

Chapter 2

Cryopreservation Theory

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2.1 Introduction

Cryopreservation protocols contain components which are usually developed empirically using plant-specific strategies that enhance survival. The theory of cryopreservation encompasses several interconnected disciplines ranging from the physiological to cryophysical. Water status and cryoprotection are the most influential determinants of survival in combination with physiological factors. This chapter introduces the basic principles of cryopreservation theory and aspects of biological chemistry pertinent to protocol development. This review is targeted at researchers new to the field and emphasis is placed on understanding cryoprotection, the physiological role of water and the manipulation of its different states. A theoretical understanding of cryobiology will help cryoconservationists to optimize their storage methods and enhance the long-term preservation of plant and algal collections in a stable cryogenic state.

2.2 The Biological Chemistry of Water

Water, nature's biological solvent, is highly influential in determining survival after exposure to liquid nitrogen (LN). For its small size, H₂O is a complex and behaviourally anomalous molecule, possessing special properties without which the existence of life would not be possible. These

pertain to its polar, electrostatic, cohesive, solvent, buffering and thermal stabilizing characteristics.

The behaviour of water in liquid and frozen states is largely dictated by its *bipolar chemistry*. Its hydrogen atoms carry a slight net positive charge and act with unpaired “negatively charged” electrons of oxygen to make water dipolar. The different parts of its *tetrahedron geometry* carry both positively and negatively charged regions. The lone pairs of electrons on water’s oxygen atom and its two polarized hydrogens enable the creation of *hydrogen bonds*. How water molecules associate with one another is influenced by H-bonding. Neighbouring molecules orient so that partially positively charged H atoms align with the partially negatively charged oxygen. As liquid water participates in electrostatic interactions with other neighbouring water molecules it is less disordered than most liquids.

Molecules drawn to water are hydrophilic; whilst those that are repelled are *hydrophobic*. The organization of hydrophobic lipids in membranes dictates the compartmentalized structure of cells. Water bodies are highly cohesive, having a robust capacity to resist rupturing if placed under tension, due to H-bonds. In plants these *cohesive forces* support the integrity and movement of water in vascular tissues; these are biological pipelines which, via transpiration and surface evaporation, deliver nutrients from the soil to the plant.

Because of its electrical nature, water clusters in “hydration shells” around individual ions or molecules; this specifies the *hydration state*, the number of H₂O molecules associated with solutes. Water thus maintains dissolved substances in a fluid state. The relationship between water and solutes defines the *colligative properties* of a system and specifically the *concentration of dissolved solutes* in the cell.

In contrast, the *osmotic properties* of a system are defined by flow of a solvent across a *semipermeable membrane*, which permits the passage of the solvent, but not the solutes. Osmotic pressure is the pressure that must be applied to a solution to prevent the flow of a solvent across the semipermeable membrane. Solutions separated in this way must equilibrate with respect to their molecular concentration. Water flows from a weaker to a stronger solution: when equilibrated, solutions are termed *isotonic*. Tonicity measures the extent to which a concentrated solution causes water to move in or out of cells. Solutions that cause cells to swell are *hypotonic*, as the bathing solution has a lower solute content than the cell. In plants, cellular swelling caused by hypotonic solutions creates *turgor pressure*. This is observed as an expansion of the cytoplasm and the pushing of the plasma membrane against the rigid cell wall. This process is affected by the swelling of the central vacuole which gains water. Unlike animal cells, hypotonic plant cells do not usually burst because the cell wall holds

them in place. Turgor pressure is structurally important in plants because it maintains them in an erect condition. Solutions that cause cells to shrink are *hypertonic*; they have a higher solute level than the cell. When a plant cell is placed in a hypertonic solution the plasma membrane recedes from the wall as the vacuole loses water; this process is plasmolysis.

To summarize, H₂O, water has unique physio-chemical properties essential for cell function. Cryobiological manipulations influence the different states of water placing it in an important and central role in cryopreservation and storage stability (as summarised in Fig. 2.1).

2.3 Thermal Properties of Water and Ice

The temperature of a given substance may be considered a measure of its molecular motion or “energy”. High numbers of H-bonds in liquid water “buffer” large intracellular thermal fluctuations and stabilize the impacts of temperature on organisms living in aquatic environments. Liquid water is dynamic and, at its interface with other molecules or at exposed surfaces, H-bonds constantly break and re-form. If a large amount of energy impacts the system or it is exposed to differential humidities (e.g. powerful desiccants and air-drying), H-bonds remain broken and individual molecules are expelled by evaporation. The energy applied converts liquid water to vapour and when molecules are discharged they take energy with them and this lowers surface temperature.

In contrast, at temperatures at or below 0°C, H₂O resists the breaking of H-bonds and the molecules lock together in a lattice-like symmetry. This creates pockets of “open space” between more tightly associated parts of the structure. Ice is formed, which is less dense than liquid water. The anomalous density of solid, compared to liquid water is the reason why ice sheets occur near the surface of aquatic bodies. Ice acts as a “frozen duvet” insulating the liquid phase of water beneath and protecting aquatic organisms from freezing.

2.3.1 Ice Nucleation

How liquid water associates to form ice is a point of debate (Benson et al. 2005; Mazur 2004; Franks 1972). Critical to the development of cryopreservation protocols is the control and/or avoidance of intracellular *ice nucleation*. This is also termed “*seeding*”, the point at which ice crystals are initiated. It occurs when local thermal properties of a system make it energetically possible for H₂O molecules to come together to form sufficient H-bonds

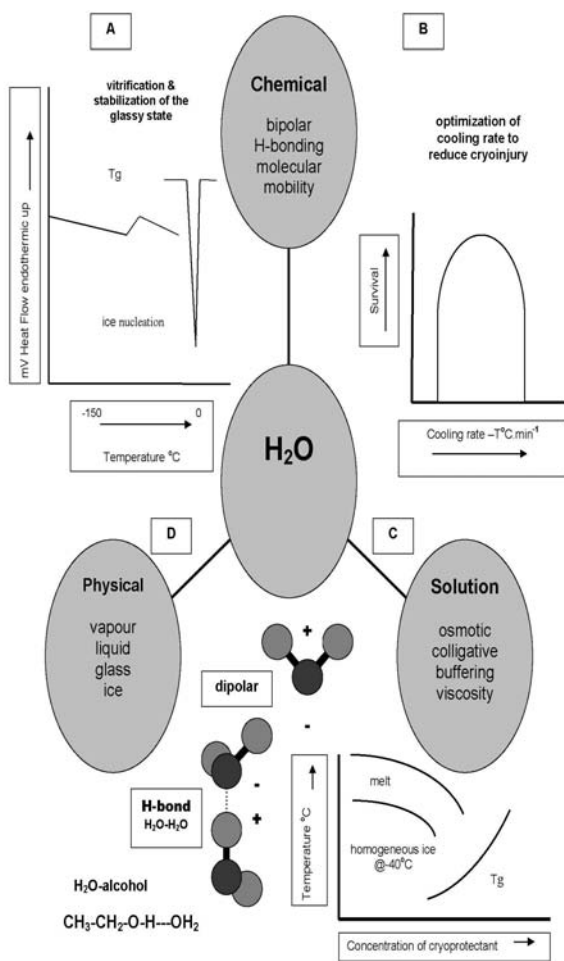


Fig. 2.1 Properties of water and their relevance to cryoprotection: **(A)** Thermal analysis used to confirm stabilities of vitrified states, ice nucleation on cooling and rewarming, and glass transition temperatures (T_g); **(B)** Optimization of cooling rate to reduce cellular damage and enhance survival as depicted by the inverted “U” and two-factor theory of cryoinjury proposed by Mazur (2004); **(C)** Water as a biological solvent exemplified by a phase diagram demonstrating the effects of cryoprotectant concentration on ice nucleation, melting and T_g; **(D)** Chemical properties, of water, bipolar characteristics, and hydrogen bonding (H-bonds) with self and other molecules (e.g. cryoprotective additives such as alcohols, polyols and sugars)

to enable initiation of an embryonic ice crystal. It is popularly believed that water freezes at 0°C, but this is rarely the case. In the absence of templates that allow the coming together of H₂O molecules, water supercools to freezing points below zero. The lowest possible supercooling temperature in most biological systems is the point of homogeneous ice nucleation, around or at -40°C. At this temperature, water molecules form an “ice embryo” of a critical size that is thermodynamically capable of growing a crystal. This creates an ordered matrix and energy is released as the latent heat of fusion and as ice is formed heat is produced.

Ice nucleation has important consequences. When liquid water is removed from the system to form ice, solutes become more concentrated. This lowers the temperature at which further ice is formed (Fig. 2.1a, c). One way of considering this is that the solute molecules become increasingly concentrated and impede the ability of the remaining water molecules to interact to form crystals. Above the temperature of homogeneous ice formation the freezing temperature of water becomes increasingly depressed to a point known as the eutectic. At this stage, the whole system solidifies and there is no further change as all available water has “frozen out”. The eutectic has coordinates for temperature and concentration and it applies to two or more substances that are able to form solid solutions with one another and lower each other’s freezing point to a minimum temperature.

After the initial ice nucleation event, ice crystals have the capacity to grow into complex clusters forming networks that can grow exponentially as more and more water molecules participate in complex alignments with each other. The implications of both extra- and intracellular ice nucleation were discussed by Muldrew et al. (2004) and Mazur (2004). Ice nucleation affects the structural, osmotic and colligative integrity of cells causing physical ruptures and mechanical injury. Colligative damage results from excessive solute concentrations that jeopardize cellular function.

In cryopreserved plants, the dynamics of the two factors involved in cryoinjury are moderated by differentials in the ability of the intracellular and extracellular components to initiate ice crystals. Freezing usually occurs when it is energetically and physically (in terms of the availability of ice templates) more favourable. With the exception of very rapid freezing temperatures, ice preferentially forms *extracellularly*. In this scenario, a deficit is created between the partially frozen outside of the cell and the unfrozen inside and water molecules migrate to the outside to establish osmotic equilibrium. Cells continually exposed to this process shrink and the rate of water loss can be expressed as a function of cell size. This is more applicable to animal as compared to plant cells which normally have a rigid cell wall that defines size and shape. The osmotic gradient and

permeability of the plasma membrane also affect water movement, through diffusion and the passage of water molecules across aquaporin pores (Tyerman et al. 2002).

2.3.2 *Controlled Rate Cooling*

Controlled cooling aims to optimize two injurious components: damaging colligative solution effects and ice formation (Fig. 2.1b). These are moderated by the precise control of cooling rate, terminal freezing parameters (transfer and holds at intermediate subzero temperatures), ice nucleation and the application of colligative protectants (Chap. 5). The underlying principle that determines cell survival in this type of protocol is the rate of cooling (Fig. 2.1b), figuratively described as the inverted “U” of cryoinjury (Mazur 2004). If the rate is too fast or too slow, the cells do not survive and balancing these two cryoinjuries is the key to success. When a controlled, gradual rate of cooling is applied to cells, extracellular ice first forms, a differential water gradient is created across the cell membrane and intracellular water moves to the outside. This has an important cryoprotective effect as it reduces the amount of water available to form ice. Although it is a counterintuitive concept, extracellular ice has a vital cryoprotective role as its formation indirectly reduces the potential for ice nucleating inside the cell. Cells can survive extracellular ice formation, although it can be harmful, particularly for complex multicellular tissues. Intercellular ice is almost always lethal. This accounts for one arm of the “U” (Fig. 2.1b), but if cooling is too slow, the cell’s solutes become excessively concentrated causing colligative damage and this accounts for the other side. The key is to balance the two injuries by optimizing cooling rates so that just the right amount of water is removed to prevent colligative injury. Any water that does remain is so minimal that ice formation is inhibited and the cytoplasm most likely vitrifies.

Generally, methods for controlled rate cooling include a sequence of manipulations by which the sample is precultured or acclimated and then cryoprotected. Usually, DMSO is the main penetrating colligative cryoprotectant and it may be combined with non-penetrating osmotically active additives such as sugars or polyethylene glycol. Samples are exposed to a specific cooling rate (range -0.1 to $5^{\circ}\text{C}\cdot\text{min}^{-1}$) usually called a “ramp” during which a step may be incorporated for the operator or the machine to “seed” extracellular ice, and initiate an exothermic reaction. The ramp then continues to an intermediate or *terminal transfer temperature* (usually at or above the temperature of homogeneous ice nucleation, -35 to -40°C). One or more ramps with different rates of cooling may be incorporated into a

protocol. Samples may be plunged at this point, or a *hold* (of 30–45 min) at the terminal transfer temperature may be instituted, followed by a direct plunge into LN. Hence, this type of protocol is also called *two-step cooling* or freezing. The slow rates of cooling may also be achieved using alcohol baths, or self-contained units (containing methanol or isopropanol) placed in a -20 or -80°C freezer. For example, isopropanol contained in a Mr Frosty unit provides a $1^{\circ}\text{C}\cdot\text{min}^{-1}$ cooling rate. These methods are sometimes termed traditional because they were some of the first techniques developed (Benson 2004; Day and McClellan 1995; Kartha 1985; Sakai 1966; Uemura and Sakai 1980; Withers 1975; Withers and King 1980). However, this is a general term that could also apply to early vitrification techniques, so it should be avoided.

2.4 Vitrification: The Glassy State

So far cryopreservation has been considered in terms of the liquid and solid (ice) phases of water. It is also possible to cryopreserve plants by the process of “*vitrification*”, the solidification of liquids without crystallization. This comprises a “glassy state” as the system is *amorphous*, lacks organized structure but possesses the mechanical and physical properties of a solid (Taylor et al. 2004). Vitrification of water in biological systems is dependent on increased cell *viscosity*, occurring as cell solutes become concentrated. Increased viscosity inhibits the coming together of water molecules to form ice. The vitrified state is *metastable*, meaning that it can relatively easily revert back to a liquid and/or devitrify to form ice (Fig. 2.1a, c). Vitrified systems are particularly unstable during rewarming as changes in molecular mobility and energy may be sufficient to allow water molecules to rearrange (relax) and form ice. Vitrification can be achieved in a number of ways but all usually have the end result of increasing solute concentration to a critical viscosity. For temperature-induced glasses, the point at which this occurs is called the *glass transition temperature* (T_g); molecular motion nearly ceases and the liquid becomes a glassy solid (Fig. 2.1a).

Ice-free cryopreservation was pioneered for animal cells (Fahy et al. 1986) and extensively applied to plants by Sakai (2004). This cryoprotective regime completely eliminates ice formation inside and outside the cell. Vitrification is used to advantage in cryopreserving complex, heterogeneous tissues for which it is difficult to achieve optimum cooling rates. Plant materials may be preconditioned on special media or with specific growth conditions, precultured with cryoprotective chemicals just prior to cryopreservation, and protected with vitrification solutions prior to plunging in

LN (Chap. 3). Alternatively, plant samples are encased in an alginate solution, osmoprotected with sugar solutions, and dried to low moisture contents before plunging in LN (Chap. 4).

2.5 Water State Transitions and Instabilities

Thermal analysis using *differential scanning calorimetry* (DSC) is used in fundamental cryobiology to assist cryopreservation protocol development (Fig. 2.1a). Heat flow and water state transitions are monitored in DSC samples as a function of time and temperature, and changes can be detected during the cooling and heating of samples. This approach is used to evaluate the efficacy of cryoprotectants, particularly in stabilizing the glassy state (Benson et al. 2005) and the development of protocols for highly recalcitrant plants. *Cryomicroscopy* is also a useful tool which explores real-time transitions between water states in the presence of cryoprotectants (Fleck et al. 2006).

The behaviour of water in different state transitions and its influence on survival was evaluated by Taylor et al. (2004) with respect to the relative stabilities of amorphous, liquid and solid phases and their potential for causing thermo-mechanical stresses in biological tissues. Glasses are usually considered to be more unstable and their labile properties are often observed as cracking, or fractures which are particularly damaging to larger organs and tissues such as seeds. Glasses can also undergo relaxation during rewarming, which can be innocuous, but may lead to devitrification and ice crystal formation. This is observed in cryovials as the transparent glasses become opaque when ice is formed. For many vitrification systems, rapid rates of rewarming are prescribed to avoid the possibility of ice nucleation when the samples pass through their T_g . This practice should be undertaken with caution as stress cracks and fractures can occur if rewarming is too rapid. Two phases may be required, first a short (e.g. 1–2 s) slow phase (e.g. at ambient temperatures) to allow for glass relaxation without stress fracturing, followed by a rapid warming (in a water bath at 45°C) to ensure the speedy transition from glass to liquid without passage through an ice phase. Empirical optimization of these phases may be required for each biological system and its cryoprotective regime.

Liquid-ice-liquid transitions common to controlled rate cooling protocols have different biological consequences compared to those of totally vitrified systems. It is important to insure the initial transition from liquid to frozen state by manually or automatically “seeding” ice nucleation. This nucleation prevents supercooling of the sample with resulting spontaneous nucleation at –40°C and death of the sample. Control of the point at which

extracellular ice is first formed consequentially controls the sequence of events key to colligative cryoprotection and freeze dehydration.

Rewarming can also affect ice stability and is dependent upon the type of tissue, rates of cooling and the cryoprotective additives used. In general, higher survival is achieved when slow cooling is followed by rapid re-warming (e.g. in a water bath at 40–45°C). Very small and innocuous ice crystals may grow into larger, more damaging crystals if a frozen system is rewarmed too slowly. These ice-liquid transitions can be potentially damaging as expansions and contractions may occur non-uniformly across cells, tissues and organs; these can be induced by a combination of thermal, colligative and osmotic stresses (Taylor et al. 2004).

2.6 Cryoprotection

Many temperate and cold-adapted plants are naturally equipped to withstand freezing in their natural environments (Hirsh 1987; Meryman and Williams 1985; Pearce 2004; Sakai 1956, 1960) and an appraisal of their behaviours provides a logical starting point.

2.6.1 *Biophysical Aspects of Natural Freezing Tolerance*

In the absence of ice nucleators, it is potentially possible for water in biological systems to supercool to around -40°C , the point of homogeneous ice formation. This is an avoidance strategy used by plants to resist freezing at natural subzero temperatures. Thus supercooling is an important adaptation in cold-tolerant tree species and it accounts for the location of tree line on mountains, the point above which vegetation cannot survive (-40°C). This adaptation allows survival of *dormant* tissues. It is termed *cold hardening* or *acclimation* and generally involves the synthesis of intracellular solutes which increase cell osmolality and reduce plasmolysis injury at freezing temperatures.

Meryman and Williams (1985) cautioned that freeze avoidance by these mechanisms would only permit tolerance to temperatures around -15°C . It was therefore necessary to explore how, and why, cold acclimation (CA) adaptations might be applied to recover plants from much lower cryogenic temperatures (as demonstrated by Sakai 1956, 1960). Hirsh et al. (1985) pioneered fundamental DSC and freeze-etch/fracture electron microscopy studies on a cultivar of *Populus balsamifera*. They formulated the first definitive explanation as to why resistance to LN was possible in naturally cold-acclimated woody plants. Their findings support their hypothesis that

aqueous glasses form intracellularly. Three distinctive Tgs (glass transitions) were detected using thermal analysis, these remained stable at relatively high, subzero temperatures and vitrification was concomitant, with hardened tissue having high levels of free sugars (Hirsh 1987). Hirsh went on to postulate that proteins in conjunction with sugars caused the solutions to have Tgs at temperatures lower than those of water and sugars alone. This pointed the way towards a bio-molecular understanding of natural cold tolerance in plants.

2.6.2 Aspects of Natural Freeze Tolerance

It is outside the scope of this chapter to provide a comprehensive review of the molecular biology of natural freezing and CA responses. The reader is directed to Thomashow (1999), Xin and Browse (2000), Pearce (2004), Thorlby et al. (2004) and Chen et al. (2006) for further insights. Xin and Browse (2000) report the involvement of reduced hydration status; growth; increased antioxidants, osmotic regulation; the accumulation of polyols, sugars, proline, betaine, increased transcription, stability and down regulation, changes in hormone status, and lipid composition of membranes; fatty acid unsaturation and cell wall modification from CA. Pearce (2004) proposed that an array of molecular mechanisms is involved in CA and that these are likely to be related to dehydration tolerance. This is not a surprising assumption, considering the links between freezing injury, colligative and osmotic damage. Gaining an understanding of natural cold-acclimation mechanisms is highly pertinent to cryopreservation protocol development.

In temperate species and naturally cold-hardy plants, acclimation can enhance the ability to withstand LN and is usually achieved by exposing donor plants to low, near zero temperatures. Standard CA is often at 4°C in the dark, but CA is more effectively applied in a diurnal temperature cycle (Chang and Reed 2000; Reed 1988). CA is also affected by environmental parameters other than cold *per se*, as demonstrated by Bourion et al. (2003), who demonstrated that CA in *Pisum sativum* L. plants did not occur at low temperatures if light intensity was low. However, light was not a significant factor for the CA of pear or blackberry shoots. Shoots developed significantly better cold tolerance when grown under a 12–16 h cold period and a short warm period than with a constant temperature, either with a light cycle or in the dark (Chang and Reed 1999, 2000). Future explorations of CA will undoubtedly be dependent upon molecular and transgenic technologies (see Chen et al. 2006).

2.6.3 Artificial Cryoprotection

The cryobanking era commenced with the serendipitous and milestone discovery of Polge et al. (1949) that glycerol protects avian sperm cells against freezing. After this breakthrough, mammalian and health care cryobiological research explored the mode of action of cryoprotectants in terms of colligative theory (Lovelock 1953; Meryman and Williams 1980, 1985). In Lovelock's key study, red blood cell injury occurred when a critical concentration of applied extracellular salt was exceeded, independent of glycerol being applied or the freezing temperature required for achieving the critical salt concentration. This was interpreted that glycerol penetrates the cell, and on a colligative basis reduces the amount of ice formed at any temperature. That is, it acts as antifreeze by reducing the concentration of extracellular salt and water lost due to osmosis. The cell is protected against reaching a *minimum lethal cell volume* with the additional benefit of lowering the temperature at which freezing actually occurs. A *colligative property* of a solution depends only on the ratio of the number of particles of solute and solvent in the solution, not the identity of the solute. The *theory of colligative cryoprotection* has two essential attributes:

1. Cryoprotectants must be able to penetrate the cell, or else they will cause osmotic dehydration and cause the very injury they are used to protect against.
2. Cryoprotectants must be non-toxic to the cell at the concentrations required for their efficacy.

Penetrating cryoprotectants make a contribution to the overall osmolality of the cell. A colligative additive such as glycerol increases the initial osmolality of the cell before the freezing process is initiated. Hence, the level of water that needs to be frozen out to achieve osmotic equilibrium is far less, and the extent of the dehydration that occurs in the cell is better tolerated (Meryman and Williams 1985). There is an additional protective advantage in that this occurs at a lower temperature as the additive depresses the freezing point. The most common colligative (penetrating) cryoprotectants used in plant cryopreservation are glycerol, dimethyl sulphoxide (DMSO), methanol (and sometimes ethanol) and the smaller molecular weight glycols.

It is important to consider colligative cryoprotection in relation to the dynamics of the freezing process and revisit (Fig. 2.1b) Mazur's "inverted U theory" of cryoinjury (Mazur 2004). The aim of controlling cooling rate is to optimize cell dehydration between too little (resulting in ice damage) and too much (resulting in colligative damage). Colligative agents moderate

the extent to which excessive and insufficient dehydration occurs. For example, if glycerol is present, damage to the cell will only occur at a higher osmolality. It is necessary to apply a sufficiently slow cooling rate to ensure water has enough time to exit the cell. Operator-induced extracellular seeding of ice is important and creates a gradient. A “hold” in the programme at a terminal transfer temperature (the end of the slow cooling phase) allows for the completion of water efflux before exposure to LN. Colligative additives also enhance viscosity of the cell and reduce the efflux of water from the cytoplasm. Cell viscosity may be elevated to such a level that ice nucleation is inhibited (Meryman and Williams 1980, 1985). Thus, although extracellular freezing occurs, cells do not freeze intracellularly and they are preserved in a “glassy state”.

Completely ice-free cryogenic storage is the newest approach to plant cryoconservation (Benson 2004; Sakai 2004). The key to developing cryoprotective vitrification strategies is to increase cell viscosity to the point at which ice formation is inhibited and water becomes vitrified on exposure to cryogenic temperatures. Cell viscosity enhancement is achieved using two main approaches:

1. Addition of cryoprotective additives at very high concentrations.
2. Water removal by evaporative desiccation and osmotic dehydration.

In practice, many plant vitrification protocols integrate both. Vitrification achieved through the use of additives can result in cryoprotectant toxicity, osmotic injury (for non-penetrating components) and devitrification. In the case of dehydrating processes where water is removed through osmotic dehydration and/or by evaporative means (air drying or drying agents such as silica gel), there is the additional problem of desiccation sensitivity. It is advantageous to use a mixture of different additives (Fahy et al. 1986). This reduces the toxicity of any single additive, limits the impacts of extreme evaporative drying and helps to stabilise the glasses formed. It is recommended that mixtures of penetrating and non-penetrating cryoprotectants are used (Fahy et al. 1984). Most vitrification solutions applied to plants include a mixture of penetrating and non-penetrating cryoprotectants.

Whereas cooling rate is critical to the success of colligative cryoprotection, it is rewarming and glass stabilisation that is key to successful vitrification (Fahy 1987). Glass stability may be dependent upon how the glass was initially formed, particularly in relation to the water potential within the whole system. In the case of plants it may be expected that cells that have been vitrified at very low water contents through evaporative desiccation will have fewer water molecules available to participate in nucleation,

devitrification and ice growth on rewarming. In contrast, those vitrified using a combination of penetrating and non-penetrating cryoprotectants may form less stable glasses on rewarming. Comparative thermal analysis studies of the stabilities of glasses in dehydrated, desiccated alginate beads and Plant Vitrification Solution 2 (PVS2) provides some evidence for this (Benson et al. 1996). In vitrification by alginate encapsulation dehydration, glasses remained stable on passive rewarming at ambient temperatures. For vitrification solutions, the stringently controlled addition and removal of cryoprotectants and rapid rewarming is required to achieve thermal and osmotic stability.

2.7 New and Retrospective Insights in Cryoprotection

Research on both plants and animals has resulted in a number of fundamental investigations, which question and expand upon existing cryoprotection theory (Fuller 2004; Benson 2004). It is important to integrate physical and biological knowledge of cryoconservation particularly for difficult-to-preserve plants. As reviewed by Fuller (2004), polyols and sugars (particularly sucrose and trehalose) increase the glass-forming tendency of aqueous solutions which, when applied in combination with other cryoprotectants, reduce the cryoprotectant concentrations required to achieve a stable glass. Sugars offer several advantages in that they form H-bonds with water and have high molecular weights, contributing substantially to the elevation of cell viscosity. For example, desiccation tolerance in seeds has been associated with glass-formation tendencies of sugars (Koster 1991) even at ambient temperatures.

Turner et al. (2001) explored an alternative theory as to the basis of cryoprotectant efficacy by comparing different polyols and sugars with regard to the stereochemical arrangement of their hydroxyl groups. They postulated that the mode of action is based not on molarity but on the total number of OH groups present in the medium. Not too surprisingly, glycerol was the most effective. Similarly, Fahy et al. (2004) present a new theory pertaining to the basis of non-specific cryoprotectant toxicity. Their studies on mammalian cells showed that cryoprotectant solutions designed to be at the minimum concentrations needed for vitrification at moderate cooling rates were toxic in proportion to the average strength of water H-bonding by the polar groups on the permeating cryoprotectants in the system. This finding is highly relevant to plant cryobiologists, particularly for those working on desiccation-sensitive plants. The theory proposes that vitrification solutions based on achieving a minimal disturbance of intracellular

water may be superior and Fahy et al. (2004) provide an elegant explanation for this. This starts with the premise that for a solution to vitrify at attainable cooling rates, water–water interactions must be inhibited. It also considers the observations that despite the equivalence of the physical tendencies of different types of vitrification solutions to inhibit ice nucleation, they do have very different levels of toxicity. Fahy et al. (2004) propose that the toxicity of vitrification solutions increases as the strength of the water-cryoprotectant H-bonding increases. The non-specific toxicity of vitrification solutions is dictated by the availability (using a statistical prediction model) of water molecules for hydrating bio-molecules. Competition between cellular components and cryoprotectants for hydration will affect water availability for cellular constituents and will damage the cell; although how this occurs is yet to be elucidated. The future development of controlled rate cooling and vitrification protocols will benefit from this type of fundamental research.

In plants, progress in cryoprotection must also take into account their special properties. Tao and Li (1986) present the distinctive attributes of plant, compared to animal cells, classifying a range of frequently used plant cryoprotectants on their ability to penetrate cells:

1. *Unable to penetrate the cell wall* (high molecular weight polymers (PEG₆₀₀₀, PVP), polysaccharides and proteins)
2. *Penetrable through the cell wall only* (oligosaccharides, mannitol amino acids (proline) and low molecular weight polymers PEG₁₀₀₀)
3. *Penetrable through the cell wall and membrane* (DMSO and glycerol)

Importantly, the plant cell wall is often overlooked, particularly as animal models of freezing and cryoprotective theory are presented as standard. Tao and Li (1986) propose that each classification of cryoprotectant has a different function and to provide optimum protection, cryoprotectants should be used in combination in plants. Penetrating cryoprotectants act colligatively, but they may also produce temporary plasmolysis as they penetrate the wall and loosen adhesion between the wall and cell membrane. Those that penetrate the wall, but not the plasmalemma prevent ice crystals from damaging the cell membrane and also buffer the cytoplasm from excessive dehydration. Cryoprotectants that remain in the intercellular spaces and do not penetrate either structure become concentrated at the ice crystal front, inhibit the rate and extent of ice growth and protect against mechanical deformation.

A similar approach is now required to critically evaluate the *modus operandi* of vitrification in plants (Sakai 2004). There are now a number of permutations of the two main approaches to creating a glassy state in plants. Usually, these are alginate encapsulation dehydration (using osmotic dehydration with sugars and evaporative desiccation) and the application of additives such as PVS2. Volk and Walters (2006) recently applied DSC to help elucidate the mode of action of the PVS2 cryoprotectant mixture developed by Sakai and colleagues (Sakai 2004). They propose that PVS2 operates through two cryoprotective mechanisms: (1) it replaces cellular water, and (2) it changes the freezing behaviour of water remaining in the cells. Volk and Walters (2006) also put forward the theory that the penetration of some of the components (e.g. DMSO) of PVS2 into the cell is central to its cryoprotective efficacy. Significantly, the assumption that the mode of action of PVS2 is primarily due to osmotic dehydration cannot therefore constitute a full explanation for its high efficacy. Rather, cell-penetrating constituents of PVS2 replace water as the cells become dehydrated and prevent injurious cell shrinkage caused by dehydration (Volk and Walters 2006). Penetrating PVS2 cryoprotectants therefore have a colligative role, moderating the effects of osmotically active, non-penetrating components of the mixture, which includes high concentrations of sucrose and glycerol. The solution is effective because protection is imparted before exposure to cryogenic treatments as some components of PVS2 are sufficiently mobile to permeate cells at 0°C and displace water. When the temperature is subsequently reduced, the penetrating components of PVS2 cryoprotect the cells by restricting the molecular mobility of water molecules and prevent them from nucleating ice crystals. Buitink and Leprince (2004) present a similar view, such that to fully understand the glassy state it is important not only to measure thermal behaviour (e.g. as Tgs) but also to elucidate the molecular mobility and H-bonding properties of the systems.

Cryoconservationists are increasingly exploiting and amalgamating cryoprotectant modalities which combine various vitrification, colligative and biophysical/molecular attributes. These resulted in the cryopreservation of a diverse range of species. Recent studies of plant cryoprotectant behaviour suggest that a greater theoretical understanding of their mode of action is required. This is particularly important for affirming the long-term stability of stored plants using different cryoprotective strategies, which may potentially and differentially affect their stability.

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