that high levels of MDA in seedlings reduced the capacity for regrowth following

cryogenic treatment, with the 72 h seedlings (MDA increased almost 2-fold) suffering serious membrane damage from lipid peroxidation and subsequent cell death (Ren *et al*. 2013). Chen *et al*. (2015) also examined the survival tolerance of *A. thaliana* seedlings, which were germinated for 48 or 72 h, at five steps of cryopreservation (desiccation, cryoprotection, rapid warming, unloading and recovery), in order to determine the role of ROS (superoxide, H_2O_2 and $OH^{\bullet}$) in cryo-injury. The main cause of cryo-injury in the 48 h seedlings and 72 h seedlings (no survival), was that H_2O_2 (generated mainly in cotyledons, shoot tips and roots) induced oxidative stress at the cryoprotection step (with PVS2) and rapid rewarming steps (Chen *et al*. 2015). MDA content was higher throughout the steps in the 72 h seedlings but showed a similar trend to that seen in the 48 h seedlings (Chen *et al*. 2015). Other studies also found increases in MDA content, including a 3-fold increase in MDA detected during the desiccation and freezing of maize embryos (Wen *et al*. 2010), and substantially more MDA detected at each step of the cryopreservation of untreated blackberry shoot tips compared with shoot tips treated with antioxidants (Uchendu *et al*. 2010). The initial recovery period following cryopreservation is a critical stage: increased levels of oxidative stress have been linked to a decrease in post-cryogenic regrowth, with increased detection of superoxide burst during rehydration of cryopreserved *Trichilia dregeana* (Whitaker *et al*. 2010), increased MDA content in *A. thaliana* during 24 h recovery (Chen *et al*. 2015), and detection of 4-HNE and hydroperoxide in *Coffea arabica* (coffee), indicating lipid peroxidation during the first hour of seed rehydration following desiccation (Dussert *et al*. 2003). The above indicate lipid peroxidation can cause substantial damage to cryopreserved plant material unless controlled with remedial or mitigating treatments (e.g. antioxidants).

Impact of cryopreservation on mitochondrial function

Although there are few studies on plant mitochondria in cryopreservation, studies on how plant cells in general are physically affected by cryopreservation have been conducted. Vacuolisation, abnormalities and rupture of the nuclear envelope, cell lysis, and autophagic decomposition were all observed following exposure to LN (Helliot *et al*. 2003; Wesley-Smith *et al*. 2015). Helliot *et al*. (2003) determined that this damage occurred during either osmotic dehydration using PVS2 or freezing and thawing. The finding by the same study that surviving cells (1 week post-thaw) showed that the presence of numerous organelles, including mitochondria, is of particular significance as it suggests a high level of metabolic activity in the plant cells during the recovery period (Helliot *et al*. 2003). Despite the evidence that suggests cryopreservation often causes damage to mitochondria in animal cells, as well as to plant cells as a whole, Skyba *et al*. (2012) found that exposure to cryogenic temperatures in the plant species *H. perforatum* caused no significant observable structural damage to mitochondria, although they did not test for impacts on mitochondrial function. Ren *et al*. (2013) found that cryogenic treatment caused the downregulation of transporters related to oxidative phosphorylation in *A. thaliana* seedlings, which also suggests that the negative effect seen during cryopreservation on animal cell mitochondrial function may also apply to plant cells. However, the full extent (and duration) of this damage, and whether or not mitochondria can repair cryo-induced damage and resume normal function, is still unresolved.

Recovery medium

Recovery methods are an often overlooked but crucial aspect of cryopreservation protocols (Reed 2008). Optimal recovery methods vary greatly among species. Although most protocols employ a standard basal medium for recovery from cryopreservation (Gonzalez-Arnao and Engelmann 2006), several studies have found altering the contents of the medium to be beneficial. The addition of growth regulators, for example, stimulates shoot production in some species (Paulet *et al*. 1993). Uchendu *et al*. (2013) found that the addition of melatonin to the recovery medium improved regrowth of American elm when used in conjunction with a melatonin preculture stage. Conversely, some species benefit from the removal of growth hormones such as benzyl adenine (BA) from the recovery medium (Chang and Reed 1999; Gupta and Reed 2006), which can reduce callus formation, thus maintaining genetic integrity.

The addition of growth regulators to recovery media is common for cryopreservation of many Australian species (Touchell *et al*. 2002; Menon *et al*. 2012; Whiteley *et al*. 2016). Shoot tips of *Grevillea scapigera*, for example, fully recovered only in the presence of zeatin whereas, in the same study, *Anigozanthos viridis* showed greatest recovery levels with the addition of choline chloride (Touchell *et al*. 2002). Environmental conditions are also an important aspect of recovery protocols. Both *A. viridis* and *G. scapigera* showed increased recovery rates when stored in darkness for 7 and 14 days following thawing respectively (Touchell *et al*. 2002). Sugarcane and cassava protocols also include a period of darkness during recovery (Paulet *et al*. 1993; Escobar *et al*. 1997). However, both *Carica papaya* and *Vasconcellea pubescens* showed decreased recovery with the exclusion of light for any period of time after cryopreservation (Ashmore *et al*. 2007).

Progress in understanding and ameliorating cryo-injury

Role of antioxidants

Plants have complex antioxidant systems, which are their natural defence against the accumulation of ROS and their by-products, and which enable them to resist external stress (Chen *et al*. 2015; Funnekotter *et al*. 2017*b*). Although antioxidants within the cells can balance the build-up of ROS (Mittler 2002), it has been shown that concentrations of antioxidants like glutathione (GSH) and ascorbic acid (AsA) decrease in plant tissues with each successive step of the cryopreservation process (Fig. 4) (Funnekotter *et al*. 2017*b*). Although Chen *et al*. (2015) also found a reduction in GSH and AsA antioxidants in thawing and recovery stages, levels of these antioxidants were found to increase in the desiccation period. However, the same study also showed a decrease in superoxide dismutase (SOD) over all stages of the cryopreservation process (Chen *et al*. 2015). Funnekotter *et al*. (2016) also examined the influence of abiotic stress preconditioning on antioxidant enzymes in shoot tips of an Australian species, *Lomandra sonderi*, before cryo-storage. A weak positive correlation was observed between SOD

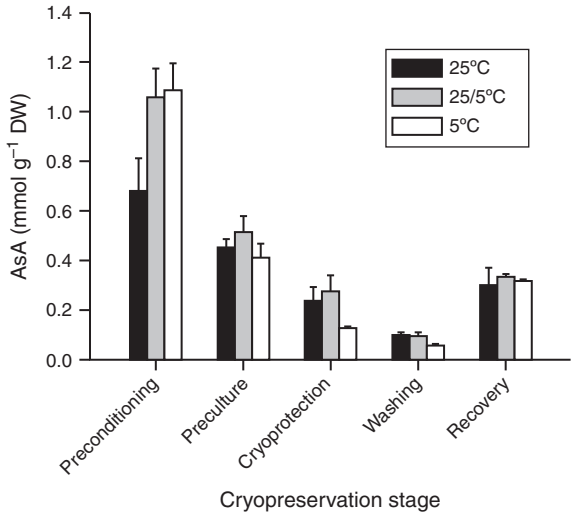

Fig. 4. Reduction in ascorbic acid (AsA) content in *Lomandra sonderi* during the five stages of the cryopreservation process. Three *in vitro* preconditioning temperatures (constant 25°C, 5°C and an alternating 25/5°C) for 3 weeks were tested before cryopreservation (Funnekotter *et al*. 2017*b*).

activity and post-cryogenic survival; however, the activities of glutathione reductase, glutathione peroxidase and catalase showed no correlation. Analysis of the antioxidants found that glutathione reductase activity decreased significantly after cryopreservation, but glutathione peroxidase and catalase did not change (Funnekotter *et al*. 2016).

The damaging effects of ROS formation can be modulated in two ways: through treatment to reduce ROS production, or by exogenous application of antioxidants which can exert a direct effect or may enhance endogenous antioxidant activity (Berjak *et al*. 2011). Several studies have shown that the application of exogenous antioxidants during the cryopreservation process can increase survival and regeneration post-cryopreservation, such as the use of vitamin C and E at critical steps during vitrification, which significantly improved regrowth of blackberry shoot tips following exposure to LN (Uchendu *et al*. 2010), and the use of low concentrations of glutathione in the preculture step of cryopreservation of citrus shoot tips, which was beneficial in post-thaw recovery (Wang and Deng 2004). Alternatives to antioxidants can also be used to mitigate oxidative stress damage to plant cells during cryopreservation. The use of desferrioxamine (a powerful Fe^{3+} chelator which interferes with OH• generation from H_2O_2 in the Fenton reaction), for example, promoted post-thaw recovery after cryopreservation of rice cell cultures (Benson *et al*. 1995), whereas cathodic water has been used to reduce oxidative stress and promote shoot development after cryopreservation of *Strychnos gerrardii* axes (Berjak *et al*. 2011).

Transcriptome profiling

A more recent area of study is that of transcriptome changes that occur due to the stresses imposed throughout cryopreservation. Studies by Ren *et al*. (2015) and Gross *et al*. (2017) explored the regulation of transcripts in response to exposure to CPAs and LN on *A. thaliana*. Ren *et al*. (2015) primarily looked at the effect of addition of vitamin C to PVS2, and its effect on genes related to ROS stresses. It was seen that, compared with controls, plant tissue incubated in PVS2 with vitamin C upregulated genes associated with antioxidant formation as well as genes associated with ROS-scavenging. During osmo-protection, receptors for drought, osmotic, cold and salt stresses were upregulated. Gross *et al*. (2017) characterised transcriptional regulation due to exposure to CPAs, PVS2 or PVS3 before cryopreservation, compared with a control of exposure to LN only. They found that 180 transcripts exhibited modified expression due to treatment with CPAs or cryopreservation. Of these, 67 were related to general stress responses, defence, wounding, lipid and carbohydrate production, abscisic acid regulation, oxidation, temperature stress and osmoregulation. It was observed that PVS2-regulated genes were involved in an oxidative response, whereas transcripts of PVS3-regulated genes were involved in a more general metabolic response. Transcriptional analysis allows for in-depth understanding of the stresses imposed on plant material during cryopreservation protocols and may help to identify changes in protocols that could result in greater survival.

Advancements in cryopreservation protocols

The vitrification protocol is currently the most commonly used cryopreservation technique, and hundreds of plant species have been successfully cryopreserved in this way (Sakai and Engelmann 2007). The vitrification method has continued to develop and improve over time, with approaches such as vacuum infiltration vitrification (VIV) (Nadarajan and Pritchard 2014; Funnekotter *et al*. 2015), cryo-plate protocols (Yamamoto *et al*. 2011, 2012), and a new cryo-mesh (Funnekotter *et al*. 2017*a*) protocol created to increase success in cryopreservation.

VIV as an alteration to cryopreservation protocols involves applying a vacuum to plant material during the incubation phases, whether that involves loading solutions, CPA solutions or a combination of both. The vacuum is thought to reduce trapped air bubbles on the surface of plant material, thus increasing the surface interactions between plant membranes and the surrounding solution and resulting in a more uniform infiltration and a potentially greater intake of CPAs (Nadarajan and Pritchard 2014). Using the VIV method, Nadarajan and Pritchard (2014) achieved greater post cryopreservation viability and regrowth in embryos of *Carica papaya*, *Passiflora edulis* and *Laurus nobilis* seeds, with a 10-fold reduction in PVS2 exposure times at both pretreatment temperatures of 0 and 25°C. Funnekotter *et al*. (2015) also investigated the use of VIV as an addition to cryopreservation protocols on shoot tips of species native to Western Australia. It was observed that the use of a vacuum during PVS2 incubation resulted in increased post-cryopreservation survival, along with a reduction in CPA incubation times and regeneration times. Of great interest were shoot tips of *L. cinerea*, which did not achieve shoot tip regeneration under a standard protocol but achieved up to 10% regeneration with the addition of vacuum conditions during PVS2 incubation.

The cryo-plate protocol developed by Yamamoto *et al*. (2011) is a modification of the encapsulation-vitrification protocol. The

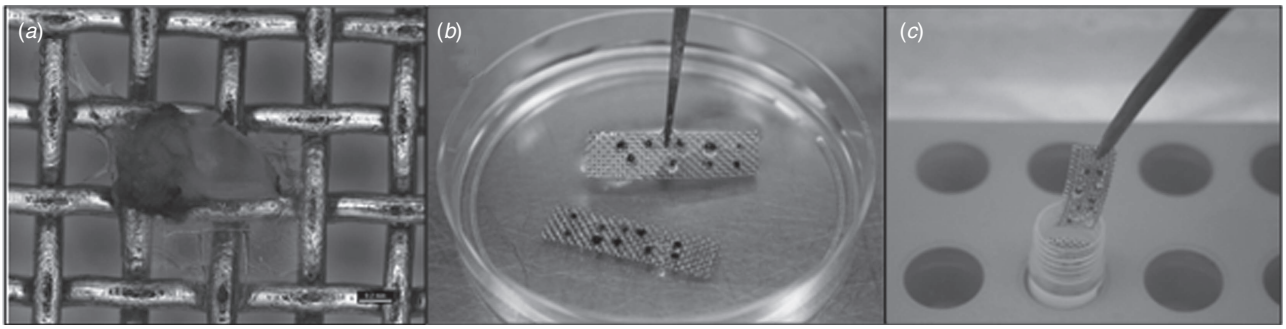

Fig. 5. Cryo-mesh cryopreservation method. (*a*) Germplasm material (shoot tip) is anchored onto 25 × 7 mm mesh strip using sodium alginate encapsulation. (*b*) Multiple shoot tips on each cryo-mesh can then be exposed to cryoprotective agents. (*c*) Cryo-mesh plunged into liquid nitrogen for cryo-storage (Funnekotter *et al*. 2017*a*).

cryo-plate is an aluminium strip with wells of set diameter in which excised shoot tips can be placed. The excised shoot tips are embedded in wells with alginate to secure them to the well. Having multiple shoot tips attached to a cryo-plate helps to reduce mechanical damage through reduction of direct handling of shoot tips during the transition between solutions and LN (Yamamoto *et al*. 2011). The cryo-plate protocol has been further optimised, and in the case of *Mentha* spp. Yamamoto *et al*. (2012) achieved post-cryopreservation regrowth between 73–100% across 16 species. Funnekotter *et al*. (2017*a*) investigated an alternative to the cryo-plate protocol in which an aluminium mesh, deemed cryo-mesh, was used in place of the cryo-plate with fixed well sizes. Cryo-plates require specific and consistent production of wells or shoot tips may not be able to adhere. Cryo-mesh overcomes this by allowing the alginate to act as a glue between the mesh and the shoot tips (Fig. 5) (Funnekotter *et al*. 2017*a*). Cryo-mesh efficacy was tested in comparison to droplet-vitrification with cryopreservation of the West Australian native species *A. viridis*. No significant difference in post-cryogenic generation was observed between the two protocols: 78% for droplet-vitrification and 83% for cryo-mesh. Cryo-mesh, however, showed efficacy in reducing the manufacturing precision required over cryo-plates, as well as reducing the operator skill required in comparison to the droplet-vitrification protocol (Funnekotter *et al*. 2017*a*).

Meristem shoot tips from tissue cultured plants are commonly used in cryopreservation and are desiccated by osmotic means; however, excised zygotic embryos and embryonic axes are the preferred materials for cryopreservation as they allow for whole plants to be generated without complex tissue culture protocols (Ballesteros *et al*. 2014). It is known that the partial drying of material pre-cryopreservation is pivotal to survival. However, the loss of freezable water in recalcitrant species affects metabolism, with damage being dependent on the rate at which water is removed (Varghese *et al*. 2011). New desiccation methods, such as flash drying, followed by addition of CPAs, have been shown to reduce water contents further than slow drying without loss of survival (Wesley-Smith *et al*. 2001). The positive effect of flash drying has been shown in embryonic axes of jackfruit (*Artocarpus heterophyllus*) in which Wesley-Smith *et al*. (2001) found that axes that underwent rapid drying (<90 min) maintained 100% survival at WC of 0.4 g g^{-1} DW, in contrast to total mortality of embryonic axes brought down to this WC by 2–3 days of osmotic desiccation. Ballesteros *et al*. (2014) looked at the effect of flash drying on various species in relation to dehydration rates. Large differences between species were observed, with dehydration between 18–92% after 30 min at constant air flow. It was reported that, in general, the species with the highest rate of drying exhibited the highest rate of post-cryopreservation survival. Flash drying provides a novel technique that has the potential to be incorporated into cryopreservation protocols with Australian recalcitrant-seeded species, reducing desiccation times without having to risk additional stress and damage due to exposure to toxic CPAs or highly osmotic solutions.

Conclusions

The diversity and complex range of different physiologies exhibited by Australian plants, especially in regard to recalcitrant-seeded species (and other groups that have unusual seed characteristics), are major impediments to seed banking as the sole means of *ex situ* plant conservation. In addition, the time available to develop effective *ex situ* germplasm conservation for threatened species (and indeed the wider floras of threatened plant communities) before they become extinct is likely to shrink even further with the added threats of diseases such as myrtle rust. Cryopreservation is currently the only viable alternative for long-term biosecure germplasm storage of seeds from species that cannot be seed-banked effectively. Progress has been made in developing a more holistic understanding of the stresses that cryopreservation imposes on plant germplasm, and in developing novel cryopreservation protocols to counteract them. This has greatly improved the scope for conservation of rare, threatened and recalcitrant Australian species. However, this progress requires continued dedicated research to enable further development of viable and efficient cryopreservation protocols applicable to species in highly biodiverse but threatened ecosystems, such as Australian sub-tropical and temperate rainforest remnants, where a relatively high proportion of species possess recalcitrant seeds, and for species with special storage requirements such as the short-lived seeds of orchids. Failure to appreciate the urgency of the precarious future of many threatened Australian plant taxa will inevitably hasten their permanent demise from the unique palette of our biodiversity heritage.

Conflict of interest

The authors declare no conflicts of interest.

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