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PRETREATMENT STRATEGIES FOR THE CRYOPRESERVATION OF PLANT TISSUES

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Abstract: Preconditioning of plant material prior to cryopreservation is beneficial for increasing the survival of plant tissues. Pretreatment of plant cells and tissues can be divided into three categories. Cultural pretreatments may involve growth medium, cold acclimation or consideration of the plant part used. Chemical treatments may be osmotic or penetrating cryoprotectants. Desiccation treatments may involve air flow or desiccants. Pretreatments are crucial to the survival of plant tissues from cryopreservation. Since at present, cryoprotectants alone can not provide enough protection to untreated cells or tissues for high rates of survival. One or more of these pretreatment techniques is needed to condition the cells to withstand the stresses imposed by freezing in liquid nitrogen. This paper will review the pretreatments used in plant cryopreservation.

I. Introduction:

The first successful attempts to cryopreserve plant tissues occurred in the late 1960's.

Undifferentiated plant tissues were placed in 10% DMSO and plunged into liquid nitrogen (Quatrano, 1968). The few cells which survived could be cultured as undifferentiated cells. In the 1970s, organized plant tissues were used in cryopreservation. Dormant branches of cold-hardy trees were frozen in liquid nitrogen and thawed; some very hardy types survived the treatment and grew (Sakai and Nishiyama, 1978). Apical meristems, the growing tip of plants, were also tested. These actively growing tissues required special treatments before they could withstand freezing stresses (Sakai et al., 1978; Kartha et al. 1979; 1980). Even with cryoprotectants many did not survive. They needed more than cryoprotection; they required special conditioning. Some pretreatments were borrowed from the techniques used for animal cells. Other techniques mimic those required to produce natural dormancy and cold hardiness in plants. This article will outline the pretreatment techniques used for cryopreservation of plant cells and tissues and discuss some of the underlying principles involved. Pretreatments for plant cells include: 1) chemical 2) cultural 3) desiccation.

II. Cultural Pretreatments:

Growing plants on a suitable medium or with preconditioning chemicals may improve survival percentages following cryopreservation. The base growth medium is important in the overall vigor of the plants. Plants which are healthy tend to survive at a higher rate than those that are struggling to grow (Reed, 1993; Dereuddre, 1988). We found that most of nine raspberry and blackberry cultivars tested produced higher survival rates on Murashige and Skoog medium than on Anderson's medium (Reed, 1993)(Figure 1).

Changes in recovery medium may also affect the recovery of plant tissues following freezing. The exact reasons for these effects are not well documented.

Chemicals are commonly used in growth medium for several weeks or days of pregrowth. Osmotically active chemicals, DMSO, and growth regulators such as abscisic acid are often used for

preconditioning cells, tissues, or mother plants. Early work by Withers and Street (1977) showed that preculturing cells in highly osmotic medium improved survival. During the first 10 days of preculture both control and cryopreserved cell survival was high, then declined. The pretreated cells were smaller than the controls but that was not the only important criterion noted: negative water potential enhanced sugar and overall solute levels in the cells. These effects resemble changes involved in natural or artificial cold acclimation.

A similar response to increased osmotic pressure was observed in carnation shoot tips by Dereuddre et al., 1988. Survival rates following cryopreservation increased greatly with 24 hour pretreatment in 0.3 to 0.75 molar sucrose. Microcalorimetric studies of palm somatic embryos by Dumet and associates (1993) also showed that resistance to freezing increased for highly dehydrated embryos if they were pregrown with sucrose. The critical water level for dried palm embryos was 2.9 grams water per grams dry weight (about 15% moisture) but sucrose-treated palm embryos survived all survived. It was originally thought that sugars had only an osmotic effect outside the cell, but now sucrose and other disaccharides have been shown to enter the cells and stabilize proteins and membranes during desiccation.

DMSO is the most common chemical pretreatment. It is used both for pregrowth and during cryoprotection. The most typical DMSO pretreatment of plant cells is with 5% DMSO in the medium for 48 hours. This was determined by Kartha et al. for pea and strawberry meristems (1979; 1980) Meristems exposed to levels lower than 5% DMSO did not survive cryopreservation while those at higher concentrations survived but produced undifferentiated tissue. DMSO has been used successfully to pretreat cells and tissues of many different plant species.

Cold acclimation pretreatments are used to trigger a plants natural resistance mechanisms to cold weather. Acclimation may include growing the plant at low temperatures for one to several weeks, providing a short daylength similar to the winter season, or treating with chemicals such as abscisic acid which mimic these effects (Reed, 1988, 1990, 1993; Steponkus and Bannier, 1971).

Not all plants respond to cold acclimation. It is most effective for temperate plants and may be totally ineffective or detrimental to tropical plants.

Cold acclimation treatments increase starch grains, lipid bodies, sugar content and dry weight (Tanino, et al., 1990). Acclimation increases the capacity of canola tissue to synthesize phosphatidylcholine (Itshaki et al., 1991). It causes an increase in the ratio of unsaturated to saturated fatty acids, and increases in phospholipids and linolenic acid. Cytoplasmic membranes from acclimated plants have a higher phospholipid content and a lower sterol to phospholipid ratio than membranes from control plants. Changes in the amount and composition of the membrane lipids may protect against freezing-induced injury. Improvement in cold tolerance can be seen in whole plants after fairly brief periods of acclimation. After 14 days of cold acclimation, canola plants showed improved cold tolerance (Itshaki et al., 1991). Half of the acclimated plants survived to -14°C , while all of the control plants were killed by -10°C . In order to explain this phenomenon, Itshaki et al. studied cell membrane lipids. The uptake of choline, a major lipid of plant membranes, was studied using leaf discs; both its uptake and incorporation into phosphatidylcholine were monitored. There were no significant differences in choline uptake between control and acclimated plants but incorporation of choline into phosphatidylcholine increased in acclimated control plants and acclimated, frozen leaf discs. The biosynthetic pathway in the acclimated tissue was found to be less sensitive to freezing than in the non-acclimated tissues, as shown by increased incorporation of choline into phosphatidylcholine by frozen leaf tissue from acclimated plants.

These same effects of acclimation are seen with isolated plant organs such as meristems. Acclimation can double the survival rates of meristems following cryopreservation (Reed, 1988). In blueberry we found that the survival rate doubled following 5 weeks of acclimation, then decreased (Figure 2). The amount of acclimation required can also vary. Blueberry needs 5 weeks of hardening for maximum survival while strawberries, pears and raspberries require only one week (Reed, 1989).

Membrane damage is one of the first symptoms observed during freezing injury. This was clearly shown by Steponkus et al. (1983). Acclimation treatments that increase freezing resistance are shown to cause membrane modifications. In isotonic (0.53 osmolar) and hypertonic (1 osmolar) sorbitol, nonacclimated protoplasts of plant cells have a smooth surface. Acclimated rye protoplasts have some irregularities in the membrane in isotonic solutions and have many extrusions in hypertonic solutions. These extrusions are reversible with return to isotonic conditions. During a freeze-thaw cycle, lysis occurs in nonacclimated protoplasts because during dehydration the cell membranes lose material to form small vesicles, these vesicles are not reincorporated at thawing, causing lysis. In the acclimated protoplasts the extrusions are reincorporated into the membrane and the cell does not lyse as it regains its normal complement of water after thawing. Membrane lipids of hardened plants were shown to be more fluid than those of control plants. The physical properties of membranes are strongly affected by alterations in the relative amounts of their constituent lipids. The lipid content of membranes may increase relative to the protein content. A decrease in sterol content contributes to a decrease in viscosity in cell membranes. Changes also occur in membrane proteins during hardening.

Combinations of cold acclimation and DMSO preculture also affect survival. With pea shoot tips, either preculture or hardening was as effective or better than the combination (Sakai, 1985). In other plant types, preculture and hardening were both needed for survival (Reed, 1988). The effect of combination treatments may also depend on genotype. Combinations of one week of acclimation and 48 hours of 5% DMSO pretreatment significantly improved survival in three of four blackberry genotypes (Figure 3). The duration of acclimation can be important, as was noted earlier with blueberry meristems.

Abscisic acid (ABA) is a growth regulator which is used to induce cold tolerance or dormancy in plants. *Brassica napus* suspension-cultured cells are hardened in 6 days by the addition of 50 μ M abscisic acid to the culture medium (Johnson-Flanagan et al, 1991). A combination of

13% sucrose and ABA was most successful. Freezing tolerance can also be induced by mefluidide, a growth regulator known to increase abscisic acid synthesis in the plant. Increases in abscisic acid paralleled the freezing tolerance of cells. Exogenous ABA also increased cell hardiness for *B. napus*. Fluridone, an inhibitor of carotenoid biosynthesis, blocks mefluidide-induced increases in abscisic acid accumulation and decreases tolerance of cells to freezing.

While abscisic acid is very effective for cell cultures, it is less effective for whole plant organs. *Rubus* meristems have a markedly different response to ABA than do *Canola* cell cultures (Reed, 1993). We found that the survival of *Rubus* plantlets grown at 25°C was not improved by a seven-day abscisic acid pretreatment (Figure 4). Cold acclimation significantly improved survival of most *Rubus* genotypes either with or without abscisic acid. This lack of abscisic acid acclimation may be due to inhibition of transport to apical buds in stressed plants or inherent differences in response to abscisic acid by diverse genera. The addition of 50 µM abscisic acid failed to produce significant increases in total survival for three of five cold-acclimated *Rubus* plants. Two genotypes showed significantly greater total survival and shoot survival with a combination of cold acclimation and abscisic acid than with any other treatment. This appears to be a synergistic effect for *R. caesius* since it was unaffected by cold acclimation alone or by abscisic acid under warm conditions, but total survival and shoot survival tripled following pretreatment with the combination of abscisic acid and cold acclimation.

The last of the cultural pretreatments is the choice of plant parts to be cryopreserved and the manner in which they are grown. Plant shoot cultures are divided and transferred to new growth medium at three to four week intervals. The size of the plant, the time it has spent on the medium since the last division and the size of the piece frozen can all affect survival rates following cryopreservation. All of these are decisions that must be made to minimize variability in experiments.

The survival of carnation shoot tips following cryopreservation is related to the time since

the last transfer to new medium (Dereuddre et al., 1988). One week of culture produced poor survival while two or more weeks produced 90% survival. Shoot tips have traditionally been used for cryopreservation because buds growing from lower on the stem give variable results. Dereuddre et al. (1988) found the lateral meristems nearest the shoot tip had higher survival rates than those from 6 nodes lower on the stem. The farther from the apex, the lower the survival rate. These results may be due to physiological stages of the buds. The apical bud produces growth hormones which inhibit the growth of the side buds. Even when the apical bud is removed the inhibition may remain for some time. Endogenous growth regulators may also influence survival rates.

III. Chemical pretreatments:

Chemical treatments can be used as a part of cultural treatments by adding the chemical to the growth medium for various periods of time, or chemicals may be used immediately before the actual freezing process. Chemical and cultural pretreatments are often used together for the best results. The addition of 5% DMSO in the growth medium for two days prior to freezing is a standard practice.

Many chemicals have been tested as pretreatments for plant cell and tissue cryopreservation. The effect of pretreatment chemicals was explored by Nag and Street (1975). A one hour pretreatment with 5% DMSO or a combination of 10% Glycerol and 10% DMSO was successful. DMSO, sugars and sugar alcohols protect living cells against damage during freezing and thawing. These compounds lower the temperature at which freezing first occurs and can alter the crystal size of ice. The colligative properties of the cryoprotectants minimize the harmful effects of electrolyte concentration resulting from the loss of liquid water to ice formation. High solubility and low toxicity are essential for cryoprotectants. DMSO and glycerol are penetrating, while sugars may or may not penetrate, and sugar alcohols, polyvinylpyrrolidone and polyethylene glycol do not.

Mixtures of permeating and non-permeating cryoprotectants may allow both colligative and permeating protection of cells or tissues. DMSO treatment decreases ice formation below -30 and sorbitol decreases the amount of ice formed above -25. The combination of the two is more effective than either alone.

Morisset et al. (1993) demonstrated that the actin cytoskeleton of carrot cell suspensions is affected by DMSO and cytochalasin D. DMSO treated cells show weaker fluorescence of microfilaments as DMSO concentration increased. Cells loaded with 5, 10 and 15% DMSO, frozen and thawed have few and fragmented microfilaments. The effect of pretreatments has been noted for cell membranes but little attention has been paid to other cellular components. The behavior of actin filaments during freezing and thawing appears to be related to survival from cryopreservation. Cryomicroscopic studies by Morisset show that cellular microfilaments are dependent on the ionic equilibrium. After a freeze-thaw cycle, DMSO-treated carrot-cell microfilaments were fragmented.

Cryoprotectants may also protect the conformation of the actin. Actin filament abundance is reduced after freezing with DMSO, but the structure of the remaining microfilaments is undisturbed. Microfilaments were maintained by pretreatment with 0.5M sorbitol but the cells did not survive freezing. This same pretreatment followed by incubation with cytochalasin D greatly reduced microfilaments and the cells survived liquid nitrogen exposure. With sorbitol and DMSO pretreatment, cells appear plasmolyzed and crystals form outside the cells. Removal of membrane-bound actin filaments may modify the properties and behavior of the plasmalemma. Propanediol and DMSO have complex cryoprotective effects due to colligative properties, metabolic effects and perhaps their effect on microfilament-linked proteins.

IV. Desiccation Pretreatments:

Dehydration is another natural mechanism for cryopreservation. Many seeds can be safely stored in liquid nitrogen when they have low moisture contents (Stanwood, 1985). Some seeds,

however, and most actively growing plant parts can not tolerate drying. Dehydration pretreatments provide ways to overcome the lack of natural tolerance to drying.

Rubber tree seeds can not be stored because they do not tolerate drying. Methods to store plants such as rubber now use embryos with the cotyledons removed (Normah et al., 1986). The embryo without the cotyledon is referred to as the embryonic axis. Slowly drying the embryonic axes of rubber seeds in laminar flow air decreased the axis moisture content from 55% to about 20% in two hours, then to 16, 15 and 14% at further hourly desiccation intervals. Viability increased from 20% at 2 hours to 87% at 3 hours. Actual survival after cryopreservation was 69% at 3 hours and lower at longer and shorter dehydration times. A critical optimum moisture content of axes appears to be a requirement for a high cryopreservation survival rate. In the case of rubber seeds, 16% moisture gave the highest survival rates.

Another drying method is a directed stream of air, referred to as flash drying (Vertucci et al., 1991). *Landolphia* is a South African plant with short lived seeds. Individual embryonic axes with 2 grams of water per gram dry weight were flashed dried for 35 or 40 minutes to 0.3 (15% moisture) to 0.45 (22% moisture) grams of water per gram dry weight; 90% survived freezing to -70 C. Differential scanning calorimetry showed the water in most of the surviving axes melted as a broad endothermic peak at about -12 to -25C. In several cases there was freezable water present in the axes but they survived anyway. The critical moisture level for pea seeds is similar to that for *Landolphia*. Tissues dried to moisture contents less than 0.29 grams (14.5%) per gram of dry weight suffer additional stress on exposure to -70C.

In both laminar flow drying and flash drying the critical steps appear to be the removal of the cotyledons and the determination of the critical moisture level. This same principle holds for drying over desiccants such as silica gel. Desiccation of palm somatic embryos was combined with pregrowth in sucrose solutions to improve survival (Dumet et al., 1993). Embryos pregrown in 0.75 M sucrose had 4 g H₂O/g dw moisture compared to 14.6 g for the controls. Dehydration for 12 to 16

hours was optimal for the survival of embryos precultured in sucrose but none of the controls survived. At 12 hours the moisture of pregrown embryos was 0.7 g and at 16 hours 0.5 g. The temperature of ice formation in the embryos decreased progressively with the desiccation duration. After 16 hours dehydration only glass transitions were noted upon cooling and rewarming of pregrown embryos. All embryos frozen without pretreatment had crystallization and melting peaks, and dehydration did not result in survival. Genotype differences may be due to physiological differences such as the absorption of sucrose at different rates or to different concentrations.

Dehydration of growing plant tissues is more complicated. Where embryos are often dormant or not actively growing and have low moisture contents, actively growing tissues have much higher water contents. Desiccation treatments are possible for some drought hardy or resistant plants.

Single node buds of asparagus dried over silica gel for 24 hours decreased in water content from 80% to 14-17% (Uragami, 1991). Maximum survival following freezing was at 16.4% water content and required a 48 hour, 0.75 M sucrose pretreatment. Segments were damaged by desiccation to less than 13% of fresh weight. The critical water content for seeds is usually 16-20%, but may vary from 10 to 25%, while survival of dehydrated buds was highest in precultured buds with 20% moisture content. During a two-day pretreatment, water content decreased and dry matter increased; a large increase in sugars within the cells was also noted.

V. Conclusions:

Pretreatments of plant cells and tissues can be divided into three categories. Cultural pretreatments may involve growth medium, cold acclimation or consideration of the plant part used. Chemical treatments may be osmotic or penetrating cryoprotectants and desiccation treatments may involve air flow or desiccants. Pretreatments are crucial for the survival of plant tissues from cryopreservation since, at present, cryoprotectants alone can not provide enough protection to untreated cells or tissues for high rates of survival. The use of one or more of these pretreatment techniques is needed to condition the cells to withstand the stresses imposed by freezing in liquid nitrogen.

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Figure 1: Growth medium effects on the survival of raspberry and blackberry genotypes following cryopreservation. *Rubus* hybrid cvs. Hillemeier, Kotata, Mandarin, ORUS 1362, and Silvan, *R. grabowskii* Weihe ex. Gunther et.al., *R. leucodermis* Doug. ex Tor. & Gray, *R. ulmifolius* f. *bellidiflorus* Schott, and *R. hirsutus* Thunb. From Reed, 1993.

Figure 2: Cold hardening effects on percent shoot production by blueberry meristems four weeks following cryopreservation. From Reed, 1989.

Figure 3: Survival of cryopreserved blackberry and raspberry meristems following pretreatment on 5% DMSO for 48 hr. and with or without one week of cold acclimation, and cryopreservation. From Reed, 1988.

Figure 4: The effect of combinations of cold acclimation and ABA on survival of meristems of four *Rubus* genotypes following cryopreservation. From Reed, 1993.