Dissertation

DESICCATION AND FREEZING TOLERANCE OF EMBRYONIC AXES AND LATERAL BUDS OF *CITRUS* SPP: IMPLICATIONS FOR GERMPLASM CONSERVATION

Submitted by
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY IZULMÉ R. I. SANTOS ENTITLED **DESICCATION AND FREEZING TOLERANCE OF EMBRYONIC AXES AND LATERAL BUDS OF *CITRUS* SPP: IMPLICATIONS FOR GERMPLASM CONSERVATION** BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

DESICCATION AND FREEZING TOLERANCE OF EMBRYONIC AXES AND LATERAL BUDS OF *CITRUS* SPP: IMPLICATIONS FOR GERMPLASM CONSERVATION

Citrus is the second most important fruit crop in the world in terms of economic value. Germplasm conservation of commercial cultivars as well as of wild relatives is a priority. Presently, *Citrus* germplasm has been preserved mainly in field gene banks. This approach is only a medium-term conservation system. Cryopreservation (conservation in liquid nitrogen at -196°C) is a technique that can ensure long-term storage of plant material and maintain its biological and genetic integrity.

This research examined several aspects of tolerance to desiccation and freezing in an attempt to develop cryopreservation protocols for 'Pineapple' sweet orange (*Citrus sinensis* [L.] Osb.) embryonic axes and lateral buds and for embryonic axes of *Citrus limon* Burm. F. and *Citrus reticulata* Blanco. The cryoprotective effect of sucrose was tested as an exogenous supplement in the pre-culture medium. *Citrus* tissue carbohydrate analysis was carried out to help gain some insight on the role of sucrose as a cryopreservation stabilizer. The interrelationship

between water content and thermodynamic properties of water during freezing and thawing, which are critical in achieving survival in liquid nitrogen, were also studied.

Fully hydrated control embryonic axes cultured *in vitro* had 100% seedling recovery, whether they had been encapsulated and pre-cultured with sucrose or not. Surviving axes produced normal seedlings, without intermediary callus formation, within three weeks of culture *in vitro*.

Fully hydrated axes did not survive exposure to liquid nitrogen regardless of the pre-treatment used, that is, encapsulation in alginate gel, pre-culture with sucrose, or pre-culture with sucrose combined with glycerol and proline. Desiccation was mandatory to obtain seedling recovery after cryopreservation. Encapsulation and sucrose pre-treatment influenced desiccation tolerance of embryonic axes. For encapsulated control axes, the critical water content was about 0.146 g $H_2O.g^{-1}$ dry mass; for encapsulated pre-treated axes it was 0.25 g $H_2O.g^{-1}$ dry mass. For bare, untreated embryonic axes the critical water content was in the range of 0.2 mg $H_2O.g^{-1}$ dry mass; axes pre-cultured with sucrose retained regeneration potential even following dehydration to 0.116 mg $H_2O.mg^{-1}$ dry mass

Sucrose pre-treatment had a stabilizing effect on cryopreserved axes of 'Pineapple' sweet orange, maintaining a high recovery percentage over a broader range of water content. However, higher post-thaw recovery was obtained when glycerol and proline were added to sucrose

in the pre-culture medium. In the case of lemon, the best seedling recovery (63%) was observed when axes were pre-cultured in medium containing 0.8M sucrose plus 0.5 M glycerol and subsequently desiccated to approximately 0.146 g $H_2O.g^{-1}$ dry mass prior to directly plunging in liquid nitrogen. Only 23% recovery was obtained for mandarin axes pre-cultured with sucrose alone, but for axes pre-cultured with sucrose combined with proline and glycerol there was over a three-fold increase in seedling recovery (86%) after cryopreservation. Dehydration of axes pre-cultured with sucrose occurred at a much slower rate as compared to controls.

Tissue sucrose and fructose increased when embryonic axes were pre-cultured with sucrose, regardless of drying time. Tissue raffinose and stachyose levels dropped as embryonic axes were dried. Higher tissue levels of glucose, raffinose and stachyose, as compared to untreated samples, suggest that sucrose from the pre-culture medium may have entered the metabolic pathway following hydrolysis.

DSC analysis revealed the presence of freezable and unfreezable water in embryonic axes of 'Pineapple' sweet orange. A broad melting peak was observed in fully hydrated axes; this melting peak reduced and eventually disappeared as water was removed by desiccation. Minimum or no melting of water was observed at the point axes survived cryopreservation. Occurrence of a glass transition was not a condition for axes post-thaw recovery.

Fully hydrated lateral buds had water content in the range of 1.8 to 2.0 mg $H_2O.mg^{-1}$ dry mass; 100% shoot growth was obtained when buds were cultured *in vitro*. Similar shoot regeneration percentages were maintained as the water content in the buds was reduced by desiccation over silica gel from 1.8 to 0.6 mg $H_2O.mg^{-1}$ dry mass. However, shoot recovery consistently decreased as the water content was reduced below this level, up to the point where no regeneration was observed when the water content was dropped to 0.198 mg $H_2O.mg^{-1}$ dry mass. Buds isolated from acclimated plants had a similar range of desiccation tolerance, suggesting that acclimation did not enhance desiccation tolerance. No shoot recovery was obtained for buds from acclimated plants when water content dropped in the range of 0.17-0.20 mg $H_2O.mg^{-1}$ dry mass. Lateral buds of 'Pineapple' sweet orange did not survive cryopreservation, regardless of the pre-treatment or water content to which they were desiccated. Sucrose pre-treatment did not improve bud tolerance of desiccation and freezing. Electrolyte leakage tests conducted on buds isolated from plants grown in the greenhouse and *in vitro* grown plants provided evidence that desiccation caused severe damage to cells. Leakage from 'Pineapple' orange lateral buds isolated from greenhouse or *in vitro* grown plants and desiccated to different water contents was negatively correlated with shoot recovery in culture. Removal of water caused a significant decrease in shoot recovery while a steep increase in electrolyte leakage was observed.

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Chapter 1

Literature Review

1. Plant germplasm: the need for conservation

As the world's population increases and agricultural frontiers expand those areas that have for centuries been the natural habitat for plant and animal species are threatened by expansion of agriculture and urban boundaries (Eberhart *et al.*, 1991; Stushnoff and Seufferheld, 1995; Villalobos, 1991). This phenomenon has caused rapid and profound erosion of the genetic diversity of existing wild plant species and provoked total extinction of others we did not have the opportunity to know (Eberhart *et al.*, 1991). Genetic erosion has also been caused by the replacement of domestic cultivars of crop plants by improved cultivars with a narrow genetic base (Kartha, 1985). Numerous domestic cultivars, or landraces, selected by farmers and indigenous peoples over thousands of years due to their nutritional value, high productivity, and resistance to diseases and environmental stresses have been lost in this process (Villalobos *et al.*, 1991). Conservation of landraces, domestic cultivars and crop's wild relatives is the key to detain genetic depletion and to improve agricultural productivity (IBPGR, 1993).

2. Methods of germplasm conservation

Since the 1970's researchers around the world have been working to develop techniques to maintain the genetic variability of plant species with as much genetic and biological integrity as possible. The strategies defined so far can be classified into two broad categories: *in situ* and *ex situ* conservation. *In situ* conservation refers to the maintenance of the selected species in their natural habitats (CGIAR, 1993). This type of conservation is mainly done in parks and biological or ecological reserves. *Ex situ* conservation is the conservation of plant species out of their natural environment, maintained as collections in field gene banks, seed gene banks, and *in vitro* gene banks (CGIAR, 1993).

2.1. Field gene banks

The conservation of plants in field gene banks is desirable for evaluation and characterization of plant germplasm, mainly of vegetatively propagated crops and tree species (Duran-Vila, 1995). However, plants maintained in the field are under risk of losses due to natural disasters, pests, and pathogens; and are subjected to pressures from urban development (Engelmann, 1991; Stushnoff, 1987; Withers, 1991). Besides, the required land and trained personnel necessary to maintain the plants (pruning, disease eradication, weed control, and propagation) are quite costly, imposing a limit on the number of accessions that can be conserved (Duran-Vila, 1995; Stushnoff, 1987).

2.2. Seed gene banks

Seed gene banks were developed to store the so called orthodox seeds, that is, seeds that can be desiccated to about 5% of their initial fresh weight and stored in chambers at about -18°C (Roberts, 1973). Seeds can in theory retain viability for many decades under these storage conditions and longevity is increased in a predictable manner. In contrast, seeds of many species are injured and lose viability when stored under the same conditions mentioned above. They are called recalcitrant seeds (Roberts, 1973; Roberts and King, 1982; Roberts and Ellis, 1989). Many crop species of great economic importance such as oil palm, coconut, rubber and cacao have recalcitrant seeds. There are several other plant species that are best preserved using some means other than the conventional seed conservation method. For example, they may be exclusively vegetatively propagated, and do not produce viable seeds, or they may have such a high degree of heterozygosity that seed derived plants are of little value to breeders. Likewise, with some species segregation may result in the expression of undesirable traits in the population of seeds, making them unsuitable for seed conservation.

2.3. In vitro gene banks

The possibility of obtaining whole functional plants from isolated cells, tissues and organs of plants through tissue culture techniques led to the establishment of *in vitro* gene banks. This technique was conceived

to be a safer alternative to field gene banks for the conservation of species with recalcitrant seeds and those vegetatively propagated (fruit trees, root and tuber crops) which have traditionally been maintained in field gene banks (Eberhart *et al.*, 1991; Engelmann, 1991; Kartha, 1985). It involves the maintenance of actively growing cultures by periodic subculture of proliferating shoots or nodal segments. *In vitro* conservation has been successfully applied to numerous species such as coffee (*Coffea arabica*), strawberry (*Fragaria* spp.), banana (*Musa* spp.), cassava (*Manihot esculenta* Crantz), potato (*Solanum* spp.), sweet potato (*Ipomea batatas*), grapevine (*Vitis* spp.) (Marin and Duran-Vila, 1991).

High multiplication rates, production of pathogen-free stocks, reduction of space requirements, decrease of genetic erosion, reduction of labor costs, and simplification of international exchange of germplasm, are some of the advantages of *in vitro* gene banks (Engelmann, 1991; Stushnoff and Seufferheld, 1995). However, there are also disadvantages related to the necessary frequent subcultures associated with *in vitro* conservation, such as the possibility of identification errors and/or contamination with microorganisms due to frequent handling, and increase in the cost of the germplasm maintenance. Slow growth conservation methods have been developed to reduce the growth rate and to extend subculture interval. Growth limitation in culture has been achieved mostly by the application of osmotica (e. g. mannitol and sorbitol), by supplying the carbon source at sub- or supra-optimal levels,

by reducing temperature and photoperiod, and by incorporating a growth retardant into the culture medium (Engelmann, 1991). However, this creates more problems because cultures maintained for long periods of time under slow growth conditions can be exposed to some level of stress leading to detrimental effects upon their health, regeneration potential, and clonal uniformity (Withers, 1991).

Another important concern associated with *in vitro* conservation is the possibility of occurrence of genetic variation among tissue culture-derived plants (Constabel, 1985). This variation was termed somaclonal variation by Larkin and Scowcroft (1981). Mutation and chromosomal rearrangements have been associated especially with two tissue culture systems: callus cultures and somatic embryogenesis (Bajaj, 1990; Karp, 1991; Müller *et al.*, 1990; Ogura, 1990). The case of somaclonal variation induced by somatic embryogenesis is well known and attempts have been made to use it as a tool in crop improvement to produce plants with novel genotypes and phenotypes (Fourré *et al.*, 1997; Smulders *et al.*, 1995). Therefore, the utilization of these approaches for germplasm conservation should be avoided to minimize the risk of genetic variation, since maintenance of trueness-to-type of each accession preserved is of crucial importance. Organized structures like zygotic embryos, shoot tips, lateral buds and meristems, are considered the best starting material for *in vitro* conservation. The constituent cells of these explants are less differentiated and genetically more stable, so they can develop

directly into a new plant with much less risk of genetic instability when compared to other methods of *in vitro* culture (Kartha, 1985; Villalobos *et al.*, 1991).

In summary, in spite of the advantages, *in vitro* storage methods under slow growth conditions can only ensure short- to medium-term conservation. Long-term storage is highly desirable to reduce problems associated with germplasm regeneration, loss due to contamination and identification errors that can result from handling, and somaclonal variation, which can occasionally happen in conventional tissue culture methods. Therefore, it was realized that a satisfactory, long-term storage system for organized structures, particularly shoots, was necessary for vegetatively propagated crops and species with recalcitrant seeds. In recent years attention has been focused on cryogenic techniques.

3. Cryopreservation: definition and methods

Cryopreservation is defined as the preservation of biological material in liquid nitrogen at -196°C or in its vapor phase at -150°C (Kartha, 1985; Stanwood, 1985). This technique has the potential to ensure long-term preservation of biological material with high genetic and physiological stability (Stushnoff, 1987; Engelmann, 1991). At the ultra-low temperature of liquid nitrogen there is no liquid water, molecular kinetic energy is very low and no thermally driven reaction will occur (Kartha, 1985; Stanwood, 1985). The only physical states that do

exist below about -130°C are crystalline or glassy, and in both states the viscosity is so high that diffusion is insignificant (Kartha, 1985). Under such conditions metabolic reactions occur very slowly or not at all. Thus, storage longevity can be extremely long and genetic stability very high (Stushnoff and Seufferheld, 1995). In addition, cryogenic storage is cost-competitive with other conservation systems (Eberhart *et al.*, 1991).

Over the last ten years, cryopreservation techniques have been developed for the long-term conservation of over a 100 plant species. These include *in vitro* culture as protoplasts, cell suspensions, calluses, shoot tips, meristems, seeds, lateral buds and somatic and zygotic embryos (Chaudhury and Chandel, 1995; Stushnoff and Seufferheld, 1995; Yongjie *et al.*, 1997). Cryopreservation has also been used for the conservation of biotechnological products including metabolite-producing cultures and genetically engineered cell strains (Sakai, 1995).

Whether or not plant tissues will survive cryopreservation depends on their tolerance of reduced moisture and freezing temperature stresses, which are inherent to the cryopreservation procedure. Accordingly, the development of a cryopreservation protocol requires some knowledge of the biochemical and biophysical mechanisms associated with tissue response to these stresses (Stushnoff and Seufferheld, 1995).

3.1. Slow freezing

Methods of freezing plant tissues slowly are based on the physicochemical events occurring during freezing identified by Mazur (1963 and 1969). As the temperature decreases, the cell and its external medium remain unfrozen both because of supercooling and because of the depression of the freezing point by the protective solutes (compatible osmolites) that are present in the cell. With further decrease in the temperature ice subsequently forms in the extracellular medium, either spontaneously or as a result of seeding the solution with an ice crystal. The supercooled cell contents remain unfrozen presumably because the cell wall and plasma membrane act as barriers, preventing the ice from seeding the cytoplasm. The aqueous vapor pressure of the supercooled cell exceeds that of the frozen exterior, so with further decrease in temperature, water from inside the cell diffuses to the extracellular solution and is converted into ice on the surface of the cell or between the protoplast and the cell wall. Subsequent physical events in the cell depend on the cooling rate. If cooling is slow the cell looses water rapidly and as a result, the concentration of the cellular solute increases and the cell shrinks. This phenomenon is called freeze-induced desiccation. In such cases, the cell dehydrates and intracellular ice formation is avoided. When the cellular water potential of the partially dehydrated cell equals that of the extracellular ice, an equilibrium is established and further dehydration will not occur provided the temperature remains constant.

After warming intact cells can reabsorb water and regain full turgor (Sakai and Larcher, 1987). But, if the cell is cooled too rapidly it is not able to lose water fast enough to maintain equilibrium with the extracellular water. It becomes increasingly supercooled and eventually freezes intracellularly and mechanical injury to the cell is then observed. After warming these cells will show signs of freezing injury.

Under experimental conditions, when the slow freezing process is used the cells are cooled to –30 or -40°C (pre-freezing temperature) at a defined cooling rate, using a programmable freezer, and then directly plunged into liquid nitrogen (-196°C). When cells reach the pre-freezing temperature all the freezable water has escaped to become external ice and exposure to liquid nitrogen temperature has very little adverse effect (Kartha, 1985; Sakai, 1995). The cooling rate is critical in this procedure. If it is too slow the cells will be over dehydrated and in addition will be exposed to damaging effects of increased concentration of electrolytes (solution effects). In contrast, if the rate is to fast cells will not be sufficiently dehydrated and intracellular ice formation may occur. In summary, the slow freezing method is a complex multi-step procedure in which cryoprotectant concentration, cooling rate, and pre-freezing temperature play a critical role in preserving the viability of the cryopreserved material (Kartha, 1985; Sakai, 1995). The requirements for these components need to be precisely determined for different types of plant species, which complicate the procedure even further.

3.2. Vitrification

Vitrification, or glass formation, is the creation of a solution with the viscosity of a solid (Fahy *et al.*, 1984; Koster, 1991; Williams and Leopold, 1989). A glass is defined as an amorphous, highly viscous, supersaturated and metastable state (Sun and Leopold, 1993; Williams and Leopold, 1989). The vitrification process of cryopreservation is based on the dehydration of plant material with a highly concentrated vitrification solution, which consists of a mixture of chemical cryoprotectants, before plunging directly into liquid nitrogen. Cryoprotectants are organic compounds such as dimethyl sulfoxide (DMSO), ethylene glycol (EG), methanol, glycerol and propylene glycol. These compounds can permeate through cellular membranes, and as a result of their accumulation the cell solution becomes very concentrated and can undergo a vitrification transition when an appropriate freezing rate is applied, thus avoiding ice formation during exposure to freezing temperatures. Unfortunately, most cryoprotectants exhibit varying degrees of cytotoxicity, ranging from total killing to modification in the morphogenetic response of cells in culture, which creates some concerns in relation to their use (Kartha, 1985).

The fast freezing method eliminates the need for cellular dehydration by freeze-induced desiccation. Cells are osmotically dehydrated with cryoprotectants prior to freezing in liquid nitrogen. During rapid decrease in temperature, in contrast to slow cooling, the

cells do not have sufficient time to equilibrate with the external ice or the vapor pressure deficit by efflux of intracellular water. A rapid reduction in temperature prevents the growth of intracellular ice crystals by rapidly passing the temperature zone in which lethal ice crystal growth occurs (Luyet, 1937).

Recently, the vitrification procedure has been applied to a wide range of plant tissues (Sarkar and Naik, 1998). However, low levels of survival was observed for some species, suggesting that direct exposure to highly concentrated vitrification solution may lead to harmful effects due to osmotic stress or chemical toxicity (Sakai, 1995).

3.3. Encapsulation-dehydration

An alternative to freeze-induced cell dehydration before immersion in liquid nitrogen was proposed by Dereuddre *et al.* (1990), in a preliminary report for pear shoot tips. This process involves encapsulation of explants in alginate gel beads, which are then pre-cultured in medium containing high levels of sucrose, dehydrated in air, directly immersed in liquid nitrogen, and slowly re-warmed. This process has been referred to as "encapsulation-dehydration" (Dereuddre *et al.*, 1990; Scottez *et al.*, 1992). Since Dereuddre's report on this procedure, it has been used with success for the cryopreservation of a variety of plant species. For example, cultured shoot tips of pear (Scottez *et al.*, 1992), potato (Fabre and Dereuddre, 1990), grapevine (Plessis *et al.*, 1993) and

chicory (Vandenbussche *et al.*, 1993), and of somatic embryos of carrot (Dereuddre *et al.*, 1991).

Encapsulation-dehydration offers several advantages over conventional cryopreservation techniques (two-step slow cooling and vitrification), for instance, easier handling of explants of reduced dimensions, simplification of cryoprotective media, elimination of costly programmed freezers, independence of survival from cooling rates, and increased size of explants surviving liquid nitrogen exposure (Bachiri *et al.*, 1995). Encapsulation protects the structure imbibed and makes it resistant to treatments which otherwise could be lethal (Paulet *et al.*, 1993). Exogenous treatment with sucrose has been shown to increase tolerance to desiccation and freezing stresses (Stushnoff and Seufferheld, 1995).

The choice of any of the conservation approaches discussed above will depend on the nature of the plant species to be preserved, namely its form of reproduction, the size of the structure to be preserved, the availability of material, and its origin, to mention a few.

4. General factors affecting cryoability

Irreversible changes in the structure of membranes are one of the primary causes of injury to living cells subjected to desiccation and freezing. Injury and the mechanisms by which plants avoid or tolerate

these stresses are subject to fundamental physical and biochemical principles.

4.1. Water-deficit-related damage

4.1.1. Desiccation injury

Biological systems are based on hydrated structures within which primarily aqueous reactions occur in aqueous media (Leopold, 1990). Water has many biological roles in the cell of living organisms. It is the major solvent and transport medium, an important evaporative coolant, and an essential constituent and stabilizer of the structure of macromolecules and organelles, which is maintained by hydrophilic and hydrophobic interactions between them (Kramer and Boyer, 1995). When water is removed from the cell, a series of deleterious effects can be observed. First, solutes become more concentrated, possibly increasing the rate of destructive chemical reactions; some solutes may crystallize, changing the ionic strength and pH of the intracellular solution. Second, biologically active macromolecules such as nucleotides, phospholipids, proteins and carbohydrates are denatured, in many cases irreversibly, since water is implicated in the maintenance of their conformation. Thirdly, membranes are disrupted leading to loss of cell compartmentation (Koster, 1991; Kramer and Boyer, 1995).

Although most vascular plants cannot tolerate dehydration of their vegetative tissues, desiccation tolerance is a feature of many plant

structures or even of entire plants (Leopold, 1990; Leprince *et al.*, 1994). Mature seeds of angiosperms, pollen, and spores of ferns and mosses are common examples of plant structures that can tolerate considerable desiccation (Bewley and Oliver, 1992; Oliver, 1996). At the whole plant level, desiccation tolerance is exhibited by some species of ferns (*Polypodium virginianum*), mosses (*Tortula ruralis*), algae, yeasts, lichens, fungi, and angiosperms (*Craterostigma plantagineum*) (Leopold, 1990). Such species are called resurrection plants, because they can withstand complete desiccation of their tissues and readily restore photosynthetic and metabolic activities upon re-hydration. Here desiccation tolerance is an adaptive trait, that is, it allows plants to endure exposure to environmental stresses such as drought, heat, and freezing; in the case of reproductive structures such as seeds, spores and pollen it is an adaptation evolved to facilitate dispersion by wind or water.

Ultrastructural studies of desiccated tissues have revealed that cellular membranes are the primary sites of injury (Koster, 1991; Leprince *et al.*, 1993). In biological membranes of unstressed cells the hydrated lipid bilayer is in a liquid-crystalline state at physiological temperatures (Leprince *et al.*, 1993). Drying causes alterations in membrane structural integrity and function, alterations in membrane physico-chemical properties (changes in membrane phase behavior and relative composition) (Leprince *et al.*, 1993). Desiccation causes a general collapse of membranes and partial loss of membrane semi-permeability.

A hydrated lipid that is in the liquid crystalline phase at physiological temperatures will undergo a phase transition to a gel phase at the same temperature if water is removed from its phospholipid head groups. This transition is fully reversible upon rehydration. An irreversible and lethal type of alteration in the membranes is the change in lipid composition as a consequence of dehydration. Recent evidence suggest that a de-esterification of the acyl chains from the head group-glycerol moieties of membrane phospholipid together with the release of degradation products in the medium had occurred during desiccation (Senaratna *et al.*, 1987). The formation of the gel-phase and the lateral-phase is highly damaging for the membrane and the membrane-protein organization and contribute to the loss of membrane functions, such as permeability, compartmentation and membrane-bound enzyme activity (Leprince *et al.*, 1993).

Desiccation induced alterations in membrane structural integrity and function can be indicated by leakage of various cytoplasmic solutions (ions, sugars and proteins) that occurs upon rehydration. Leakage reflects a partial loss of membrane semi-permeability, suggesting that desiccation injury is closely associated with membrane disfunction. The rate and extent of cytoplasmic leakage is positively correlated with the degree of desiccation sensitivity.

Reducing the water content in cells prior to freezing is fundamental to improve their survival following freezing and thawing. If

moisture levels are too high during liquid nitrogen exposure, ice will form and damage to the tissue will occur. However, if the water content is too low, the tissues will be damaged by desiccation. There is a narrow window of moisture content at which maximum survival through liquid nitrogen exposure is obtained.

Macroscopically water is the substrate that transports essential nutrients and waste products within living organisms. Each cell, and organelle within a cell, requires a critical balance of water so that it can function properly. Flow of water into and out of cells across the plasma membrane is coupled to various ion fluxes. In metabolism, water is the necessary reagent in any process that involves hydrolysis, condensation and redox reactions, that is, most biochemical reactions (Kramer and Boyer, 1995). Hence, it is not surprising that desiccation has profound effects not only on the structural organization of the cells, but also on the molecular organization within them (Leopold, 1990).

4.1.2. Freezing injury

Freezing injury is a complex process that involves a diversity of aspects. It is mainly due to excessive dehydration or ice formation inside cells. In both cases membrane disruption, toxic solute concentration in the cytoplasm and nucleic acid and membrane denaturation will occur. Ice formation and the associated injuries take different courses

depending on the plant species, state of hardiness and the freezing conditions. Therefore, cell death is most probably caused by several rather than a single, general mechanism (Sakai and Larcher, 1987). However, it seems to be of general consensus that freezing injury is primarily a consequence of disturbance of the semipermeable characteristics or lysis of the plasma membrane resulting from freeze-induced desiccation (Steponkus, 1984; Steponkus and Webb, 1992). That is, the major damage caused to the living cell by freezing temperatures is related to water deficit.

The plasma membrane plays a central role in cellular behavior during freeze-thaw cycles. Although all cellular membranes are vulnerable to freeze-induced destabilization, the plasma membrane is of primary importance because it is the principal interface between the extracellular medium and the cytoplasm, acting as a semipermeable barrier allowing for the efflux/influx of water during a freeze/thaw cycle (Uemura and Steponkus, 1998). The cell membrane also prevents seeding of the intracellular solution by extracellular ice. Thus, whether the cell survives during a freeze/thaw cycle is ultimately a consequence of the stability of the plasma membrane.

Freezing tolerance in plants is a multifaceted trait and cryostability of cell membranes depends on many factors. For instance, it has been reported that increase in endogenous cryoprotectants (sugars), synthesis

of certain proteins and alterations in lipid composition of the plasma membrane during cold acclimation result in increased cryostability of membranes (Uemura and Steponkus, 1998).

4.2. Role of sugars

Freezing tolerance of temperate plants increases from autumn to winter allowing them to survive harsh winter sub-zero temperature conditions. This phenomenon is referred to as cold hardening or cold acclimation (Levitt, 1956). Accumulation of soluble carbohydrates in temperate plants has been demonstrated during cold acclimation, either in nature or under experimental conditions. Many studies have shown that the accumulation of these soluble carbohydrates in plant tissues correspond to the period when they were most tolerant to freezing (Imanishi *et al.*, 1998; Stushnoff *et al.*, 1993). For instance, the levels of raffinose family oligosaccharides (RFO), namely raffinose and stachyose, have been shown to be lowest in summer and highest in autumn and winter (Imanishi *et al.*, 1998). Similarly, sucrose concentration increases in the tissues of some species in response to low temperature (Imanishi *et al.*, 1998). Sugars like sucrose, trehalose, and larger olygosaccharides, such as raffinose and stachyose, have been found to accumulate in large amounts in many desiccation tolerant organisms. For instance, fungal spores, yeast, mosses, ferns, and in seeds and pollen of most

angiosperms which can survive for long periods of exposure to extremely low water contents (Leopold, 1990; Oliver, 1996).

These results suggest that carbohydrates are indeed involved in the acquisition of freezing tolerance. Although a causal relationship has not been demonstrated yet, it is generally accepted that soluble sugars play a significant role in freezing and desiccation tolerance (Imanishi *et al.*, 1998; Sun and Leopold, 1994). The protective effect of sugars seems to be a function of both their concentration and molecular weight, *i.e.*, on a molar basis trisaccharides are more effective than disaccharides and the latter more than monosaccharides (Dumet *et al.*, 1993; Santarius, 1973). The extensive use of sugars like sucrose as cryoprotectants is linked to these findings. It is also due to the their lower toxicity and higher efficiency in stabilizing the membranes during freezing, as compared to standard cryoprotectants like DMSO and glycerol.

The mode of action of sugars involves multiple components. First, sucrose and other sugars act as external osmoticum by removing excessive intracellualr water through osmotic gradient. It was assumed initially that the protective role of sugars was due to this osmotic effect only, but more recently it was observed, both directly and indirectly, that sugars enter cells in large quantities (Finkle *et al.*, 1985; Dumet, 1993).

Secondly, soluble sugars are major vitrifying agents in plants so the protective effect of sugars may be associated with cytoplasmic vitrification (Koster, 1991). Vitrification, or glass formation, is the

production of a supersaturated liquid with the mechanical properties of a solid (Koster, 1991). Interchanges between the two states involve no chemical changes, but only physical changes in the viscosity of the liquid (Koster, 1991; Leopold, 1990). Another proposed role for sugars is in the induction of vitrification of the cytoplasm (Hirsh, 1987; Leopold, 1990; Leopold and Vertucci, 1986; Sun and Leopold, 1997; Sun *et al.*, 1994). The vitrified state has many beneficial effects to the desiccated cell, such as limitation of water loss, limitation of crystallization of salts and proteins in the cytoplasm, protection against pH changes as water is removed, and prevention of cellular collapse during extensive water loss (Koster, 1991; Sun and Leopold, 1997). Vitrification promotes a state of metabolic quiescence by restricting diffusion of substrates and products within the cell, causing a preclusion of chemical reactions requiring diffusion (Koster, 1991). In the vitreous state deterioration of dry biological systems is suppressed, thus ensuring stability during the period of quiescence. It is interesting to mention that the vitreous state is thermodynamically unstable. Its physical stability depends on the constant maintenance of the extremely high viscosity of the system. Increasing temperature or water content can disrupt the glassy state, leading to crystallization and cell damage (Sun and Leopold, 1997).

A third hypothesis is that carbohydrates may substitute for water in maintaining hydrophilic structures in their hydrated orientation even after water has been removed (Crowe *et al.*, 1992). Studies with model

membranes demonstrated that the hydroxyl groups of sugars like sucrose and trehalose are able to replace water molecules on hydrophilic (polar) end groups of membrane phospholipids and functional groups of proteins. With that they would prevent changes in selective permeability due to lateral phase separation of phospholipids in the bilayer and phase transition from liquid crystalline to gel (Carpenter *et al.*, 1990; Crowe *et al.*, 1988; Crowe *et al.*, 1992; Kermode, 1997). The water replacement hypothesis was proposed by Crowe and collaborators based on experiments with model membrane systems (Crowe *et al.* 1988; Crowe *et al.* 1990; Leslie *et al.*, 1995). They observed that the structural integrity of membranes under dry conditions was maintained in the presence of sugars, especially sucrose and trehalose. They proposed that sugars replace the water molecules removed by dehydration, inserting themselves between the polar head groups of the phospholipids present in the membrane bilayer. The hydroxyl groups of the sugars can bind to the polar head groups of the phospholipids by means of hydrogen bonds, providing the hydrophobic interactions necessary to avoid fusion of the polar head groups and consequently membrane phase transition from a liquid crystalline phase into a gel phase. As a result, denaturation of membranes is avoided.

A fourth explanation has recently emerged to support observations that disaccharides are effective stabilizers. Branca *et al* (1999) demonstrated with Raman spectroscopy, that disaccharides obstruct the

crystallization process by destroying the network of water compatibility with ice, thus effectively reducing the amount of freezable water.

Under experimental conditions it has been observed that pre-culture with highly sugar-enriched medium, especially with sucrose, results in increased tolerance to dehydration and liquid nitrogen temperatures (Dereuddre *et al.*, 1990; Paulet *et al.*, 1993; Stushnoff and Seufferheld, 1995). It has been hypothesized that sugars like sucrose, when added exogenously before dehydration and low temperature stress would act by stabilizing the structural integrity of membranes and proteins during desiccation by preventing membrane fusion, phase transition and phase separation (Crowe *et al.*, 1990).

In spite of these recent evidences, the exact role of sugars in the acquisition of tolerance to desiccation and freezing remains to be established (Bachiri *et al.*, 1995).

4.3. Cold acclimation

During cold acclimation the cryostability of the plasma membrane is increased (Uemura *et al.*, 1995). It has been demonstrated that this increased freezing tolerance results in part from alterations in the lipid composition of the plasma membrane during cold acclimation (Uemura and Steponkus, 1994; Uemura *et al.*, 1995). Phospholipids (PL) are known to increase in the plasma membrane during cold acclimation of

many plant species (Uemura and Steponkus, 1994). In many cases the increase in PL during cold acclimation is associated with an increase in the proportion of di-unsaturated species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and a corresponding decrease in mono-unsaturated species (Uemura and Steponkus, 1994). The proportion of cerebrosides (CER) in the plasma membrane before and after cold acclimation varies among the plant species studied, but there is a general tendency to decrease during cold acclimation (Uemura *et al.*, 1995). Changes in the relative proportions of free (FS) and glucosylated forms (SG and ASG) of sterols during cold acclimation are generally rather small (Uemura and Steponkus, 1994).

However, membrane lipid alterations are not the only factors responsible for the increased freezing tolerance during cold acclimation. Other factors such as the accumulation of cryoprotective substances (*e. g.* sugars and amino acids), and the expression of the cold responsive (COR) genes play a significant role in this enhanced freezing tolerance of plants during cold acclimation.

5. Why *Citrus*?

Citrus is the second major fruit crop in economic importance, being cultivated in about 90 countries in tropical and subtropical climates for production of fresh fruits and processed juice. Among *Citrus* cultivars,

sweet oranges account for 70% of the world production, followed by mandarins (12%) and lemons and limes (10%). Most *Citrus* species are both heterozygous and apomitic. Thus plants obtained from seed germination may be either genetically identical or distinct from the mother plant. Therefore, commercial *Citrus* cultivars, that have unique gene combinations, which must be preserved as such are vegetatively propagated, generally by air layering or grafting on seedling rootstocks (Marin and Duran-Vila, 1991).

Germplasm preservation of *Citrus* involves the conservation of samples representative of wild species and of cultivated material. Conservation through seeds is limited by a decrease in viability when they are stored for extended periods (King *et al.*, 1981; Mumford and Panggabean, 1982). There are also practical problems in applying seed storage for long-term conservation of long-lived woody plants such as *Citrus:* plants obtained through seed germination have a very long juvenile period and do not produce fruits (and seeds) for several years (Engelmann, 1991). Presently, *Citrus* has been conserved mainly through the maintenance of collections in field gene banks (Marin and Duran-Vila, 1991). The conservation of woody plants in field gene banks is desirable, however, plants maintained in the field are exposed to risk of losses due to biological and climatic hazards (Duran-Vila, 1995; Engelmann, 1991; Stushnoff, 1987; Withers, 1991). Besides, the land requirement, labor costs and personnel necessary to maintain the plants

make this form of conservation very costly (Duran-Vila, 1995; Stushnoff, 1987). Moreover, *Citrus* species are heterozygous and it would be necessary to preserve large sample numbers to maintain as much as possible the genetic variability within a population, which makes the maintenance of field collections even more expensive (Engelmann, 1991).

Citrus seeds are among those that are injured by drying and hence deteriorate rapidly under conventional storage conditions (King and Roberts, 1980). Their sensitivity to desiccation varies among the different species, and depends on many factors such as drying temperature, desiccation rate, testa scarification and atmosphere humidity, to name a few (Barton, 1943; Edwards and Mumford, 1985; King and Roberts, 1980; Mallareddy *et al.*, 1977). Early reports of loss of viability on drying led to the classification of *Citrus* seeds as recalcitrant (Roberts, 1975; King and Roberts, 1980). Subsequent studies showed that although drying does lead to some viability loss, there is the possibility that some *Citrus* species could show orthodox behavior, *i. e.*, their longevity increases with a reduction in moisture content and storage temperature (Edwards and Mumford, 1985; King *et al.*, 1981; Mumford and Grout, 1979). However, the true relationship between desiccation and *Citrus* seed viability remains obscure and optimal methods for the long-term maintenance of *Citrus* seeds have yet to be established. Currently, research efforts are aiming at the development of alternative methods, namely tissue culture and cryopreservation techniques.

A method based on the establishment of primary cultures from nodal stem segments, recovery of plants *in vitro* and successive cycles of secondary cultures of nodal stem segments from *in vitro* grown plants was developed for several species and varieties of *Citrus* (Marin and Duran-Vila, 1991). As mentioned previously, maintenance of plant germplasm *in vitro*, under slow growth conditions, have several advantages. However, it is not a long-term conservation procedure. Therefore, a satisfactory, long-term storage system for organized structures, particularly shoots, has yet to be developed for *Citrus* species.

Attempts to apply cryopreservation techniques to *Citrus* are restricted to a few reports. Whole seeds (Mumford and Grout, 1979), ovules (Bajaj, 1984), excised uncoated seeds (Radhamani and Chandel, 1992), somatic embryos (Marin and Duran-Vila, 1988; Marin *et al.*, 1993), or nucellar cells (Kobayashi *et al.*, 1990; Sakai *et al.*, 1990 and 1991a and 1991b), have been used. It is interesting to notice that the *in vitro* culture approaches used in the referred works (ovules, somatic embryos, cell suspension and callus cultures) are not normally the systems recommended for germplasm conservation of plants in general, and for woody plants in particular. This is due to the long period needed to obtain a mature, productive plant, and the risk of somatic variation associated with these tissue culture systems. Another aspect deserving mention is that vitrification with cryoprotectants was used in almost all cases. This process involves the pre-treatment of explants with highly

concentrated cryoprotectant mixtures. The utilization of such treatments have been commonly included in protocols for freezing plant tissues, although survival of frozen tissues not treated with cryoprotectants has also been reported for many species and explants (Dumet *et al.*, 1993; Hatanaka *et al.*, 1994; Uragami *et al.*, 1990). Recently, there has been a tendency to develop cryopreservation techniques that do not depend on cryoprotectants for explant survival, because these compounds are often very toxic to plant cells (Kartha, 1985). They also complicate, tremendously, the freezing procedure, and in many cases do not improve survival, as reported by Marin and Duran-Vila (1988).

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Chapter 2

Cryopreservation of embryonic axes from three *Citrus* species by encapsulation-dehydration

Abstract

Na-alginate coated embryonic axes of three *Citrus* species (*C. sinensis*, *C. limon*, *C. reticulata*) were cryopreserved using direct freezing in liquid nitrogen. The effect of pre-culture medium and desiccation duration on water content and freezing resistance was examined. Generally, 70% seedling recovery was achieved for 'Pineapple' sweet orange axes desiccated to 0.146 g $H_2O.g^{-1}$ dry mass. Axes pre-cultured with sucrose (stepwise increase from 0.5-0.75M) and dehydrated for the same period of time had 0.25 g $H_2O.g^{-1}$ dry mass and showed about 60% seedling recovery. Over 80% post-thaw recovery was obtained when glycerol and proline were added to sucrose in the pre-culture medium. In the case of lemon, the best recovery rate (63%) was observed when axes were pre-cultured in medium containing 0.8M sucrose plus 0.5 M glycerol and subsequently desiccated to approximately 0.146 g $H_2O.g^{-1}$ dry mass prior to directly plunging in liquid nitrogen. Only 23% recovery was obtained for mandarin axes pre-cultured with sucrose alone, but for axes pre-cultured with sucrose combined with proline and glycerol there was over a three-fold increase in seedling recovery (86%) after

cryopreservation. Surviving axes produced normal seedlings, without intermediary callus formation, within three weeks of culture *in vitro*.

Introduction

Citrus are evergreen plants included in the family Rutaceae that are cultivated mostly for their fruits. Presently, conservation has been done mainly through the maintenance of field genebanks (Duran-Vila, 1995; Marin and Duran-Vila, 1991), but this approach has limitations. It is costly, exposes germplasm to climatic and biological hazards and is not a long-term solution (Duran-Vila, 1995; Engelmann, 1991; Stushnoff, 1987).

Seeds are generally used for the long-term storage of plant germplasm. Seeds that can withstand desiccation and sub-freezing temperatures without loosing viability (orthodox seeds) can be stored for long periods in seed gene banks, under conventional storage conditions (-18°C), in a practical and economical way (Roberts, 1973). Currently seed conservation is not a feasible option in the case of *Citrus* species. There is a considerable variation in seed storage behavior among these species (Hong and Ellis, 1995). Seeds were initially classified as recalcitrant, but they were thought to be orthodox after Mumford and Grout (1979) showed that *Citrus limon* L. seeds from which the testa had been removed could be dehydrated to very low moisture contents and still remain

viable. More recently Hong and Ellis (1995) classified *Citrus* species as orthodox, recalcitrant or intermediate. *Citrus sinensis* [L.] Osb. seeds are classified as intermediate. The sensitivity of intermediate seeds to desiccation varies with maturity stage, provenance and imbibition (Ellis *et al.*, 1991). The critical moisture content below which intermediate seeds die varies from species to species, generally within the range 10-12% (Ellis *et al.*, 1990 and 1991). At this relatively high moisture content the seeds are not able to survive freezing temperatures. For this reason traditional seed conservation methods have not been applied to citrus due to viability loss with storage time (Hong and Ellis, 1995; King *et al.*, 1981; Mumford and Panggabean, 1982). Therefore, a reliable long-term conservation technique for *Citrus*, especially to provide a viable backup to field gene banks, has yet to be established.

Cryopreservation, storage of biological material at ultra low temperature using liquid nitrogen (-196°C) can facilitate long-term storage of plant germplasm. At the temperature of liquid nitrogen there is no liquid water, molecular kinetic energy is very low, and diffusion is extremely slow. Under such conditions metabolic reactions occur very slowly or not at all and time-dependent changes are eliminated. Thus, storage longevity can be extremely long and genetic stability very high. In addition, cryogenic storage is cost-competitive with other conservation systems (Eberhart *et al.*, 1991).

Cryopreservation techniques have been applied to *Citrus* species in the recent past. Ovules, somatic and zygotic embryos, nucellar cells and callus survived liquid nitrogen after being subjected to vitrification using chemical cryoprotectants such as DMSO, ethylene glycol, propylene glycol, etc. (Kobayashi *et al.*, 1990; Marin and Duran-Vila, 1988; Marin *et al.*, 1993; Mumford and Grout, 1979; Radhamani and Chandel, 1992; Sakai *et al.*, 1990 and 1991a and 1991b). However, the plant material used (ovules, somatic embryos, cell suspension cultures and callus) in these studies are not normally the systems of choice for conservation of plant species in general, and of woody plants in particular, mostly because of the latent risk of somaclonal variation associated with these procedures. Additionally the long period needed to obtain a mature, productive plant from such systems make them less attractive for conservation purposes.

Zygotic embryos on the other hand are highly organized, small systems and can produce a whole plant from the meristematic tissues contained therein. Therefore, they are likely to carry lower risk of somaclonal variation in culture when compared to other methods of *in vitro* plant regeneration such as those using embryogenic calluses or cell suspensions. Embryo culture has been used with success to cryopreserve the germplasm of many plant species that have recalcitrant or intermediate seeds. While the mature seeds of these plants are damaged when dehydrated or stored at sub-zero temperatures, the

excised embryos exhibit orthodox characteristics and can be desiccated without viability loss (Abdelnour-Esquivel *et al.*, 1992a and 1992b; Berjak and Dumet, 1996; Chin *et al.*, 1988; Engelmann *et al.*, 1995; Fu *et al.*, 1990; Normah and Vengadasalam, 1992; Pritchard and Prendergast, 1986; Wesley-Smith *et al.*, 1992)

The other aspect involved in the approaches mentioned above that requires careful consideration is the dependence on chemical cryoprotectants. Recently there has been a tendency to develop cryopreservation protocols that do not depend on chemical cryoprotectants (dimethylsulfoxide, polyethylene glycol, ethylene glycol, etc.) for explant survival. The reason is that they are toxic to plant cells, complicate the freezing procedure and in many cases do not improve plant recovery significantly (Arakawa *et al.*, 1990; Marin and Duran-Vila, 1988). Fabre and Dereuddre (1990) developed the encapsulation-dehydration method that uses sucrose as a cryoprotective substance coupled with partial desiccation prior to exposure to liquid nitrogen. Pre-culture with sucrose and subsequent dehydration avoids the use of chemical cryoprotectants such as DMSO. This procedure has since been used successfully for the cryopreservation of a variety of plant species (Dumet *et al.*, 1993; Panis *et al.*, 1996; Suzuki *et al.*, 1997). Alginate encapsulation of plant tissues had been used before in research of plant micropropagation and artificial seed production (Piccioni and Standardi, 1995). Results obtained with these studies led to the suggestion that the

alginate bead may protect the tissue during handling and most importantly reduce the fluctuations of environmental conditions (temperature, osmolarity, nutrients, relative humidity) for the encapsulated tissue. In terms of microprogation it was observed that the growth rate of plants regenerated from apices improved after coating. As a result the time needed to obtain plants fit to be transferred to the greenhouse for acclimatization was reduced (Draget *et al.*, 1988; Fabre and Dereuddre, 1990; Mathur *et al.*, 1989; Piccioni and Standardi, 1995). This increase in development rate may be due to a greater availability of mineral and hormonal nutrients in the extracellular environment (Fabre and Dereuddre, 1990).

The primary objective of this study was to develop a cryopreservation protocol for embryonic axes of 'Pineapple' sweet orange, *Citrus sinensis* [L.] Osb.using the encapsulation-dehydration method. Embryonic axes of mandarin (*Citrus reticulata* Blanco) and lemon (*C. limon* L.) were used to test the protocol developed for 'Pineapple' sweet orange axes. These studies were based upon the hypothesis that tissues can be stabilized against injury from loss of all freezable water using an alginate gel encapsulation and non-toxic cryoprotectants, such as sucrose. We therefore combined encapsulation, sucrose pre-treatment and controlled dehydration in order to develop a successful cryopreservation procedure for embryonic axes of the three *Citrus* species.

Material and Methods

Plant Material and embryonic axes excision procedures

Fruits of 'Pineapple' sweet orange (*Citrus sinensis* [L.] Osb.) were harvested at Lake Alfred Citrus Experiment Station, in Lake Alfred, Florida. Fruits received in the laboratory were immediately stored in a growth chamber at 15±2°C. For embryonic axes excision, fruits were surface disinfested with a 10% (v/v) solution of commercial sodium hypochlorite (Clorox) containing 4-5 drops of polyoxyethylene sorbitan monolaurate (Tween 20) for 15 min, and rinsed 3-4 times with sterilized distilled water in a laminar flow hood. Seeds were extracted from the fruits and embryonic axes were excised from the seeds under a stereo microscope. Embryonic axes were transferred to Petri dishes, over moist filter paper, until used.

Fruits were purchased at a grocery store and fruit sterilization and embryonic axes excision done the same way described above for 'Pineapple' sweet orange.

Cryopreservation

Alginate gel beads containing embryonic axes were prepared as follows. Axes were transferred to a 3% Na-alginate (Sigma, medium viscosity) aqueous solution and then dipped with a spatula in a 0.1 M

$CaCl_2$ aqueous solution for one hour to cross-link the alginic acid into a stable encapsulation gel. Capsules containing one axis each were pre-cultured in liquid MS medium (Murashige and Skoog, 1962) containing sucrose alone or in combination with glycerol and proline. Pre-culture with medium containing sucrose alone consisted of liquid MS medium supplemented with 0.5 up to 0.75 M sucrose, in 0.25 increments, in a stepwise series, 24 hours in each step.

Beads were also pre-cultured for 16 hours in liquid MS containing 0.8 M of sucrose plus 0.5 M glycerol or 0.8 M sucrose plus 0.5 M glycerol and 0.5 M proline. During pre-treatment, beads were kept at ambient temperature (25±2°C), in darkness, under agitation (100 rpm) in a horizontal shaker.

Pre-cultured beads were briefly blotted on filter paper and batches of 10 were dehydrated by placing them in a monolayer on sterilized filter paper upon a layer of dry silica gel (85 g) within a tightly closed container (500 mL capacity) for 0-8 hours, at ambient temperature (25±2°C). Water content was determined gravimetrically following oven drying at 80±°C for 5 days at the end of each desiccation period. Water contents are expressed on a dry weight basis, that is, g H_2O.g dry mass ($g.g^{-1}$).

After each dehydration period the axes were enclosed in 2.0 ml cryotubes (10 axes *per* vial serving as an experimental unit) which were plunged into liquid nitrogen and maintained at -196°C for at least 30 min. Three replications were used *per* treatment assigned at random.

Axes were thawed by plunging cryotubes in a water bath at 40±2°C, under agitation, for 3 min. Axes were immediately transferred to recovery medium and cultured as described below.

Survival assessment

Viability after both desiccation and cryopreservation tests was determined by plating beads on basic MS media solidified with 0.7% agar and dispensed in Magenta boxes. Encapsulated axes were cultured in a growth chamber at 25±2°C with a photoperiod of 16 hours light/8 hours dark and 62 $\mu mol.m^{-2}.s^{-1}$ light intensity. Axes exposed to liquid nitrogen were kept in the dark for 48 hours before they were transferred to the lighting conditions described above. Survival was assessed weekly and data were recorded after four weeks of culture and expressed as the percentage of axes that produced seedlings with normal morphology (with both shoots and roots) compared to untreated controls. Seedlings with abnormal morphology, for instance, with normal shoots and no roots, or vice-versa, were not included in the results because we considered that they would not survive transplant to *in vivo* conditions.

After six months in culture, 10 plants obtained from each treatment were transplanted into pots (500 mL capacity) containing growing mix # 2 (Fafard) composed of Canadian *Sphagnum* peat moss (70%), perlite and vermiculite. For the first three weeks after

transplanting, plants were maintained in the laboratory under natural light conditions and temperature in the range of 25±2°C. They were covered with a transparent plastic bag to avoid dehydration. On the fourth week, plastic bags were removed gradually and plants were then transferred to the greenhouse with temperatures in the range of 25±5°C and a photoperiod of 12 hours/day or longer obtained by natural conditions or by supplemented lighting. They were fertilized every two weeks with a water soluble fertilizer concentrate (15-30-15, GroBest) at a final concentration of approximately 0.84 g/L.

Statistical analysis

All percentage data were arcsine transformed prior to statistical analysis. Data in the figures are actual percentage values. The relationship of encapsulation and sugar pre-treatment to dehydration and survival were analyzed by logistic regression.

Results and Discussion

Generally, 90 to 100% of control embryonic axes cultured on recovery medium defined for these studies, resumed growth producing normal seedlings, that is, seedlings with roots and shoots with one or two pairs of leaves, in 14-21 days, without callus formation. Avoidance of callus proliferation as a step in plant regeneration after cryopreservation

was a very important concern in this study because somaclonal variation is most likely to occur when an intermediary callus growth occurs (Fourré *et al.*, 1997; Karp, 1991; Smulders *et al.* 1995).

Fully hydrated beads containing embryo axes but not treated with sucrose had average water content of 14.6 g $H_2O.g^{-1}$ dry mass (Fig. 1). Those pre-treated with sucrose showed much lower water contents (around 2.85 $g.g^{-1}$) when fully hydrated (Fig. 2), probably because water binding by sucrose removed moisture from treated beads by means of osmotic dehydration. Similar decrease in moisture content due to sucrose pre-treatment was observed in other cases (Dumet *et al.*, 1993; Fabre and Dereuddre, 1990; Panis *et al.*, 1996).

Regeneration of embryonic axes was not affected by encapsulation and remained close to 100%. Removal of the axes from the beads was not necessary since growing embryos were able to rupture the capsule as they grew. Encapsulation is said to protect the structure contained in the capsule and to make it resistant to treatments which otherwise could be lethal (Draget *et al*, 1988; Paulet *et al.*, 1993). It seems that encapsulation can in many cases promote growth of the encapsulated structure. Mathur *et al.* (1989) reported that shoot tips of *Valeriana wallichii* DC. grew and reached the acclimatization stage in half the time required by unencapsulated controls. Likewise, Draget *et al.* (1988) reported that the time required for plant regeneration from protoplasts of *Brassica napus* encapsulated in calcium alginate beads was halved when

compared to protoplasts grown in suspension. According to Fabre and Dereuddre (1990) the increase in development rate is due to a greater availability of mineral and hormonal nutrients in the environment surrounding the plant tissue inside the bead.

Seedling recovery following desiccation and cryopreservation

Desiccation of both control and pre-treated beads decreased the water content rapidly within the first two hours of desiccation, and slightly but steadily from 2 to 8 hours during desiccation over silica gel (Fig. 1 and 2). Survival percentages for sucrose pre-treated and untreated axes after dehydration to different water contents, and dehydration followed by cryopreservation are shown in Fig. 1 and 2. Viability of axes that were encapsulated but not pre-cultured with sucrose decreased with desiccation, indicating that encapsulation alone did not improve desiccation tolerance of the axes. Desiccation tolerance for encapsulated axes that were pre-treated with sucrose in a stepwise manner is shown in Fig. 2. Sucrose pre-treatment stabilized viability at about 70% following dehydration for 1 to 6 hours (1.5 g $H_2O.g^{-1}$ dry mass), whereas survival of untreated axes decreased steadily during the time course of dehydration (Fig. 1).

Untreated, fully hydrated axes did not survive exposure to liquid nitrogen (Fig. 1). Survival increased significantly after axes were desiccated and the highest survival for the untreated axes after

cryopreservation (70-80%) was attained for encapsulated axes that were desiccated for 4 and 5 hours. The water content of these axes was reduced to 0.173 and 0.146 g $H_2O.g^{-1}$ dry mass, respectively (Fig. 1). Dehydration improved freezing tolerance for pre-treated axes as well, and the best survival (60-70%) was attained with axes dehydrated for 4-5 hours, resulting in water content of 0.25 - 0.30 g $H_2O.g^{-1}$ dry mass, respectively.

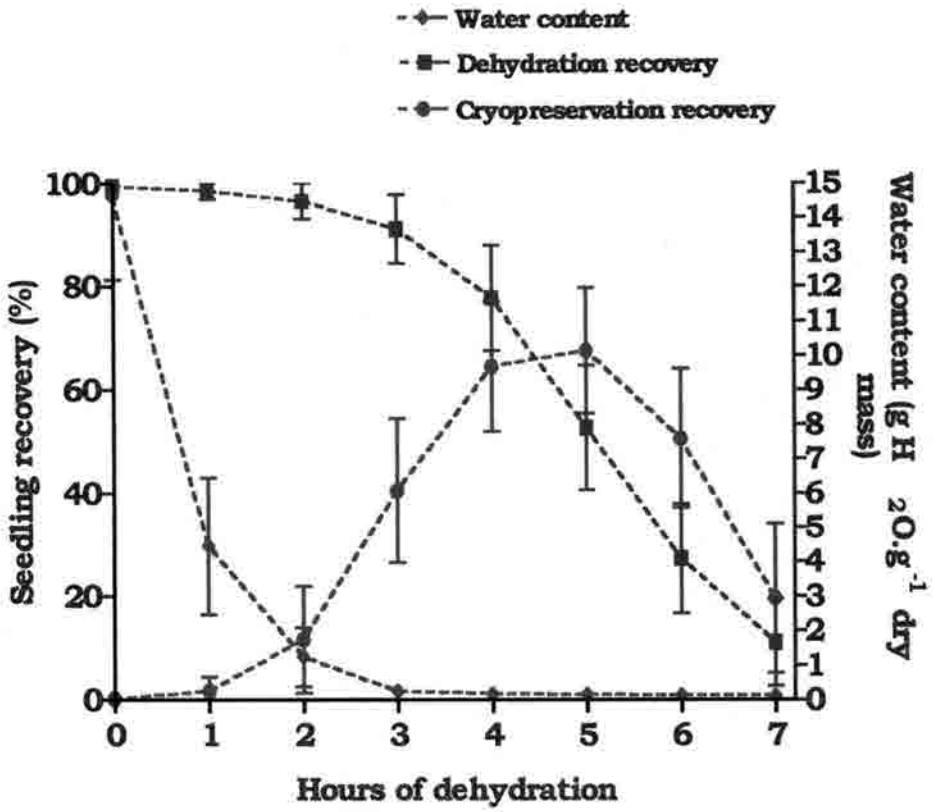

Figure 1. Effect of dehydration duration on the water content (◆), seedling recovery after dehydration alone (■), or followed by cryopreservation (●). Embryonic axes of 'Pineapple' sweet orange were encapsulated in Na-alginate, but received no sucrose treatment, prior to desiccation over silica gel for various periods of time. Vertical bars represent means of three observations ± SEM ($P<0.05$).

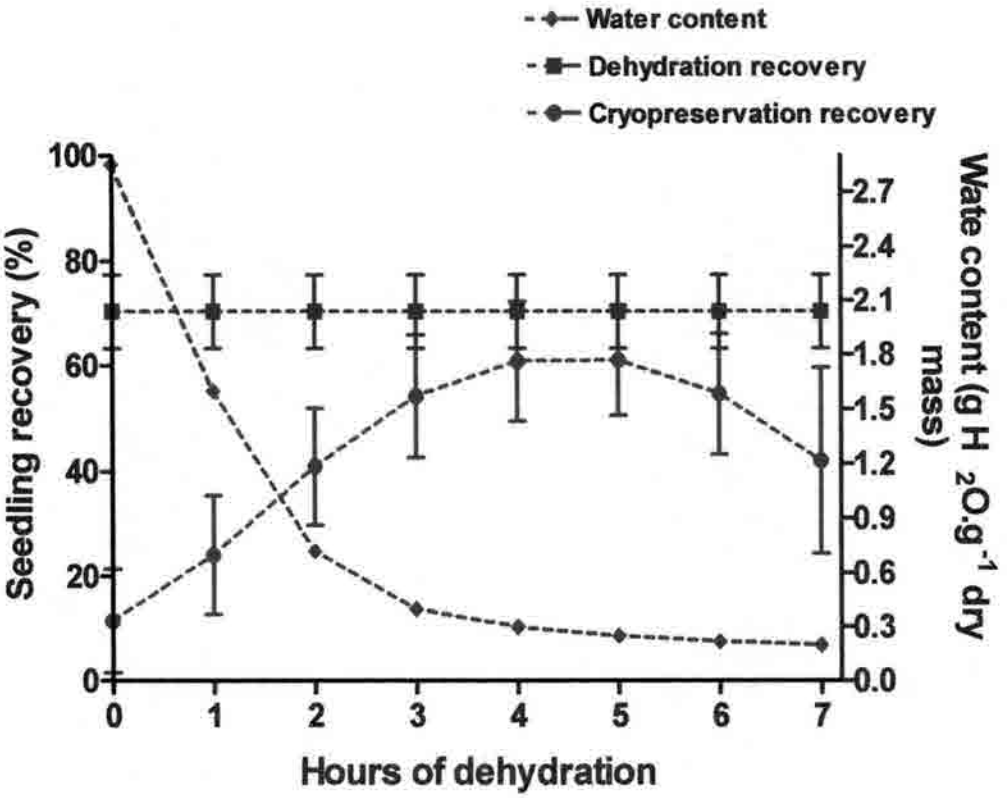

Figure 2. Effect of dehydration duration on the water content (◆) and seedling recovery after dehydration alone (■), or dehydration followed by cryopreservation (●). 'Pineapple' sweet orange embryonic axes were encapsulated in Na-alginate gel prior to pre-treatment with 0.5 and 0.75 M sucrose, 24 hours each, in a stepwise manner. Vertical bars represent means ± SEM of three observations ($P<0.05$).

Dehydrated, encapsulated, and pre-cultured axes survived slightly, but significantly better at lower and higher water contents than untreated axes (Fig. 2). Sucrose pre-culture conferred cryoprotection to highly hydrated axes (0.72-2.85 g $H_2O.g^{-1}$ dry mass), and at lower moisture content (0.20 - 0.22 g $H_2O.\ g^{-1}$ dry mass), sucrose increased desiccation tolerance (Fig. 2). Similar enhancement of tolerance to dehydration and liquid nitrogen temperatures for tissues pre-cultured in highly sugar-enriched media, especially with sucrose, has been observed for other plant species (Dereuddre *et al.*, 1990; Paulet *et al.*, 1993; Stushnoff and Seufferheld, 1995). Initially it was assumed that the protective role of sugars was due to an osmotic effect only, but more recently it has been observed, both directly and indirectly, that sugars enter cells in large quantities (Dumet *et al.*, 1993; Suzuki *et al.*, 1997). It has been reported that as a result of sucrose uptake the freezing point is lowered and the amount of freezable water in the tissues decreases and with that the formation of ice crystals upon exposure to sub-zero temperatures is reduced, improving the post-thaw recovery rates (Panis *et al.*, 1996). Sugars like sucrose, when added before dehydration and low temperature stresses, are also known to stabilize membranes and proteins during desiccation (Crowe *et al.*, 1990; Plessis *et al.*, 1993), which would improve recovery following freezing. According to recent reports carbohydrates provide protection because they serve as water substitutes in desiccated biomolecules satisfying the hydrogen bonding

requirements of polar head groups and thus maintaining their structure and function (Carpenter *et al.*, 1990; Crowe *et al.*, 1990). However, in spite of these recent insights, the exact role of sugars in the acquisition of tolerance to desiccation and freezing remains to be established (Bachiri *et al.*, 1995).

Effect of sucrose pre-culture

The level of sucrose in the pre-culture medium did not influence the re-growth of embryonic axes following desiccation to various water contents (Fig 3). However, an inhibitory effect of sucrose at concentrations above 0.75M was observed when axes were exposed to liquid nitrogen following dehydration to various water contents (Fig. 3). A similar effect of sucrose on encapsulated shoot tips of potato and banana meristems was observed elsewhere (Fabre and Dereuddre, 1990; Panis *et al.*, 1996). Survival of potato apices pre-treated with sucrose concentrations of 1M or higher decreased significantly before and after freezing in liquid nitrogen (Fabre and Dereuddre, 1990). Panis *et al.* (1993) reported that media containing more than 0.3 M sucrose retarded growth of proliferating meristems of banana and caused intense browning of the tissues. They concluded that sucrose at such high concentrations induced an osmotic shock, which caused browning and reduced growth. Their results indicate that although sugars may play a very important role in the acquisition of resistance to desiccation and

freezing in liquid nitrogen, and although they are not toxic compounds by nature, tissues of different plant species have different thresholds of intracellular sugar concentration they can tolerate. Thus, in order to increase resistance to desiccation and freezing in liquid nitrogen without affecting tissue integrity the sugar concentration in the pre-culture medium must be determined carefully, on a species-specific basis.

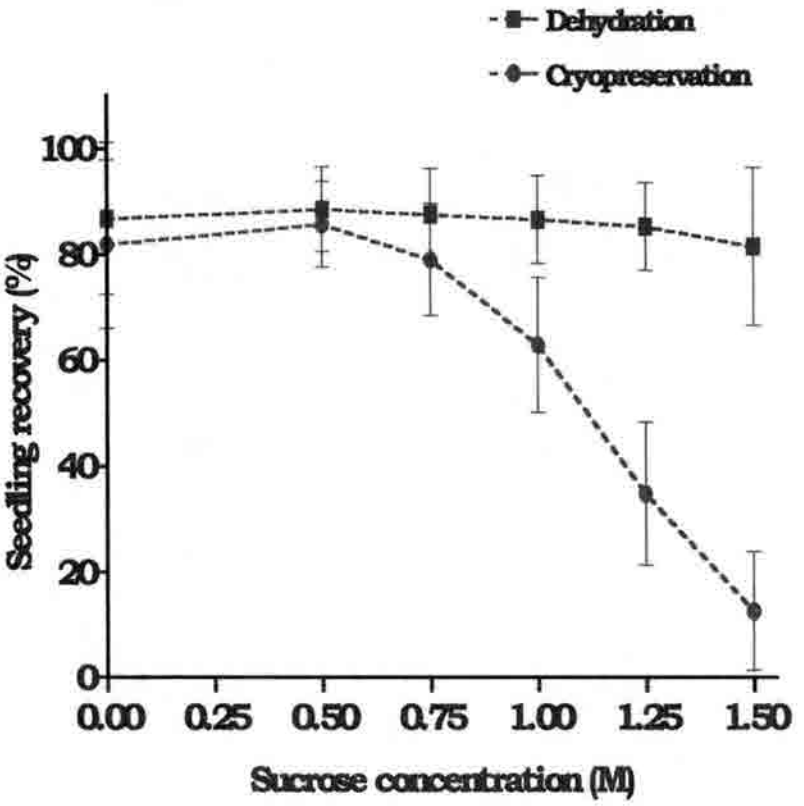

Figure 3. Effect of increasing sucrose concentrations on 'Pineapple' orange embryonic axes tolerance of dehydration (■) or dehydration followed by cryopreservation (●). Axes were encapsulated in alginate gel prior to pre-treatment with sucrose. Points represent means ± SD of three replications (P=0.05).

Figure 4 shows the effect of pre-culture duration on seedling recovery. Axes pre-treated for 12 and 24 hours showed similar germination percentages.

Effect of glycerol and proline

The cryopreservation protocol defined for 'Pineapple' sweet orange. embryonic axes was also tested with axes of lemon and mandarin Embryonic axes of these two species were isolated, encapsulated, pre-treated with sucrose, dehydrated over silica gel to various water contents and then plunged in liquid nitrogen. Percent seedling recovery for these two species and also for 'Pineapple' sweet orange is displayed in figure 5. Additionally, axes were pre-cultured with glycerol (a permeating cryoprotectant) and proline which were used in an attempt to improve post-thaw seedling recovery.

Combination of 0.5 M glycerol and 0.045 M proline with 0.8 M sucrose increased seedling recovery of *C. sinensis* embryonic axes, producing over 83.3% seedling recovery compared to 60.97% for sucrose alone (Fig. 5). Pre-treatment with sucrose, glycerol and proline caused a three fold plus increase (from 23 to over 86.7%) in post-thaw recovery of embryonic axes of mandarin. There was a similar but much less pronounced increase in the case of lemon axes. A significant interaction (Table 1, Fig. 5) occurred when sucrose plus glycerol or sucrose plus

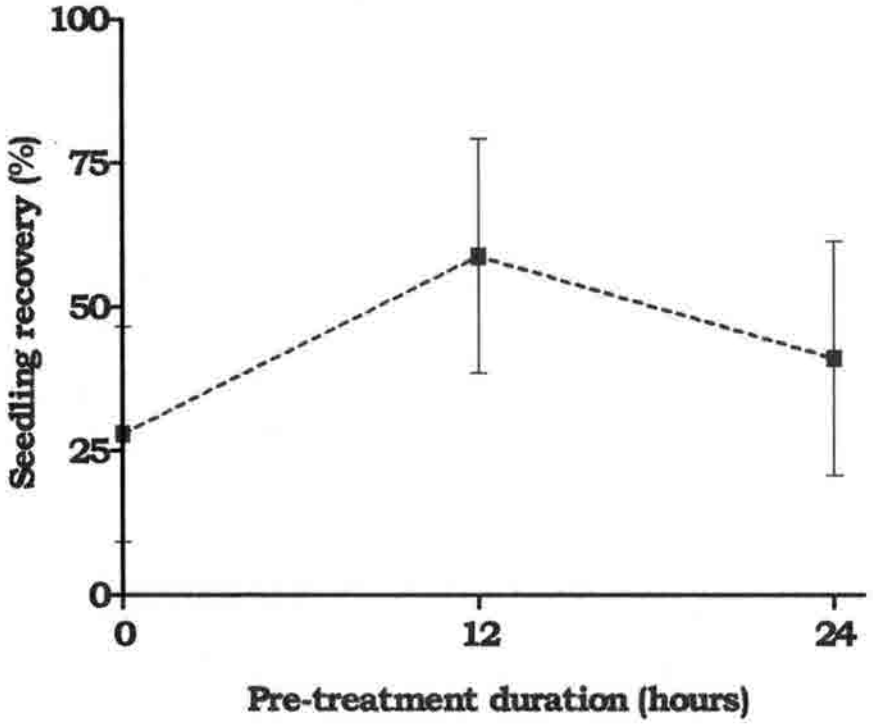

Figure 4. Effect of duration of pre-culture with sucrose on seedling recovery. 'Pineapple' orange embryonic axes were encapsulated in alginate gel and pre-cultured with 0.5 and 0.75 M sucrose, in a stepwise manner. Points represent means ± SD of three observations (P=0.05).

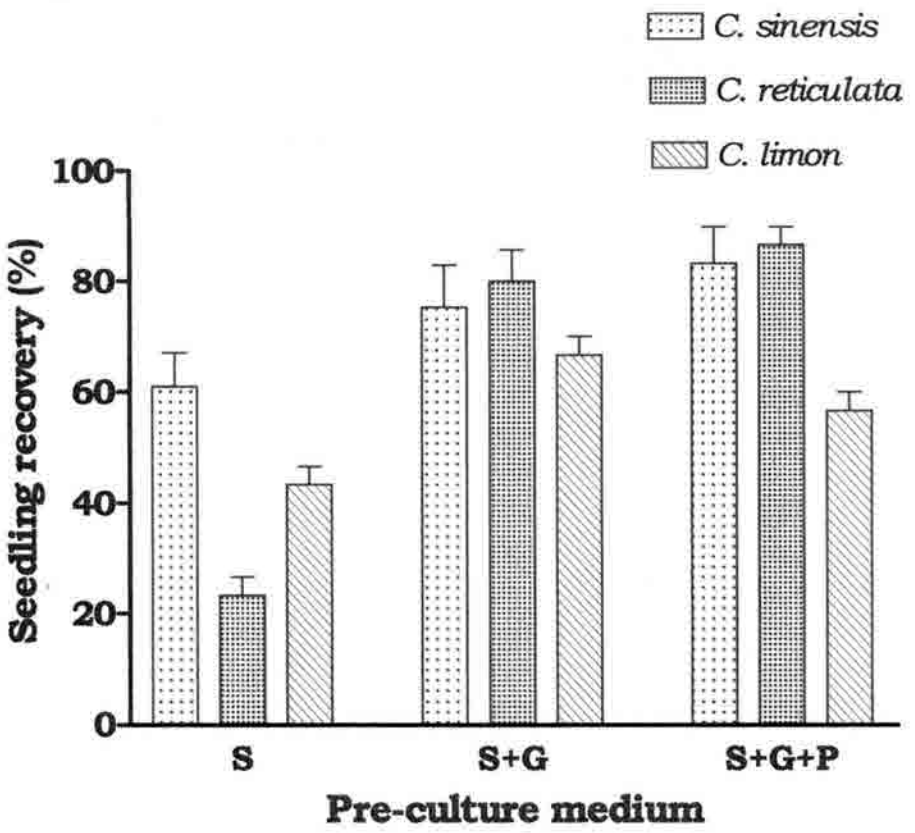

Figure 5. Seedling recovery following cryopreservation of excised embryonic axes of three citrus species. Axes were encapsulated with NA-alginate gel, pre-treated with sucrose (S; 0.5 and 0.75 M, stepwise) or in combination with glycerol (S + G; 0.5 M) and proline (S + G + P; 0.045 M) and dehydrated for up to 5 hours to a water content of approximatelly 0.14 g $H_2O.g^{-1}$ dry mass.

Table 1. Results of two-way analysis of variance on the effect of pre-culture medium, species and the interaction between both factors on seedling recovery after cryopreservation.

Source of variation		**Results**
Species (*C. sinensis*, *C. reticulata*, *C. limon*)	F	11.26
	P	0.0007 (***)
Pre-treatment (sucrose, sucrose+glycerol, sucrose+glycerol+proline)	F	20.34
	P	<0.0001 (***)
Interaction (species x pre-treatment)	F	8.548
	P	0.0005 (***)

glycerol plus proline was tested with the three species compared to sucrose alone. Survival of both *C. reticulata* and *C. limon* improved when glycerol alone and glycerol plus proline were added to sucrose. Comparing the addition of glycerol plus proline suggests none or only slight added benefit from proline for any of the species, compared to sucrose plus glycerol.

Proline has been shown to accumulate in halophytes exposed to salt stress, and in a wide range of plants exposed to cold and desiccation stresses (Alberdi *et al*, 1993; Liu and Zhu, 1997; Xin and Li, 1993). It is a highly soluble, neutral, amino acid that exerts high osmotic pressure, and is not toxic at high concentrations (Liu and Zhu, 1997). The mechanism(s) by which proline induces stress tolerance is not clear. However, it has been suggested that proline avoids membrane damage by preventing lipid peroxidation, acting as an antioxidant, and in doing so counter-acting stress-induced free radical accumulation (Xin and Li, 1993). Thus, it was hypothesized that exogenously applied proline might be an effective cryoprotectant of plant cells, and it was used in a few cryopreservation studies with good results (Withers and King, 1979). That is the reason why proline was included in the present work. Addition of proline to the pre-culture medium caused a noticeable increase in seedling recovery from axes of lemon and mandarin (Fig. 5). However, in the case of 'Pineapple' sweet orange axes this amino acid proved to be ineffective (Fig. 5). Thomas and James (1993) reported that

although proline levels increased in *Lolium perenne* L. plants during drought and cold treatments, this accumulation did not enhance freezing and water stress tolerance in this species. They question whether accumulation of proline and amino acids is the consequence of reduced protein synthesis or increased protein degradation during stress conditions rather than a marker of increased stress tolerance.

In conclusion, dehydration before freezing in liquid nitrogen was key to increasing seedling recovery in the cryopreservation method described here. Fully hydrated axes did not survive freezing in liquid nitrogen and the best recovery rate was attained by axes with water content in the range of 0.146 to 0.173 g $H_2O.g^{-1}$ dry mass. Sufficiently dehydrated embryonic axes that were cooled to -196°C developed into seedlings without any intermediary callus formation, within one month after transfer to recovery medium (Fig. 6).

Encapsulation in Na-alginate beads and addition of sucrose alone to the pre-growth medium did not have a significant role in increasing seedling recovery. However, axes with water contents lower or higher than the optimal range benefited from pre-treatment with sucrose alone, showing some improvement in seedling recovery after cooling to -196°C. The addition of 0.5 M glycerol to 0.8 M sucrose significantly improved survival of *C. reticulata* and *C. limon*, but not *C. sinensis*.

Figure 6. Plant recovered after cryopreservation, one year after transplanting to soil.

This cryopreservation process offers several advantages, namely easy handling of explants, simplification of cryoprotective media, elimination of costly programmed freezers, independence of survival from cooling rates and increased size of explants surviving liquid nitrogen exposure (Bachiri *et al.*, 1995). This is also a non-toxic and simple process that could be used on a routine basis for the three *Citrus* species tested, if dehydration tolerance can be achieved. Long-term storage tests are still needed to determine if deterioration occurs during storage.

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Chapter 3

Desiccation and cryopreservation tolerance of bare embryonic axes from 'Pineapple' sweet orange (*Citrus sinensis* [L.] Osb.) pre-treated with sucrose

Abstract

Essentially 100% germination was obtained with control 'Pineapple' sweet orange axes that were not subjected to dehydration or freezing. Axes pre-cultured with sucrose and dehydrated to 0.116 mg $H_2O.mg^{-1}$ dry mass had good mean seedling recovery of 75 to 80% after which there was a steady decline until total loss of viability was observed.

Embryonic axes of *C. sinensis* were successfully cryopreserved, attaining up to 70% viability after their water content was reduced to approximately 0.2 mg $H_2O.mg^{-1}$ dry mass. Sucrose pre-treatment had a stabilizing effect on cryopreserved axes, maintaining a higher seedling recovery (80-95%) over a broader range of water content (from 0.2 to 0.4 mg $H_2O.mg^{-1}$ dry mass), as compared to untreated axes. Axes pre-cultured with sucrose retained regeneration potential even following dehydration to 0.116 mg $H_2O.mg^{-1}$ dry mass

Sucrose pre-treatment had a stabilizing effect, maintaining a high recovery percentage over a broader range of water content. Dehydration of axes pre-cultured with sucrose occurred at a much slower rate as compared to controls.

Tissue glucose and fructose increased when embryonic axes were pre-cultured with sucrose, regardless of drying time. Tissue raffinose and stachyose levels dropped as embryonic axes were dried. Higher tissue levels of glucose, raffinose and stachyose, as compared to untreated samples, suggest that sucrose from the pre-culture medium may have entered the metabolic pathway following hydrolysis.

Introduction

Citrus species are extensively cultivated in tropical and subtropical regions an are among the most important fruit crops in the world. The need for conservation of commercial cultivars and rootstocks of *Citrus*, as well as wild relatives, has been demonstrated by growers associated with the *Citrus* industry and by breeders that depend on a wide array of genetic resources to carry on with their breeding programs. Traditionally *Citrus* species have been conserved as living collections in field gene banks because seeds of *Citrus* species have intermediate or recalcitrant storage behavior (Hong and Ellis, 1995). Recalcitrant seeds are defined as being sensitive to both dehydration and low temperatures, as opposed to orthodox seeds which are tolerant of desiccation and freezing temperatures (Hong and Ellis, 1995; Roberts, 1973). Intermediate seeds are those moderately desiccation-tolerant that are injured by freezing temperatures (Ellis *et al.* 1990 and 1991).

On one hand, maintenance of tree crops in the field has the advantage of simplifying the evaluation and characterization of plant germplasm. On the other hand such collections are constantly exposed to pests, diseases, and environmental stresses which may cause vigor loss and eventually lead to death of the plant, or the need to eliminate it from the genebank (Duran-Vila, 1995; Marin *et al.*, 1993; Stushnoff, 1987 and 1991; Towill, 1988). To complicate the scenario, expansion of urban and agricultural frontiers, as well as reduced resources to face the high financial costs associated with the maintenance of field gene banks could put them at risk (Stushnoff, 1987 and 1991). Therefore, an alternative method for long-term preservation of *Citrus* valuable germplasm needs to be developed. A system that would allow conservation of unique individuals under controlled, stable and disease-free conditions, at low cost, and with high genetic stability.

Currently, cryopreservation is the technique with the potential to fulfill all these requirements. Cryopreservation is the storage of biological material in the vapor or liquid phase of liquid nitrogen, at the ultra low temperatures of –150°C and -196°C, respectively. At the temperature of liquid nitrogen (-196°C) almost all the metabolic functions of living cells are greatly reduced or completely arrested and they can be preserved in such a state for extended periods without viability loss or genetic alteration (Kartha, 1985). In recent years cryogenic techniques have become important tools for the long-term preservation of genetic

resources of numerous plant species. More recently cryopreservation has also been used for the conservation of genetically modified cells and plants bearing unique characteristics (Duran-Vila, 1995; Forsline *et al.*, 1998; Kobayashi *et al.*, 1990; Sakai *et al.*, 1990 and 1991a and b).

There have been reports on cryopreservation of *Citrus*, where ovules (Bajaj, 1984), somatic embryos (Marin and Duran-Vila, 1988) or nucellar cells (Kobayashi *et al.*, 1990; Sakai *et al.*, 1991a and b) have been used. However, these systems are not highly recommended for conservation of plant germplasm. Somaclonal variation, that is, uncontrolled genetic or epigenetic variability that arises through the *in vitro* plant regeneration obtained adventitiously through cell and callus cultures, is frequently associated with this methodology (Kartha, 1985; Karp, 1991; Larkin and Scowcroft, 1981). Navarro *et al.* (1985) reported the production of aberrant plants of *Citrus clementina* obtained by somatic embryogenesis of nucelli cultured *in vitro*. They observed changes in internode length, and leaf, petiole and fruit shape and size in 29% of the plants obtained. Abnormalities remained through seven years of greenhouse growth and after several cycles of propagation through grafting. Such risk must be avoided if one's interest is to preserve true-to type individuals.

Plant organs such as whole seeds, zygotic embryonic axes, axillary buds, meristems, and shoot tips would be a better choice of explant. They are organized plant organs and given the appropriate conditions

would allow the recovery of whole plants with minimal risk of somaclonal variation (Karp, 1991; Navarro *et al.*, 1985; Stushnoff, 1987; Towill, 1988). Zygotic embryonic axes offer the possibility of increasing the genetic pool in a base collection (Stushnoff, 1991).

The most important factor that is very critical in cryopreservation is the water content in the cells prior to freezing. When moisture levels are high, freezing is lethal as a result of membrane disruption due to fast growth of ice crystals in the protoplast. Hence, reducing the water content in cells before they are exposed to liquid nitrogen can considerably improve their survival subsequent to freezing and thawing. Extensive drying, however, must be avoided since it causes desiccation damage. The importance of water for living cells and the effect of dehydration must be considered and reconciled with the need to remove freezable water, so as to avoid desiccation injury. The range of moisture content that supports viability must be defined in order to develop a successful cryopreservation protocol. Extracellular freezing and evaporation can remove freezable water present in plant tissues. Under experimental conditions cells are pre-frozen slowly to -20 to -70°C using a programmable freezing device prior to plunging in liquid nitrogen (Chen *et al.*, 1984). Freeze-dehydrated cells are not injured by rapid immersion in liquid nitrogen or subsequent re-warming as long as enough freezable water was removed. This pre-freezing method is effective with cold hardy species, however it depends on the availability of a programmable freezer

and can only be used with cold tender materials if they are pre-treated with cryoprotectants. Cryoprotectants are organic compounds such as glycerol, DMSO (dimethyl sulfoxide), and ethylene glycol, to name a few. A programmable freezer is an expensive piece of equipment and is not always available. As for cryoprotectants, most of them are known to be toxic to plant cells. More recently there has been a tendency to avoid using programmable freezers and cryoprotectant pre-treatment. Potentially toxic cryoprotectants have been replaced with compounds that were found to exhibit cryoprotectant characteristics and do not exhibit cytotoxicity. Sucrose is presently the most widely used non-toxic cryoprotectant.

In most of the cryopreservation protocols reported in the current literature water is removed from plant tissues by evaporation, achieved by exposing tissue to an air current or to a desiccant, such as silica gel or saturated salt solutions.

Our hypothesis for this research was that there is a range of water content that would allow embryonic axes of 'Pinneapple' sweet orange to survive cryopreservation without any deleterious effect upon seedling recovery. It was also hypothesized that pre-treatment with sucrose would enhance tolerance of embryonic axes to desiccation and freezing in liquid nitrogen. Therefore, the purpose of the study presented here was to test the effect of desiccation to different water contents on the viability of excised embryonic axes of 'Pineapple' sweet orange frozen in liquid

nitrogen. The effect of pre-culture with different sucrose concentrations on seedling recovery of cryopreserved axes was investigated as well.

Materials and methods

Plant material

Fruits of 'Pineapple' sweet orange (*Citrus sinensis* [L.] Osb.) were obtained from Lake Alfred Citrus Research Station, University of Florida, Florida, and from Brownsville, Texas. They were surface sterilized by dipping them into a solution of 10% (v/v) sodium hypochlorite (commercial *Clorox*) containing 4-5 drops of Tween 20 (polyoxyethylene sorbitan monolaurate), Mallinckrodt OR, for 15 minutes. They were rinsed 3-4 times with sterilized water in a laminar flow hood and seeds were extracted from the fruits and cleaned thoroughly to remove mucilaginous aril from the seed coat. Embryonic axes were excised aseptically from the seeds under a stereo microscope. The axes were transferred to Petri dishes lined with moist filter paper until used.

Sucrose pre-treatment

Embryonic axes were pre-cultured on MS basal medium containing 0.5 and 0.75 M sucrose. They were pre-cultured for 24 hours on each sucrose concentration, in a stepwise manner. Twenty mL of semi-solid medium containing different levels of sucrose was dispensed in Petri dishes, and a sterile filter paper was placed on top of the medium after it solidified to avoid direct contact of the embryonic axes with the medium. Following pre-treatment axes were subjected to desiccation to different water contents.

Desiccation, cryopreservation and thawing procedures

Excised embryonic axes pre-cultured or not (control) with sucrose were desiccated over silica gel (*ca.* 80 g) in an airtight closed container (500 mL capacity), at ambient temperature (25±2°C), for 0-300 minutes. Water content was determined gravimetrically at the end of each desiccation period following drying in a oven at 100±2°C for 24 hours, and expressed on a dry weight basis. For freezing, after each desiccation period a sample of ten axes was collected and transferred into 2 mL sterile cryotubes which were immersed rapidly in liquid nitrogen (-196°C) for 30 minutes. Axes were subsequently thawed rapidly by plunging the cryotubes into a water bath at 40±2°C, under agitation, for 3 minutes. Axes were removed from the cryovials and immediately transferred to culture medium. Desiccated axes transferred to culture medium without exposure to liquid nitrogen provided as the control treatment.

***In vitro* culture**

Following desiccation and/or freezing, embryonic axes were placed in magenta boxes containing 30 ml MS solid basal medium (Murashige and Skoog, 1962) and incubated in a growth chamber at 25±2°C, with a 16 hour daylength photoperiod and a photosynthetic photon flux (PPF) of 62 $\mu molm^{-2}s^{-1}$. Axes that had been frozen were kept in darkness for 48 hours prior to exposure to light.

Assessment of recovery

Survival was assessed daily up to two weeks following transfer to recovery medium. Axes were considered viable when re-growth in the form of greening, root elongation and shoot growth was observed after one week of culture. After one month in culture survival was defined as the percentage of embryonic axes that produced a normal seedling, with shoot and root emergence. The cultures were kept for at least 3 months before being discarded as non-survivors. Each experiment was repeated three times.

Thermal analysis

Differential scanning calorimetry (DSC) analysis was performed using a DSC 50 ISI calorimeter. The instrument was calibrated using indium and mercury standards. Samples were placed in 100 µL Mettlar aluminum pans and sealed with the aid of a Mettlar sealer. An empty aluminum pan was used as a reference sample. A nitrogen gas flow of 40-50 mL/minute was used to purge moist air and to prevent condensation in the measuring cell. DSC warming profiles were constructed using a scanning rate of 10°C.min^{-1}. Samples sealed in aluminum pans were quenched in liquid nitrogen then immediately transferred to the sampler which had been previously cooled to -100°C. Upon transfer to sampler temperature was always less than -130°C and was then allowed to warm to -100°C before initiation of the warming

scan. Scans were performed from -100°C to +50°C. Analysis was done on six samples per treatment. Representative profiles are presented for each treatment.

Carbohydrate analysis

Embryonic axes were freeze-dried and stored in airtight closed containers at room temperature, in desiccators containing silica gel. Axes that were pre-treated with sucrose were quickly rinsed three times with nanopure water to remove residues of sugar accumulated on their surface. For extraction, four axes (*ca.* 1.45 mg) were ground into a fine powder using a mortar and pestle. Soluble carbohydrates were extracted for one hour at 4°C using 3 mL of 0.1 M NaOH solution. The extract was centrifuged at 7000 rpm for 5 minutes, at 4°C. The supernatant was collected, filtered using a 0.22 µm nylon syringe filter to remove particulates and to bind soluble proteins, and used immediately or stored at -70°C for future use. Sugar concentrations were analyzed by a Dionex DX-300 (Dionex Co., Sunnyvale, CA) high performance liquid chromatography (HPLC) apparatus equipped with a 25 µL injection loop. Sugars were separated on a Carbopac PA 100 column (4.6 X 250 mm) equipped with a Dionex guard column (3 X 25 mm), using a flow rate of 1 mL/min, at ambient temperature (25±2°C). A pulsed electrochemical detector was used for the detection of the oligosaccharides. An eluant gradient was used to optimize separation. NaOH concentration was

linearly increased from 70 mM to 120 mM in 18 minutes, combined with sodium acetate at 100 mM concentration held at 3 mM for 10 minutes, then increased to 35 mM for the last 8 minutes. Sugar concentrations are expressed as mg of sugar per mg of dry weight. Analysis was performed on three samples per treatment.

Statistical analysis

Arcsine transformations were done on percentage data prior to running analysis of variance (ANOVA) with Graph Pad Prism software (GraphPad Software Inc., San Diego, CA, 92121). Analysis of variance was used to compare the differences in the mean survival at different water contents. Non-linear regression analysis was used in some cases to determine the relationship between survival following desiccation and cryopreservation, and water content level.

Results and Discussion

The experiments reported here describe the effect of desiccation to different water contents and sucrose pre-treatment on seedling recovery after cryopreservation in liquid nitrogen (-196°C) of 'Pineapple' sweet orange (*C. sinensis*) embryonic axes.

During dehydration the water content of the embryonic axes decreased rapidly within the first hour of desiccation and more gradually thereafter. Up until 30 minutes of dehydration, water contents of pre-treated and untreated axes was similar; after 30 minutes of dehydration

the water content of untreated axes was consistently lower than in the sucrose pre-cultured axes (Table 1). This could be an indication of water binding by sucrose.

Essentially 100% germination was obtained with control embryonic axes that were not subjected to dehydration or freezing (Fig. 1). Following dehydration for 30 minutes the water content of axes decreased from about 2.0 mg $H_2O.mg^{-1}$ dry mass to 0.205 mg $H_2O.mg^{-1}$ dry mass (control, Fig. 1). After 60 minutes of dehydration seedling recovery of control embryonic axes decreased significantly from 100% to 70% as water contents dropped from 2.043 mg to 0.097 mg dry mass (Fig. 1, Table 1). According to Berjak and Dumet (1996) the fact that excised axes remain viable and show good germination percentage at water contents below 0.28-0.31 $g.g^{-1}$, where generally only non-freezable water is present, is an indication of intermediate storage behavior. Thus, these results seem to support the classification of *C. sinensis* seeds in the intermediate category (Hong and Ellis, 1995).

After pre-culturing with sucrose the proportion of axes that resumed growth also remained at a 100% (Fig. 2). Thereafter, a steady decline in seedling recovery until total loss of viability was observed, when the water content reached 0.104 mg $H_2O.mg^{-1}$ dry mass. Axes pre-cultured with sucrose retained regeneration potential over a broader range of water content and 70% seedling recovery was retained even

Table 1. Changes in water content (mg $H_2O.mg^{-1}$ dry mass) of control and pre-cultured embryonic axes of 'Pineapple' sweet orange as a function of duration of dehydration, in minutes. Values on the right represent standard deviation of the mean (P=0.05).

Dehydration duration (min.)	Water content (mg $H_2O.mg^{-1}$ dry mass)	
	Control	Pre-cultured
0	2.043 ± 0.049^{a}	1.727 ± 0.261^{a}
6	0.951 ± 0.209^{b}	0.748 ± 0.202^{b}
12	0.563 ± 0.369^{bc}	0.492 ± 0.100^{c}
18	0.369 ± 0.177^{cd}	0.375 ± 0.049^{cd}
24	0.263 ± 0.122^{cde}	0.297 ± 0.029^{de}
30	0.205 ± 0.083^{cde}	0.251 ± 0.023^{de}
36	0.159 ± 0.061^{cde}	0.229 ± 0.027^{de}
42	0.133 ± 0.044^{de}	0.201 ± 0.014^{de}
48	0.116 ± 0.310^{de}	0.185 ± 0.014^{de}
54	0.105 ± 0.023^{de}	0.172 ± 0.013^{de}
60	0.097 ± 0.021^{de}	0.162 ± 0.012^{de}
90	0.068 ± 0.013^{de}	0.138 ± 0.009^{e}
120	0.058 ± 0.008^{de}	0.126 ± 0.008^{e}
150	0.049 ± 0.010^{de}	0.116 ± 0.008^{e}
180	0.049 ± 0.01^{e}	0.110 ± 0.007^{e}
210	0.045 ± 0.008^{e}	0.106 ± 0.008^{e}
240	0.043 ± 0.006^{e}	0.104 ± 0.006^{e}
270	0.042 ± 0.006^{e}	0.101 ± 0.008^{e}
300	0.036 ± 0.003^{e}	0.100 ± 0.008^{e}

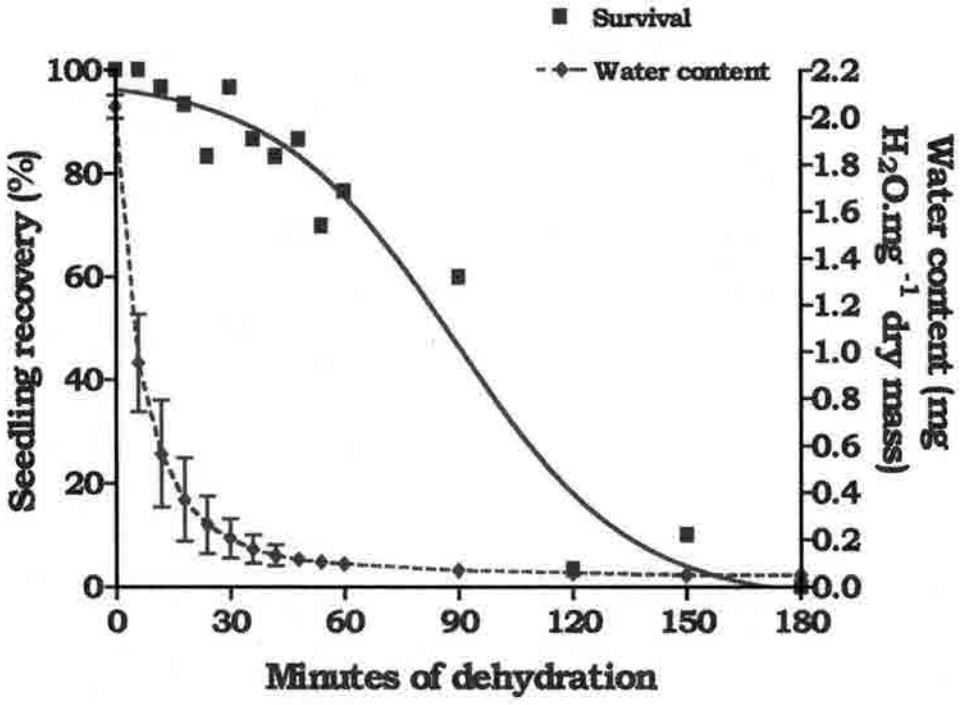

Figure 1. Effect of duration of dehydration on the water content (◆) and on the percentage of seedling recovery (■). Embryonic axes of 'Pineapple' sweet orange were dehydrated over silica gel for various periods of time prior to tranfer to growth medium. Vertical bars represent SD (P=0.05). Points represent means of three observations; ANOVA shows that means are significantly different (P<0.0001). Solid line represents non-linear regression of the data (r^2=0.8590).

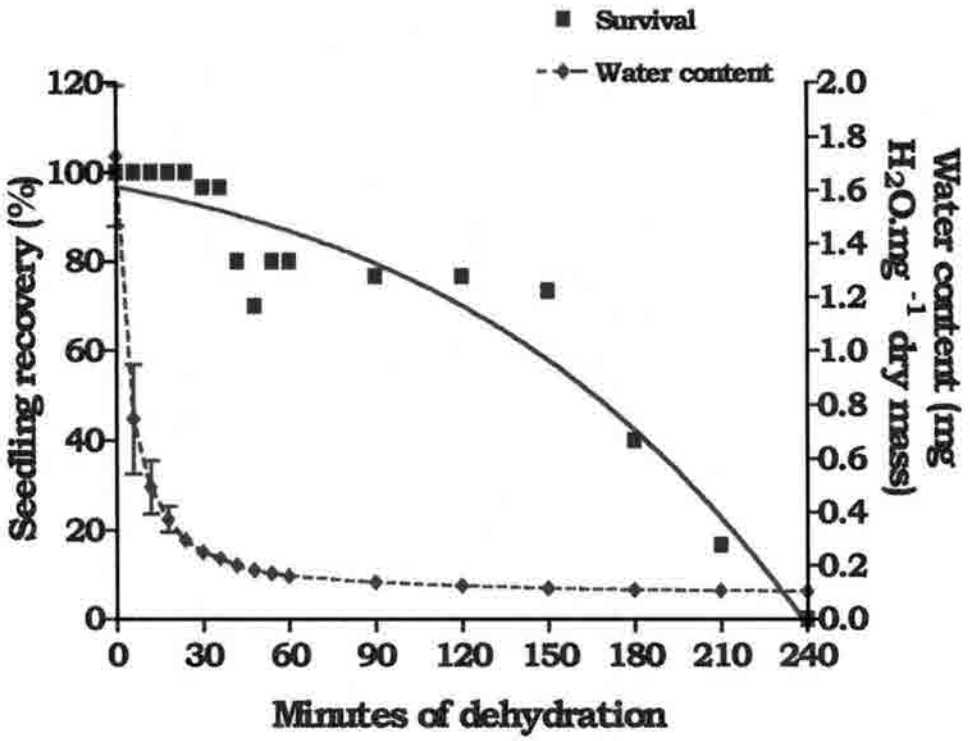

Figure 2. Effect of sucrose treatment on the water content (◆) and the percentage of seedling recovery after desiccation to various water contents (■). Vertical bars represent SD of the means (P=0.05); missing bars are within the limit of the symbol. Points represent means of three observations ($P < 0.0001$); solid line represents the regression of the data ($r^2 = 0.9114$).

following 150 minutes of dehydration when the water content dropped to 0.116 mg $H_2O.mg^{-1}$ dry mass (Table 1). These results indicate that sucrose pre-culture had a positive effect on axis tolerance of dehydration and freezing. It is generally accepted that sucrose supplied during cryoprotective treatments has an osmotic dehydration effect and is also able to enter cells and to exert cryoprotection of cellular structures (Dumet *et al.*, 1993). Histological observations have shown intracellular accumulation of starch during pre-culture with sucrose, an indirect evidence that sucrose entered cells (Bagniol *et al.*, 1992; Gonzalez-Arnao *et al.*, 1993). Direct evidence has been provided by measurement of sucrose concentration in samples of different plant materials.

Fully hydrated control and pre-treated axes (2.043 and 1.727 mg $H_2O.mg^{-1}$ dry mass, respectively) did not survive exposure to liquid nitrogen (Fig. 3 and 4). Most plant tissues do not withstand freezing at such high water contents. At least 12 minutes dehydration (0.56 mg $H_2O.mg^{-1}$ dry mass) was required to begin recovering viable axes after cryopreservation of control samples (Fig. 3). Likewise, 12 minutes dehydration resulted in 0.492 mg $H_2O.mg^{-1}$ dry mass and 60% recovery of pre-cultured axes. Highest seedling recovery (100%) for control axes was obtained when they reached approximately 0.2 mg $H_2O.mg^{-1}$ dry mass. However, axes with water content as low as 0.042 mg $H_2O.mg^{-1}$ dry mass showed about 20% seedling recovery.

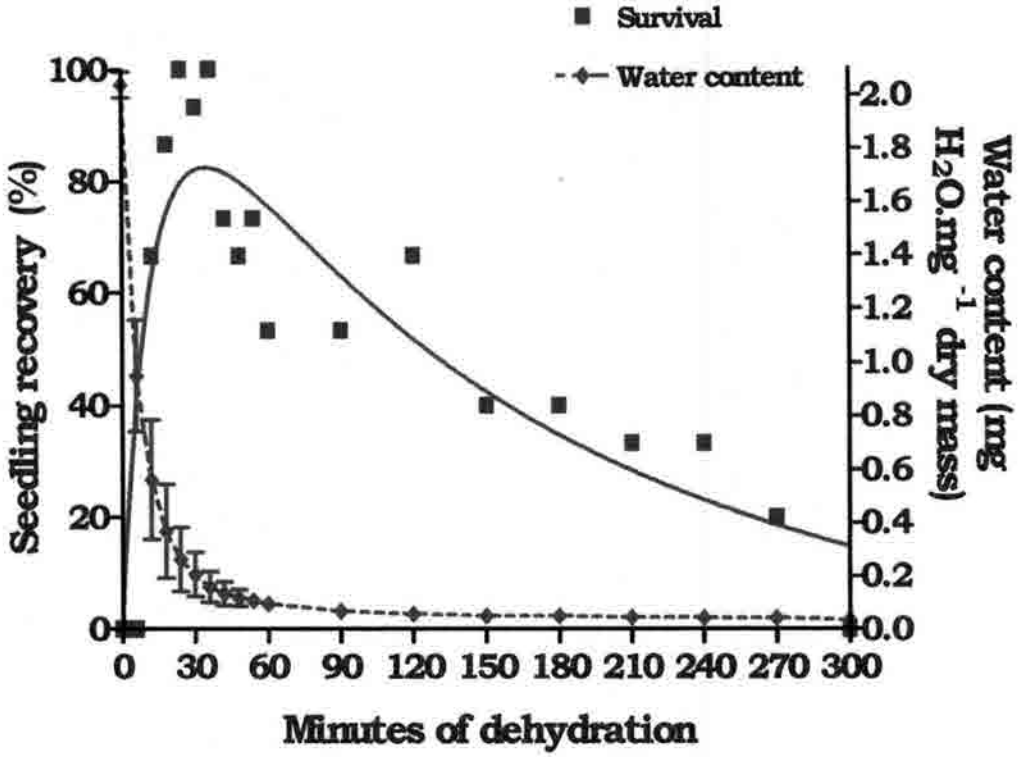

Figure 3. Effect of the duration of dehydration on the water content (◆) and on the percentage of seedling recovery after cryopreservation (■). Vertical bars represent SD of the mean (P=0.05). Points are means of three replications (means are significantly different (P<0.001). Solid line is the non-linear regression of the data (r^2= 0.6589).

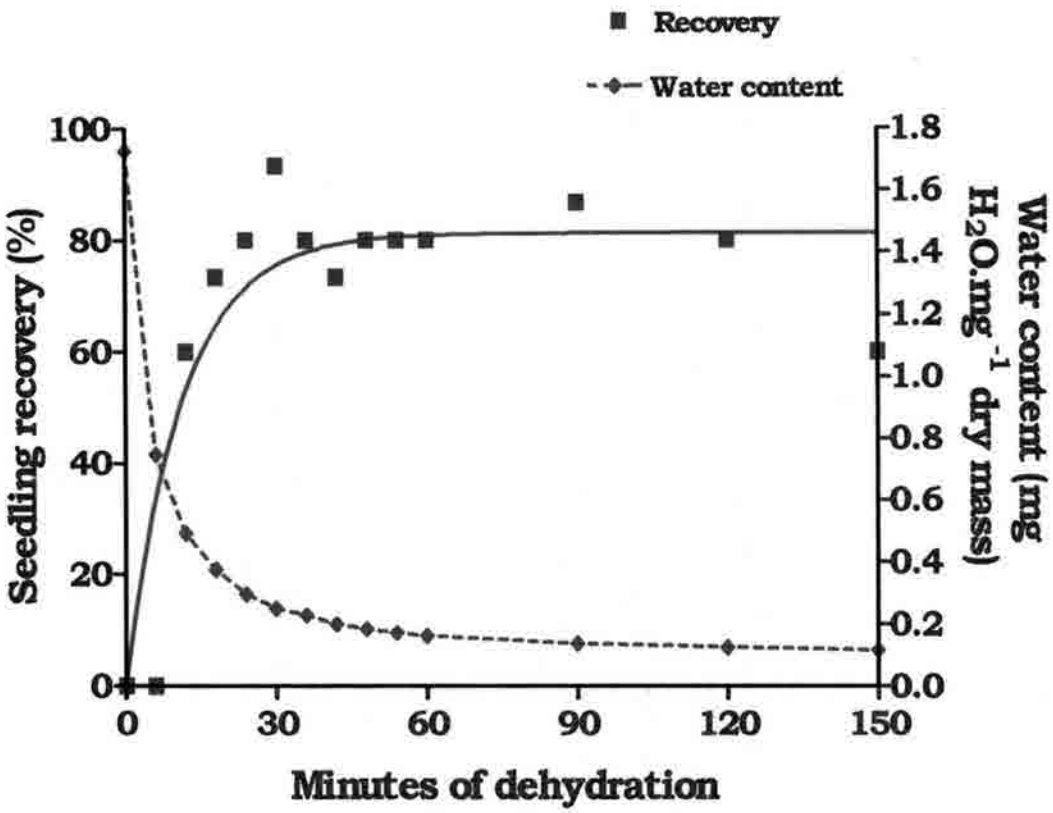

Figure 4. Effect of dehydration duration on water content (◆) and seedling recovery after cryopreservation (■). Embryonic axes were pre-treated with sucrose (0.5 and 0.75M, stepwise) prior to desiccation and freezing in liquid nitrogen. Points represent means of three replications ($P < 0.0001$); solid lines represent the regression of the data ($r^2 = 0.6033$).

Pre-cultured axes showed high recovery percentages after cryopreservation (80-95%) over a broader range of water contents (Fig. 4). Evidence of a role for sugars on freezing tolerance of plant material have been reported. For example, it was observed that pre-culture with sugars (*e. g.* sucrose and fructose) caused an increase in freezing tolerance of cell suspensions of two *Eucalyptus* species, one resistant and the other sensitive to freezing (Travert *et al.*, 1997). However, pre-culture in a high sucrose concentration alone was insufficient to guarantee the viability of cryopreserved embryonic axes (Fig. 4). Partial dehydration down to 0.492 mg $H_2O.mg^{-1}$ dry mass was necessary to allow seedling recovery after cryopreservation. Thus the most efficient strategy was to combine dehydration and sucrose pre-culture. Similar results were reported on studies with sugarcane apices; highest survival rates were obtained with pre-growth in 0.75 M of sucrose followed by desiccation (González-Arnao *et al.*, 1996). Combination of these two conditions avoids the use of vitrification solutions which are generally toxic for plant material (Engelmann, 1991). Seedling recovery of 100% was obtained when axes were pre-cultured with 0.5 and 0.75 M sucrose (24 hours each, stepwise manner); higher concentrations of sucrose did not improve tolerance of desiccation and was apparently toxic. González-Arnao *et al.* (1996) also observed a detrimental effect of sucrose at concentrations higher than 0.75 M on survival of sugarcane apices following cryopreservation. Our interpretation is that higher sucrose concentration caused extensive

osmotic dehydration of the axes, and they were damaged by such severe desiccation. Different species may absorb sucrose at different rates or up to different levels. This might explain why some species might require higher sugar concentration or longer pre-growth duration to achieve optimal survival. Re-growth of embryonic axes subjected to dehydration alone or dehydration followed by cryopreservation occurred without callus formation. Viable axes swelled and turned green within one week of culture.

Differential scanning calorimetry (DSC) can be used to predict critical moisture contents for desiccation and freezing damage (Vertucci *et al.*, 1994). In our study DSC was used to assess the phase changes of water when 'Pineapple' sweet orange embryonic axes at different water contents were exposed to sub-zero temperatures. Representative DSC profiles for warming at 10°C/minute, from -100°C to 50°C for control and pre-cultured embryonic axes desiccated to different water contents are shown in figures 5 and 6, respectively. Freezable and non-freezable water were present in the thermograms of both control and pre-cultured embryonic axes. Fully hydrated control and pre-cultured axes showed a broad melting peak at about -5°C. These axes did not survive exposure to liquid nitrogen. Axes dried to 0.205 mg $H_2O.mg^{-1}$ dry mass (control axes) and 0.251 mg $H_2O.mg^{-1}$ dry mass (pre-cultured) showed the highest seedling recovery; they turned green and produced a normal seedling in about three weeks after transfer to culture medium. At this water content

there was very little or no freezable water present and a small melting transition was observed. Axes at higher water contents (with more freezable water available) were also able to survive freezing in liquid nitrogen, however with a lower seedling recovery percentage. The presence of melting transitions was also observed in embryonic axes of *Landolphia kirkii* that survived storage at -70°C, indicating that certain amount of water froze in the tissues with no apparent ill-effect on viability; (Vertucci *et al.*, 1991). On a subsequent study Vertucci *et al.* (1995) confirmed that to obtain maximum survival at subfreezing temperatures *Zizania* embryos should remain partially hydrated. The optimum water content depending on the maturity stage and storage temperature This result indicates that viability is not affected by intracellular ice formation if water content is less than a critical value (Vertucci *et al.*, 1991). At water contents below these critical levels the ice crystals that form are too small to cause injury (Vertucci *et al.* 1995). Lethality in such cases is associated with formation of pure ice or desiccation of the tissues to a level where no freezable water is available (Vertucci *et al.*, 1991). For both control and pre-cultured axes the size of the transition was proportional to the water content of the tissues (Fig. 5 and 6). Sucrose pre-culture did not change the thermal characteristics of the axes, that is, pre-cultured axes undergo a major nucleation and ice melt event similar to those observed in control axes. It has been proposed that glass transition is fundamental to obtain survival following freezing

in liquid nitrogen (Benson *et al.*, 1996). In this study a glass transition was not a necessary to ensure pot-thaw recovery. It is important to mention that glasses might be unstable and could revert to the crystalline phase, releasing water (Vertucci *et al.*, 1994).

Cryopreservation is dependent upon removal of water from plant tissues using osmotic dehydration or air desiccation. The success of the cryopreservation method depends upon desiccation tolerance and the ability to prevent ice nucleation during cooling and warming. DSC data can be instrumental in optimizing cryopreservation procedures.

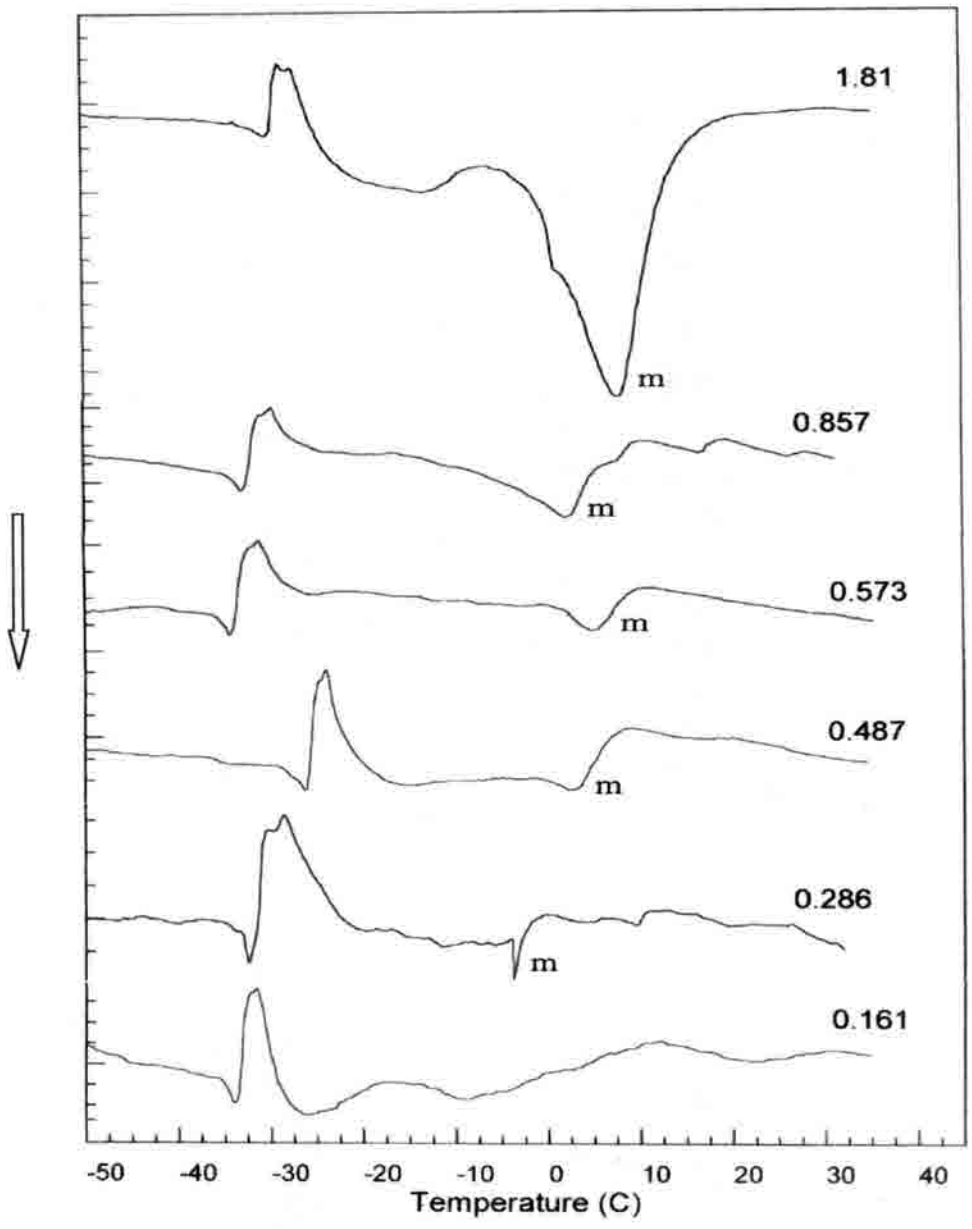

Figure 5. Representative warming Differential Scanning Calorimetry profiles of thermal transitions in bare embryonic axes of 'Pineapple' sweet orange dried to indicated water content (on a mg $H_2O.mg^{-1}$ dry mass basis). The "m" indicates melting transitions. Profiles were recorded as axes were warmed at a scanning rate of 10°C min^{-1}. Y axis arrow indicates endothermic transitions.

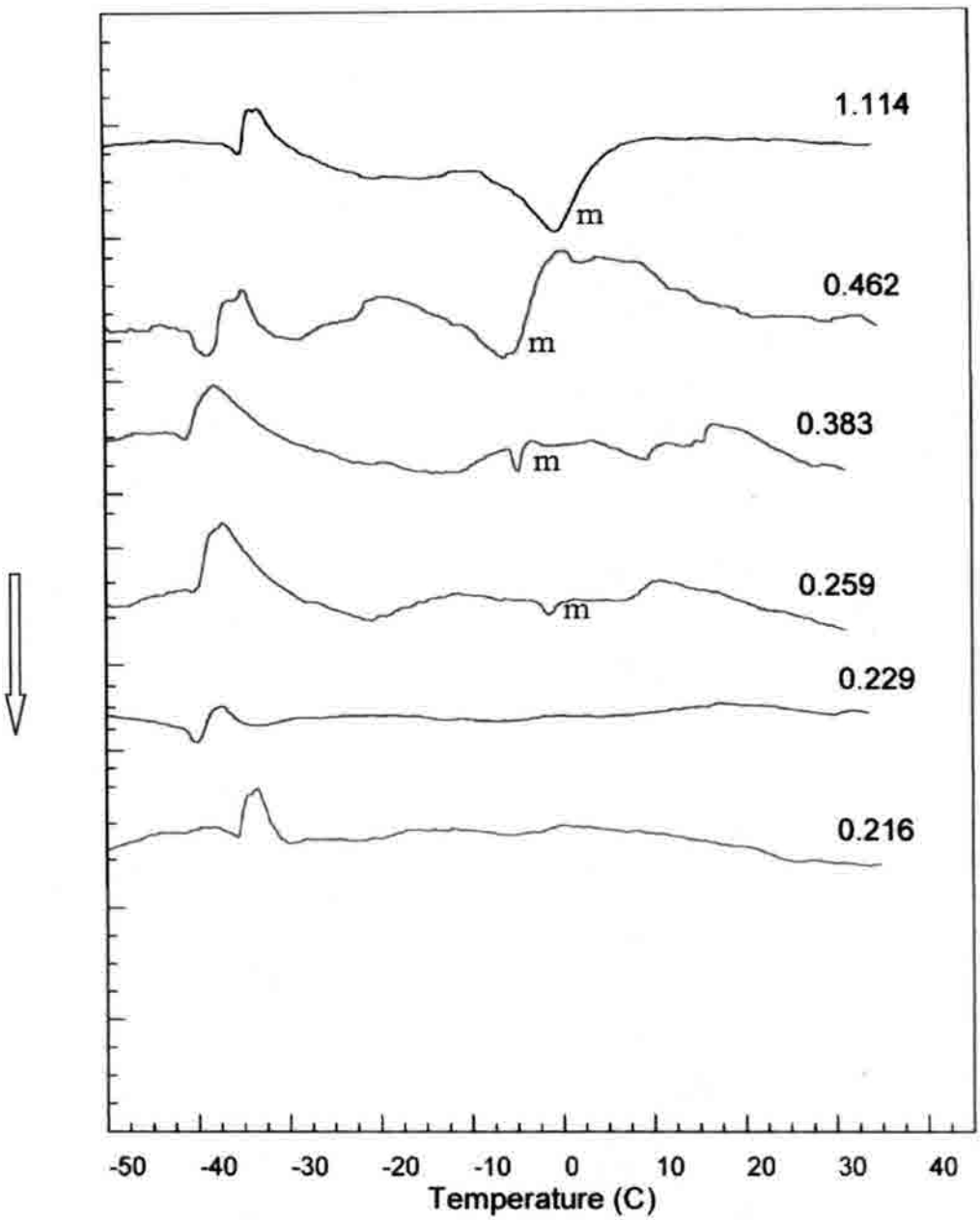

Figure 6. Representative warming Differential Scanning Calorimetry profiles of thermal transitions in embryonic axes of 'Pineapple' sweet orange pre-treated with sucrose 0.5 and 0.75 M (24 hrs each, stepwise) and dried to indicated water contents (on a mg $H_2O.mg^{-1}$ dry mass basis). The "m" in the curve indicates melting transitions. Profiles were recorded during warming at a scanning rate of 10°C min-1. Y axis arrow indicates endothermic phase transitions.

Carbohydrate analysis

Changes in carbohydrate concentrations for control and sucrose pre-cultured embryonic axes desiccated over various periods of time are shown in figure 7. Tissue sucrose and fructose increased over drying time for embryonic axes pre-cultured with sucrose, but remained unaltered for control axes that were not pre-treated with sucrose. Tissue raffinose and stachyose levels dropped as embryonic axes were dried, for both pre-cultured and control axes. Glucose concentrations changed very little with sucrose pre-culture. Glucose increased in non-sucrose treated samples when dried up to 45 minutes. Similar carbohydrate profiles were detected in desiccated leaf samples of *Ramonda nathaliae* (Müller *et al.*, 1997). It was observed that in this resurrection plant desiccation of the leaf lead to an increase in sucrose but a decrease in raffinose. Travert *et al.* (1997) reported a positive effect of sugars on freezing tolerance of cell suspensions of two *Eucalyptus* species, one resistant and the other sensitive to freezing. They observed that freezing tolerance of cells increased after sugar pre-culture. Fructose and sucrose were the most abundant carbohydrates in cell suspensions during cold exposure. The protective effect of sugars was observed in both resistant and sensitive genotypes.

Our data indicate that sucrose supplied in the pre-culture media may have entered the metabolic pathway following hydrolysis, accumulating in the cells. It is also suggested by our data that sucrose

increase could have happened at the expense of raffinose since the concentration of this carbohydrate decreased considerably over drying time, for both pre-treated and control axes. Part of the sucrose accumulated could have been produced by *de novo* carbohydrate synthesis as well. Sucrose and fructose seem to be the main sugars involved in cold acclimation of eucalyptus cells (Travert *et al.*, 1997). Higher tissue levels of glucose, raffinose and stachyose, compared to untreated samples

Likewise, fructose and glucose accumulation probably results from raffinose and stachyose hydrolysis. It is interesting to note the high amounts of raffinose and stachyose in control, undesiccated axes. Glucose levels did not increase with sucrose pre-culture in the same fashion that fructose did.

Sucrose pre-treatment had a stabilizing effect, maintaining a high percentage recovery rate over a broader range of water content. Since dehydration of axes pre-cultured with sucrose occurred at a much slower rate as compared to controls, one can interpret the stabilizing effect of sucrose observed here as a consequence of the axes retaining a higher water content over a broader range of desiccation periods. However, the nature of the effect of sugars on the subcellular structures of embryonic axes of "Pineapple' sweet orange needs to be investigated further. The plasma membrane, often reported as the primary site of freezing injury, could be one of the structures cryoprotected by sugars. Cryoprotection

by carbohydrates during freezing can be achieved through the protection of membranes and proteins from the damages induced by dehydration. Some sugars are effective in preserving the structure and function of labile proteins in the absence of water by helping to sustain ordered water around them (Guy, 1990). Certain sugars are capable of inhibiting fusion between adjacent vesicles during drying and by maintaining the lipids in a fluid phase in the absence of water thus avoiding changes in permeability and lateral phase separations (Crowe *et al.*, 1987). This protection might be achieved through interaction with the polar head groups of phospholipids or hydrophobic interactions

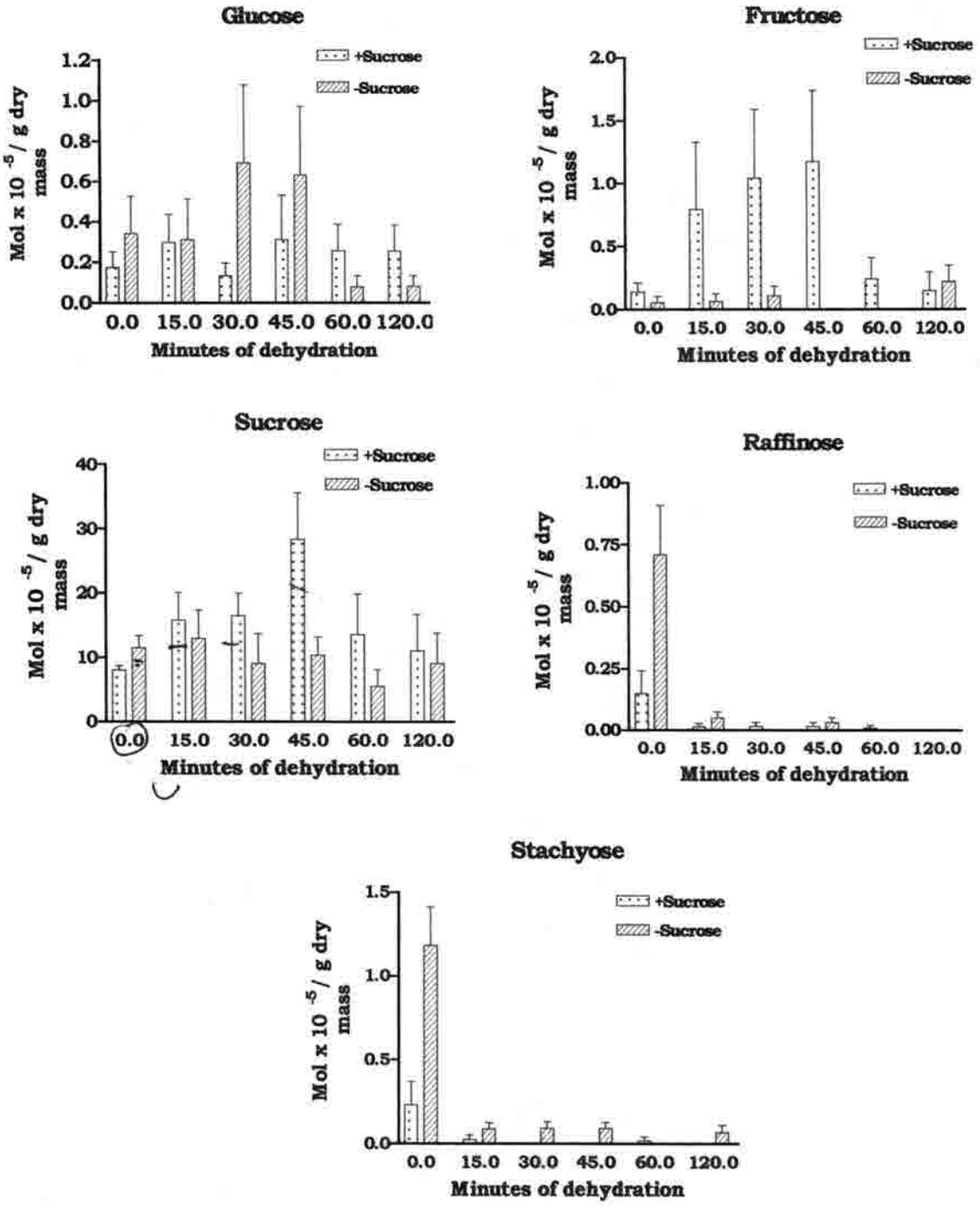

Figure 7. Soluble carbohydrate content of embryonic axes from 'Pineapple' sweet orange seeds, pre-cultured or not with 0.5 and 0.75 M sucrose (24 hours each, stepwise manner). Axes were dried for 0 to 120 minutes prior to carbohydrate extraction.

Table 2. Summary of Analysis of Variance (ANOVA) for soluble carbohydrate content of embryonic axes from 'Pineapple' sweet orange, dried for 0, 15, 30, 60 and 120 minutes, with and without stepwise pre-culture in 0.5 and 0.75 M sucrose.

Carbohydrate	Source of variation	df	MS	P
Fructose	Time (T)	5	0.5143	ns
	Sucrose (S)	1	3.995	0.0032
	T x S	5	0.6268	ns
Glucose	T	5	0.1576	ns
	S	1	0.2077	ns
	T x S	5	0.2088	ns
Sucrose	T	5	145.4	ns
	S	1	506.8	0.0278
	T x S	5	130.6	ns
Raffinose	T	5	0.2881	<0.0001
	S	1	0.1423	0.0128
	T x S	5	0.1282	0.0002
Stachyose	T	5	0.7462	<0.0001
	S	1	0.6477	<0.0001
	T x S	5	0.3390	<0.0001

The results reported here show that embryonic axes of *C. sinensis* can successfully be cryopreserved, attaining up to 100% viability after thawing. Presently, the preservation of *Citrus* germplasm is by the establishment of plantations. This requires vast areas of land, trained personal and risk from biological and climatic disasters. Excised embryonic axes have been used for the cryopreservation of many desiccation sensitive species (Boucaud *et al.*, 1991; Pense, 1990; Radhamani and Chandel, 1992). Excised axes provide a convenient alternative for cryostorage because they easily develop into independent plants and retain viability after storage in liquid nitrogen when the whole seed failed to survive. Thus, the cryopreservation system described here for embryonic axes can be used for the long-term conservation of 'Pineapple' sweet orange germplasm. Our studies also indicate the potential involvement of sucrose on improved freezing tolerance of embryonic axes, which opens new research prospects.

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Chapter 4

Desiccation and freezing tolerance in vegetative intercalary buds of 'Pineapple' sweet orange (*Citrus sinensis* [L.] Osb.)

Abstract

Fully hydrated buds had water content in the range of 1.8 to 2.0 mg $H_2O.mg^{-1}$ DW, and regenerated 100% in culture. Similar high regeneration percentages were maintained as the water content in the buds was reduced by desiccation over silica gel from 1.8 to 0.6 mg $H_2O.mg^{-1}$ DW. However, shoot recovery consistently decreased as the water content was reduced below this level, up to the point where no regeneration was observed when the water content was dropped to 0.198 mg $H_2O.mg^{-1}$ DW. Buds isolated from acclimated plants had a similar range of desiccation tolerance, thus it appears that acclimation did not enhance desiccation tolerance. No shoot recovery was obtained for buds when water content dropped in the range of 0.17-0.20 mg $H_2O.mg^{-1}$ DW. Buds isolated from *in vitro* grown plants had much higher initial water content (3.6 to 4.0 mg $H_2O.mg^{-1}$ DW). Total loss of viability was observed when the water content of buds from *in vitro* grown plants dropped to 0.79 mg $H_2O.mg^{-1}$ DW. No buds survived pre-culture in medium

containing 0.75 M sucrose. Shoot recovery percentages were higher for buds pre-treated with medium containing 0.1 and 0.5 M sucrose and buds pre-cultured for 24 hours. Neither buds from greenhouse grown plants nor from *in vitro* grown plants survived direct plunging in liquid nitrogen, regardless of the pre-treatments used. Electrolyte leakage tests conducted on buds isolated from plants grown in the greenhouse and *in vitro* grown plants provided evidence that desiccation caused severe damage to cell membranes and decreased bud viability upon rehydration. Leakage from 'Pineapple' orange lateral buds isolated from greenhouse or *in vitro* grown plants and desiccated to different water contents was negatively correlated with shoot recovery in culture. Removal of water caused a significant decrease in shoot recovery while a steep increase in electrolyte leakage was observed.

Introduction

The genus *Citrus* (Rutaceae family) includes several species that comprise one of the most important groups of cultivated fruit trees. The center of origin of the genus is Southeast Asia but the domesticated species are now cultivated in tropical and subtropical regions of the world (Gmitter and Hu, 1990). Domestication started very early in that region and propagation of hybrids and mutants contributed to increasing the complexity of the group (Bouharmont and Beloualy, 1996). Genotypes used in breeding programs of cultivated citrus cultivars are

generally maintained in field gene banks and propagated vegetatively by means of grafting (Duran-Vila, 1995).

The development of alternative methods to field collections for the genetic conservation of citrus is highly desirable. Most of the methods proposed are based on *in vitro* culture techniques, such as ovule culture, embryo culture, cell culture and somatic embryogenesis. *In vitro* storage under reduced growth conditions has been developed for citrus species (Marín and Duran-Vila, 1991). The establishment of *in vitro* cultures with these genotypes allows them to be maintained under disease-free conditions, but in the particular case of the genus *Citrus* this approach has a limited application because the proliferating shoots and buds fail to grow after a few subcultures (Marin and Duran-Vila, 1988).

Cryopreservation studies conducted over the last two decades have enabled the regeneration of entire plants from cells, protoplasts, meristems, embryos, etc. of a variety of genera belonging to diverse families. These studies have demonstrated that cryopreservation can be applied for the conservation of germplasm of vegetatively propagated crops, of plants with recalcitrant seeds and of cell cultures with desirable traits, especially those of high-yielding alkaloids or secondary metabolites of pharmaceutical interest (Bajaj, 1995). Cryopreservation of *Citrus* has been achieved with seeds, ovules, somatic embryos and cell cultures. The cryoconservation protocols described involve several approaches. Desiccation followed by fast cooling was used for seeds

(Mumford and Grout, 1979), ovules (Bajaj, 1984), and embryonic axes (Radhamani and Chandel, 1992); dehydration and cryoprotection followed by slow cooling for somatic embryos (Marín and Duran-Vila, 1988; Marín *et al.*, 1993) and embryogenic cell lines (Kobayashi *et al.*, 1990; Sakai *et al.*, 1991b); and vitrification and fast cooling for embryogenic cell lines (Sakai *et al.*, 1990, 1991a). However, the recovered plants are always juvenile and several years must elapse before adult and productive trees are available (Duran-Vila, 1995). In principle, the cryopreservation of embryogenic cell lines and somatic embryos may be applied to a wide range of polyembryonic citrus species and cultivars, however its implementation for the conservation of wild species may not be feasible (Duran-Vila, 1995). In such cases, the conservation of buds, ovules and seeds would be a more desirable approach (Duran-Vila, 1995; Niino, 1995; Stushnoff and Seufferheld, 1995). Cryopreservation of mature vegetative buds is a promising method for conserving germplasm of vegetatively propagated species and establishing base collections because it is inexpensive, space-efficient and simple (Niino, 1995; Stushnoff and Seufferheld, 1995). This approach ensures clonal integrity, requires low maintenance, and reduces vulnerability to environmental and biological hazards. Cryopreserved buds can easily be thawed and grafted, producing flowering plants in a short period of time.

Survival following cryopreservation depends on the tolerance of desiccation to very low levels of water content as well as the ultra-low

temperature of liquid nitrogen (-196°C). There is a critical water content that must be defined which is high enough to prevent desiccation damage and low enough to prevent freezing injury.

Membrane damage can be caused in a plant cell by freezing, chilling, heating, drought and other environmental stresses (Levitt, 1980). Electrolyte leakage from cells has long been used as a means of determining the amount of injury to plasma membrane. The electrical conductivity method is based on the measurement of the amount of electrolytes released from damaged tissues to deionized water, usually used as the test medium. The greater the damage to the membranes, the greater the loss of electrolytes (Oliver *et al.*, 1993). The leakage of electrolytes from plant tissues is a function of time. At first, electrolytes are mainly released from cells injured by cutting (at preparation) and from cell walls and intercellular spaces. During a second phase, the release of electrolytes is a result of cell injury or death caused by a stressful event (Prášil and Zámečník, 1998).

Our hypothesis for this research was that lateral buds of 'Pineapple' sweet orange could be cryopreserved in liquid nitrogen provided that appropriate water content was identified. Thus, the objectives of these studies were: (a) to develop a procedure for the preservation of lateral buds of 'Pineapple' sweet orange at the ultra-low temperatures (-196°C) of liquid nitrogen; (b) to test the effect of cold acclimation and desiccation to different water contents on survival after

slow cooling and fast cooling cryopreservation procedures; (c) to study the composition of soluble sugars in the tissues following various treatments; and (d) to evaluate the effect of dehydration and freezing procedures on cell membrane integrity using electrolyte leakage.

Materials and Methods

Plant material

Vegetative intercalary (lateral) buds used in these studies, were isolated from 'Pineapple' sweet orange (*Citrus sinensis* [L.] Osb.) plants grown in the greenhouse with and without cold acclimation treatment, and from plants obtained through the *in vitro* germination of excised embryonic axes. Potted plants, as well as fruits were obtained from the Lake Alfred Citrus Research Station, University of Florida, Florida, and from the USDA Citrus Research Station, Brownsville, Texas (fruits only).

Plants maintained in the greenhouse received natural sun light year round and ambient temperature (25±5°C). An extended photoperiod was provided by supplementing artificially with cool white fluorescent lights during the cold, short days of autumn and winter, when day length would normally fall below 12-14 hours.

To induce cold acclimation, plants were transferred to a walk-in cooler under controlled temperature and short photoperiod conditions. The temperature was initially set at 25±5°C and it was reduced by 5°C *per* week until it reached 0°C. Plants were kept at 0°C for a week before

the buds were isolated for the experiments. A twelve-hour photoperiod was provided with cool white fluorescent lights during the entire cold acclimation period.

For production of *in vitro* cultured plants embryonic axes were isolated aseptically from seeds in the laminar flow hood, under a stereo microscope. Seeds were extracted from fruits that had been sterilized with a 10% sodium hypochlorite solution containing 4-5 drops of polyoxyethylene sorbitan monolaurate (Tween 20, Mallinckrodt OR) for 15 minutes and washed 3 times with sterilized water. Axes were transferred to MS basal medium (Murashige and Skoog, 1962) containing 30 g/L sucrose and 8 g/L agar. Cultures were maintained in a growth chamber at 25±2°C with a 16-hour photoperiod under 62 $\mu mol.m^{-2}.s^{-1}$ light intensity. Lateral buds were isolated with the help of a stereo microscope from 8 months old seedlings that contained approximately 10-12 nodes each.

Encapsulation and pre-treatment conditions

For encapsulation, buds were dipped in a 3% Na-alginate (Sigma, medium viscosity) aqueous solution, collected individually with a spatula and dropped in a 0.1 M Ca Cl_2 aqueous solution. After one hour in $CaCl_2$, the alginic acid cross-linked forming a stable capsule enclosing the bud. Capsules containing one bud each were pre-treated with sucrose or PVS2 vitrification solution. Pre-culture with sucrose consisted of liquid MS

medium containing 0.25 up to 0.75 M sucrose, in 0.25 increments, in a stepwise series, 24 hours in each step. A vitrification procedure based on Pennycooke (1997) was used as well. Beads were pre-cultured overnight with liquid MS medium containing 0.3 M sucrose, then loaded with a solution containing 0.4 M sucrose plus 2 M glycerol for 0.5, 1.0 and 2.0 hours. At the end of each loading period beads were treated with PVS2, solution containing 15% ethylene glycol (w/v), 30% glycerol (w/v), 15% dimethyl sulfoxide (w/v) and 0.4 M sucrose in liquid basic MS for 0.5, 1.0 and 2.0 hours. Capsules were washed with liquid MS medium containing 1.2 M sucrose before they were transferred to recovery medium.

Desiccation regimes

Isolated lateral buds, both encapsulated or non-encapsulated were desiccated at ambient temperature (25±2°C) over sterilized filter paper placed over 80 g of dry silica gel, inside an air tight closed container (500 mL), for different periods of time. Bud moisture content was expressed on a dry weight basis. After each desiccation period dry weight was determined gravimetrically at the end of 48 hours by drying in an oven at 100°C. Buds pre-cultured with PVS2 were not dehydrated over silica gel.

Freezing regimes

At the end of each desiccation period buds were enclosed in 2.0 mL cryovials and subjected to two freezing regimes: fast cooling in liquid nitrogen or slow cooling with a programmable freezer (Tenney Jr 942, South Brunswick, NJ). For fast cooling, cryovials were directly submerged in liquid nitrogen and maintained in liquid nitrogen for at least 30 minutes prior to thawing. For slow freezing cryovials were placed inside the freezer chamber at 25°C, and after equilibrating for 30 minutes the temperature in the chamber was gradually reduced; a cooling rate of 1°C/hour was used. When the target temperature (-20°C) was reached a sample of slow cooled buds was collected, thawed out, and transferred to recovery medium. Thawing was done by plunging cryovials in a water bath at 40±2°C, for 3 minutes, under agitation. Buds were immediately placed on the recovery medium and cultured as described previously. Ten buds *per* vial comprised one experimental unit and three replications were used per treatment assigned at random. Each experiment was repeated three times.

Electrolyte leakage

Electrolyte leakage was measured from buds isolated from greenhouse and *in vitro* grown plants by an electrical conductivity meter (Neogen ASAC 1000-B, Neogen Food Tech. Corp., Lansing, MI). Two mL

of nanopure water was dispensed in each cell of a clean conductivity meter tray and samples were submerged simultaneously in the water filled cells using an acrylic sample dispenser. Each cell contained one sample bud. Five buds at each moisture content were used for conductance measurement. Initial conductance (C_o) of the leachate was measured at ambient temperature (25±2°C) at 6 minutes interval for the first two hours and every 30 minutes for the next two hours. The tray containing the samples was frozen overnight at -70°C to lyse cellular membranes, and thawed at ambient temperature for 30 minutes before maximum conductivity (C_t) was measured. These maximal conductivity data were used as reference values for the calculation of the relative conductivity of the samples. Percent electrolyte leakage was expressed as (C_o/C_t) x 100. All leakage data in the figures are expressed as means of three replicates ± SEM.

Recovery from desiccation/freezing

After all desiccation and freezing treatments, buds were transferred to basic MS solidified with 8 g/L agar and dispensed in Magenta boxes, for recovery assessment. Buds were cultured in a growth chamber at (25±2°C) with a photoperiod of 16 hours light/8 hours darkness and 62 $\mu mol.m^{-2}.s^{-1}$ light intensity. Cryopreserved buds were maintained in darkness for 48 hours prior to transfer to light. Survival was assessed weekly and data collected on the fourth week of culture, was expressed

as the percentage of buds that regenerated a shoot. Buds that became green but started senescing after two weeks in culture did not produce a shoot and thus they were not included as survivors in the data shown in the figures.

Carbohydrate analysis

Lateral buds and leaves of cold acclimated plants were freeze-dried and stored in airtight closed containers at room temperature, in desiccators containing silica gel. Freeze dried buds and leaves were ground to a fine powder with a Wiley Mill using a 40-mesh screen and passed through a 100-mesh stainless steel screen. Soluble carbohydrates were extracted by adding 1.0 mg powder to 3 mL of a 0.1 M NaOH solution. After one hour at 4°C the extract was centrifuged at 7000 rpm for 5 minutes, at 4°C. The supernatant was collected, filtered using a 0.22 μm nylon syringe filter to remove particulates and to bind soluble proteins, and used immediately or stored at -70°C for future use. Sugar concentrations were analyzed by a Dionex DX-300 (Dionex Co., Sunnyvale, CA) high performance liquid chromatography (HPLC) apparatus equipped with a 25 μL injection loop. Sugars were separated on a Carbopac PA 100 column (4.6 X 250 mm) equipped with a Dionex guard column (3 X 25 mm), using a flow rate of 1 mL/min, at ambient temperature (25±2°C). A pulsed electrochemical detector was used for the detection of the oligosaccharides. An eluant gradient was used to optimize separation. NaOH concentration was linearly increased from 70

mM to 120 mM in 18 minutes, combined with sodium acetate at 100 mM concentration held at 3 mM for 10 minutes, then increased to 35 mM for the last 8 minutes. Sugar concentrations are expressed as mol x 10^{-5} of sugar per g of dry weight. Analysis was performed on three samples per treatment.

Statistical analysis

Percentage data were arcsine transformed before one way analysis of variance was done (ANOVA). Graph Pad Prism software version 3 (GraphPad Software Inc., San Diego, CA, 92121), was used to compare the differences in the mean survival and the amount of leakage at different water contents. The significance of differences between means at the 5% level was examined using a Bonferroni mean separation test. Data in the figures are actual percentage values. The relationship between water content and shoot recovery was analyzed by non-linear Boltzman regression.

Results

Desiccation tolerance

Isolated buds in the fully hydrated state had water content in the range of 1.8 to 2.0 mg $H_2O.mg^{-1}$ DW, and regenerated 100% in culture. Similar high regeneration percentages were maintained as the water content in the buds was reduced by desiccation over silica gel from 1.8 to

0.6 mg $H_2O.mg^{-1}$ DW. However, shoot recovery consistently decreased as the water content was reduced below this level, up to the point where no regeneration was observed when the water content was dropped to 0.198 mg $H_2O.mg^{-1}$ DW (Fig. 1). Buds isolated from acclimated plants had a similar range of desiccation tolerance, thus it appears that acclimation did not enhance desiccation tolerance. No shoot recovery was obtained for acclimated buds when water content dropped in the range of 0.17-0.20 mg $H_2O.mg^{-1}$ DW (Fig. 2).

Buds isolated from *in vitro* grown plants (Fig. 3) had a much higher initial water content (3.6 to 4.0 mg $H_2O.mg^{-1}$ DW) compared to those isolated from greenhouse grown plants. When not desiccated below 2 mg $H_2O.mg^{-1}$ DW their recovery was 90% in the growth medium. However, buds from this source had very little desiccation tolerance. Shoot recovery percentage started decreasing when the water content decreased to 2.2 mg $H_2O.mg^{-1}$ DW, reaching zero when the water content dropped to 0.79 mg $H_2O.mg^{-1}$ DW (Fig. 3).

Sucrose treatment of encapsulated beads improved the desiccation tolerance of buds taken from *in vitro* grown plants (Fig. 4). Encapsulated, sucrose treated buds with water content as low as 0.25 mg $H_2O.mg^{-1}$ DW still had 30% shoot recovery.

Effect of sucrose pre-treatment

Greenhouse lateral buds were also pre-cultured for 24 and 48 hours on solidified basic MS medium supplemented with 0.0-0.75 M sucrose. After pre-culture, buds were transferred to recovery medium and the percentage of shoot recovery observed is shown in Figure 5. No buds survived pre-culture in medium containing 0.75 M sucrose. Shoot recovery percentages were higher for buds pre-treated with medium containing 0.1 and 0.5 M sucrose and buds pre-cultured for 24 hours. Analysis of variance revealed that sucrose concentration had a significant effect on shoot recovery, whereas pre-culture duration had no effect on the recovery rates observed (Table 2).

Shoot recovery of buds pre-cultured with sucrose for 24 and 48 hours, desiccated over silica gel for various periods of time (0-120 minutes), and following dehydration is shown in Figure 6 and 7. Buds pre-cultured for 24 hours with 0.1 M sucrose could withstand some desiccation, but those pre-cultured with higher sucrose concentrations were damaged by all levels of water contents. When buds were pre-cultured for 48 hours, 0.3 M sucrose produced the best shoot recovery (Fig. 7). Analysis of variance showed that the interaction of both variables dehydration duration and sucrose concentration had a significant effect on shoot recovery after dehydration (Table 3).

Bewley and Oliver (1992) concluded that desiccation tolerance can be achieved by mechanisms that are based on the protection of cellular

integrity or are based on the repair of desiccation- and rehydration-induced cellular.

Cold acclimation and freezing tolerance

Neither buds from greenhouse grown plants nor from *in vitro* grown plants survived direct plunging in liquid nitrogen, regardless of the pre-treatments used (Table 4). Non acclimated buds from greenhouse grown plants, cooled slowly using the programmable freezer survived -5°C (LT50) but not much lower temperatures (Fig. 8). Acclimated buds survived –12°C (LT50), Figure 9, indicating that the acclimating conditions used were effective in increasing freezing tolerance. Acclimated buds that were desiccated over silica gel prior to slow cooling did not survive as well as the non-desiccated ones (Fig. 10). Encapsulation and sucrose pre-treatment of buds from acclimated plants had no appreciable effect on shoot recovery following slow cooling to –10°C as compared to non-acclimated buds (Fig. 8 and 11). However, none of these buds were capable of surviving exposure to liquid nitrogen.

Electrolyte leakage as an indicator of desiccation damage

Reduction in water content caused a significant decrease in shoot recovery *in vitro.* It was hypothesized that such decrease in shoot recovery was a result of damage caused to the cell membranes by desiccation. Electrolyte leakage tests were conducted on buds isolated from plants grown in the greenhouse and *in vitro* in order to assess for

desiccation injury. Time course curves of electrolyte leakage from desiccated 'Pineapple' sweet orange lateral buds during rehydration provided evidence that dehydration had a profound effect on electrolyte leakage from the tissues. Electrolyte leakage was nonlinear during the initial two hours of imbibition and became linear after the initial two hours. Extrapolation of the slope of the linear section of the curve to the ordinate was used to estimate the net leakage during the initial nonlinear phase (Appendix I and II). Buds isolated from greenhouse grown plants that were dehydrated to water contents lower than 1.15 mg $H_2O.mg^{-1}$ DW leaked up to 90% of their cell contents into the imbibition medium at the end of 2.0 hours of re-hydration (Fig. 12; Table 5). The correlation between leakage rate, water content and shoot recovery for buds from greenhouse grown plants is shown in Figure 13 and Table 5. Buds obtained from *in vitro* grown plants that were fully hydrated (3.96 mg $H_2O.mg^{-1}$ DW) or desiccated to 2.39 mg $H_2O.mg^{-1}$ DW, showed less than 10% leakage after re-hydration (Fig. 14). Desiccation to lower water contents caused a considerable increase in leakage from the cells, and at the lowest water content tested (1.18 mg $H_2O.mg^{-1}$ DW) total leakage was observed (Fig. 14). Similar to buds from greenhouse grown plants, leakage from buds of *in vitro* grown plants increased up to two hours and then reached a linear, steady phase after two hours of re-hydration in deionized water.

Leakage from 'Pineapple' orange lateral buds isolated from greenhouse or *in vitro* grown plants and desiccated to different water contents was negatively correlated with shoot recovery in culture, as can be seen in figures 12 through 15. Removal of water caused a significant decrease in shoot recovery while a steep increase in electrolyte leakage was observed. Thus correlation of electrolyte leakage was negatively correlated with water content (r^2=0.8311, P=0.0043) and shoot recovery was positively correlated with water content (r^2=0.5695, P=0.0499) for greenhouse grown plants. Similar correlation of the same variables was observed for plants grown *in vitro*. Water content was negatively correlated to electrolyte leakage (r^2=0.7953, P=0.0029), and positively correlated to shoot recovery (r^2=0.6560, P=0.0148). These data support our hypothesis that desiccation caused severe damage to cell membranes and decreased bud viability upon re-hydration.

Sugar content

Except for elevated glucose at warm temperature, the only significant change observed was a large increase in raffinose at low temperature acclimating conditions.

This is consistent with many reports in the literature that clearly link raffinose accumulation with low temperature and cold acclimation.

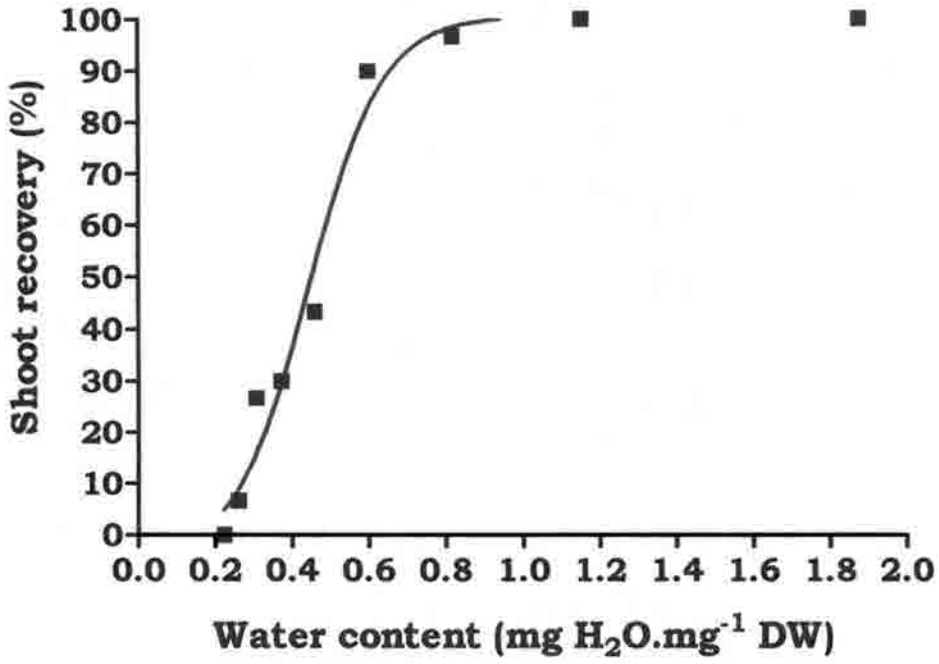

Figure 1. Desiccation tolerance of non-acclimated greenhouse grown 'Pineapple' sweet orange buds. Points represent means of three replications ($P < 0.0001$); solid line represents the Boltzman regression of the data ($r^2 = 0.9254$).

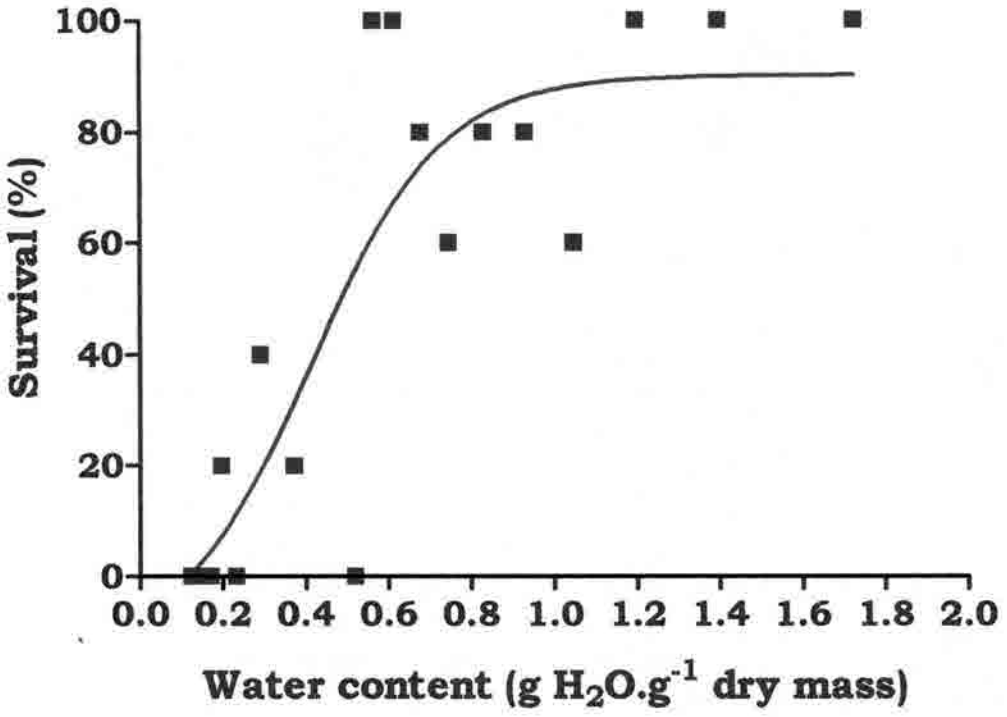

Figure 2. Desiccation tolerance of lateral buds from greenhouse grown acclimated 'Pineapple' orange plants. Points represent means of three observations ($P<0.0001$); solid line represents the Boltzman regression of the data ($r^2 = 0.7505$).

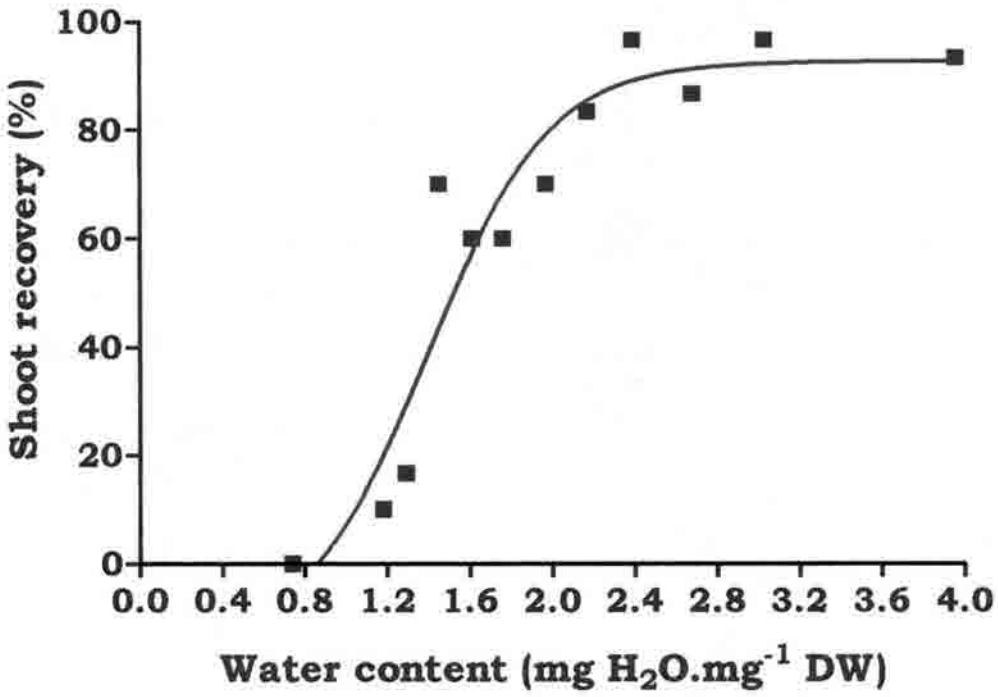

Figure 3. Desiccation tolerance of lateral buds from untreated *in vitro* plants. Points represent means of three observations ($P < 0.0001$); solid line represents the Boltzman regression of the data ($r^2 = 0.7028$).

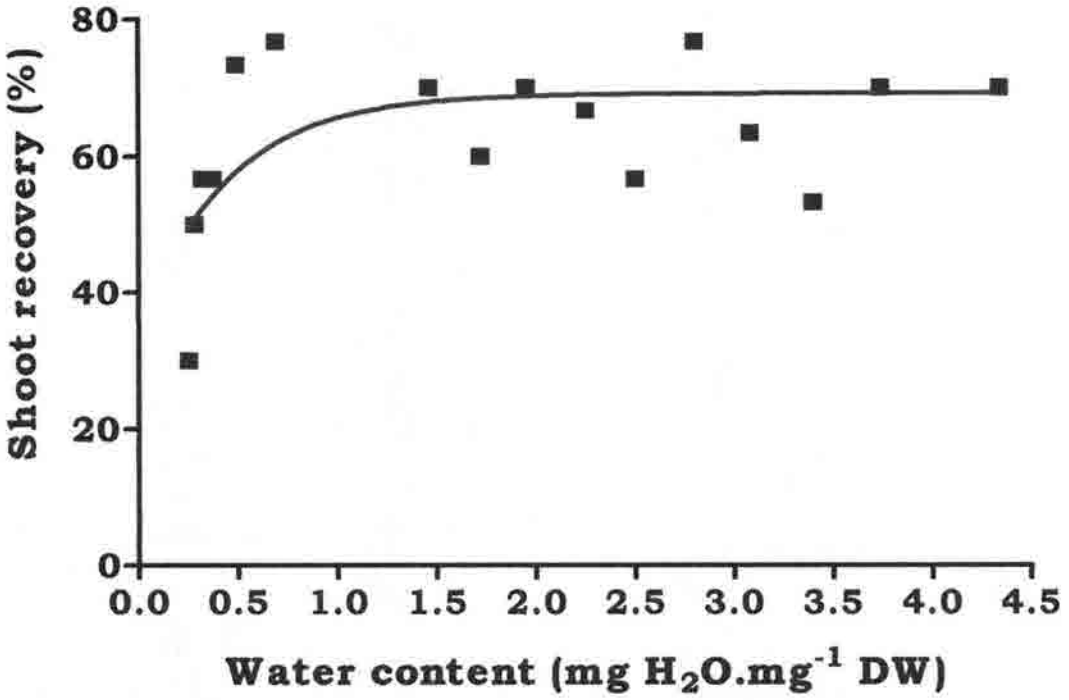

Figure 4. Desiccation tolerance of encapsulated lateral shoots from *in vitro* plants pre-treated with sucrose. Points represent means of three observations ($P<0.0001$); solid line represents the Boltzman regression of the data ($r^2 = 0.6417$).

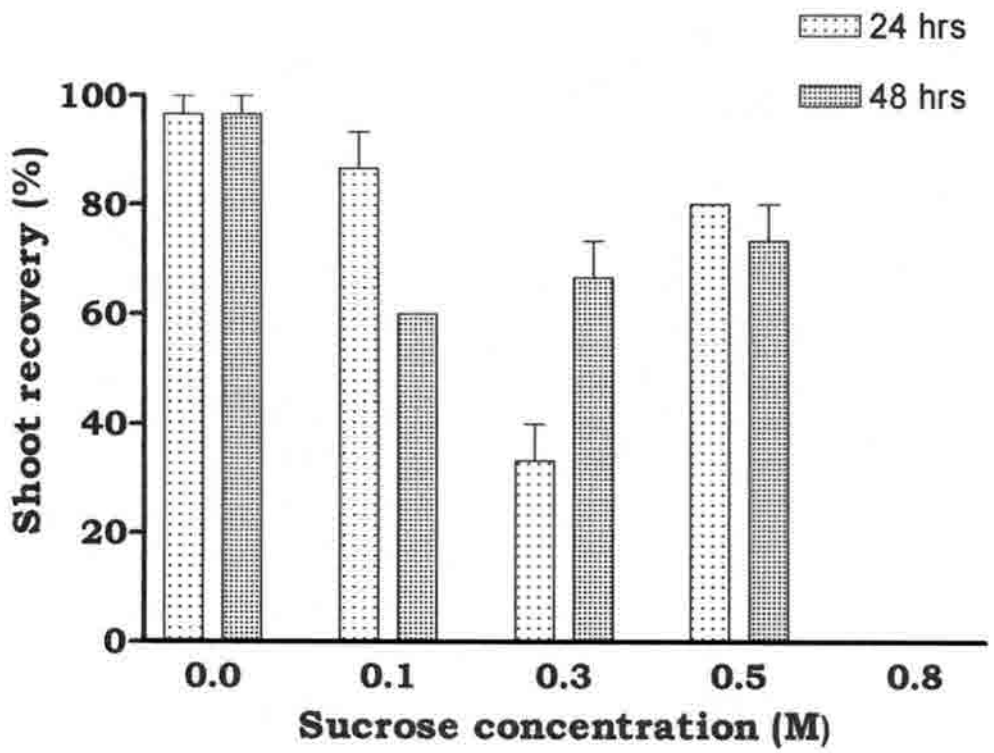

Figure 5. Effect of sucrose concentration on shoot recovery. Lateral buds were pre-cultured for 24 and 48 hours in solidified basic MS containing 0.0 - 0.75 M sucrose and then transferred to recovery MS medium. Vertical bars represent means of three observations ± SEM.

Table 1. Results of two-way analysis of variance on the effect of sucrose concentration, pre-culture duration and the interaction between both factors on shoot recovery *in vitro*.

Source of variation		**Results**
Sucrose concentration (0.0-0.75 M)	F	94.76
	P	< 0.0001 (***)
Pre-culture duration (24/48 hrs)	F	0.1599
	P	0.6935 (ns)
Interaction	F	5.266
	P	0.0046 (**)

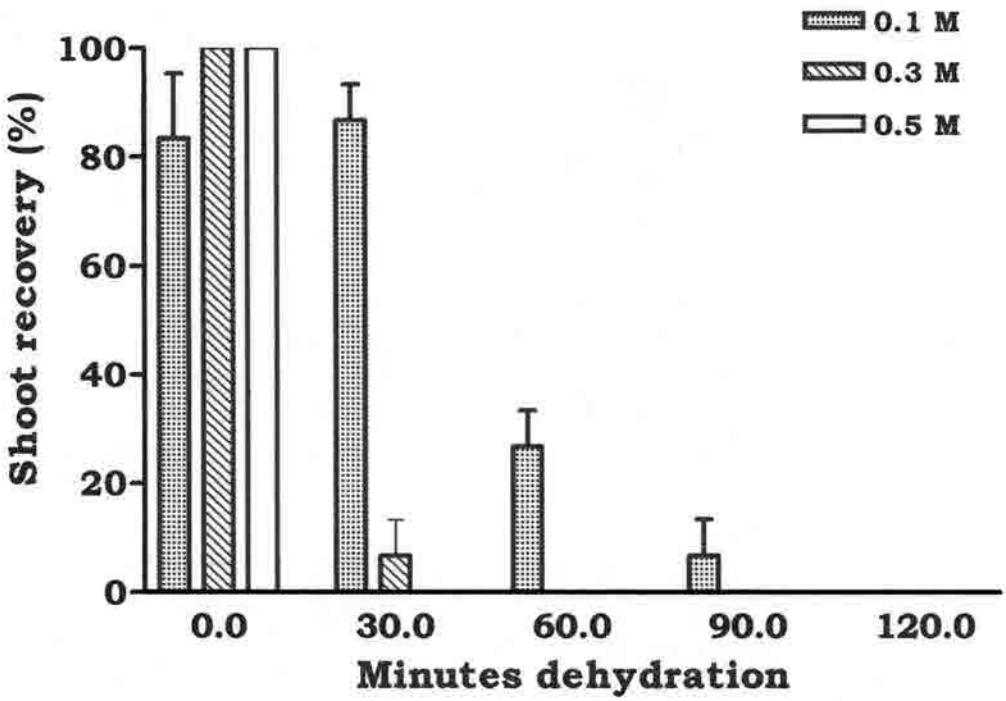

Figure 6. Effect of desiccation on lateral buds from *in vitro* plants pre-treated with sucrose . Buds were pre-cultured for 24 hours with solid basic MS containing 0.1 and 0.3 M sucrose prior to desiccation over silica gel. Points represent means of three observations ± SEM. Missing bars represent 0% shoot recovery.

Table 2. Results of two-way analysis of variance on the effect of dehydration duration, sucrose concentration and the interaction between both factors on shoot recovery of buds from *in vitro* grown plants.

Source of variation		**Results**
Dehydration duration (0-120 minutes)	F	140.6
	P	< 0.0001 (***)
Sucrose concentration (0.1, 0.3 and 0.5 M)	F	50.59
	P	< 0.0001 (***)
Interaction	F	14.72
	P	< 0.0001 (***)

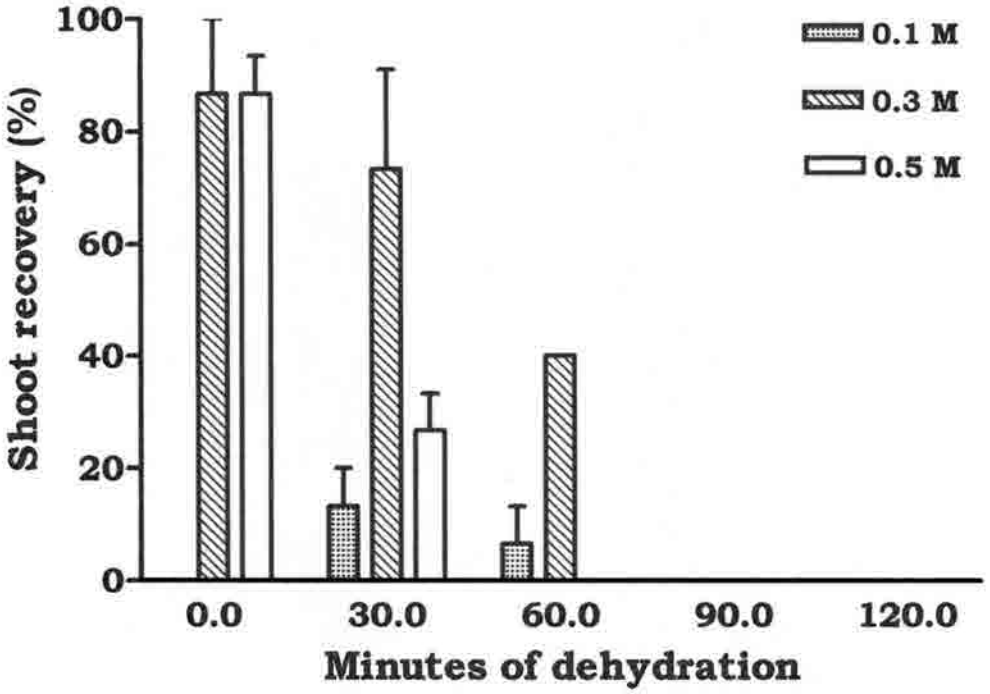

Figure 7. Effect of dehydration on greenhouse grown lateral buds pre-treated with sucrose. Buds were pre-cultured for 48 hours on solid basic MS containing 0.1 - 0.5 M sucrose prior to desiccation over silica gel. Points represent means of three observation ± SEM.

Table 3. Results of two-way analysis of variance on the effect of dehydration duration, sucrose concentration and the interaction between both factors on shoot recovery for greenhouse grown lateral buds.

Source of variation		**Results**
Dehydration duration (0 – 120 minutes)	F	35.33
	P	< 0.0001 (***)
Sucrose concentration (0.1, 0.3 and 0.5 M)	F	27.77
	P	< 0.0001 (***)
Interaction	F	9.841
	P	< 0.0001 (***)

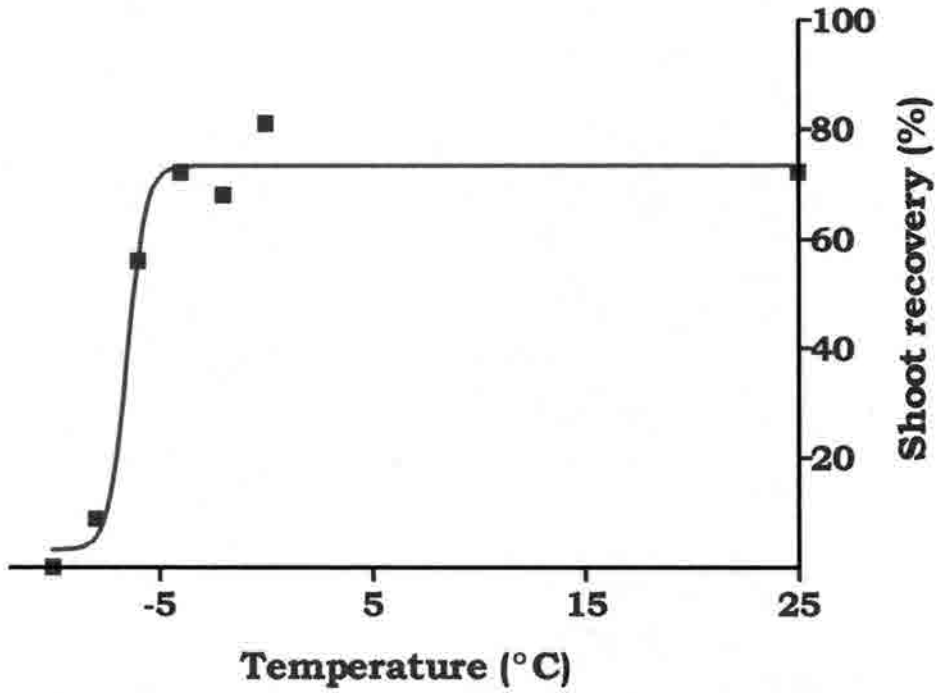

Figure 8. Slow cooling of bare buds of 'Pineapple' sweet orange. Points represent means of three observations ± SEM. Solid line represents the sigmoidal dose response regression of the data (r^2=0.8019).

Table 4. Shoot recovery after freezing in liquid nitrogen. Lateral buds from greenhouse and *in vitro* grown plants were isolated and pre-treated in various ways prior to direct plunging in liquid nitrogen.

Treatment	**Shoot recovery (%)**		
	Non-acclimated	Acclimated	*In vitro*
Control	0	0	0
Dehydrated (D)	0	0	0
Encapsulated (E)	0	0	0
Sucrose pre-culture (S)	0	0	0
E + D	0	0	0
E + S + D	0	0	0
Vitrification (PVS 2)*	0	0	0

* Pennycooke, 1997

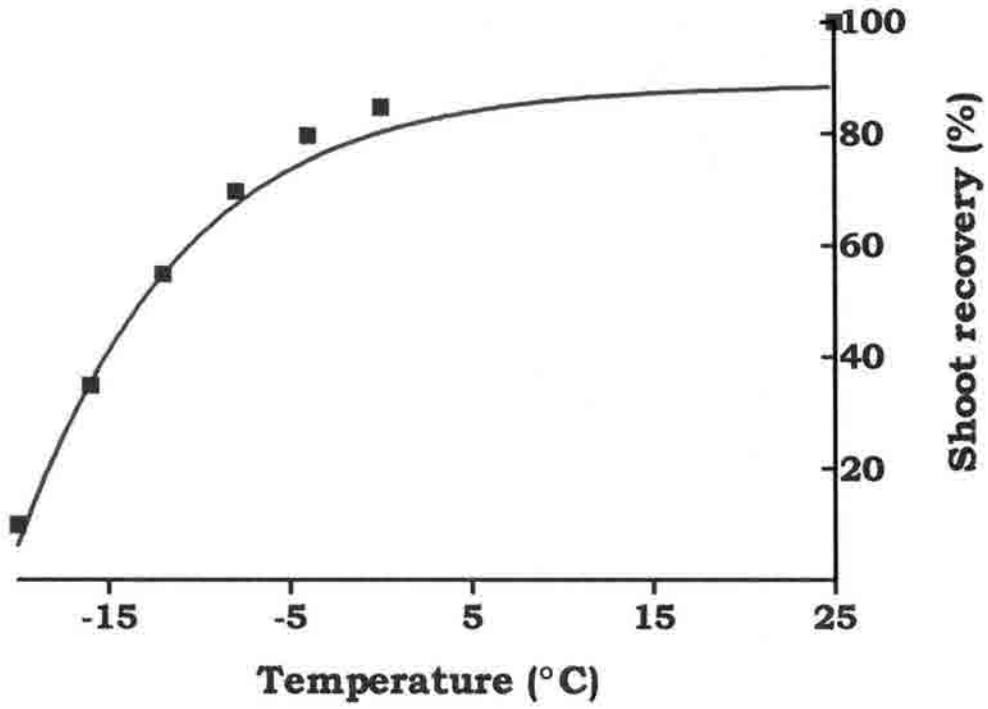

Figure 9. Freezing tolerance of acclimated lateral buds from greenhouse grown plants. Points represent means of three observations. Solid line represents the Boltzman regression of the data (r^2 = 0.9658).

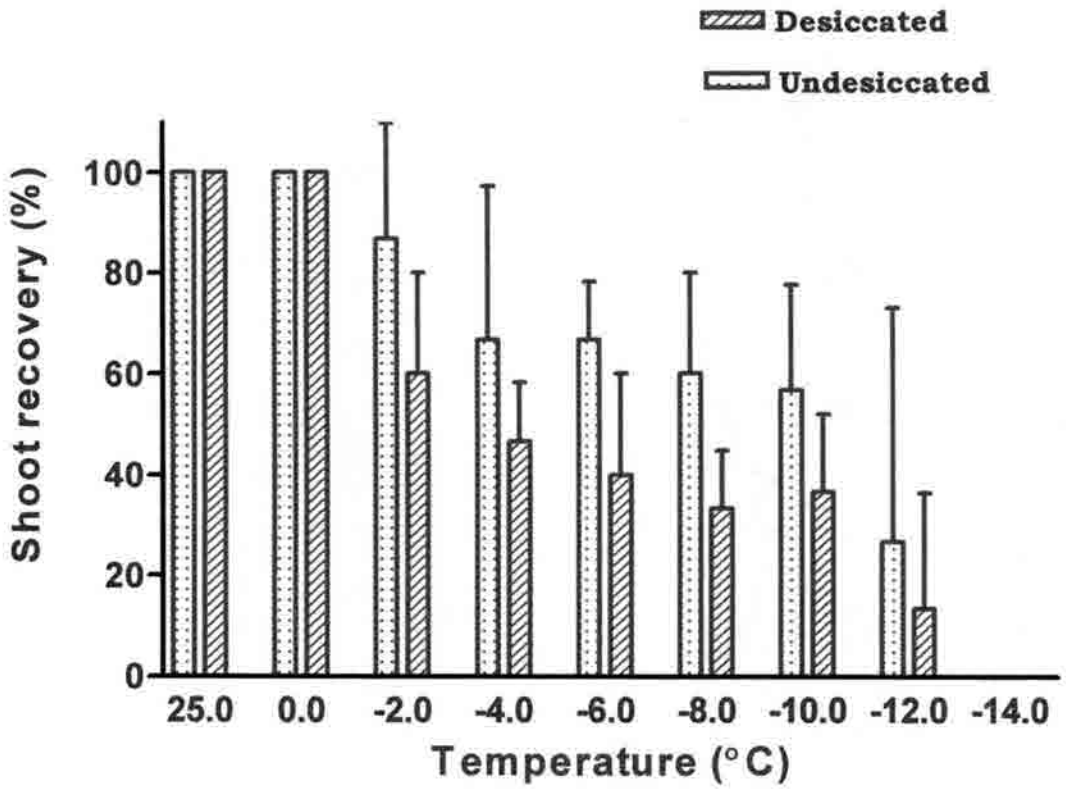

Figure 10. Freezing tolerance of 'Pineapple' orange lateral buds from acclimated greenhouse grown plants. Buds were desiccated to approximately 0.9 mg $H_2O.mg^{-1}$ dry mass prior to freezing.

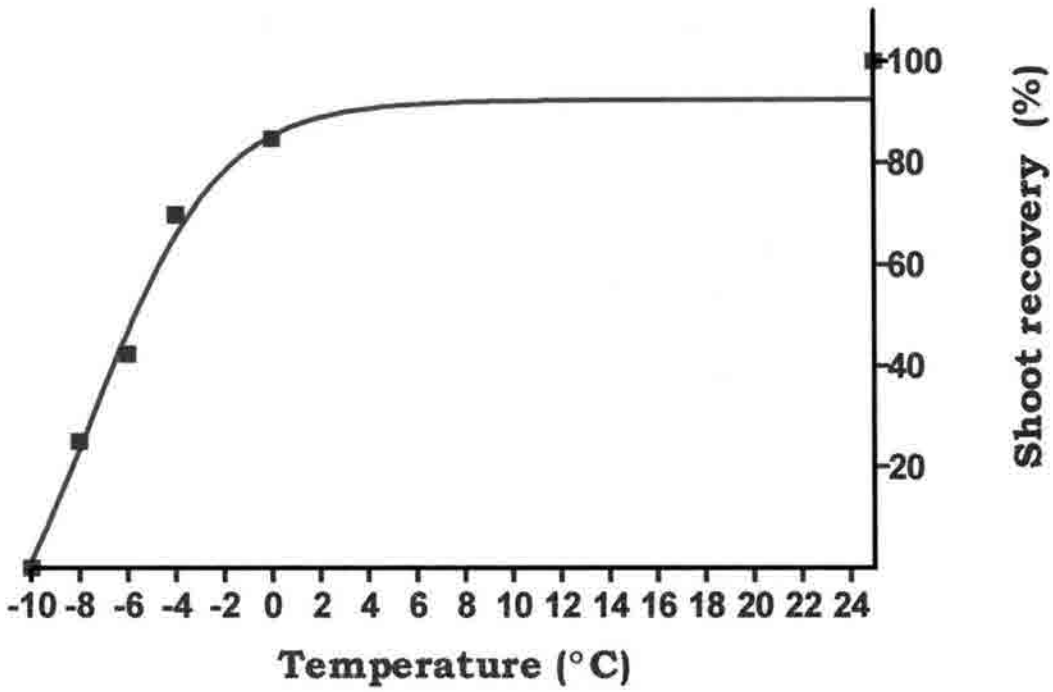

Figure 11. Shoot recovery after slow cooling of 'Pineapple' orange axillary acclimated buds encapsulated and pre-treated with sucrose (0-0.75 M, stepwise). Points represent means of three observations ± SEM; solid line represents the Boltzman regression of the data (r^2 = 0.9925).

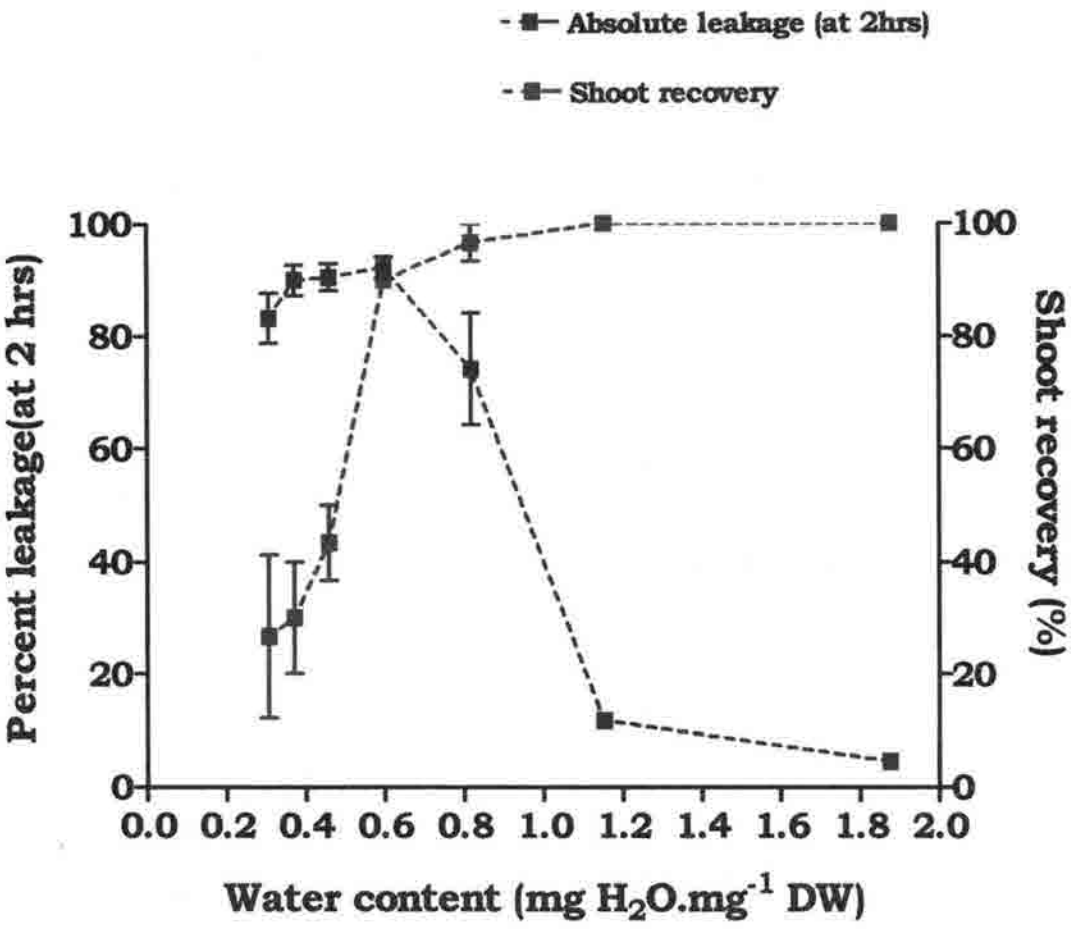

Figure 12. Relationship of water content, leakage and shoot recovery for buds from greenhouse grown plants after drying and rehydration. Points represent means of three observations and vertical bars represent ± SEM; missing bars are within the limits of the symbol.

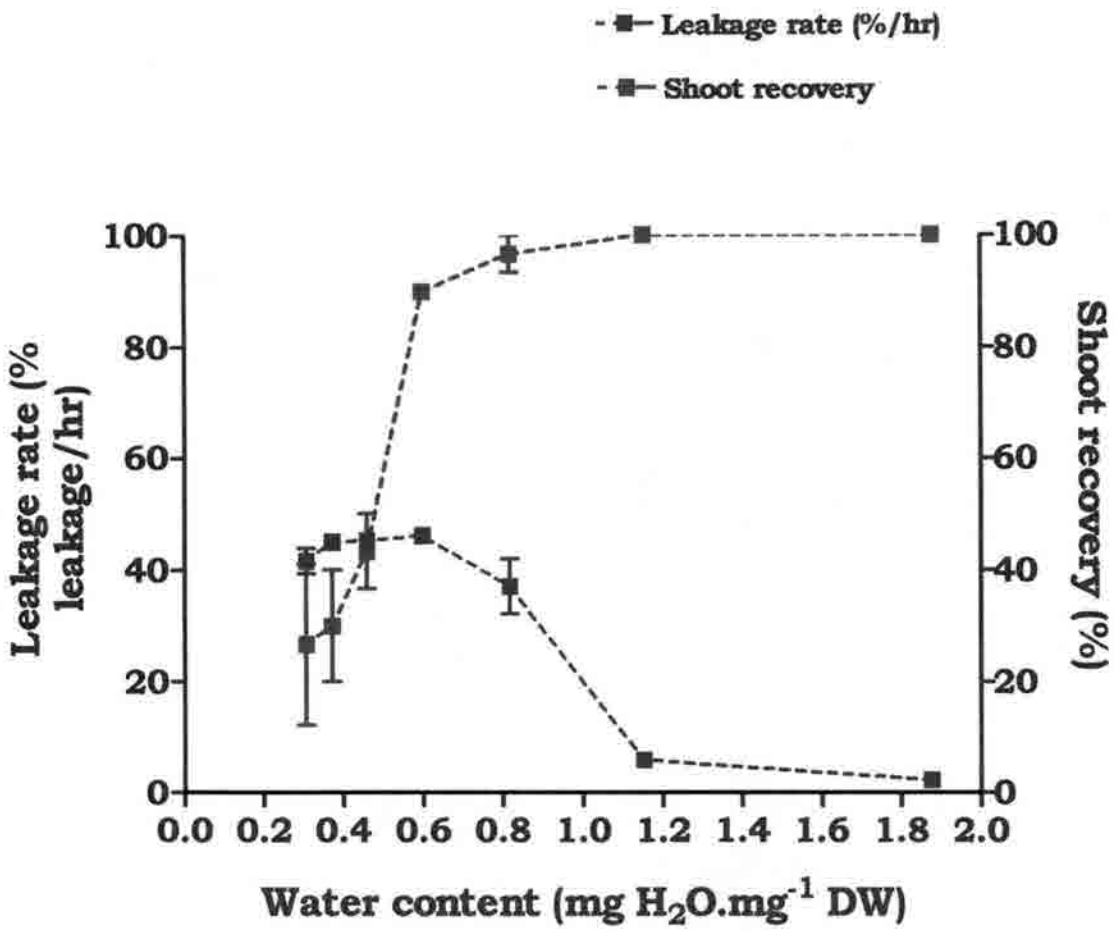

Figure 13. Correlation between water content, leakage rate and shoot recovery for buds from greenhouse grown plants. Points represent means of three observation and vertical bars represent ± SEM; missing bars are within the limits of the symbols.

Table 5. Correlation coefficient matrix for desiccated greenhouse grown lateral buds at 2 hours of leakage and for leakage rate over the 1st 2 hours.

Leakage at 2 hours		
Factor	Water content	Electrolyte leakage
Water content		
Electrolyte leakage	r^2=0.8311 P=0.004 (**)	
Shoot recovery	r^2=0.5695 P=0.05 (*)	r^2=0.3922 P=0.1834 (ns)
Leakage rate over the 1st 2 hours		
Electrolyte leakage	r^2=0.8310 P=0.004 (**)	
Shoot recovery	r^2=0.5695 P=0.05 (*)	r^2=0.8658 P=0.0008 (***)

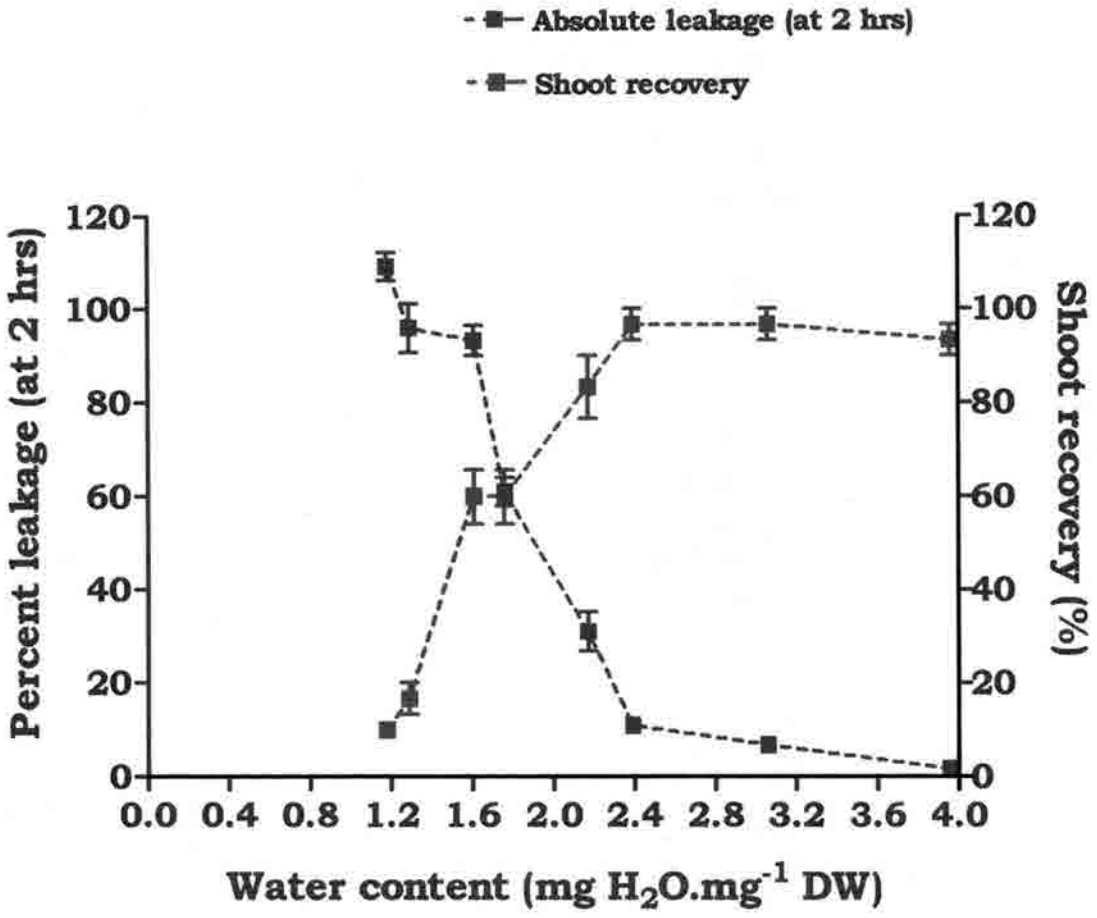

Figure 14. Correlation between water content, electrolyte leakage and shoot recovery for buds from *in vitro* grown plants after drying and rehydration. Points represent means of three observations and vertical bars represent ± SEM; missing bars are within the limits of the symbol.

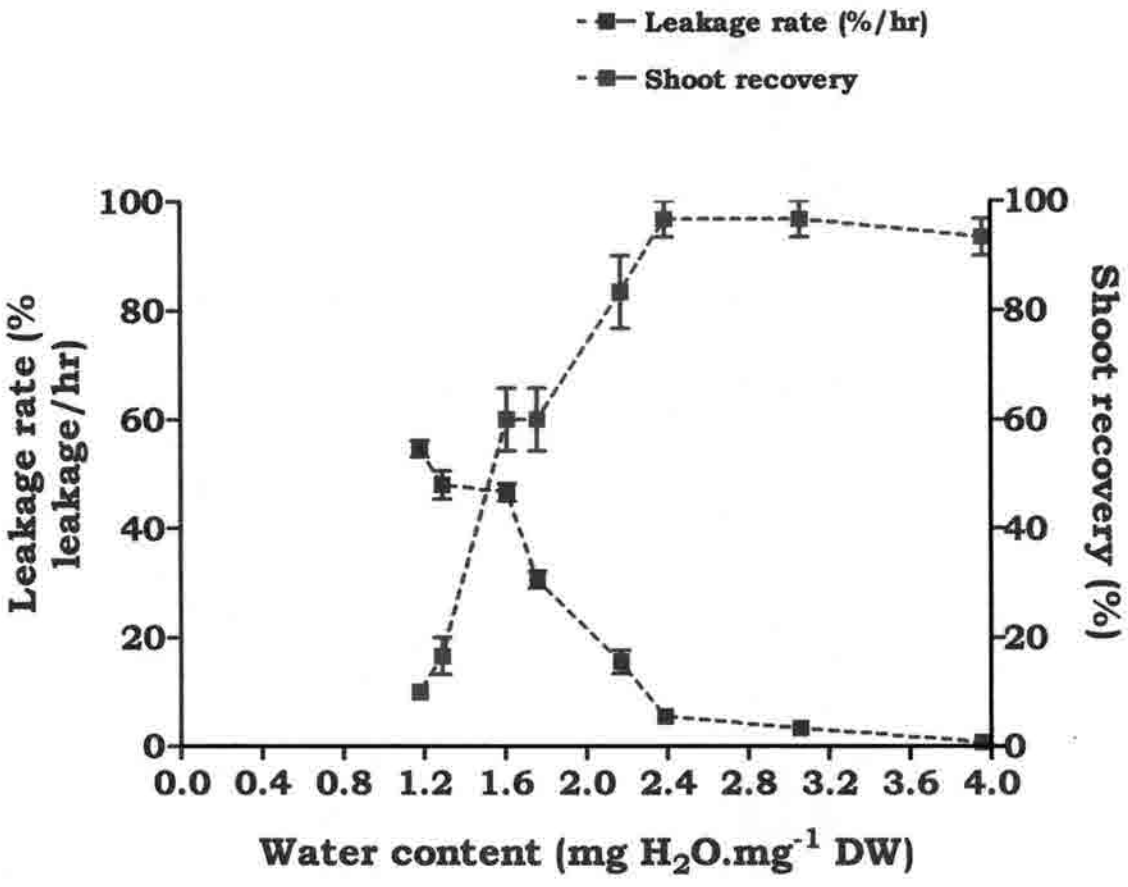

Figure 15. Correlation between water content, leakage rate and shoot recovery of buds from *in vitro* grown plants after drying and rehydration. points represent means of three observations and vertical bars represent ± SEM; missing bars are within the limits of the symbol.

Table 6. Correlation coefficient matrix for desiccated *in vitro* grown lateral buds at 2 hours of leakage and for leakage rate over the 1st 2 hours.

Leakage at 2 hours		
Factor	Water content	Electrolyte leakage
Water content		
Electrolyte leakage	r^2=0.7953 P=0.0029 (**)	
Shoot recovery	r^2=0.6560 P=0.0148 (*)	r^2=0.8658 P=0.0008 (***)
Leakage rate over the 1st 2 hours		
Electrolyte leakage	r^2=0.7958 P=0.0029 (**)	
Shoot recovery	r^2=0.6560 P=0.0148 (*)	r^2=0.8658 P=0.0008 (***)

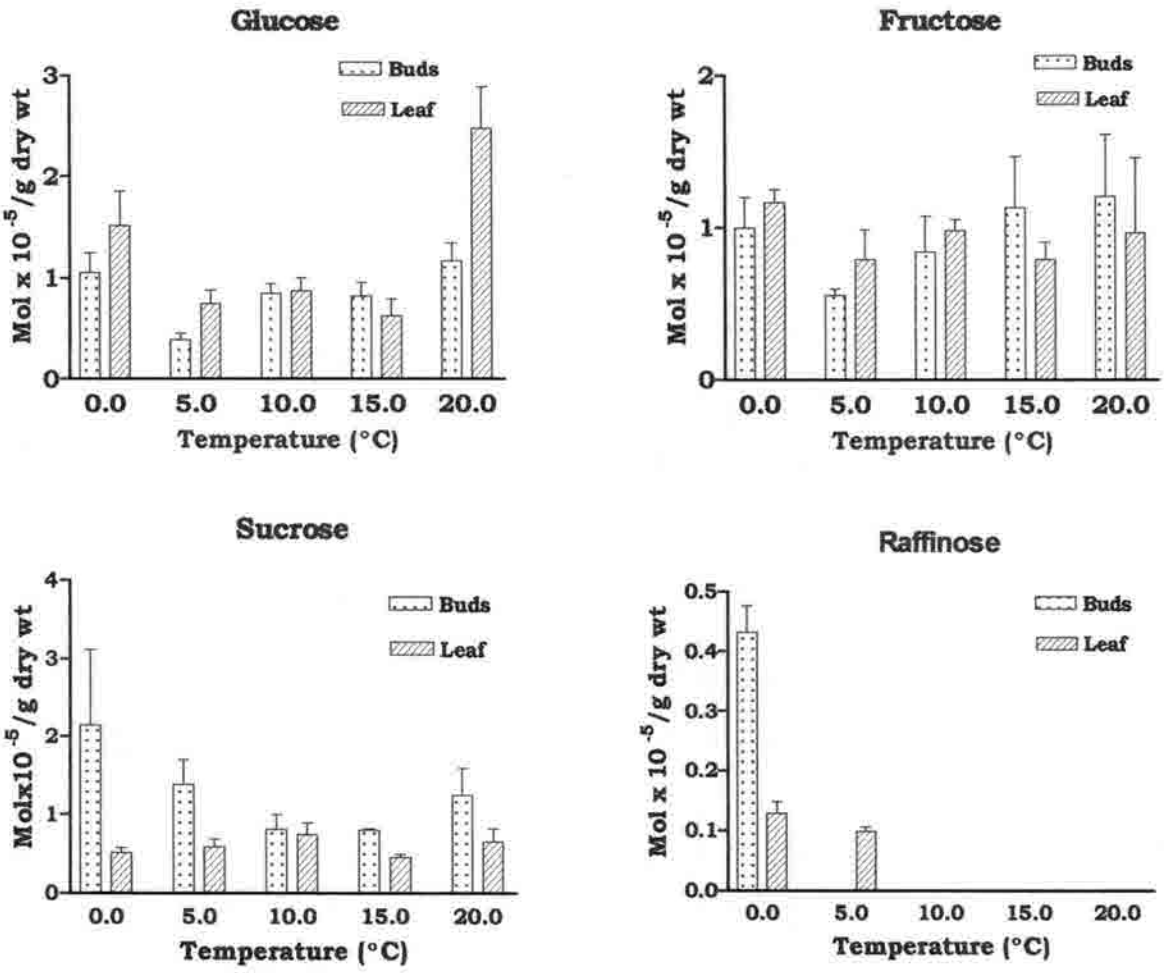

Figure 16. Soluble carbohydrate content extracted from buds and leaves of 'Pineapple' sweet orange plants grown at five different temperatures (0, 5, 10, 15 and 20°C).

Table 7. Summary of Analysis of Variance (ANOVA) for soluble carbohydrate content extracted from buds and leaves of 'Pineapple' sweet orange plants grown at five different temperatures.

Carbohydrate	Tissue	df	MS	P
Fructose	Bud	4	0.1993	ns
	Leaf	4	0.0723	ns
Glucose	Bud	4	0.3354	<0.05
	Leaf	4	1.7580	<0.01
Sucrose	Bud	4	0.7298	ns
	Leaf	4	0.0388	ns
Raffinose	Bud	4	0.1120	<0.0001
	Leaf	4	0.0119	<0.0001

Discussion

Dehydration, cold acclimation, sucrose pre-culture, vitrification, and tissue culture techniques were tested in these studies, in an attempt to develop a cryopreservation protocol for lateral buds of 'Pineapple' sweet orange.

'Pineapple' orange vegetative tissue proved to be intolerant of desiccation. Shoot recovery decreased consistently for buds with 0.6 mg $H_2O.mg^{-1}$ DW or less, and total loss of viability was observed when buds were dehydrated to 0.198 mg $H_2O.mg^{-1}$ DW. At this level of water content all freezable water has supposedly been lost and only non-freezable water remains (Berjak and Dumet, 1996). Some seeds, classified as orthodox by Roberts (1973), can maintain high viability when dehydrated to such water contents. However, vegetative tissues in general cannot withstand similar levels of desiccation although some plants are capable of surviving total removal of water from their vegetative tissues (Berjak and Dumet, 1996).

Buds of 'Pineapple' sweet orange did not survive exposure to the low temperatures of liquid nitrogen (-196°C), regardless of the pre-treatment to which they were subjected (Table 4). Cryopreservation of lateral buds of cold hardy tree fruits has been accomplished by slow cooling to about -40°C prior to plunging in liquid nitrogen, a procedure denominated two-step cooling (Bajaj, 1995; Niino, 1995; Sakai, 1995;

Stushnoff and Seufferheld, 1995). In some instances plants have been cold acclimated in order to enhance survival following exposure to liquid nitrogen temperatures. Cold hardiness of cold acclimated trees of 'Valencia' sweet orange (*C. sinensis*), exposed to progressively colder temperatures from 30 to 0°C, was studied by Yelenosky and Guy (1977). They observed that acclimated trees did not survive exposure temperatures below –6.7°C. This evidence of cold acclimation in *C. sinensis* led us to hypothesize that buds isolated from cold acclimated plants would stand a better chance to survive freezing in liquid nitrogen. Although they did withstand subzero temperatures as low as –10 to -14°C after the cold acclimation treatment, they did not survive -196°C. Thus, the two-step cooling could not be applied for the cryopreservation of 'Pineapple' orange tissues. Nevertheless, these results indicate that isolated buds can cold harden to some extent after they have been cold acclimated and that they can withstand much lower temperatures than whole trees as compared to the results reported by Yelenosky and Guy (1977). Cold hardiness of whole trees was not evaluated in these studies since this research focused on the use of isolated lateral buds.

Carbohydrate concentrations are known to change in *Citrus* trees in association with new growth, fruit set and harvest, and cold acclimation. Total carbohydrate accumulated in 'Valencia' orange leaves and stems at progressively colder temperatures (Yelenosky and Guy, 1977). Carbohydrate accumulation is believed to be one of the

mechanisms that allows vegetative tissues and most mature angiosperm seeds to survive desiccation to extremely low water contents (Blackman *et al.*, 1992; Crowe et al., 1984, 1987 and 1988; Hoekstra *et al.*, 1994; Oliver, 1996). This study hypothesized that cold acclimation might induce a similar carbohydrate increase in 'Pineapple' orange lateral buds, and that carbohydrate accumulation could improve resistance to freezing temperatures, allowing them to survive exposure to liquid nitrogen. However, any increase in carbohydrate content promoted by cold acclimation did not increase cold hardiness of 'Pineapple' orange tissues to the extent that they could withstand freezing to -196°C. It is interesting to note that although total carbohydrates increased in leaves and stems of 'Valencia' orange, the starch-sugar conversion in this species was not good enough to favor sugar increase and maximum cold hardiness in the trees (Yelenosky and Guy, 1977). While sugars are known to have a protective role under freezing conditions, starch is a non-protective carbohydrate. Supposedly a greater starch-sugar conversion would grant greater freeze protection (Yelenosky and Guy, 1977).

Cellular damage associated with dehydration and freezing stresses was manifested immediately upon rehydration of lateral buds. Evidence of this damage was provided by leakage of cellular ions into the surrounding test medium (distilled water) as a function of time. Similar results have been obtained with other species elsewhere (Becwar *et al.*,

1982; Borowski *et al.*, 1995; Prášil and Zámečník, 1998). At first, electrolytes are mainly released from cells injured by cutting and from cell wall and intercellular spaces. Then, the release of electrolytes is a result of injury to cell membranes and indicates that the membrane lost its integrity and its effectiveness as a barrier to solute diffusion (Becwar *et al.*, 1982; Borowski *et al.*, 1995). Thus, electrolyte leakage has been used as a measure of the extent of membrane damage resulting from desiccation/rehydration and freezing stresses; increase in leakage reflects an increased level of membrane disruption in desiccation and freezing sensitive tissues (Becwar *et al.*, 1982; Prášil and Zámečník, 1998). Our results do not provide direct evidence on the mechanism of membrane damage associated with dehydration stress, however they do indicate that membranes of 'Pineapple' orange lateral buds were severely damaged by dehydration. 70% of the cellular electrolytes leaked from buds dehydrated below 0.6 mg $H_2O.mg^{-1}$ DW water content. Less than 5% of electrolytes leaked from fully hydrated buds during the same period of time. Thus, the effect of dehydration on 'Pineapple' orange buds was evidenced by a nearly total diffusion of solutes out of the cells. Becwar *et al.* (1982) observed that leakage from silver maple (*Acer saccharinum* L.) and areca palm (*Chrysalidocarpus lutescens* [Bory] Wendl.) seeds increased markedly as seed moisture contents decreased from 45 to 35% and 84 to 53%, respectively, whereas germination percentage decreased significantly as seeds were dehydrated. Likewise,

Prášil and Zámečník (1998) have shown that electrolyte leakage increased in rape leaf discs and wheat tiller segments after freezing tests. They also showed that leakage values were affected by the shapes and sizes of segments in a sample and by the time allowed for electrolyte leakage. Thus, these results are in agreement with the concept that membranes of desiccation-sensitive tissues are injured by dehydration below the critical water content and cannot effectively function as a barrier to solute diffusion during imbibition. Once the membrane loses its integrity, viability loss follows (Hoekstra *et al.*, 1989).

It is concluded from the electrolyte leakage study that in the case of 'Pineapple' sweet orange water content is directly related to leakage and shoot recovery, thus electrolyte leakage of samples can be used to predict accurately and reliably shoot recovery following desiccation. Both absolute leakage and rate of leakage for the first two hours are excellent predictors of tissue recovery. This correlation does apply to both greenhouse and *in vitro* grown plants.

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