AN ABSTRACT OF THE THESIS OF

Jie Luo for the degree of Master of Science in Horticulture presented on April 5, 1996. Title: Improvement of Cryopreservation Regrowth of In Vitro Ribes by Pretreatment.

Abstract approved: ________________________________

Barbara M. Reed

Improved recovery of cryopreserved meristems and calli from in vitro currant (Ribes aureum Pursh and R. ciliatum Humb. & Bonpl.) plants was obtained by two-hour pretreatment with sucrose, proline, abscisic acid-responsive protein (RABP), and bovine serum albumin (BSA) solutions. Meristems and calli cold-acclimatized for one week were immersed in pretreatment solutions for two hours prior to cryopreservation by vitrification. Pretreatment with 5% proline, 1% crude RABP, 0.2% dialyzed RABP, and 1% BSA significantly increased the regrowth, compared to pretreatment with 0.4 M sucrose liquid NCGR-RIB medium, and non-pretreated controls. There were no significant differences among proline, crude and dialyzed RABP, and BSA pretreatments. Meristems in pretreatment groups resumed growth three days after thawing, and reached the maximum regrowth at one week, compared to two weeks for non-pretreated controls. Pretreatment by
hourly immersion provides a simple and effective approach for improving the recovery of cryopreserved meristems and calli.

Improved regrowth of cryopreserved meristems was also obtained by cold acclimatization (CA) of plantlets before dissection of meristems. Meristems acclimatized for 3 or 4 wk had more regrowth than those acclimatized for 1 or 2 wk. No significant differences were found between 1 and 2 wk, and 3 and 4 wk CA pretreatments.
Improvement of Cryopreservation Regrowth of *In Vitro* *Ribes* by Pretreatment

by

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Jie Luo, Author
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CONTRIBUTION OF AUTHORS

Dr. Barbara M. Reed participated the entire experimental designs, analyses, discussions, support, and critical editing. Dr. M. K. Walker-Simmons provided crude and dialyzed RABP for the experiments and information about the proteins.
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Cryopreservation

Cryopreservation (freezing preservation) is a cryogenic storage technique for the long-term preservation of cells, tissues, and organs. It involves exposure and maintenance of cells or tissues in liquid nitrogen (LN) without loss of viability. The theories and techniques are reviewed by Kartha (ed., 1985), Benson (1993) and Bajaj (ed., 1995). This technique is based upon the theory that the metabolism of plant materials is reduced to such a low level that biochemical processes and biological deterioration almost completely cease. The advantages of cryopreservation over maintaining collection of growing plants or tissue cultures include savings in space and labor, lower losses due to contamination during in vitro storage, maintaining clonal integrity, and storing genetic resources.

Cryogenic research dates from 1912 when Maximow (1912) first discovered that leaf tissue of Tradescantia discolor, a tropical plant
normally killed at -2 °C, could survive temperatures several degrees lower in the presence of juice from hardened red cabbage leaves. Early research explored methods to decrease the freezing point of cells but only a few degrees of cold tolerance were achieved. Quatrano (1968) first reported cryopreservation of plant cell suspension cultures of *Linum usitatissimum* L. with recovery viability at 14% detected by 2,3,5-triphenyl tetrazolium chloride (TTC), and this showed that it is possible to cryopreserve cell cultures in liquid nitrogen. Nag and Street (1973) later reported the regeneration of plantlets from a frozen and thawed embryogenic culture of *Daucus carota* L.; Sakai and Sugawara (1973) described the regrowth of a frozen and thawed callus culture of *Populus euramericana* L. Since then, cryopreservation techniques have been developed and applied to cell suspensions, protoplasts, pollen, somatic embryos, and meristems (Bajaj, 1995).

**Freezing injury**

Normally plant cells and tissues can not withstand freezing at a few degrees below 0 °C. One of the major problems encountered in cryopreservation is freezing injury to plant cells or tissues (Withers, 1980). When cells freeze, intracellular or extracellular ice may form. Intracellular freezing mechanically damages the unity and function of the cell, particularly the functions of plasma membranes (Burke et al., 1976). Extracellular freezing is also harmful because of the concentration of
solutes by extracellular ice formation, called "solution effects" (Mazur, 1965). Because water moves out of the cell and is crystallized extracellularly, solutes both outside and inside cells are concentrated, resulting in osmotic dehydration or plasmolysis. This process will reduce cell volume, surface tension, and membrane area to a certain extent, and exert forces on plasma membrane during plasmolysis (Meryman and Williams, 1985). Because plant cells have a mechanical resistance generated by the cell wall, cell contents, cell membrane and its structure, and membrane-cell wall attachment (Nobel, 1969), there is a potential energy discrepancy between intracellular and extracellular osmolality. This discrepancy is only expressed when the membrane becomes unstable, resulting in the loss of membrane structure and materials, especially lipid components. The cells from very hardy plants can resist cell volume reduction and collapse of cell structures and contents, whereas less hardy species have a limited tolerance and less energy is required to collapse the membrane structure (Meryman and Williams, 1985).

Freezing injury can be detected through anatomical, biochemical, and physiological responses of plant materials. Freezing injury in rice cells can be detected by the lipid peroxidation product, malondialdehyde (MDA), and by the thiobarbituric acid (TBA) assay (Benson et al., 1992). Osmiophilic granules found in freeze-damaged cells are also an
indication of injury (Gnanapragasam and Vasil, 1992). Taxol, a microtubule-stabilizing compound, prevented the depolymerization of microtubules of cells in response to freezing, and the amount of stabilization could be detected by electrolyte leakage, the visual appearance of cells, and a microtubule repolymerization assay (Bartolo and Carter, 1991).

**Pretreatments**

**Cold acclimatization**

Cold acclimatization (CA) is one method for preventing cell or tissue injury. CA comprises a myriad of biochemical and physiological processes leading to an increase of cold tolerance. CA has two main functions: the adjustment of metabolism and basic cellular function to the biophysical constraints imposed by low temperature, and the induction of freezing tolerance. During cold acclimatization, new proteins are synthesized. Soluble protein content, enzyme activities and levels of glycoproteins or glycopeptides with high molecular weight increase while free water decreases (Guy, 1990). Numerous molecular studies indicate that mRNAs in cold-acclimated tissues encode proteins that are translated and accumulated at low temperature (Lang et al., 1989; Lin et al., 1990).
During cold acclimatization of pea, the cell wall hydroxyproline content increases as well as cell wall glycosyl residues and cellulose (Weiser et al., 1990). The lipid composition in the plasma membrane of cold-acclimatized oat and rye leaves is different from non-acclimatized treatments; that is, the proportion of the phospholipids, phosphatidylcholine and phosphatidylethanolamine, increases after four weeks of cold acclimatization (Uemura and Steponkus, 1994). Similar effects were also found in Solanum species (Palta et al., 1993). The permeability of the membrane increases such that the amount of freezable cellular water decreases while sugars or other disaccharides in the cells increase. In apple callus culture, the activities of ascorbate peroxides and catalase and the levels of glucose-6-phosphate were higher during cold acclimatization (Kuroda et al., 1991). Accumulation of proline, asparagine and aspartic acid was also found in immature maize embryos during freezing (Delvallee et al., 1989). Cold acclimatization improves the percentage survival from cryopreservation of in vitro meristems of Vaccinium and Rubus genotypes (Reed, 1988; 1989).

Abscisic acid (ABA) plays a role in mediating plant response to CA and other environmental stresses. The first indication of a relationship between CA and ABA was observed in woody plants (Irving and Lanphear, 1968). They demonstrated that increased freezing tolerance in Acer negundo may involve an ABA-like substance. Lee and Chen (1993)
summarize the evidence that ABA is involved in plant freezing stress, including an increase in endogenous levels of ABA during CA treatment, induction of CA by ABA, and the effects of ABA on metabolic processes which are similar to cold treatment (Chen and Gusta, 1983; Chen et al., 1983; Lee et al., 1991). Both CA treatment and ABA application change gene expression and induce new cDNAs which encode specific proteins corresponding to ABA or CA (Lee et al., 1991). These specific proteins are ABA-responsive proteins (RABP) which will be addressed in the later section of cryoprotective proteins.

**Osmotic stress**

Increasing osmolarity dehydrates cells and reduces freezable water. The decrease of free water in cells increases the free energy gradient, thus reducing the formation of intracellular ice (Johnson-Flanagan and Singh, 1988). Depressing the freezing point and increasing intracellular osmolality protects cells during freezing. For example, introduction of osmotic stress in sycamore cells by the addition of mannitol in the preculture medium improves survival following cryopreservation with the highest survival rate obtained during the first 10 days of preculture with 3.3% mannitol in the medium (Withers and Street, 1977).

Increased osmolality reduced cell wall thickness and vacuolar volume (Pritchard et al., 1986a), decreased the water potential, and produced changes in the cell contents of total and soluble protein,
respiratory activity, and free cellular proline in soybean and sycamore cells (Pritchard et al., 1986b). The reduction of total protein due to protein hydrolysis during osmotic stress (Levitt, 1980) increased the free proline levels in stressed soybean cells (Pritchard et al., 1986b). However reduced free proline and increased total protein content in stressed sycamore cells could be explained as a result of the increased incorporation of proline into newly synthesized protein during osmotic stress (Pritchard et al., 1986b). Increased respiratory activity in both sycamore and soybean cells during osmotic stress was due to the increased need of energy input for the maintenance of metabolic pathways or for the synthesis of some specific cell solutes (Pritchard et al., 1986b). Ultrastructural studies indicate that the central vacuole of Panicum maximum was redistributed as smaller vesicles during pregrowth in mannitol. Invagination of the plasma membrane, swelling of organelles, and dilation of cisternae of the endoplasmic reticulum occurred following cryopreservation (Gnanapragasam and Vasil, 1992).

Sucrose is also useful for osmotic treatments. It plays an important role in stabilizing membranes and proteins during desiccation (Crowe et al., 1990). Survival of carnation shoot tips following cryopreservation increased from 75% to 99% with 24 hr pretreatment with 0.3M to 0.75M sucrose (Dereuddre et al., 1988). Sugars and other disaccharides not only have osmotic effects outside the cells, but also
enter the cells and stabilize proteins and membranes during desiccation (Dumet et al., 1993).

**Cryoprotectants**

The success of cryoprotection is dependent upon the effectiveness of avoidance or minimization of intracellular freezing and reduction of damage to the cells from the concentrated solutes during cooling (McGann, 1978). This can be fulfilled by pretreatment with cryoprotectants. Polge, Smith and Parkes (1949) discovered that glycerol could protect living cells from freezing injury. Ten years later, Lovelock and Bishop (1959) introduced dimethyl sulfoxide (DMSO) as a cryoprotectant for animal cells. DMSO was first used for plant cells in 1968 (Quatrano, 1968).

Traditional cryoprotective agents (CPA) are classified into three groups according to their permeability into cells (Morris, 1980; Farrant and Ashwood-Smith, 1980). The first class is the penetrating type, which includes DMSO, glycerol, ethylene glycol, methanol, and dimethyl acetamide. The second non-penetrating type includes polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES), dextrans, albumin, and polyethylene glycol (PEG). The third type is a mixture of penetrating and nonpenetrating compounds, including glucose + DMSO (Sugawara and Sakai, 1974), PGD (PEG + glucose + DMSO) (Ulrich et al.,
1979), and plant vitrification solution #2 (PVS2) (glycerol + ethylene glycol + DMSO) (Sakai et al., 1990).

The most commonly used cryoprotectant for both animal and plant cells is DMSO (Meryman, 1966), although there are a large number of alternative compounds such as glycerol, methanol, glucose, and proline (Heber et al., 1971; Finkle and Ulrich, 1979; Morris and Canning, 1978; Withers and King, 1979a, b).

DMSO, as a penetrating agent, has colligative properties - the ability to reduce the concentration of toxic solutes below the limit of toxicity during dehydration or freezing (Heber et al., 1971). It reduces the amount of ice formation at any temperature during cooling, thus postpones increased ionic concentrations to lower temperatures; that is, damage to cells is retarded (McGann, 1978). Furthermore, DMSO prevents lethal intracellular freezing by decreasing the growth rate of intracellular ice crystals (Sakai, 1985).

Glycerol maintains hydrophobic forces that are critical to the tertiary and quaternary structures of macromolecules and the stability of membrane bilayers (Tanford, 1973). Colligative retention of intracellular water, and the contribution of its mass further slow cell volume reduction. However glycerol has a relatively slow rate of passage across the membrane, and this may lead to possible damage of cells or tissue during extended exposure (Meryman and Williams, 1985).
HES (hydroxyethyl starch), one of the non-penetrating agents, creates an osmotic stress on cells which results in a loss of cell water and is only concentrated in the extracellular region at lower temperatures (McGann, 1978). Sucrose and PVP also cause osmotic dehydration, rather than protecting against it (Meryman et al., 1977).

Proline, an amino acid, can inhibit membrane mixing during freezing and stabilize bilayers by preventing the aggregation of intramembranous particles (Rudolph and Crowe, 1985). Proline is water soluble and at certain concentrations participates in colloid-like associations, interacts with biomacromolecules, and stabilizes them in solution especially during water stress (Schobert and Tschesche, 1978). Proline also acts as a nontoxic intracellular solute, protecting the cells from denaturation. The incorporation of proline into cell wall hydroxyproline may affect cell wall flexibility and tensile strength (Withers and King, 1979a). Thylakoids can be protected against inactivation by the addition of some amino acids such as proline, hydroxyproline, glycine, threonine, and lysine-HCl, but other amino acids such as phenylalanine, tyrosine, valine, leucine, and methionine are not effective. These protective compounds act by colligative action - the ability to reduce the concentration of toxic solutes below the limit of toxicity (Heber et al., 1971). Specific interaction between cryoprotectants
and membranes may play an important role in membrane preservation during the freeze-thaw cycle.

The characteristics of individual types of CPAs can be summarized as follows: penetrating CPAs are beneficial for reducing cell water content and protecting intracellular organelles when cells or tissues are exposed to additives for short intervals (Morris, 1980). They exhibit a colligative action that increases the volume of the cellular solution and avoids an excessive concentration of toxic electrolytes in both the cellular and external solutions. These compounds prevent the cellular salt concentration from reaching a toxic level and slow the damaging reactions at low temperature (Finkle et al., 1985). Penetrating solutes decrease the homogenous nucleation temperature and the rate of crystal growth, and increase glass transformation temperature (Meryman et al., 1977).

Non-penetrating CPAs create osmotic potential around the cells and force the freezable cell water to exit during freezing (McGann, 1978). The remaining unfrozen water is highly structured and acts as a diluting solvent to diminish salt toxicity. Non-penetrating CPAs do not function by intracellular action or by interaction with cell membranes, but instead alter the properties of the external solution to protect external membranes during the freeze-thaw cycle (Connor and Ashwood-Smith, 1973). The application of concentrated cryoprotectants further increases
solution viscosity and speeds cell dehydration, which allows more cells to avoid injury (Meryman et al., 1977).

Mixtures of cryoprotectants decrease the toxic effects to plant cells and tissues compared to using single cryoprotective compounds (Ulrich et al., 1979) because they reduce the damaging effect of individual cryoprotectants by dilution (Herber et al., 1971). Mixtures of some major cryoprotectants, glucose and DMSO for example, produced successful recovery in sycamore cells (Sugawara and Sakai, 1974) and Saccharrum sp. cells (Finkle and Ulrich, 1979).

The main protective functions of cryoprotectants are decreasing the ice crystal size and growth rate by reducing the rate of diffusion of cell water into the ice crystal, depressing the freezing point of the cytoplasm, avoiding intracellular ice formation, stabilizing cell membrane composition and permeability, and substituting freezable water with mono- or polysaccharides to protect and maintain cell structure and osmotic equilibrium during the freeze-thaw cycle (Yoshida and Sakai, 1974; Wiest and Steponkus, 1978; Meryman and Williams, 1985).

The application of cryoprotectants (concentration and duration) is important because of their cytotoxicity. Medium to long-term exposure to DMSO may damage the cells because of intracellular effects (Morris, 1980), and inhibition of photosynthesis (Morris, 1976). Glycerol may damage the integrity of membranes (Morris, 1980) and may cause severe
plasmolysis because of decreased uptake at certain application temperatures (McGann, 1978). Glycerol or other cryoprotectants may also predispose cells to deplasmolysis injury during and after thawing (Withers, 1980). The normal concentration of cryoprotectants is between 0.5 M and 2 M, but the concentration and type of cryoprotectants used are determined by the type of materials to be cryopreserved (cell, meristem, callus, suspension, embryo, protoplast, pollen) (Withers, 1980).

A number of different cryoprotectants have been used in the cryopreservation of different plant materials under slow freezing and vitrification protocols. With slow freezing, mixtures of glucose and DMSO produced successful recovery in sycamore cells (Sugawara and Sakai, 1974) and Saccharrum sp. cells (Finkle and Ulrich, 1979). A mixture of polyethylene glycol (PEG), glucose, and DMSO (PGD) was successfully applied for cryopreservation of sugarcane callus and cell suspension culture (Ulrich et al., 1979) and of apical meristems of Rubus (Reed and Lagerstedt, 1987; Reed, 1988, 1993), Pyrus (Reed, 1990), and Vaccinium species (Reed, 1989). With respect to vitrification, the following cryoprotectants have been used to cryopreserve different species including: 1.5 M ethyl glycol solution for cell suspensions of Brassica campestris (Langis et al., 1990); PVS2 (Sakai et al., 1990) for nucellar cells of Citrus sinensis and white clover (Yamada et al., 1991); a mixture
of glycerol, ethylene glycol, propylene glycol, DMSO, and 0.5 M sorbitol in MS (Murashige and Skoog, 1962) medium for somatic embryos (SE) of asparagus (Uragami et al., 1989), and for some dormant buds of apples (Seufferheld et al., 1991); and 0.85-1 M sucrose for SE of carrot and coffee (Tesserreau et al., 1991).

Cold-hardy winter-dormant buds can be prefrozen to about -30 °C, or air-desiccated or dehydrated in alginate to low moisture contents followed by direct immersion in LN without the application of cryoprotectants (Sakai and Nishiyama, 1978; Tyler and Stushnoff, 1988; Niino et al., 1992).

**Cryoprotective proteins**

Many physiological and biochemical changes occur during the induction of freezing tolerance and cold acclimatization of plants, especially the induction and accumulation of certain specific new proteins. These changes also occur in some overwintering fish and insects at low temperatures. In plants, these proteins are abscisic acid-responsive proteins (RABP) which include cold-regulated (COR) proteins, late embryogenesis abundant (LEA) proteins, and dehydrins (DHN), while in animals, they are glycoproteins or glycopeptides and antifreeze proteins.

Glycoproteins/glycopeptides are hydrophilic, causing a decrease in the water content in cells, providing resistance to cell volume loss, and
lowering the glass transition temperature (Williams, 1973). Damage from dehydration effects of extracellular ice formation would be eliminated because of the increase of cellular osmotic potential when proteins are present (Johnson-Flanagan and Singh, 1988).

**Cryoprotective proteins in animals: antifreeze proteins**

Antifreeze proteins (AFPs) isolated from a variety of fish (Arctic and Antarctic) and insects, prevent ice crystal growth in body fluid, stabilize the supercooled state of the organisms, and enhance survival in freezing temperatures (Storey and Storey, 1988; Chang et al. 1991). The antifreeze protein was first discovered by Ramsay (1964) in the hemolymph of common mealworms (*Tenebrio molitor* L.). Fish antifreeze proteins and glycoproteins were found during the 1970’s (DeVries et al., 1970; DeVries and Lin, 1977). In fish it was found that winter-acclimatized winter flounder were more freezing tolerant than summer-acclimatized fish, and that injection of the AFP directly into rainbow trout enhanced their freezing resistance (Fletcher et al., 1986). Some insects survive supercooling below -25 °C by producing proteinaceous antifreeze agents which prevent the growth of ice crystals (Zachariassen and Husby, 1982).

Proteins lower the freezing point of body fluid without changing the melting point. This protective ability, called thermal hysteresis, was first observed in the blood plasma of polar teleost fishes (DeVries, 1982).
These antifreeze proteins or glycoproteins are referred to as thermal hysteresis factors (THFs). The noncolligative ability of AFPs is defined as the difference between the melting and freezing temperatures of the solution (Feeney et al., 1979).

One of the early mechanisms of THF antifreeze effects was proposed by Raymond and DeVries (1977). They suggested that the proteinaceous molecules attached to the surface of an ice crystal and confined the growth of the ice crystal to the spaces between adjacent molecules. This increased the ice front curvature and ice formed at lower temperatures. The continuous increase of ice front curvature further decreased the freezing point. Burcham et al. (1986) pointed out that antifreeze glycoprotein (AFGP) molecules could create an activation energy barrier for water molecules entering the ice lattice. With the increase of supercooling, the barrier resulting from AFGP adsorption diminishes, then vanishes, and ice formation occurs. The energy barrier does not form upon melting.

One of the functions of AFPs is to inhibit the recrystalization of thawing extracellular ice (Knight and Duman, 1986), protecting organisms during freeze-thaw cycles. Another function of AFPs is the protection of membranes at low temperatures. Increased survival of mammalian oocytes was achieved by incubating them in fish AFP
solutions at 4 °C for 24 hr because the AFP maintained the integrity of the oolemma (Rubinsky et al., 1991).

The application of fish AFP to leaves of potato, canola, and Arabidopsis thaliana and cells of bromegrass demonstrated that the AFPs from winter flounder lowered freezing temperatures, decreased the quantity of freezable water, and the rate of ice crystal formation (Cutler et al., 1989). However, AFPs are toxic to ram spermatozoa (Payne et al., 1994) and spinach thylakoid membranes (Hincha et al., 1993). These results may be due to the different lipid compositions of the membranes and their response to AFPs (Hincha et al., 1993).

**Cryoprotective proteins in plants**

Weiser (1970) proposed a possible relationship between cold acclimatization and gene expression. Since then, studies with a variety of plant species demonstrated that CA is closely correlated with changes in gene expression. Specific proteins are produced as a result of changes in gene expression in response to different stresses. Most of these cryoprotective proteins are abscisic acid-responsive proteins (RABP) including some of the cold-regulated (COR) proteins (Gilmour et al., 1988), the late embryogenesis abundant (LEA) proteins (Dure et al., 1981), and the dehydration-inducible proteins (dehydrins, DHN) (Close et al., 1989; Jacobsen and Shaw, 1989). All these proteins are hydrophilic and heat-stable.
Studies of cryoprotective proteins in plants date from 1968 when Heber et al. (1968) found that 0.1% of the protein fractions (10-16 kDa) from the extract of cold-hardy spinach leaves was effective in protecting isolated chloroplast membranes against freezing. Later research indicated that the combination of a protein with sucrose was necessary for protection against freezing (Volger and Heber, 1975). These specific proteins, called cold-regulated (COR) proteins, are regulated and synthesized in response to low temperature and ABA application (Chen and Gusta, 1983; Lang et al., 1989). The cor genes can be found in *Arabidopsis*, wheat (Lin et al., 1990), alfalfa (Mohapatra et al., 1988), *Brassica* (Johnson-Flanagan and Singh, 1987), potato (Chen et al., 1983), and spinach (Guy and Haskell, 1988). These genes encode polypeptides that remain soluble upon boiling (Lin et al., 1990). However, not all cor genes are ABA-responsive (Lang et al., 1989; Mohapatra et al., 1989; Nordin et al., 1991). This may suggest that expression of some cor genes is regulated by a pathway that is independent of ABA. Because all of these COR proteins are hydrophilic and boiling-stable, they could possess cryoprotective ability. COR15, on the molar basis, was found to be more effective in protecting lactate dehydrogenase (LDH) against freeze inactivation *in vitro* than sucrose or bovine serum albumin (BSA) (Lin and Thomashow, 1992). The possible function of the COR proteins may not be colligative, such as AFPs in fish
and insects, but rather may affect a specific locus of the outer membrane (Volger and Heber, 1975). Proteins extracted from cold-acclimated cabbage and spinach leaves protected membranes of isolated thylakoids from non-hardy spinach against mechanical rupture during the freeze-thaw cycle by reducing solute permeability during freezing, increasing expandability during thawing, and increasing resistance against the osmotic stress exposed during a freeze-thaw cycle (Hincha et al., 1990).

LEA proteins accumulate in late embryogenesis and in dehydrated seedlings and are proposed as desiccation protectants (Lane, 1991; Ried and Walker-Simmons, 1993; Thomann et al., 1992). They also accumulate in plant tissue under environmental stress (Skriver and Mundy, 1990). LEA proteins are induced by ABA and desiccation. Study of cotton seed showed that six of the LEA genes coded for polypeptides with a hydrophobic character. Two of the LEA polypeptides were higher in glycine and hydroxylated amino acids. They could bind water molecules and act as hydrating agents. Two other LEA polypeptides could bind ionic species which played an important rôle in binding excess ions to prevent cytotoxicity due to the increase of ion concentration during desiccation (Baker et al., 1988). The wheat Group 3 LEA cDNA, for example, contains an 11-amino acid segment which repeats ten times and is a major characteristic of Group 3 LEA proteins (Curry et al., 1991).
Dehydration-inducible proteins (dehydrins, DHN) are induced during embryogenesis as well as under plant stresses. Desiccation, like cold hardiness induction, increases the endogenous levels of ABA (Guerrero and Mullet, 1986), which mediates a number of physiological, biochemical, morphological, and molecular changes. The pattern of protein synthesis is also altered in response to dehydration, and new translatable RNA is expressed (Bartels et al., 1990). These dehydrins are rich in glycine and threonine and lack cysteine and tryptophan (Close et al., 1989).

**Freezing protocols**

Three main protocols are available for the cryopreservation of actively growing meristems: slow freezing, vitrification, and alginate encapsulation-dehydration. Generally, the procedures include: selection, preculture and pre-conditioning → pregrowth or pretreatment → cryoprotection → freezing (slow, vitrification) → thawing (slow, rapid) → recovering culture.

**Slow freezing**

Slow freezing is based on the physicochemical events which occur during freezing. With decreasing temperature, the cell wall and plasma membrane act as barriers and prevent ice formation in the cell interior at temperatures above -10 °C, so the cell cytoplasm remains unfrozen but
supercooled. With further reduction of temperature, more and more water from the inside of the cells moves out and is crystalized in the extracellular spaces, resulting in the increased concentration of both extracellular and intracellular solutes (dehydration); in such cases, the cytoplasm becomes so viscous that crystallization can not occur and it vitrifies when plunged into liquid nitrogen (Kartha, 1985).

Some of the factors that influence the success of the slow freezing method include cooling rates, type and concentration of cryoprotectants, terminal freezing temperature, and the physiological state of plant materials (Kartha, 1985). The optimum cooling rate varies from 0.3 to 10 °C/min. until -50 °C or -60 °C (Farrant and Ashwood-Smith, 1980), but it is genus-dependent. In Pyrus, for example, the cooling rates were 0.1 to 0.8 °C/min. to -40 °C with the highest survival at the slower cooling rates (Reed, 1990). The advantages of the slow freezing method are that freezable water from cells escapes to become external ice and a subsequent drop in the temperature of liquid nitrogen has very little adverse effect on the dehydrated cytoplasm. One main drawback of the slow freezing procedure is the need for a controlled rate freezer.

**Vitrification**

Vitrification is the physical process by which a highly concentrated solution is supercooled to a very low temperature and forms a glasslike, noncrystalline solid at or below the temperature of the freezing point of
the liquid solution (Meryman and Williams, 1985). Vitrification, also
called rapid freezing, was initially discussed in the 1930’s by Luget
(1937). He proposed that the viability of cells or tissues could be
maintained by rapidly passing through the temperature where lethal
intracellular ice-crystal formation occurred. This technique was first
applied in the cryopreservation of animal cells (Fahy et al., 1984).
Vitrification occurs at or near the equilibrium freezing point but well
above the temperature of homogeneous nucleation, so intracellular ice
formation is avoided because the aqueous solution is too concentrated or
viscous to permit ice crystal nucleation, then both the solution and
cytoplasm are vitrified when quenched into liquid nitrogen (Benson,
1993).

Vitrification is relatively simple because samples are submerged
directly in liquid nitrogen after pretreatment and in the presence of
cryoprotectants. Disadvantages of vitrification include possible
recrystalization during thawing, toxicity of prolonged exposure to
cryoprotectants (PVS2), and osmotic damage to the cells or tissues
(Benson, 1993).

**Alginate encapsulation-dehydration**

This technique was first applied to cryopreserve potato shoot-tips
(Fabre and Dereuddre, 1990), grapevines (Plessis et al., 1991), carnations
(Tannoury et al., 1991) and *Ribes* (Reed and Yu, 1995).
The tissues (shoot-tips, meristems, and somatic embryos) are encapsulated in calcium alginate beads, followed by conditioning in a high concentration of sucrose for several hours, and air desiccation. After these treatments, the tissues withstand exposure to liquid nitrogen without chemical cryoprotectant additives so cytotoxicity and osmotic damage of cryoprotectants to cells and tissues can be avoided (Fabre and Dereuiddre, 1990).

Moisture content of the tissues is crucial to the success of this protocol. Study of the encapsulation-dehydration of *Ribes* meristems indicated that a 3 hr dehydration period with a 39% moisture content had a higher percentage survival than did a 2 hr dehydration with 50% moisture content (Reed and Yu, 1995). With the decreasing moisture level in the tissues, the concentration of the cytoplasm increases, and becomes so viscous that it is vitrified when quenched into liquid nitrogen. Study of the cryopreservation of oil palm somatic embryos using differential scanning calorimetry (DSC) indicates the complete transition into the amorphous glassy state after quenching into liquid nitrogen (Dumet et al., 1993). The advantage of this protocol is that no cryoprotectant is necessary, resulting in avoidance of toxicity of cryoprotectant to cells and tissues. However, encapsulation dehydration is tedious, time consuming, and beads need individual handling.
The applications of cryopreservation

Cryopreservation of apical meristems

Apical meristems have been used for clonal propagation and for the production of virus-free plants. They have several characteristics that favor their use for cryopreservation. The cells in the meristems are less differentiated and more compact, have larger nuclei, small vacuoles, thin cell walls, and less intercellular space than cells in other tissues (Sakai, 1985). Because of the relative genetic stability of meristematic cells, plants regenerated from in vitro shoot apices without a callus formation stage, result in the recovery of genetically identical progeny (Sakai, 1984). Thus, genetically stable or true-to-type germplasm can be maintained after recovery from liquid nitrogen storage.

Several factors affect the survival of meristems after the freeze-thaw cycle. The concentration of the cryoprotectant is very important. The first cryopreserved isolated meristems were of carnation cryoprotected with 10% dimethyl sulfoxide (DMSO) (Seibert, 1976). Five to 15% DMSO maintained maximum survival of carnation apices frozen slowly to -40 °C, but increasing the concentration of DMSO to 25% decreased the survival. A mixture of DMSO and glucose gave higher survival than a mixture of DMSO and glycerol (Uemura and Sakai, 1980). Preculturing with DMSO before liquid nitrogen treatment greatly improved the survival of apical meristems of pea and strawberry (Kartha
et al., 1979; 1980). Cold acclimation for one week also improved the percentage survival of Rubus sp. (Reed, 1988).

With the development of cryopreservation protocols such as slow freezing, vitrification, and alginate encapsulation-dehydration, meristems or shoot-tips from more than 30 different plant species, e.g. apple, brambles (Rubus sp.), cassava, carnation, currants (Ribes sp.), date palm, mint, pea, peanut, pear, potato, strawberry, sugarbeet, sugarcane, sweet potato, Vaccinium sp., have been successfully cryopreserved (Bajaj, 1995).

**Cryopreservation of dormant buds**

Cryopreservation of dormant buds is also a reliable method for conserving base clonal collections. The first successful experiment in the cryopreservation of non-desiccated plant materials was winter willow twigs prefrozen at -30 °C followed by quenching into LN (Sakai, 1960). This prefreezing method is applied to several hardy trees such as apple (Sakai and Nishiyama, 1978; Tyler and Stushnoff, 1988) and mulberry buds (Niino et al., 1992). A slow cooling rate allows water to move through membranes to extracellular spaces: a freeze-desiccation procedure (Stushnoff and Seufferheld, 1995). Air dehydration over silica gel was also applied to desiccate bud tissues followed by liquid nitrogen treatment (Niino et al., 1992). For less cold hardy or insufficiently acclimated taxa, pretreatment with sugars followed by encapsulation-
dehydration, and the application of cryoprotectants, such as MPVS (modified plant vitrification solution), can enhance survival during the freeze-thaw cycle (Seufferheld et al., 1991). Cryopreservation of dormant winter buds maintains clonal integrity and avoids toxicity from cryoprotectants.

Cryopreservation of callus and cell suspension cultures

Callus and cell suspension cultures are used as model systems to study anatomical, morphological, biochemical, and physiological responses during the freeze-thaw cycle. They are invaluable resources for genetic variability. Many mutant cultures have been selected for their economic importance: high production, high quality, resistance to diseases and stresses, and for their pharmaceutical importance (Kurz and Constabel, 1979). However, long-term in vitro culture causes declines in growth rate and morphogenetic potential for both wheat callus and cell cultures (Ahloowalia, 1982). Several years of subculture changed the alkaloid content and spectra in Hyoscyamus niger (Dhoot and Henshaw, 1977). Because it takes several years to evaluate these mutant plants, and in order to stabilize callus and cell lines, storage in liquid nitrogen would be an ideal method for establishing and maintaining these cultures for plant evaluation and improvement. Callus and cell suspension cultures are convenient to use in physiological and biochemical research.
Plant cells or protoplasts can be successfully cryopreserved with a high recovery rate. Cells in the late lag phase or early exponential phase are superior to larger, highly vacuolated cells for cryopreservation (Withers and Street, 1977). Pretreatment includes the addition of DMSO (Nag and Street, 1975) or mannitol (Withers and Street, 1977). Pretreatment with 1% bovine serum albumin (BSA) followed by adding 15% DMSO produced 60% survival for *Rubus* cell suspension cultures (Lett and Schmitt, 1992). The achievement of a high freezing tolerance depends upon growth stage, cell volume, membrane permeability, physiological conditions and the level of metabolite reserves (Pritchard et al., 1986c).
References


Reed, B.M. 1993. Responses to ABA and cold acclimation are genotype dependent for cryopreserved blackberry and raspberry meristems. Cryobiology. 30: 179-184.


Chapter 2

Proline, BSA, and RABP Pretreatments Improve Recovery of *In Vitro* Currant Meristems and Calli Cryopreserved by Vitrification

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Abstract

Improved recovery of vitrified currant (Ribes aureum Pursh and R. ciliatum Humb. & Bonpl.) meristems and calli was obtained following two-hour pretreatment in sucrose, proline, abscisic acid-responsive proteins (RABP), and bovine serum albumin (BSA). Two-hour immersion in 0.4 M sucrose liquid NCGR-RIB medium prior to vitrification greatly improved the regrowth of meristems compared to 0, 1, 3, and 4 hour immersion. Two-hour immersion of meristems in 5% and 10% proline dissolved in 0.4 M sucrose liquid medium significantly improved regrowth following vitrification. Initial tests with extracts of crude RABP from wheat seedlings found that regrowth of vitrified Ribes apical meristems improved after two-hour immersion pretreatment with the highest survival at 1%. No significant difference in regrowth was found between 1% crude RABP and 0.2% dialyzed RABP, indicating the effectiveness was from the proteins rather than the carbohydrates in crude RABP. Pretreatments of meristems and calli with 5% or 10% proline, 1% crude RABP, and 1% BSA in 0.4 M sucrose solutions produced similar results, and there were no significant differences among them. Meristems in pretreatment groups resumed growth three days after thawing, and reached the maximum regrowth at one week, compared to two weeks for non-pretreatment controls.
Introduction

Cryopreservation as a long-term storage method for germplasm has developed rapidly over the last decade. The development and modification of cryopreservation protocols is important for successful preservation of new plant species and cultivars.

Cryoprotectants are often a source of cell injury. Toxic effects of cryoprotectants [glycerol, dimethyl sulfoxide (DMSO)] used in slow freezing protocols are minimized by pretreatment with sugars, sugar alcohols, and amino acids introduced in solid or liquid medium (Withers and Street, 1977; Withers and King, 1979; Pritchard et al., 1986). Pretreatment reduces the cell size and the cytoplasm to vacuole ratio, enhances the ability of cells or tissues to take up cryoprotectants during prolonged exposure, or modifies cell walls and membranes to resist dehydration injury and deformation during freezing (Withers and King, 1979). Sucrose and proline stabilize membrane bilayers and enzymes during desiccation and freezing (Rudolph and Crowe, 1985; Crowe et al., 1990; Dumet et al., 1993). They act in a colligative manner by preventing the toxic accumulation of compounds in membranes during dehydration and freezing. These colligative cryoprotectants must be present at high concentrations to be effective (Heber et al., 1971; Volger and Heber, 1975).
Vitrification, a newly developed cryopreservation protocol which uses highly viscous solutions to form a glass at low temperature, is now used to preserve plant cells and tissues in liquid nitrogen (LN). Solutions required for vitrification are highly toxic and excessive exposure may damage membrane integrity, inhibit photosynthesis, cause severe plasmolysis, and predispose cells to deplasmolysis injury (Morris, 1976, 1980; McGann, 1978; Withers, 1980). Present vitrification protocols use only limited pretreatment regimes, and modifications to improve regrowth are needed.

Natural cryoprotective mechanisms are present in many seeds and plants. New proteins are synthesized by changes in gene expression in response to stress. Some of these proteins are abscisic acid-responsive proteins (RABP) including some cold-regulated (COR) proteins, late embryogenesis abundant (LEA) proteins, and dehydration-inducible proteins (dehydrin, DHN) (Gilmour et al., 1988; Dure et al., 1981; Close et al., 1989; Jacobson and Shaw, 1989). These proteins are hydrophilic and heat-stable. One such protein, COR15, was more effective on a molar basis in protecting lactate dehydrogenase (LDH) against freeze inactivation \textit{in vitro} than sucrose or bovine serum albumin (BSA) (Lin and Thomashaw, 1992). Cryoprotective effects of RABP include the protection of isolated chloroplast membranes, and isolated thylakoid membranes against mechanical rupture by reducing solute permeability...
during freezing, increasing expandability during thawing, and increasing resistance against the osmotic stress (Heber, 1968; Hincha et al., 1990). RABP, like other cryoprotective proteins, are probably non-colligative and may affect specific loci of the outer membrane (Volger and Heber, 1975). RABP may act as hydrating agents and also bind excess ions to prevent cytotoxicity due to the increase of ion concentration during desiccation (Baker et al., 1988). These ABA-responsive proteins may possess unique biochemical properties that could protect cells or tissues during environmental stresses.

The effect of RABP on the cryopreservation of meristems and calli has not been previously studied. The objective of this research was to investigate the effectiveness of an RABP extract from wheat seedlings as a pretreatment agent to improve the recovery of cryopreserved meristems and calli. In addition, we compared the effectiveness of RABP with that of sucrose, proline, and BSA. RABP used in this research is a desiccation-tolerant protein extracted from wheat seedlings (Ried and Walker-Simmons, 1993). It is hydrophilic, heat-stable, and induced by ABA application and desiccation.
Materials and methods

Plant materials

Ribes aureum Pursh (Rib125) and R. ciliatum Humb. & Bonpl. (Rib670) in vitro plantlets were provided by the USDA/ARS National Clonal Germplasm Repository (NCGR) at Corvallis, Oregon.

General growth conditions

Plantlets

In vitro Ribes (currant) plantlets were grown in Magenta GA7 (Magenta Corporation, Chicago, IL) plastic boxes with 50 ml of NCGR-RIB growth medium composed of MS salts and vitamins (Murashige and Skoog, 1962) with 30% of the normal ammonium and potassium nitrate concentrations, pH 5.7, with (per liter): 50 mg ascorbic acid, 20 g glucose [D-(+)-glucose], 0.1 mg N\textsubscript{6}-benzyladenine (BA), 0.2 mg gibberellic acid (GA\textsubscript{3}) (Sigma, St. Louis, MO), 3.5 g agar (Bitek agar, Difco, Detroit, MI), and 1.45 g Gelrite (Kelco, San Diego, CA). Twenty-five plantlets were cultured in each box. Growth room conditions were 16-h photoperiod (25 mol.m\textsuperscript{-2}.s\textsuperscript{-1}) at 25°C. The growth cycle was three weeks.

Calli

Stems of In vitro-grown R. ciliatum were cut into segments (2-3 mm) and grown for calli in GA7 boxes with 50 ml of NCGR-RIB medium salts and
vitamins as for plantlets and 0.04 mg BA, 1 mg indole-3-butyric acid (IBA),
and 1 mg 2,4-dichloro-phenoxyacetic acid (2,4-D) (Sigma). Twenty-five calli
(4-5 mm in diameter) were induced and cultured in the dark at 25°C. The
growth cycle was two weeks.

**Cold acclimatization (CA)**

After 2 wk in the medium at general growth conditions, the boxes (25
plantlets/box) were moved into a cold acclimatization (CA) chamber for 1
wk. CA was 22°C for 8 hr (day, 3 mol m⁻² s⁻¹), and -1°C for 16 hr (night)
(Reed and Yu, 1995).

**Preculture of meristems and calli**

After cold acclimatization, 25 meristems (0.8 mm) were dissected and
precultured on NCG-RIB medium with 5% dimethyl sulfoxide (DMSO,
Sigma), 3.5 g agar, and 1.75 g Gelrite. Twenty-five calli were also
transferred to DMSO medium. All meristems and calli remained in CA on
DMSO medium for 48 hr.

**Pretreatment regimes**

**Sucrose liquid medium: determination of immersion time**

Twenty five meristems for each treatment were transferred to 1.2 ml
cryovials (Vangard Cryos, Sumitomo Bakelite Co., Ltd., Japan) with 1 ml 0.4
M sucrose liquid NCG-RIB medium and immersed for 1, 2, 3, and 4 hr.
Twenty meristems were vitrified by plunging in LN, and five were used for non-frozen controls with 3 replications (n=60). As treatment control, a non-immersed group (0 hr pretreatment) remained on NCGR-RIB medium with 5% DMSO until vitrification. All the pretreatment and control groups were followed by the vitrification procedure.

**Proline, crude and dialyzed RABPs, and BSA pretreatment**

Meristems or calli were immersed in 1 ml of pretreatment solutions for 2 hr and vitrified in LN (20 meristems, 15 calli) or used for non-frozen controls (5 meristems, 10 calli). Pretreatments included two-hour soaking in: 0.4 M sucrose liquid Rib medium; 1% (w/v), 5%, 10%, and 15% proline solutions; 0.5%, 1%, and 2% crude RABPs; 0.2% dialyzed RABPs; and 1% BSA. Crude and dialyzed RABPs were provided by Dr. Kay Walker-Simmons (USDA/ARS, 209 Johnson Hall, Washington State University, Pullman, WA). Proline, RABP (crude and dialyzed), and BSA were dissolved in 0.4 M sucrose liquid medium, and filter-sterilized (25mm, 0.20 micron, Corning Glass Works, Corning, NY). As treatment controls, non-pretreatment groups remained on NCGR-RIB medium with 5% DMSO until vitrification.

**Vitrification procedure**

General vitrification procedures were identical to those described by Reed and Yu (1995). Meristems or calli in control groups (without
pretreatment) were transferred directly to 1.2 ml cryovials with 1 ml of plant vitrification solution #2 (PVS2 - 15% DMSO, 15% ethylene glycol, and 30% glycerol (Sigma), brought to volume with 0.4 M sucrose liquid medium) (Sakai et al., 1990). Pretreatment solutions were replaced and the meristems or calli were rinsed twice with PVS2. One ml PVS2 solution was added to cryovials. Samples were incubated in PVS2 on ice for 20 min. The cryovials were plunged directly into LN and exposed for at least one hour. The controls were immediately recovered from PVS2 solution and plated as described below.

Cryovials were removed from the LN dewar, immediately plunged into 45°C water for 1 minute, and transferred to 22°C water for another minute. After thawing, PVS2 solution in the cryovials was immediately diluted with 1.2 M sucrose liquid NCG-RIB medium, and meristems and calli were rinsed three times. Meristems were drained on filter paper strips (Whatman International Ltd., Maidstone, England), and plated in individual wells of a 24-well plate (Costar Corporation, Cambridge, MA) containing NCG-RIB growth medium. All the meristems in cell-well plates were grown in the same general conditions as for plantlets. Regrowth of meristems was recorded as percentage of green and growing shoots 4 weeks after thawing. Calli were held in 1.2 M liquid NCG-RIB medium for 5 min followed by the viability test procedures described below.
Viability test for callus

A modified TTC (triphenyl tetrazolium chloride) reduction assay (DiMaio and Shillito, 1989) was used to determine viability. A 1% percent TTC solution was made by dissolving TTC in 0.05 M KH₂PO₄ (potassium phosphate) buffer (pH 5.8). Thawed calli were rinsed twice in TTC solution, and 1% TTC solution was added to an equal volume of calli. The cryovials with calli and TTC solution were held overnight at room temperature in the dark. Living cells turned red.

After staining, the calli were rinsed twice in deionized filtered water, and placed in 3 ml 100% ethyl alcohol (Nalgene, Nalge Company, Rochester, NY). The red pigment was extracted at 70°C for 30 min. Absorbance was read at 530nm (Spectronic 20, Bausch & Lomb Inc., Rochester, NY). The viability of callus after thawing was expressed as:

\[
\text{Relative percentage survival (\%)} = \frac{A_{530}\text{(frozen-thawed)}}{A_{530}\text{(non-frozen)}}
\]

Statistical analysis

The percentage regrowth (percentage survival for calli) were transformed to arcsin \( \sqrt{x} \) and analyzed with ANOVA and the Duncan's multiple range test with significance designated as \( P \leq 0.05 \) or \( P \leq 0.01 \) using STATGRAPHIC 5.0 (Statistical Graphics Corporation and STSC Inc., Rockville, MD).
Results

Meristems

Sucrose

Pretreatment of *R. ciliatum* meristems in 0.4 M sucrose liquid medium before vitrification produced higher regrowth than direct PVS2 exposure for any of four immersion times tested (Table 2.1). Two-hour immersion produced the highest regrowth (66.7%) (P<0.01). There were no significant differences among the regrowth of meristems with 1, 3, and 4 hr immersion (P>0.05). Extended immersion (3 hr and 4 hr) in the 0.4 M sucrose liquid medium significantly decreased meristem regrowth, compared to the 2 hr pretreatment.

Proline

Proline pretreatment (5 and 10%) significantly improved the regrowth of vitrified *R. ciliatum* meristems (P < 0.05) compared to pretreatment with 0.4 M sucrose, 1% and 15% proline, and non-pretreatment (Table 2.2). There were no significant differences among pretreatments with 0.4 M sucrose, 1% and 15% proline solution, and between 5% and 10% (P > 0.05).
Table 2.1

The Effect of Pretreatment Immersion Times in 0.4 M Sucrose Liquid Medium on Regrowth of Vitrified *R. ciliatum* (RIB 670) Meristems Four Weeks After Thawing

<table>
<thead>
<tr>
<th>Immersion pretreatment (hr)</th>
<th>Replication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>42.13 x</td>
</tr>
<tr>
<td>2</td>
<td>39.23</td>
</tr>
<tr>
<td>3</td>
<td>42.13</td>
</tr>
<tr>
<td>Mean y</td>
<td>41.16 c</td>
</tr>
<tr>
<td>Standard error (SE)</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Source of variation z  
Between groups 72.7539  25.48 ***  
Within groups 2.8554

x  Percentage regrowth data were transformed by arcsin $\sqrt{X}$.
y  Mean separation by Duncan’s multiple range test at $P \leq 0.05$ ($n = 60$).
z  Variation between treatments: four degrees of freedom.
*** Significant at $P \leq 0.001$. 
Table 2.2

Regrowth of Vitrified *R. ciliatum* (RIB 670) Meristems Following Pretreatment with 0.4 M Sucrose Liquid Medium Alone or With 1%, 5%, 10%, and 15% Proline Four Weeks After Thawing

<table>
<thead>
<tr>
<th>Replication</th>
<th>None</th>
<th>0.4 M Sucrose</th>
<th>1% Proline</th>
<th>5% Proline</th>
<th>10% Proline</th>
<th>15% Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45²</td>
<td>53.73</td>
<td>50.77</td>
<td>63.43</td>
<td>63.43</td>
<td>53.73</td>
</tr>
<tr>
<td>2</td>
<td>47.87</td>
<td>53.73</td>
<td>53.73</td>
<td>71.57</td>
<td>67.21</td>
<td>56.79</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>56.79</td>
<td>53.73</td>
<td>67.21</td>
<td>63.43</td>
<td>56.79</td>
</tr>
<tr>
<td>Mean</td>
<td>45.96 c</td>
<td>54.75 b</td>
<td>52.74 b</td>
<td>67.40 a</td>
<td>64.69 a</td>
<td>55.77 b</td>
</tr>
</tbody>
</table>

Standard error (SE) 0.96 1.02 0.99 2.35 1.26 1.02

Source of variation² Mean square F-ratio
Between groups 188.3620 33.98 ***
Within groups 5.5441

² Percentage regrowth data were transformed by arcsin $\sqrt{X}$.

³ Mean separation by Duncan's multiple range test at $P \leq 0.05$ (n = 60).

³ Variation between treatments: five degrees of freedom.

*** Significant at $P \leq 0.001$. 
**Crude and dialyzed RABP, and BSA solutions**

**Comparison of different concentrations of crude RABP**

Pretreatment of meristems in 1% and 2% crude RABP significantly improved regrowth (P < 0.01), compared to that without pretreatment or pretreatment with 0.4 M sucrose, and 0.5% crude RABP (Table 2.3). One percent crude RABP was significantly better than 2%, and gave the highest regrowth (83.33%) (Table 2.3). There was no significant difference between pretreatment with 0.4 M sucrose liquid medium and 0.5% crude RABP solution (P > 0.05).

**Comparison of proline, crude and dialyzed RABP, and BSA solutions**

Both crude and dialyzed RABP pretreatment produced significant improvement in regrowth of cryopreserved meristems of *R. ciliatum* following vitrification, compared to pretreatment with 0.4 M sucrose liquid medium or non-pretreatment (P < 0.01) (Table 2.4). There was no significant difference between 1% crude and 0.2% dialyzed RABP (P > 0.05).

Significant improvement in regrowth of cryopreserved meristems of *R. ciliatum* following vitrification was obtained with pretreatment solutions of 5% proline, 1% crude RABP, 0.2% dialyzed RABP, and 1% BSA, compared to that of 0.4 M sucrose liquid medium and non-
Table 2.3

Regrowth of Vitrified *R. ciliatum* (RIB 670) Meristems Following Pretreatment with 0.4 M Sucrose Liquid Medium Alone or With 0.5%, 1%, and 2% Crude RABP (cRABP) Four Weeks After Thawing

<table>
<thead>
<tr>
<th>Replication</th>
<th>None</th>
<th>0.4 M Sucrose</th>
<th>0.5% cRABP</th>
<th>1% cRABP</th>
<th>2% cRABP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42.13 x</td>
<td>53.73</td>
<td>56.79</td>
<td>63.43</td>
<td>60</td>
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<tr>
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<td>56.79</td>
<td>67.21</td>
<td>63.43</td>
</tr>
<tr>
<td>Mean</td>
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<td>54.75 c</td>
<td>57.86 c</td>
<td>65.95 a</td>
<td>62.29 b</td>
</tr>
<tr>
<td>Standard error (SE)</td>
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<td>1.02</td>
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<td>1.26</td>
<td>1.14</td>
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Source of variation²

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<td>64.05 ***</td>
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<tr>
<td>Within groups</td>
<td>3.5972</td>
<td></td>
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</tbody>
</table>

² Percentage regrowth data were transformed by arcsin $\sqrt{X}$.

³ Mean separation by Duncan's multiple range test at $P \leq 0.05$ (n = 60).

⁴ Variation between treatments: four degrees of freedom.

*** Significant at $P \leq 0.001$. 
Table 2.4

Regrowth of Vitrified *R. ciliatum* (RIB 670) Meristems Following Pretreatment with 0.4 M Sucrose Liquid Medium Alone or With 5% Proline, 1% Crude RABP (cRABP), 0.2% Dialyzed RABP (dRABP), and 1% BSA Four Weeks After Thawing

<table>
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<tr>
<th>Replication</th>
<th>None</th>
<th>0.4 M Sucrose</th>
<th>5% Proline</th>
<th>1% cRABP</th>
<th>0.2% dRABP</th>
<th>1% BSA</th>
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<tr>
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<td>50.77</td>
<td>71.57</td>
<td>67.21</td>
<td>63.43</td>
<td>63.43</td>
</tr>
<tr>
<td>3</td>
<td>42.13</td>
<td>53.73</td>
<td>67.21</td>
<td>71.57</td>
<td>71.57</td>
<td>67.21</td>
</tr>
<tr>
<td>Mean</td>
<td>42.13 c</td>
<td>53.76 b</td>
<td>67.40 a</td>
<td>76.26 a</td>
<td>68.86 a</td>
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<td>6.98</td>
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Source of variation

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<th>F-ratio</th>
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<tr>
<td>Within groups</td>
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</table>

\(x\) Percentage regrowth data were transformed by \(\text{arcsin}\sqrt{x}\).

\(y\) Mean separation by Duncan's multiple range test at \(P \leq 0.05\) (\(n = 60\)).

\(z\) Variation between treatments: five degrees of freedom.

*** Significant at \(P \leq 0.001\).
Table 2.5

Regrowth of Vitrified *R. aureum* (Rib 125) Meristems Following Pretreatment with 0.4 M Sucrose Liquid Medium Alone or With 5% Proline, 1% Crude RABP (cRABP), 0.2% Dialyzed RABP (dRABP), and 1% BSA Four Weeks After Thawing

<table>
<thead>
<tr>
<th>Replication</th>
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<th>0.4 M Sucrose</th>
<th>5% Proline</th>
<th>1% cRABP</th>
<th>0.2% dRABP</th>
<th>1% BSA</th>
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<tbody>
<tr>
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<td>53.73</td>
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<td>2</td>
<td>45</td>
<td>53.73</td>
<td>63.43</td>
<td>67.21</td>
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<td>67.21</td>
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<tr>
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<td>45</td>
<td>50.77</td>
<td>63.43</td>
<td>63.43</td>
<td>67.21</td>
<td>67.21</td>
</tr>
<tr>
<td>Mean^y</td>
<td>44.04 c</td>
<td>52.74 b</td>
<td>64.69 a</td>
<td>65.95 a</td>
<td>67.40 a</td>
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Standard error (SE) 0.96 0.99 1.26 1.26 2.35 1.45

Source of variation 2

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<th>Mean square</th>
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<td>Between groups</td>
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<td>46.51   ***</td>
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<tr>
<td>Within groups</td>
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</tbody>
</table>

^x Percentage regrowth data were transformed by arcsin√x.

^y Mean separation by Duncan's multiple range test at P ≤ 0.05 (n = 60).

² Variation between treatments: five degrees of freedom.

*** Significant at P ≤ 0.001.
pretreatment control (P < 0.01) (Table 2.4). There were no significant differences among 5% proline, 1% crude RABP, 0.2% dialyzed RABP, and 1% BSA solutions (P > 0.05) (Table 2.4). *R. aureum* treated with the same pretreatment solutions showed a similar pattern of the effectiveness (Table 2.5).

**Time course of regrowth of vitrified meristems**

Three days after thawing and plating, most of the meristems of *R. ciliatum* resumed growth (Figure 2.1). The meristems in the pretreatment groups reached maximum regrowth between three days and one week, while non-pretreatment control reached the highest regrowth in two weeks. Shoots grew directly from meristems without a callus phase.

**Calli**

Pretreatment with 5% proline, 1% crude RABP, and 1% BSA solutions significantly improved survival of cryopreserved *R. ciliatum* calli following vitrification, compared to 0.4 M sucrose liquid medium and non-pretreatment (P < 0.05) (Table 2.6). There were no significant differences among pretreatments with 5% proline, 1% crude RABP, and 1% BSA (P > 0.05). *R. aureum* treated with the same pretreatment solutions showed a similar pattern of the effectiveness (Table 2.7).
Fig. 2.1. Time course of regrowth of meristems of *R. ciliatum* (Rib 670) following 2 hr pretreatments in 0.4 M sucrose liquid medium alone or with 5% proline, 1% BSA, 0.2% dialyzed RABP (dRABP), and 1% crude RABP (cRABP), or non-pretreated control, vitrification in PVS2, and rapid thawing.
Table 2.6

Survival of Vitrified *R. ciliatum* (RIB 670) Calli Following 2 Hour Pretreatment with 0.4 M Sucrose Liquid Medium Alone or With 5% Proline, 1% Crude RABP (cRABP), and 1% BSA as Determined by the TTC Reduction Test

<table>
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<tr>
<th>Replication</th>
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<th>0.4 M Sucrose</th>
<th>5% Proline</th>
<th>1% cRABP</th>
<th>1% BSA</th>
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<tbody>
<tr>
<td>1</td>
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<td>61.12</td>
<td>62.77</td>
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<td>2</td>
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<td>3</td>
<td>43.30</td>
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<td>59.62</td>
<td>68.07</td>
<td>60.76</td>
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<tr>
<td>Mean</td>
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<td>48.74</td>
<td>60.98</td>
<td>64.30</td>
<td>64.21</td>
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<tr>
<td>Standard error (SE)</td>
<td>2.12</td>
<td>1.23</td>
<td>0.75</td>
<td>1.89</td>
<td>2.16</td>
</tr>
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</table>

Source of variation: Mean square  

- Between groups: 353.8756  
- Within groups: 8.9263

F-ratio:

- Between groups: 39.64 ***

*Percentage survival data were transformed by arcsin√x.*

*Mean separation by Duncan's multiple range test at P ≤ 0.05 (n = 45).*

*Variation between treatments: four degrees of freedom.*

***Significant at P ≤ 0.001.*
### Table 2.7

Survival of Vitrified *R. aureum* (Rib 125) Calli Following Pretreatment with 0.4 M Sucrose Liquid Medium Alone or With 5% Proline, 1% Crude RABP (cRABP), and 1% BSA as Determined by the TTC Reduction Test

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<th>Replication</th>
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<th>0.4 M Sucrose</th>
<th>5% Proline</th>
<th>1% cRABP</th>
<th>1% BSA</th>
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<td>45.00</td>
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<td>49.87</td>
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<tr>
<td>Mean</td>
<td>39.84</td>
<td>45.80</td>
<td>50.60</td>
<td>51.00</td>
<td>50.56</td>
</tr>
</tbody>
</table>

**Standard error (SE)**

- 0.52
- 0.42
- 0.84
- 0.62
- 0.46

**Source of variation**

- **Mean square**
  - Between groups: 69.5681
  - Within groups: 1.0460

**F-ratio**

- 66.51 ***

---

*Percentage survival data were transformed by* arcsin $\sqrt{x}$.

*Mean separation by Duncan's multiple range test at P ≤ 0.05 (n = 45).*

*Variation between treatments: four degrees of freedom.*

***Significant at P ≤ 0.001.*
Discussion

Many factors affect the recovery of vitrified meristems and callus including pretreatment. Early pretreatments focused on DMSO (Nag and Street, 1975; Kartha et al., 1980), DMSO plus sugar, and proline (Withers and King, 1979). DMSO or DMSO mixtures are applied in solid or liquid medium, and specimens are precultured for days. Our present research provides a simple and effective method for pretreatment with hourly immersion of specimens in pretreatment solutions after been precultured in 5% DMSO medium. Immersion facilitates the absorption into or adsorption onto the specimens.

Sucrose is known for its natural cryoprotectant qualities because it accumulates in some organisms following stress (Franks, 1985). Sakai and Yoshida (1968) found that sucrose was one of the best cryoprotectants for cabbage cells. Sucrose serves as an osmotic agent in cells and protects membranes through colligative effects and stabilizes enzymes and membrane bilayers through a preferential exclusion mechanism - the exclusion of the structure-stabilizing compounds from binding on the surface of protein (enzyme). The stabilizing compounds raise the surface tension of water so that the water with its solute is excluded from contact with the protein surface, thus preventing
denaturation of protein structure (Arakawa and Timasheff, 1982; Low, 1985).

Pretreatment with sucrose solutions reduces freezable water by osmosis and improves the survival of cryopreserved carnation shoot tips (Dereuddre et al., 1988). Our studies confirm that sucrose protects against freezing injury. Two-hour immersion in the sucrose solution was optimal for Ribes meristems and calli to adsorb adequate sucrose molecules for protection against freezing injury without adverse effects (Table 2.1). However, extended immersion (3 hr and 4 hr) of meristems in 0.4 M sucrose liquid medium decreased the regrowth. Extended immersion in sucrose solutions may overly dehydrate tissues or cells, or may exclude oxygen from the tissues and cause injury unrelated to freezing.

Proline, another natural cryoprotective substance produced following stress, is one of the cryoprotective amino acids which functions through a colligative effect and interacts with cell membranes at specific loci (Heber et al., 1971). Proline may act by a preferential exclusion mechanism at lower temperatures (Crowe et al., 1990). Proline inhibits membrane mixing during freezing and stabilizes bilayers by preventing the aggregation of intramembranous particles (Rudolph and Crowe, 1985). Post-thaw viability of Zea mays suspension cultures increased with a 3 to 4 day pretreatment of 10% proline (w/v) in the medium
Our results indicated that 2 hr immersion in 5% or 10% proline dissolved in 0.4 M sucrose liquid medium improved the recovery of vitrified *Ribes* meristems and calli. Increasing the concentration of proline (15%) significantly decreased the regrowth (Table 2.2). It could be inferred that at certain concentrations of proline (probably 5 to 10%), the permeability of cell membranes is altered to easily and adequately take up the cryoprotectant for protection during freezing. Proline acts by colligative effects to decrease the concentration of toxic solutes in cells, but also exhibits non-colligative effects of modifying membrane structure and playing an important role in maintain proper functions of cell membranes (bilayer and proteins) during freezing. We did not determine why proline at higher concentrations (15%) exerted adverse effects on meristems.

Many physiological and biochemical changes occur during the induction of freezing tolerance or cold acclimatization. The accumulation or induction of new specific proteins responsive to low temperature, dehydration, osmolarity, and ABA application is one possibility. Many of these proteins are abscisic acid- responsive proteins (RABP) which include some of the cold-regulated (COR) proteins, the late embryogenesis abundant (LEA) proteins, and the dehydration-inducible proteins (dehydrin, DHN) (Gilmour et al., 1988; Dure et al., 1981; Close et al., 1989; Jacobson and Shaw, 1989). These proteins are hydrophilic...
and heat-stable. Glycoproteins/glycopeptides are hydrophilic, causing a decrease in the free water content in cells, providing resistance to cell volume loss, and lowering the glass transition temperature (Williams, 1973). Damage from dehydration effects of extracellular ice formation would be eliminated with the addition of proteins because of the increase in cellular osmotic potential (Johnson-Flanagan and Singh, 1988).

Cryoprotective proteins or antifreeze proteins (AFP) from both plants and fish have been demonstrated to protect membranes of organelles, enzymes, cells and leaves (Heber, 1968; Hincha et al., 1990; Lin and Thomashow, 1992; Cutler et al., 1989). In this study, pretreatment with RABP from wheat seedlings was effective in protecting meristems and calli against both toxic effects of vitrification solutions and freezing injury. It took longer time for meristems of non-frozen controls in non-pretreated groups to recovery, compared to 80-100% regrowth in all non-frozen controls in pretreatment groups three days after thawing (data not shown). Preliminary experiments indicated that RABP alone was ineffective as a cryoprotectant, but significantly improved regrowth when followed by PVS2 cryopreservation (data not shown). A significant improvement in recovery of vitrified meristems and calli was obtained from pretreatment with 1% crude RABP or 0.2% dialyzed RABP, indicating that effectiveness was from the RABP rather than the carbohydrates present in crude RABP extracts (Tables 2.4-2.7).
Because of its hydrophilic character, RABP may bind water molecules from cells or tissues. Because of its high molecular weight (10-100 kD) (K. Walker-Simmons, personal communication), RABP probably does not penetrate into cells but rather attaches to the surface of cells and tissues to create a "shell", and exerts its action as an osmotic agent. The presence of RABP, like pretreatments with sucrose and proline, may modify cell structures, and stabilize membranes preparing them for dehydration and vitrification (Withers and King, 1979). In a study of cryoprotective efficiency of BSA on protecting isolated thylakoid membranes, high BSA concentrations (2 to 3%) damaged membranes, but the damage could be avoided by adding high concentrations of ethylene glycol monomethyl ether (EGMME) prior to freezing to -20°C (Santarius, 1996). This may partially explain why higher concentrations of RABP (2%) resulted in decreased regrowth (Table 2.3). Another possible cause may be related to osmotic injury to meristems.

Because RABP is a mixture of groups of proteins (K. Walker-Simmons, personal communication), the separation of protein fractions would be necessary to further investigate whether the cryoprotective effect is from the mixture of proteins or from one or several protein combinations. We did not determine the extent of RABP infiltration into the tissues. Vacuum infiltration and quantitation of radioactive RABP would indicate how much RABP was absorbed or adsorbed to meristems.
and calli. The relationship between the amount of RABP taken up and the cryoprotective effects would be more clearly illustrated. Additional study of the mode of action of RABP could be investigated using NMR and DSC.

BSA is often applied in animal cell culture as a growth factor. The main functions of albumin are to carry lipids, hormones, and minerals, provide osmotic pressure and buffering capacity, and promote cell attachment (Maurer, 1986). In plants it maintains plasma membrane integrity during dehydration (Steponkus, 1984), protects LDH activity against freeze denaturation (Tamiya et al., 1985), and has been used as part of pretreatment solution at 1% in cryopreservation of *Rubus* cell suspension cultures with 60% survival (Lett and Schmitt, 1992). Our study with *Ribes* showed improvement of regrowth of cryopreserved meristems and calli with 2 hr pretreatment with 1% BSA (Tables 2.4-2.7). The possible mechanism of BSA protection may be the same as that of RABP, because BSA also has a high molecular weight (66 kD), acts as an osmotic agent, and stabilizes membrane structure.

Another positive aspect of this pretreatment protocol was the rapid recovery of meristems with almost all pretreatment groups (Figure 2.1). Meristems resumed growth in three days and reached the maximum recovery around one week, while almost two weeks were required for non-pretreatment controls. The rapid recovery of meristems with
pretreatment indicated the effectiveness of sucrose, proline, RABP, and BSA in protecting meristems and calli against freezing or cryoprotectant injury.
Conclusion

Pretreatment in the form of 2 hr immersion is an effective procedure to improve recovery of vitrified meristems and calli of two currant species, *Ribes aureum* and *R. ciliatum*. Sucrose, proline, RABP, and BSA all protected meristems and calli during the freeze-thaw cycle. Pretreatment with 5% proline, 1% crude RABP, 0.2% dialyzed RABP, and 1% BSA significantly improved the regrowth of cryopreserved meristems and calli, compared to pretreatment with 0.4 M sucrose liquid NCGR-RIB medium or non-pretreatment. The effectiveness of pretreatment solutions is due to the osmotic effects of sucrose, RABP, and BSA solutions, colligative effects of proline and sucrose, and non-colligative effects of proline, RABP and BSA. Finally, all these solutions play an important role in maintaining integrity of cell structures and stabilizing cell membranes during the freeze-thaw cycle which result in faster recovery following thawing.
References


Bibliography


Reed, B.M. 1993. Responses to ABA and cold acclimation are genotype dependent for cryopreserved blackberry and raspberry meristems. Cryobiology. 30: 179-184.


APPENDICES
Appendix 1

Cold Acclimatization (CA) Pretreatment Improves Regrowth of In Vitro Currant Meristems Cryopreserved by Vitrification

Abstract

Cold acclimatization pretreatments are beneficial for increasing cryopreservation survival for many species. Apical meristems of in vitro Ribes plantlets cold-acclimatized for 1, 2, 3, and 4 weeks following cryopreservation had better survival than meristems from non-acclimatized plantlets (P < 0.05). Plant acclimatized for 3 and 4 wk had higher survival than those acclimated for 1 and 2 wk. There were no significant differences between 1 and 2 wk, and 3 and 4 wk CA pretreatments.
Introduction

The success of cryopreservation relies on several factors including the plant’s physiological condition, proper protocols, and pretreatment or preculture.

Cold acclimatization (CA), a pretreatment procedure, is a biochemical and physiological process which increases cold tolerance and prevents cell or tissue injury. CA has two main functions: the adjustment of metabolism and basic cellular function to the biophysical constraints imposed by low temperature, and the induction of freezing tolerance (Guy, 1990). Some cryoprotective substances are increased in response to CA, including sugar content, levels of glycoproteins or glycopeptides, cold-regulated (COR) proteins, and proline (Delvallee et al., 1989; Guy, 1990; Lin et al., 1990). CA also protects plasma membrane integrity and alters lipid compositions of membranes for increasing resistance to freezing injury (Steponkus, 1984; Uemura and Steponkus, 1994). CA improves survival of cryopreserved apical meristems of Vaccinium and Rubus in vitro plants (Reed, 1988; 1989).

The effects of CA on the survival of cryopreserved Ribes meristems has not been studied. The objectives of this study were to compare the survival of four CA regimes followed vitrification, and to determine the proper duration of CA pretreatment for Ribes cryopreservation.
Materials and methods

Plant materials

Ribes ciliatum Humb. & Bonpl. (Rib670) in vitro plantlets were provided by the USDA/ARS National Clonal Germplasm Repository (NCGR) at Corvallis, Oregon.

General growth conditions

In vitro Ribes (currant) plantlets were grown in Magenta GA7 (Magenta Corporation, Chicago, IL) plastic boxes with 50 ml of NCGR-RIB growth medium composed of MS salts and vitamins (Murashige and Skoog, 1962) with 30% of the normal ammonium and potassium nitrate concentrations, pH 5.7, with (per liter): 50 mg ascorbic acid, 20 g glucose [D-(+)-glucose], 0.1 mg N6-benzyladenine (BA), 0.2 mg gibberellic acid (GA3) (Sigma, St. Louis, MO), 3.5 g agar (Bitek agar, Difco, Detroit, MI), and 1.45 g Gelrite (Kelco, San Diego, CA). Twenty-five plantlets were cultured in each box. Growth room conditions were 16-h photoperiod (25 mol m⁻² s⁻¹) at 25°C. The growth cycle was three weeks.

Cold acclimatization (CA) regimes: comparison and determination

After 2 wk in the medium at general growth conditions, the boxes (25 plantlets/box) were moved into a cold acclimatization (CA) chamber for 1, 2, 3, and 4 wks. The non-CA controls remained at standard conditions. CA
was achieved in the condition of 22°C for 8 hr (day, 3 mol·m⁻²·s⁻¹), and -1°C for 16 hr (night) (Reed and Yu, 1995).

**Preculture of meristems**

After cold acclimatization of 1, 2, 3 or 4 weeks, 25 meristems (0.8 mm) were dissected and precultured on NCGR-RIB medium with (per liter) 5% dimethyl sulfoxide (DMSO, Sigma), 3.5 g agar, and 1.75 g Gelrite. All meristems remained in the CA chamber on DMSO medium for 48 hr. Twenty-five control meristems were dissected from non-CA plantlets and precultured on DMSO medium for 48 hr at general growth conditions.

**Vitrification procedure**

Meristems in non-CA and CA groups were transferred to 1.2 ml cryovials (Vangard Cryos, Sumitomo Bakelite Co., Ltd., Japan) with 1 ml of plant vitrification solution #2 (PVS2 - 15% DMSO, 15% ethylene glycol, and 30% glycerol (Sigma), brought to volume with 0.4 M sucrose liquid medium) (Sakai et al., 1990). General vitrification procedures were the same as described by Reed and Yu (1995). Samples were incubated in PVS2 on ice for 20 min. The cryovials with twenty meristems were plunged directly into liquid nitrogen (LN) and exposed for at least one hour, and five were used for non-frozen controls with 3 replications (n=60). The controls were immediately recovered from PVS2 solution and plated as described below.
Cryovials were removed from the LN dewar, immediately plunged into 45°C water for 1 minute, and transferred to 22°C water for another minute. After thawing, PVS2 solution in the cryovials was immediately diluted with 1.2 M sucrose liquid NCG-RIB medium, and rinsed three times. Meristems were drained on filter paper strips (Whatman International Ltd., Maidstone, England), and plated in individual wells of 24-well plate (Costar Corporation, Cambridge, MA) containing NCG-RIB growth medium. All the meristems in cell-well plates were grown in the same general conditions as for plantlets. Regrowth of meristems was recorded as percentage of green and growing shoots 4 weeks after thawing.

**Statistical analysis**

The percentage regrowth was transformed to arcsin $\sqrt{x}$ and analyzed with ANOVA and the Duncan's multiple range test with significance of $P \leq 0.05$ or $P \leq 0.01$ using STATGRAPHIC 5.0 (Statistical Graphics Corporation and STSC Inc., Rockville, MD).
**Results and discussion**

Cold acclimatization at any duration significantly improved the regrowth of *R. ciliatum* meristems cryopreserved by vitrification, compared to non-CA controls (P < 0.05) (Table 1). Within the pretreatment groups, meristems of plantlets cold-acclimatized for 3 and 4 weeks had higher percentage regrowth than those acclimatized for 1 and 2 weeks (P < 0.05), and there were no significant differences between acclimatization for 1 and 2 wk, and between 3 and 4 wk (P > 0.05) (Table 1). These results confirmed the effectiveness of CA on the regrowth of cryopreserved meristems. Because different species vary in hardiness, the duration of CA treatment may vary among and within species. At least three weeks of CA is required for *Vaccinium* species to achieve successful hardening to improve recovery following cryopreservation (Reed, 1989), but only one week is required for *Rubus* (Reed, 1988). From this study, three or four weeks of CA provided the best improvement of regrowth of currant meristems following cryopreservation. CA screening of different genotypes of *Ribes* could be done by grouping genotypes by different CA requirements to improve regrowth following cryopreservation.
Table A-1
The Effect of Cold Acclimatization on Regrowth of Vitrified *R. ciliatum* (RIB 670) Meristems Four Weeks After Thawing

<table>
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<th>Replication</th>
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<th></th>
<th></th>
<th></th>
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<td>2</td>
<td>3</td>
<td>4</td>
<td></td>
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<tr>
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<td>26.57</td>
<td>48.19</td>
<td>42.13</td>
<td>62.21</td>
<td>56.79</td>
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</tr>
<tr>
<td>3</td>
<td>29.21</td>
<td>39.23</td>
<td>45</td>
<td>58.52</td>
<td>57.69</td>
<td></td>
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<tr>
<td>Mean</td>
<td>30.68 c</td>
<td>44.14b</td>
<td>44.50b</td>
<td>60.24a</td>
<td>56.16a</td>
<td></td>
</tr>
<tr>
<td>Standard error (SE)</td>
<td>2.90</td>
<td>2.62</td>
<td>1.25</td>
<td>1.07</td>
<td>0.96</td>
<td></td>
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<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Mean square</th>
<th>F-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>434.3460</td>
<td>38.35***</td>
</tr>
<tr>
<td>Within groups</td>
<td>11.3258</td>
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</tbody>
</table>

* x Percentage regrowth data were transformed by arcsin√X.

Y Mean separation by Duncan's multiple range test at P ≤ 0.05 (n = 60).

z Variation between treatments: four degrees of freedom.

*** Significant at P ≤ 0.001.
References


Appendix 2

Comparison and Determination of PVS2 Immersion Time on Survival of Cryopreserved Currant Calli by Vitrification

Calli of *Ribes ciliatum* Humb. & Bonpl. were induced from plantlet segments, precultured on DMSO medium for 48 hr, then transferred to cryovials with 1 ml PVS2 solution, and held on ice for 5, 10, 15, 20, and 25 min. The vitrification and viability test procedures were followed as described in Materials and Methods of Chapter 2.

Twenty min incubation in PVS2 provided the highest percentage survival than any other incubation times (*P* < 0.05). The order of decreasing percentage survival was 20 > 25 > 15 = 10 > 5 min (Table A-2). Longer incubation (20 min) of calli in PVS2 at low temperature provided adequate absorption of cryoprotectants into cells, thus prevented freezing injury following vitrification. However too much incubation (25 min) in PVS2 is harmful to the cells because of the toxicity of the cryoprotectant chemicals.

Pretreatment of calli in cryoprotectant before freezing is necessary for survival of vitrified calli. Twenty min incubation in PVS2 provided higher survival than any other immersion times.
Table A-2

The Effect of Immersion Duration in PVS2 on Survival of Vitrified *R. ciliatum* (RIB 670) Calli as Determined by the TTC Reduction Test

<table>
<thead>
<tr>
<th>PVS2 immersion (min)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
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<td>Replication</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>23.14 d</td>
<td>29.96 c</td>
<td>31.58 c</td>
<td>42.06 a</td>
<td>36.42 b</td>
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<tr>
<td>Standard error (SE)</td>
<td>1.02</td>
<td>0.65</td>
<td>0.25</td>
<td>0.69</td>
<td>0.38</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Source of variation $^z$</th>
<th>Mean square</th>
<th>F-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>151.0297</td>
<td>117.49 ***</td>
</tr>
<tr>
<td>Within groups</td>
<td>1.2855</td>
<td></td>
</tr>
</tbody>
</table>

$^x$ Percentage survival data were transformed by arcsin $\sqrt{x}$.

$^y$ Mean separation by Duncan's multiple range test at $P \leq 0.05$ ($n = 45$).

$^z$ Variation between treatments: four degrees of freedom.

*** Significant at $P \leq 0.001$. 