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Current Issues in Plant Cryopreservation

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1. Introduction

Plant cryopreservation involves the storage of plant tissues (usually seed or shoot tips) in liquid nitrogen (LN) at -196°C or in the vapour phase of LN at -135°C in such a way that the viability of stored tissues is retained following re-warming (Day et al., 2008; Hamilton et al., 2009). Cryopreservation is usually applied to species with recalcitrant (i.e. dehydration sensitive) seeds that are not storable by any other means, or preservation of specific cultivars of vegetatively propagated crop plants like banana or potato, or for unique ornamental genotypes (Halmagyi et al., 2004; Kaczmarczyk et al., 2011a; Panis et al., 2005). Another reason to utilise cryostorage is to conserve endangered plant species, particularly where seeds may be extremely scarce or of doubtful quality and/or the species is threatened with imminent extinction (Decruse et al., 1999; Mallon et al., 2008; Mandal & Dixit-Sharma, 2007; Paunescu, 2009; Sen-Rong & Ming-Hua, 2009; Touchell et al., 2002).

The main advantage of cryopreservation is that once material has been successfully cooled to LN temperatures, it can be conserved in principle indefinitely, because at these ultra-low temperatures no metabolic processes occur. Replenishing a small volume of LN weekly in cryo-dewars is the only on-going maintenance operation usually required in cryostorage. There are further advantages to this approach: the low costs of storage, minimal space requirements and reduced labour maintenance compared to living collections and even when compared to maintenance of tissue cultures at room temperature. Once in storage, there is no risk of new contamination by fungus or bacteria, and cryogenically stored material has been reported to retain genetic stability (Harding, 2004). Depending on the species, small cryopreserved samples may take several weeks to re-establish shoot cultures, and several months to a year may be required to produce micropropagated plants capable of transfer to soil under greenhouse conditions and (following weaning) into the field.

Shoot tips (containing the apical meristem) are the most commonly used plant material for cryostorage. The apical meristem is composed of small unvacuolated cells served with a relatively small vascular system. The organised structure of apical meristems generally results in direct shoot formation after re-warming, thereby maintaining the genetic integrity

of the resulting propagated material. While callus tissue (unorganised wound tissue) can also be cryostored, the risk of occurrence of genetic deviations may be higher when utilising the indirect organogenesis pathway. Besides shoot tips, callus cultures, cell cultures, somatic embryos, pollen or plant buds as well as recalcitrant and orthodox seeds can be used as explants in plant cryopreservation (Reed, 2008).

Plant cryopreservation began with research on the freezing of mulberry twigs in LN (Sakai, 1965). Since then, a huge variety of plants and genotypes have been successfully cryostored for conservation of agriculture and horticultural genotypes, as well as for endangered and threatened plant species (Gonzalez-Arnao et al., 2008; Hamilton et al., 2009; Kaczmarczyk et al., 2011b; Mycock et al., 1995; Reed, 2008; Sakai & Engelmann, 2007). This chapter reviews and gives examples of different plant cryopreservation protocols that have been successfully applied. It will focus on free radical damage and membrane structure, both important topics in the cryopreservation of biological tissues. The topic of genetic and epigenetic stability in plant cryopreservation is also discussed. Recent reviews of plant cryopreservation have been written by Benson (2008), Day & Stacey (2007), Hamilton et al. (2009) and Reed (2008).

2. Plant cryopreservation methods

In the last three decades a number of different cryopreservation protocols, such as classical slow-cooling, vitrification, droplet vitrification, encapsulation/dehydration and encapsulation/vitrification protocols have been developed and utilised for germplasm storage (Reed, 2008). The choice of cryopreservation method to attain the highest survival rates is largely dependent on the plant species and tissue type that is being cryostored.

2.1 Slow cooling or controlled rate cooling

This technique involves the simple dehydration of plant material before cryogenic storage in LN. This is can be done by slow cooling of the plant tissue to a temperature of approximately -40°C (Reinhoud et al., 2000). This forces the formation of extracellular ice ahead of intracellular ice, thus causing an outflow of water from the cells due to the resulting osmotic imbalance and, consequently, dehydration. Dehydration can also be brought about by incubation of tissue material on media containing a relatively high concentration of an osmo-regulant, commonly sucrose, although other compounds can also be used (Panis et al., 2002). Usually water concentrations must be decreased to between 10% and 20% of the fresh weight for optimal cryogenic survival (Engelmann, 2004). This has the aforementioned effects of reducing the extent of ice crystal formation due to the reduced water concentration and assisting in the achievement of the vitrified state of water as a result of the increased solute concentration. These techniques do not necessarily make use of cryoprotective agents (CPAs), however they can be used in conjunction with them to further improve dehydration (Reinhoud et al., 2000), though these agents can be toxic to plant cells at high concentrations (Arakawa et al., 1990). Rapid re-warming rates are used after cryogenic storage to prevent ice crystal formation during thawing (Reinhoud et al., 2000). This approach can result in extreme rates of dehydration, which can cause cell volume reductions that are potentially lethal (Day et al., 2008). It has been suggested that slow-cooling is only suitable for non-organised tissues, as sufficient dehydration is more difficult to achieve in tissues with complex structures due to the different rates of water movement between and within plant cells with different characteristics (Gonzalez-Arnao et al., 2008).

2.2 Encapsulation-dehydration

This method, developed by Fabre and Dereuddre (1990), involves encapsulating shoot tips, somatic embryos or callus cells within alginate beads. This is followed by incubation in media with high sugar concentrations in order to raise intracellular solute concentrations and promote desiccation. Finally, silica gel or airflow is used to dehydrate the beads until the moisture content drops to 20-30%, before they are immersed in LN (Fabre & Dereuddre, 1990; Hamilton et al., 2009; Reinhoud et al., 2000). The encapsulating material is thought to promote a vitrified state in the tissue regardless of the cooling and re-warming rates, thus reducing damage from ice crystal formation (Scottez et al., 1992). Mechanical stress is also reduced because the bead protects the explants from damage during handling. The benefits of this method include avoiding the use of high concentrations of (potentially toxic) CPAs (Reinhoud et al., 2000) and the presence of a nutritive bead, which may enhance post-regeneration survival or re-growth of the material following immersion in LN and re-warming (Panis & Lambardi, 2005).

2.3 Vitrification

Vitrification involves the treatment of tissues in a mixture of highly concentrated penetrating and non-penetrating CPAs applied at non-freezing temperatures, followed by rapid cooling in LN (Gonzalez-Arnao et al., 2008; Panis & Lambardi, 2005). The combination of high intracellular solute concentrations (due to dehydration and some CPA penetration) and rapid cooling prevents the nucleation of water and the formation of ice crystals, both intracellularly and extracellularly, thus promoting the vitrification of water (Kreck et al., 2011; Mandumpal et al., 2011; Reinhoud et al., 2000).

For plants sensitive to direct exposure to vitrification solutions, due to dehydration intolerance and osmotic stresses, a loading step of 10-20 minutes can be incorporated prior to incubation within the CPA solution. This is done by incubation of the samples within a less toxic/concentrated CPA solution (a media containing 0.4 M sucrose and 2 M glycerol proved highly effective [Nishizawa et al., 1993; Sakai et al., 1990]), thereby improving dehydration tolerance. The CPAs used in vitrification usually contain high concentrations of glycerol, dimethyl sulfoxide (DMSO), ethylene glycol and various sugars (Day et al., 2008). The most commonly used mixture of CPAs for vitrification is plant vitrification solution 2 (PVS2), which consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO in basal culture medium containing 0.4 M sucrose (Sakai et al., 1990, 2008).

Exposure time to cryoprotective solutions is a vital step in the cryostorage process. Volk and Walters (2006) demonstrated that the extent of penetration of PVS2 into mint and garlic shoot tips was directly proportional to exposure time. The water content of the shoot tips also significantly decreased with an increase in exposure time to PVS2 (Volk & Walters, 2006). Greater penetration of CPAs can be useful as it helps to increase the internal solute concentration and may contribute to maintaining cell volume, thus preventing damage to the cells (Meryman, 1974). However, overexposure to CPAs may cause damage to the cells owing to the toxic nature of the CPAs or excessive dehydration.

The vitrification protocol is a more widely applied cryopreservation method than slow cooling due to its ease of use, high reproducibility and the wide range of species with complex tissue

structure (such as shoot tips and embryos) that have been successfully cryopreserved with this procedure (Takagi et al., 1997; Touchell et al., 1992; Vidal et al., 2005).

2.4 Droplet-vitrification

The droplet-vitrification technique is a modification of the basic vitrification protocol that involves placing the sample within a droplet of 1-10 µl of cryoprotective solution on a piece of aluminium foil before immersion in LN (as opposed to 1-2 mL of cryoprotective solution in the original protocol), as shown in Fig. 1.

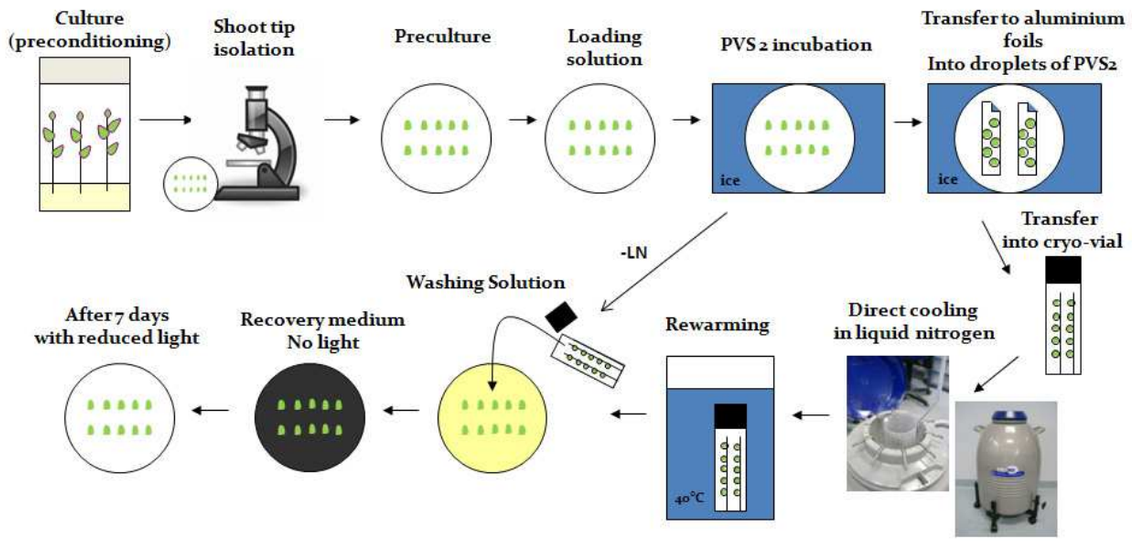

Fig. 1. Cryopreservation procedure for the droplet vitrification method.

This approach achieves higher cooling and re-warming rates, as the small volume of liquid allows a higher rate of heat transfer to and from the sample (Sakai & Engelmann, 2007). Cooling rates are increased to >130°C/sec (Panis & Lambardi, 2005), therefore facilitating the direct transition of intracellular water from a liquid state to a glassy state far more rapidly, thus minimising water crystallisation. The droplet-vitrification protocol has been successfully applied in the cryopreservation of garlic and chrysanthemum (Kim et al., 2009), yams (Leunufna & Keller, 2005), lily (Chen et al., 2011), potato (Yoon et al., 2006) and other plants (Sakai & Engelmann, 2007).

2.5 Encapsulation-vitrification

Another modification of the vitrification approach termed encapsulation/vitrification combines elements of the encapsulation/dehydration method with the vitrification method. As with the standard encapsulation method, shoot tips or calluses are first encapsulated in alginate beads and then the encapsulated material is incubated in a vitrification solution to promote sufficient dehydration and vitrification rather than dehydration under a constant airflow, which is very time consuming and relatively imprecise (Hirai & Sakai, 1999). Successful protocols have been established for potato (Hirai & Sakai, 1999), gentian (Tanaka et al., 2004), strawberry (Hirai et al., 1998) and pineapple (Gamez-Pastrana et al., 2004).

3. Free radical and oxidative damage in plant cryopreservation

While achieving an optimum cryopreservation protocol that successfully avoids ice damage is important, there are various other factors that can affect post-cryogenic survival (Fig. 2). During the cryopreservation procedure plant tissues are susceptible to a variety of stresses, including oxidative stresses (Benson & Bremner, 2004).

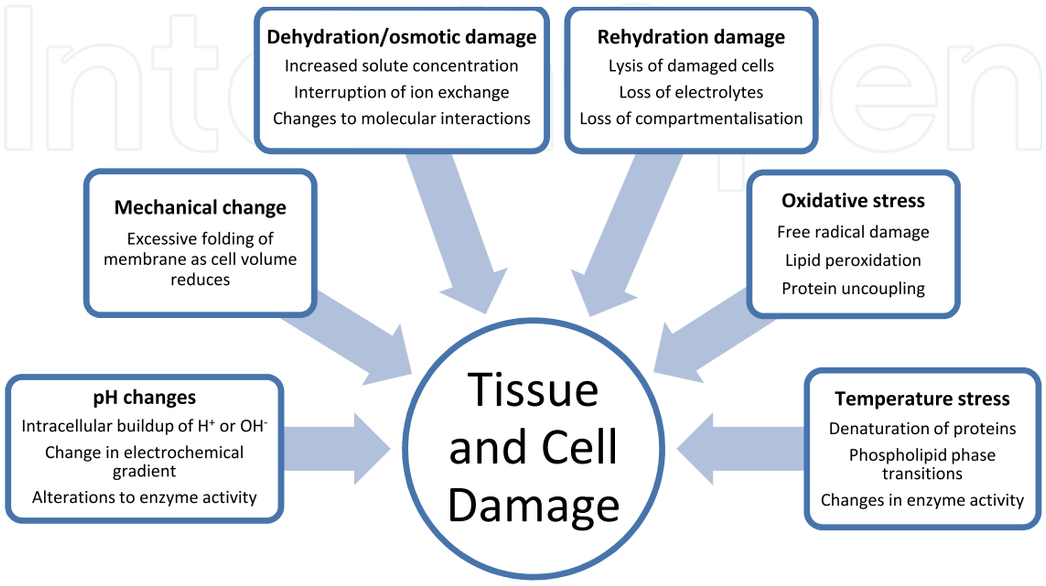

Fig. 2. Main causes of damage to plant tissues during cooling and cryopreservation (modified from [Turner, 2001]).

3.1 Reactive oxygen species (ROS)

The formation of reactive oxygen species (ROS) during cryopreservation can occur during the many steps involved in this process. For example, ROS formation has been detected in photo-oxidative stress during tissue culture, during excision of shoot apices, osmotic injury and desiccation following application of CPAs, as well as during the rapid changes in temperature when the samples are first cryostored in LN and then re-warmed (Benson & Bremner, 2004; Roach et al., 2008). ROS are highly reactive molecules that can cause a wide range of damage in cells. There is a large variety of molecules that are classified as ROS, some of which include oxygen-free radical species and reactive oxygen non-radical derivatives (Table 1).

Radicals	Non-Radicals
Superoxide ($O_2^{\bullet-}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl (OH•)	Peroxynitrite ($ONOO^-$)
Hydroperoxyl (OOH•)	Peroxynitrous acid (ONOOH)
Peroxyl (ROO•)	Hypochlorous acid (HOCl)
Alkoxyl (RO•)	Hypobromous acid (HOBr)
Carbonate ($CO_3^{\bullet-}$)	Ozone (O_3)
Carbon dioxide ($CO_2^{\bullet-}$)	Singlet oxygen ($^1\Delta g$)
Singlet oxygen ($^1\Sigma g^+$)	

Table 1. Common reactive oxygen species (ROS) (Halliwell & Gutteridge, 2007).

Many of the more active ROS are free radicals, which are molecules that contain an unpaired electron, thus being able to react non-specifically with neighbouring molecules by removing electrons and causing a self-propagating chain reaction of radical formation. The removal of electrons can lead to a loss of function and structural alterations in macromolecules like proteins, lipids and DNA (Benson, 1990; Halliwell & Gutteridge, 2007). ROS are frequently produced as by-products during cellular metabolism. The electron transport chain used in respiration and photosynthesis are the major producers of ROS, caused by the leakage of free electrons onto molecular oxygen (O_2), resulting in the formation of superoxide (Benson & Bremner, 2004; Benson, 1990; Halliwell & Gutteridge, 2007). The formation of ROS is controlled by a high concentration of antioxidants and proteins that can quench the ROS and fix the damage in these regions. Nevertheless, if there is a sudden increase in ROS formation then cellular repair processes can be overwhelmed and excessive damage can occur.

Temporarily reducing cryo-sample exposure to light immediately after cryopreservation has been shown to increase post-cryogenic survival due to the removal of photo-oxidative stress in the plants (Senula et al., 2007; Touchell et al., 2002). Photo-oxidative stress in plants can result in high levels of singlet oxygen ($^1\Sigma g^+$) and superoxide ($O_2^{\bullet -}$) being produced, either from direct UV radiation on oxygen or the leakage of light energy onto oxygen from chlorophyll when the carotenoid pigments become saturated (Wise, 1995). Plants are highly susceptible to photo-oxidative stresses at low temperatures when exposed to strong light conditions. This can be demonstrated in alpine plants, many of which display adaptations (especially to leaves, i.e. the production of carotenoid pigments) that reduce photo-oxidation damage from enhanced UV-B radiation at high altitudes (Streb et al., 1998). When the ability of antioxidants to quench the formation of ROS and the recycling of antioxidants is reduced, greater damage occurs to the chloroplast through lipid peroxidation, inactivation of photosynthetic proteins and loss of pigments (i.e. bleaching) (Wise, 1995; Wise & Naylor, 1987). Damage to the chloroplast during chilling stress has been shown to severely impede growth rates (Partelli et al., 2009); therefore, reducing the damage that occurs to plant cells due to low temperature oxidative stress is vitally important for improving survival and recovery in cryopreservation.

The most reactive ROS commonly found in plants include superoxide ($O_2^{\bullet -}$), the hydroxyl radical ($OH\bullet$), hydroperoxyl ($OOH\bullet$) and singlet oxygen ($^1\Sigma g^+$). Superoxide and singlet oxygen are often formed as by-products of the electron transport chain from both metabolism and photosynthesis, while hydroperoxyl and hydroxyl radicals are commonly formed in a process called Fenton's reaction, where hydrogen peroxide is converted into the hydroxyl or hydroperoxyl radical (1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\bullet + H^+ \quad (1)$$

The formation of the hydroxyl radical is the major cause of lipid peroxidation in membranes, but can also cause a wide range of damage to all cellular components, including proteins and DNA (Halliwell & Gutteridge, 2007). The addition of specific chelating agents (such as desferrioxamine) has been shown to reduce the levels of iron in cryopreserved tissues, with subsequent decreased levels of the hydroxyl radical observed (Benson et al., 1995; Fleck et al., 2000; Obert et al., 2005).

Damage caused by ROS is difficult to quantify as these molecules are non-specific in their interactions, reacting freely with lipids, proteins and DNA. ROS are highly reactive (and

therefore short-lived) and thus, direct measurement of the ROS present in cells is difficult due to time constraints, and does not reflect the damage that may be done prior to the ROS being quenched by antioxidants. Consequently, it is easier to measure the formation of by-products of oxidative damage or the antioxidant status of the cells. The ratio of oxidised to reduced antioxidants is a good indication of ROS formation and the ability of cells to regulate oxidative stress. Identifying end products of ROS oxidation is an indication of the damage caused and is a sign that cells have been unable to satisfactorily quench ROS activity.

3.2 Lipid peroxidation

The cell membrane represents one of the major areas where cryo-injury can occur. Any damage to the cell membrane can alter the delicate balance between intra and extracellular solutes, leading to cell death (Anchordoguy et al., 1987; Dowgert & Steponkus, 1984; Gordon-Kamm & Steponkus, 1984; Lynch & Steponkus, 1987). Lipid peroxidation of fatty acids (FA) in phospholipids can cause extensive damage to the cell membrane if the chain reaction is not controlled, leading to large areas where the semi-permeability of the membrane is altered and thus can no longer function normally (Benson et al., 1992; Halliwell & Gutteridge, 2007). Lipid peroxidation is caused when specific ROS (hydroxyl radical, peroxyl radical and singlet oxygen) interact with a FA. Polyunsaturated fatty acids (PUFA) are the most susceptible to peroxidation (Møller et al., 2007; Young & McEneny, 2001). Glutathione peroxidase can detoxify lipid peroxides by reducing them back to their lipid alcohol form and, in the process, oxidising glutathione (Benson, 1990). The formation of the toxic end-products of lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxylnonenal (HNE), can also cause damage to cells (Halliwell & Gutteridge, 2007). The toxicity of MDA is debatable (Halliwell & Gutteridge, 2007); however, there is evidence that MDA can interact with proteins and DNA, causing loss of function in proteins and mutations in DNA (Halliwell & Gutteridge, 2007; Hipkiss et al., 1997; Marnett, 1999). The formation of HNE has shown greater toxicity to cells as it can damage mitochondria, inhibit synthesis of DNA and proteins, and interfere with the action of repair proteins such as chaperones (Halliwell & Gutteridge, 2007). Identifying the formation of MDA and HNE is commonly used in cryopreservation as an indicator of oxidative stress. High levels of MDA or HNE detected have correlated with decreased survival rates in rice cell suspensions, olive somatic embryos, flax and blackberry shoot tips (Benson et al., 1992; Obert et al., 2005; Uchendu et al., 2010; Lynch et al., 2011).

Volatile headspace sampling (VHS) measures the formation of volatile compounds released from a sample in a non-destructive and non-invasive assay method. This provides an important tool for measuring oxidative stress. Free radical damage can cause the formation of volatile compounds such as methane, ethane, ethylene and pentane. Quantification of these compounds can be indicative of oxidative damage. The detection of ethylene in plants is of particular importance as ethylene is a vital hormone. Fang et al. (2008) observed that decreased levels of ethylene production correlated with decreased survival and growth. The production of the other volatile compounds is indicative of lipid peroxidation, where increased levels correlate to excessive oxidative damage. Benson et al. (1987) observed a large increase in volatile compounds produced after thawing that has since been observed in other species.

The use of DMSO as a free radical scavenger and probe for the hydroxyl radical can be utilised with VHS. The formation of methane when DMSO interacts with the hydroxyl radical can be measured if the sample is placed in an airtight container, with the levels of methane detected correlating with the formation of the hydroxyl radical. This technique has been used as a measurement of oxidative stress in multiple different plant species, such as rice, cocoa, *Daucus carota* and flax (Benson & Withers 1987; Benson et al., 1995; Obert et al., 2005; Fang et al., 2008). The production of methane is particularly strong during the initial phase of recovery, where it is predicted that antioxidant activity and production is reduced, resulting in increased ROS (Fang et al., 2008). The use of chelating agents to reduce the formation of hydroxyl radicals from the Fenton reaction has shown significant benefits in plant cryopreservation. Desferrioxamine is the most common chelating agent used. It binds to and reduces the amount of free iron. The addition of desferrioxamine to rice cells during cryopreservation showed a decreased recovery period after cryopreservation (Benson et al., 1995). Detection of methane formation was delayed in flax tissue when exposed to desferrioxamine (Obert et al., 2005), indicating a delayed production of hydroxyl radicals.

3.3 Antioxidants

An antioxidant can be defined as any molecule that "delays, prevents or removes oxidative damage to a target molecule" (Halliwell & Gutteridge, 2007). Antioxidants can be classified into two major groups: enzymes that catalytically remove ROS, or sacrificial antioxidant molecules that are preferentially oxidised to protect more important molecules by quenching ROS (Halliwell & Gutteridge, 2007). The addition of exogenous antioxidants during cryopreservation has been shown to result in increased survival in some cases (Uchendu et al., 2010; Wang & Deng, 2004). It is possible that the addition of exogenous antioxidants may also aid in reducing oxidative stresses immediately following re-warming, when cellular metabolism has not been restored to its original state.

Glutathione (GSH) is one of the main water soluble sacrificial antioxidants in plants (Kranner et al., 2006). This low molecular weight thiol is converted to its oxidised form (GSSG) upon interaction with ROS. The ratio of reduced to oxidised GSH is a good indicator of oxidative stress, as an increased amount of GSSG indicates the inability of the plant to control oxidative damage, thereby triggering premature cell death (Kranner et al., 2006). GSH is recycled by the enzyme GSH reductase using NADPH as the electron donor (Halliwell & Gutteridge, 2007). Ascorbic acid is the most abundant water-soluble antioxidant in plant cells (Foyer & Noctor, 2009). This antioxidant is able to quench free radicals, forming the stable radical semidehydroascorbate, which can be further oxidised to dehydroascorbate by another free radical. *Arabidopsis* mutants, which were unable to express ascorbic acid activity, demonstrated the important role this antioxidant plays in reducing oxidative stress, as these plants were not viable following exposure to photo-oxidative stress (Dowdle et al., 2007). Tocopherol (Vitamin E) is a lipophilic antioxidant. This is the main antioxidant involved in membrane protection against lipid peroxidation, as it is preferentially oxidised instead of PUFAs (Halliwell & Gutteridge, 2007; Uchendu et al., 2010). This antioxidant is thought to be reduced to its original, functional state by ascorbic acid (Packer et al., 1979). Carotenoid pigments are the main defence in chloroplasts, where large amounts of singlet oxygen can be produced if the activated chlorophylls transfer their energy onto oxygen. The carotenoid pigments can absorb the energy directly from the

chlorophylls, thus suppressing the formation of singlet oxygen, and they can also quench the singlet oxygen directly (Halliwell & Gutteridge, 2007).

The enzyme superoxide dismutase (SOD) catalytically removes the ROS superoxide, producing oxygen and hydrogen peroxide. The removal of superoxide is more important than the formation of hydrogen peroxide, as superoxide is a more reactive species, causing wider damage in the cells. There is potential for SOD to cause formation of ROS if levels of hydrogen peroxide are not controlled. SOD contains a metal cofactor that can cause Fenton reactions and the formation of the hydroxyl radical. Catalase is the main enzyme involved in removing hydrogen peroxide, resulting in the decomposition of hydrogen peroxide to water and oxygen. This enzyme is vital for the removal of hydrogen peroxide before it can damage the cell or be converted to the highly reactive hydroxyl radical through Fenton's reaction.

4. Plant cryopreservation and membrane structure

Membrane systems within cells are usually the site of freezing injury in plants (Steponkus, 1984). Membrane stability is therefore important for reducing such injury. There are four types of injury: (i) expansion-induced lysis, where the cells overexpand as a result of increased extracellular osmotic pressure during warming/thawing; (ii) loss of osmotic responsiveness, where there is no osmotic change during warming due to a slow cooling rate (cells remain dehydrated); (iii) altered osmotic behaviour, where cells membranes turn "leaky", resulting in the release of water and solutes into the surroundings; and (iv) intracellular ice formation, where rapid cooling causes membrane disruption due to the formation of ice crystals (Steponkus, 1984).

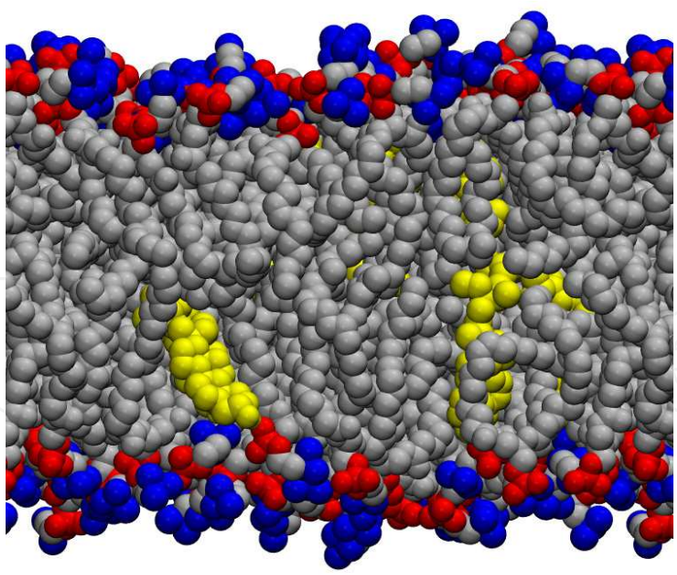

Fig. 3. Typical cell membrane structure consisting of a phospholipid bilayer with embedded sterols. Phospholipid chains are shown in grey, choline groups in blue and phosphate groups in red, while sterol molecules are shown in yellow.

The cell membrane is a bilayer consisting of different lipids and associated proteins (Fig. 3), where the lipids define the cell membrane structure and fluidity, and have a function in

signal transduction (Foubert et al., 2007; Furt et al., 2011). The three main classes of lipids found in cell membranes are glycerolipids (mostly phospholipids), sterols and sphingolipids (Furt et al., 2011).

4.1 Phospholipids

Phospholipids are amphiphilic molecules that form the core structure of the cell membrane lipid bilayer. They consist of a polar head (containing a phosphate group and simple organic molecule) and a (mostly) non-polar fatty acid tail. The most common phospholipid components of membranes include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA). Phospholipids essentially maintain the structure, fluidity and permeability of the membrane, determined to some extent by the type of phospholipid present and the level of unsaturation in their fatty acid tails (Emmelot & Vanhoeven, 1975; van Meer et al., 2008).

4.2 Sterols

Sterols are steroid alcohols that are integral components of living cells. They are formed as intermediate molecules during the metabolic production of hormones and are an integral component of cell membranes (Hartmann, 1998; Hodzic et al., 2008). Sterols are non-polar molecules with a polar hydroxyl (-OH) side chain that allows them to interact with the polar and non-polar groups of the phospholipid bilayer. They restrict the motion of the fatty acid chains in phospholipids, thus controlling the fluidity of the cell membrane (Hartmann, 1998). The major plant sterols are cholesterol, campesterol, sitosterol and stigmasterol (Grunwald, 1975). Sitosterol is usually found in higher concentration in apex tissue than stigmasterol (Grunwald & Saunders, 1978). The composition and concentration of these sterols within the membrane modify its permeability and fluidity (Grunwald, 1975; Grunwald & Saunders, 1978; Nes, 1974). Cholesterol has the greatest stabilising effect on membranes due to its small side chain (Grunwald & Saunders, 1978).

The ability of sterols to alter stability and permeability of membranes can have a large effect on post-cryopreservation survival of plant tissue. The ratio of stigmasterol to sitosterol has been found to increase after sucrose preculture and was unfavourable to obtaining high percentages of shoot regeneration after cryopreservation of banana meristems (Zhu et al., 2006). Marsan et al. (1998) investigated the interactions of sitosterol and stigmasterol with phosphatidylcholine molecules in soybean and discovered, using neutron scattering experiments, that sitosterol has a greater effect than stigmasterol on the ordering of the fatty acyl chains of PC and increasing the hydrophobic thickness of PC bilayers. Cold acclimation of winter rye (*Secale cereale*) seedlings showed an increase in free sterol content, with β-sitosterol having the largest increase (Lynch & Steponkus, 1987). Uemura and Steponkus (1994) also found an increase of β-sitosterol with cold acclimation in winter rye; however, these results were not mimicked in spring oats (*Avena sativa* L. cv Ogle), where there was no significant change in β-sitosterol, but the stigmasterol proportion increased, whilst cholesterol decreased.

4.3 Soluble sugars

Intracellular soluble sugars and sugar alcohols, such as the ones used in preculture media, reduce damage sustained by cell membranes when the cells undergo desiccation and can

improve membrane stability (Crowe et al., 1988; Steponkus, 1984). Smaller sugar molecules help membranes to osmotically retain water and may enter the interlamellar space to maintain a degree of hydration and increase the separation between membranes, thus reducing compressive stresses and, consequently, reducing the chance of a fluid-gel phase transition (Wolfe & Bryant, 1999). Furthermore, the polar hydroxyl (-OH) groups on sugars and sugar alcohols have been thought to interact with the polar membrane phospholipid headgroups and stabilise the membranes by maintaining the separation of the phospholipid molecules (Steponkus, 1984; Turner et al., 2001; Wolfe & Bryant, 1999). Turner et al. (2001) tested several sugars and sugar polyalcohols and determined that the small size of molecules such as glycerol and erythritol allowed them to 'pack' around membranes and have better bonding abilities. Additionally, the stereochemical arrangement of the -OH groups, particularly their orientation along one side of the molecules, imparted more stable hydrogen bonds with the membrane phospholipids and, consequently, resulted in more stable membranes (Turner et al., 2001). Nonetheless, recent biophysical studies have established that specific interactions of sugar molecules to phospholipid headgroups are not necessary to explain the stabilising effect of sugars on membrane gel to fluid transition temperatures (Lenne et al. 2006, 2007, 2009) and that sugars do not in fact insert between phospholipid headgroups but instead a solvation layer of water molecules separates them from the phospholipid headgroups (Kent et al., 2010).

4.4 Preconditioning and cold acclimation

Cold acclimation is the process in which plants being exposed to low non-freezing temperatures increase their freezing tolerance (Thomashow, 1999; Sakai & Engelmann, 2007). Preconditioning of raspberry and blueberry plants at 22/-1°C alternating temperatures for four weeks was essential for optimal post-cryopreservation shoot-tip re-growth using encapsulation-dehydration and vitrification protocols (Gupta & Reed, 2006). Similarly, better recovery rates were seen in mint (Senula et al., 2007) and yams (Leunufna & Keller, 2005) when they were cold acclimated for several weeks before cryostorage.

Cold acclimation is thought to activate genes that improve plant survival at low temperatures by improving stability in membranes (Guy et al., 1985; Thomashow, 1999). Cell membrane phospholipids and sterols have been observed to increase in proportion upon cold acclimation in winter rye, with a particular increase in di-unsaturated PC and β-sitosterol (Uemura & Steponkus, 1994). Changes in phospholipid and sterol composition were found in *Arabidopsis thaliana* after cold acclimation at 2°C for one week, which increased its freezing tolerance from -2°C down to -10°C (Uemura et al., 1995). Cell membrane phospholipid changes were also observed in oat leaves (Uemura & Steponkus, 1994). These changes may be related to improved membrane stability in these plants.

Preconditioning of plants has also been shown to increase antioxidant levels in plants prior to cryopreservation (Baek & Skinner, 2003; Dai et al., 2009; Harding et al., 2009; Zhao & Blumwald, 1998). Baek and Skinner (2003) analysed the expression of antioxidant genes in wheat species after cold acclimation and found increased expression of antioxidant enzymes, such as SOD and catalase. Increased levels of antioxidants may allow plants to better tolerate oxidative stress. Lynch and Steponkus (1987) observed an increase in the di-unsaturated levels of PC and PE in winter rye seedlings. Sucrose pre-treatment of banana

meristems prior to cryopreservation increased survival after warming and was related in most cases to a decrease of the double bond index in phospholipids, free fatty acids, glycolipids and sphingolipids (Zhu et al., 2006).

5. Genetic and epigenetic stability

The aim of successful cryopreservation is to maintain genetically stable plant material. While cryopreservation is now recognised as the method of choice for the long term preservation of plant material, the usefulness of cryostorage only applies if it does not lead to genetic changes in the plant species of interest (Zarghami et al., 2008). It is thus recommended to avoid the use of tissue in a non-organised dedifferentiated state, such as callus, and to use organised tissue like shoot tips instead to reduce the likelihood of non-desirable genetic mutations (Benson et al., 1996; Harding, 2004) as well as due to their higher regrowth rates (Bunn et al., 2007). Cryopreservation can cause injury at cellular level, but it is not clear if this injury can change the genetic composition of plants. The genetic stability of cryopreserved plants has nonetheless been confirmed for most of the analysed samples at morphological, histological and molecular level (Harding, 2004). Where differences between control and cryopreserved genotypes were found, it was suggested that the genetic changes might not be associated with the cryopreservation process itself, but rather that they are caused by the overall tissue culture, cryoprotection and regeneration process (Harding, 2004).

Comparisons of morphological development and analyses of the characteristics of control and cryopreserved plants have shown no differences in many species, such as *Prunus*, sugarcane, onion, kiwi, *Eucalyptus*, coffee, *Dendrobium* and *Cosmos* (Harding, 2004), as well as in the hybrid aspen (*Populus tremula*), an economically important woody plant and widely used model forest plant (Jokipii et al., 2004). Alterations in phenotype related to flower colouring have been observed in *Chrysanthemum*, which might be related to the chimeric structure of the plant (Harding, 2004). Morphological and phenotypic studies in potato, where shoot tips were used for cryopreservation, showed stable genetic integrity (Kaczmarczyk et al., 2010). Biometric studies examining morphological characters, agronomic traits or vegetal development descriptors in *Dioscorea floribunda*, sugarcane and banana revealed no significant differences between cryopreserved-derived and control plants (Harding, 2004). No abnormalities in chromosome number or cell structure were observed in cryopreserved *Vanda pumila* (*Orchidaceae*), with the regrown shoot primordia being able to induce new meristematic tissues like those of the non-cryopreserved controls (Na & Kondo, 1996). Long term storage of cryopreserved plants, such as strawberry, pea, *Rubus* and potato, did not result in any overall changes in regeneration capability and phenotype when regenerated explants were compared at the time point of storage as well as 10, 12 and 28 years later (Castillo et al., 2010; Caswell & Kartha, 2009; Keller et al., 2006; Mix-Wagner et al., 2003).

Histological studies and cytological analysis using flow cytometry have confirmed the genetic stability of plant species such as pea, oil palm, silver birch, *Rubus*, *Solanum tuberosum* and rice (Harding, 2004). Biochemical analyses have compared products of gene expression such as the formation and concentration of secondary metabolites. Examples of compounds compared in cryopreserved and control plants have been diosgenin in *Dioscorea floribunda*, chlorophyll and pyrethrin in *Chrysanthemum*, hypericin production in *Hypericum perforatum*

L. (Skyba et al., 2010; Urbanova et al., 2006), betalanin pigments in *Beta vulgaris* and nicotin alkaloids in *Nicotiana rustica*, which were all unchanged in cryopreserved plants and thus confirmed the integrity of metabolic functions after cryostorage (Harding, 2004). Similar stability was observed upon comparison of proteins and enzymes (Marin et al., 1993; Paulet et al., 1993; Wu et al., 2001).

A variety of different techniques and markers have been applied to compare genomic DNA patterns, such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPDs) fragments, simple sequence repeat (SSR) analysis and amplified fragment length polymorphism (AFLP). Most studies have confirmed the presence of genetic stability (Castillo et al., 2010; Helliot et al., 2002) and where changes in the genome have been found, such as in sugarcane and potato with RFLP markers, the changes could not be related to the process of cryopreservation itself (Castillo et al., 2010; Harding, 2004).

In contrast to genetic variations manifested by DNA nucleotide sequence alterations, epigenetic changes do not change the original DNA sequence (Boyko & Kovalchuk, 2008) but can result in heritable changes of gene expression. Typical features of epigenetic characteristics are DNA methylation, histone modification and changes in chromatin structure (Boyko & Kovalchuk, 2008). Epigenetic gene regulatory mechanisms have a function in plant development and might be influenced or changed by environmental conditions and osmotic stress during tissue culture and cryopreservation. Some recent studies have analysed epigenetic characteristics like DNA methylation in tissue culture and cryopreserved plants. Modifications in DNA methylation have been found in almond (Channuntapipat et al., 2003), papaya (Kaity et al., 2008), chrysanthemum (Martín & González-Benito, 2006), *Ribes* (Johnston et al., 2009), strawberry (Hao et al., 2002a), citrus (Hao et al., 2002b) and potato (Kaczmarczyk et al., 2010). Changes in methylation might be caused by stressful *in vitro* conditions, osmotic dehydration and the application of cryoprotective agents (Harding, 2004).

6. Conclusion

Many plant species have been successfully cryopreserved through the development of various cryopreservation methods. As a standard protocol, vitrification and droplet vitrification are widely applied. Shoot tips are the preferred material for cryostorage as they contain the meristem and an organised structure, with direct shoot development avoiding unstructured phases, which could lead to mutations. Preconditioning of plants (cold acclimation or sugar preculture) can have positive effects on survival and regeneration after cryopreservation, which could be due to increased membrane stability. Cryopreserved plants have been found to be genetically stable in most cases, but epigenetic changes have been detected, although most of the molecular analyses have only compared fractions of the genome.

Success in cryopreservation cannot be guaranteed for all plants, as some species are recalcitrant to tissue culture or the cryopreservation process. Fundamental studies looking at membrane composition, membrane damage and repair are likely to help to elucidate why some species are cryosensitive and how cryopreservation protocols can be improved for those species.

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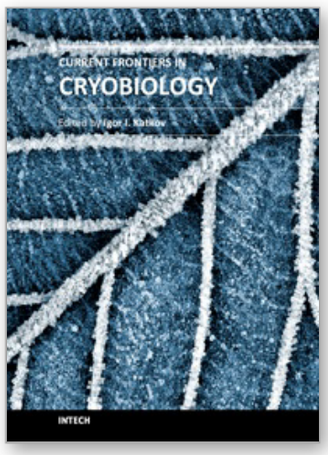

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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