THESIS

# CRYOPRESERVATION OF SWEET POTATO SHOOT TIPS BY VITRIFICATION

Submitted by

Joyce C. Pennycooke

Department of Horticulture

In partial fulfilment of the requirements

for the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 1997

COLORADO STATE UNIVERSITY

September 25, 1997

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JOYCE C PENNYCOOKE ENTITLED CRYOPRESERVATION OF SWEET POTATO SHOOT TIPS BY VITRIFICATION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

Sarah Ward

Leigh E. Towill

Harrison G. Hughes

Advisor

Department Head

## ABSTRACT OF THESIS

CRYOPRESERVATION OF SWEET POTATO SHOOT TIPS BY VITRIFICATION

The conservation of vegetatively propagated germplasm is problematic. *In vitro* conservation of sweet potato has been achieved using normal and limited growth regimes. However, although the storage of *in vitro* plants is advantageous, it is not the method of choice for long term conservation because normal growth conditions require frequent subculturing which makes it labor intensive and costly. Also, frequent subculturing over a prolonged period of time may lead to undesirable consequences such as, contamination, selection and chromosomal aberrations. Cryopreservation offers the simplest and most economical way for the conservation of plant germplasm and vitrification is the preferred method to accomplish this. Previously, Towill and Jarret (1992) reported that vitrified sweet potato shoot tips exhibited callus formation and that the vitrification protocol developed included high levels of within and between treatment variability for explant survival.

The purpose of this study was therefore to, examine the various steps of the cryopreservation procedure in order to generate a reproducible shoot tip cryopreservation protocol for sweet potato.

*In vitro* grown sweet potato shoot tips were excised at 0, 3 or 10 hr in light after an 8 hr dark period. Excised shoot tips were precultured in 2% sucrose in MS for 24 hr and 0.3, 0.5 or 0.75 M sucrose in MS for an additional 24 hr. Precultured shoot tips were loaded with two different cryoprotective solutions for 20 or 60 min at 22 °C and then dehydrated with a concentrated vitrification solution (PVS2) for various lengths of time at 22 °C. Following dehydration, shoot tips were cooled by placing them along with a

small drop of PVS2 on thin strips of aluminum foil, in 0.2 ml PVS2 in polypropylene straws or in 1 ml of PVS2 in cryo-vials. These were then plunged in nitrogen 'slush' (ca. -209 °C). Cryoprotectant-exposed shoot tips as well as vitrified shoot tips were recovered on MS media with various modifications (mineral composition, surfactant / antioxidant, hormonal content).

Another type of vitrification technique, the encapsulation / dehydration vitrification procedure, was also pursued for the cryopreservation of sweet potato shoot tips. The highest survival (67%) of vitrified encapsulated shoot tips was achieved after 4 hr dehydration corresponding to a moisture content of 18.1 %. To our knowledge, this is the first reported survival of cryopreservation of sweet potato shoot tips using the encapsulation / dehydration procedure.

For the solution–based vitrification method, shoot tip survival after both the dehydration step and cooling in nitrogen slush was strongly dependent on the preculture condition (sucrose concentration). The best survival was achieved from shoot tips (PI 290657) excised from *in vitro* plants soon after the 8 hr dark period, with a 24 hr preculture in 0.3 M sucrose prior to loading in 2M glycerol + 0.4 M sucrose for 1 hr and then dehydrated with PVS2 for 16 min at 22 °C respectively. A fast cooling rate was beneficial and this was achieved by placing the shoot tips along with a small drop of PVS2 on thin strips of aluminum foil prior to plunging in nitrogen slush. Survival of cryopreserved shoot tips was promoted significantly ($P = 0.0001$) in the absence of $NH_4^+$ in the recovery medium for 5 days prior to transfer to regular MS medium.

In this study, we report an improved vitrification protocol in that survival after dehydration and cooling results in the resumption of shoots initiated directly from treated

shoot tips. Most important for any genebank is the regeneration of shoots so as to reduce the risk of somaclonal variation. Using the same recovery medium, for all four lines tested, relatively good survival levels (ranging from 62% – 97 %) were achieved, and most important, all lines regenerated shoots after cooling to cryogenic temperatures.

Joyce C. Pennycooke

Department of Horticulture

Colorado State University

Fort Collins, Co. 80523

Fall 1997

## TABLE OF CONTENTS

Page

ABSTRACT..... iii

ACKNOWLEDGEMENTS.....vi

LIST OF FIGURES..... .....ix

LIST OF TABLES.....x

INTRODUCTION.....1

REVIEW OF LITERATURE.....7

Need for Germplasm Conservation / Cryopreservation.....7
Cryopreservation.....8
Principles of Vitrification.....12
Encapsulation /Dehydration Technique.....16
Cryopreservation Research of Sweet Potato.....20
Plant Environmental Conditions.....21
Choice of Explant.....22
Pretreatment of Excised Shoot Tips.....23
Application of Cryoprotectants.....24
Cooling and Rewarming Procedures.....26
Post-warming Treatments.....29

MATERIALS AND METHODS.....32

*In Vitro* Maintenance.....32
Ex-Plant Preparation..... 32
Growth Chamber Plant Conditions.....33
Aseptic Preparation of Shoot Tips from Growth Chamber Plants.....33
Pretreatments.....35
Solution-Based Vitrification Procedure.....35
Encapsulation / Dehydration Vitrification Procedure.....37
Recovery Media for Shoot Tips.....38
Statistical Analyses.....39

RESULTS AND DISCUSSION.....40

Solution – Based Vitrification Procedure.....40

Pretreatments.....40

Addition of Cryoprotectants.....44
Cooling.....51

Plant Environmental Conditions.....55

Age of *In Vitro* Plants.....55
Location of Shoot Tips along Stem Axis.....57
Light Conditions.....57
*In Vitro* vs. Growth Chamber Grown Source Plants.....61

Recovery Media for Shoot Tips.....61

Mineral Composition.....63
Hormonal Content.....65
Surfactant / Antioxidant.....66

Other Sweet Potato Lines.....69

Encapsulation / Dehydration Vitrification Procedure.....71

Dehydration Studies.....71
Dehydration and Cooling.....73

CONCLUSIONS.....77

REFERENCES.....80

APPENDIX.....96

## LIST OF FIGURES

Figure Page

1. General rapid cooling method for apical meristems.....10
2. Supplemented phase diagram of a hypothetical cryoprotectant.....14
3. General encapsulation / dehydration procedure.....17
4. Role of sucrose in dehydration.....19
5. Classification of buds with reference to their location along the sweet potato stem .....34
6. Effect of sucrose preculture on % survival of sweet potato shoot tips (PI 290657) cooled to ca. -200 °C.....42
7. Effect of light conditions on survival of sweet potato shoot tops (PI 290657) cryopreserved by vitrification.....60
8. Changes in moisture content and subsequent survival of encapsulated sweet potato (PI 290657) shoot tips after dehydration as a function of dehydration duration (0 to 7 hr).....72
9. Survival of vitrified sweet potato (PI 290657) shoot tips after 5 hr dehydration.....74

## LIST OF TABLES

Table Page

1. Effect of ABA conc. and preculture duration on the survival of sweet potato (PI 290657) shoot tips exposed to PVS2 (controls) and then cooled to ca. -200 °C.. .....45

2. Effect of sucrose preculture and loading solutions on the survival of vitrified sweet potato (PI 290657) shoot tips following dehydration with PVS2.....46

3. Survival of vitrified sweet potato (PI 290657) shoot tips following exposure to loading solutions and PVS2.....47

4. Effect of packaging system (cooling rate) on survival of vitrified sweet potato (PI 290657) shoot tips.....54

5. Effect of age of *in vitro* sweet potato (PI 290657) source plants on survival after vitrification.....56

6. Survival of vitrified sweet potato (PI 290657) shoot tips excised from various locations along the stem axis.....58

7. Survival of vitrified shoot tips from *in vitro* and growth chamber grown sweet potato (PI 290657) source plants.....62

8. Effect of various recovery media on the viability of vitrified sweet potato (PI 290657) shoot tips.....64

9. Initiation of shoot from vitrified sweet potato (PI 290657) shoot tips cultured on various media.....67

10. Survival of vitrified sweet potato (PI 290657) shoot tips recovered on two conc. of pluronic F-68 in the culture medium.....68

11. Survival of shoot tips from four sweet potato lines after cooling to ca. -200 °C.....70

## ACKNOWLEDGEMENTS

I would like to express my deep appreciation to Dr. Leigh Towill, my advisor for his help and guidance. I thank Dr. Harrison Hughes, my major professor and Dr. Sarah Ward for serving on my graduate committee and providing valuable suggestions.

Also special thanks to the ARS/USDA National Seed Storage Laboratory (NSSL) for allowing me to conduct my research at the laboratory, and to the NSSL staff for their support and assistance. Thanks to the professors, staff and all my friends at the Department of Horticulture.

The Food and Agriculture Organization (FAO) and ARS/USDA of the Caribbean Basin Initiative Program are acknowledged for providing the financial support.

Extra special thanks to my dear husband, Norman for his encouragement, support and sacrifice throughout my academic program. Finally, I wish to thank my family and God for their gift of love.

# INTRODUCTION

Sweet potato, *Ipomoea batatas* (L) Lam., is a trailing vine of the morning glory family (Convolvulaceae) that is characterized by its succulent, edible tuberous storage roots (Purseglove, 1988). Sweet potato production ranks seventh in world crop statistics (FAO, 1978) after wheat, rice, maize, potato, barley and cassava. It is an important food and fodder crop in the tropics where most of the production occurs. About 80 % of the crop is produced in China, although it is an important staple in the Caribbean basin, Polynesia, and in other areas of Asia, Africa and South America. It is also a high-quality luxury vegetable for some temperate countries such as the USA, Japan and New Zealand. Sweet potato is an excellent source of energy, vitamins A and C and fiber. It is also an important industrial crop for production of starch and ethanol fuels from high biomass lines.

There is considerable debate about the systematic organization of the sweet potato and its close allies. The available evidence clearly shows that the sweet potato is a hexaploid (2n = 6x = 90). It is yet to be determined whether *I. batatas* is an allopolyploid or an autopolyploid. Nishiyama (1971) suggested that it was an allohexaploid of a diploid *I. trifida* which occurs wild in Mexico. One of the difficulties with this theory is apparent nomenclature and identification problems since *I. trifida* was later found to be a feral sweet potato. More recently, Shiotani (1988) proposed that sweet potato is an autopolyploid, and that *I. trifida* is the ancestor. Yen (1976) suggested that *I. batatas* evolved to the hexaploid level through the hybridization of a diploid (2n = 2x = 30) and a tetraploid (2n = 4x = 60) to produce a triploid (2n = 3x = 45), followed by subsequent doubling of the chromosomes. The occurrence of unreduced pollen in diploid *I. trifida* (Orjeda et al., 1990) and in a tetraploid and hexaploid *I. batatas* (Jones, 1990)

provides a clue to understanding how polyploidization and gene flow between different ploidy levels can occur in nature. This mechanism is consistent with the theory that sweet potato is an autopolyploid.

Genetic studies have failed to reveal further information on this problem. This is traceable to the complexity of the genetic systems, such as self- and cross-incompatibility, operating in these species; in addition, the frequent occurrence of non-flowering types in $F_1$ and $F_2$ restricts the choice of parents in undertaking a comprehensive study involving parents representing wide genetic variability (Jones, 1990). Certainly, wild *Ipomoea* species represent possible world economic food reserves as the genetic material could be useful in future plant breeding for the improvement of sweet potato.

It is generally accepted that sweet potato is of American origin. Austin (1988) proposes that the origin of sweet potato occurred between the Yucatan and the mouth of the Orinoco River in South America. This is the area of greatest diversity for sweet potato.

Preservation of germplasm is important for providing the present and future availability of genetic materials for use in various breeding, pathological and entomological studies. Conventionally, plant germplasm is conserved through seeds, tubers, roots, bulbs, corms, rhizomes, cuttings etc. The germplasm of a number of plantation crops and fruit trees cannot be preserved as seed because they display desiccant-sensitive characteristics and the embryo degenerates early. Although sweet potato lines produce desiccant-tolerant seeds ("orthodox seeds"), propagation by seed is undesirable as the progeny is extremely variable, flowering is sporadic and segregants may produce no tubers at all.

Presently, sweet potato germplasm is mostly conserved through *in vitro* cultures. Numerous clones are being maintained by this method at international centers such as the International Potato Center (CIP), International Institute of Tropical Agriculture (IITA) and at various national genebanks.

The conservation of vegetatively propagated germplasm is problematic. To date, the *in vitro* conservation of sweet potato has been achieved using normal and limited growth regimes ( Jarret and Gawel, 1991). However, although the storage of *in vitro* plants is advantageous, it is not the method of choice for long term conservation because normal growth conditions require frequent subculturing which makes it labor intensive and costly. Also, subculturing over a prolonged period of time may have undesirable consequences such as contamination, mutation and selection, chromosomal aberrations and changes in ploidy (Scowcroft, 1984).

The value of long term conservation lies in the ability to hold materials for extended periods of time without genetic change. Thus, the base collection is a comprehensive collection of accessions that are held for the purpose of long term conservation and serves as a back up to the working or active collections (comprised of accessions that are maintained under conditions of short to medium term conservation for the purpose of study, distribution or use). The National Seed Storage Laboratory, located at Fort Collins, Colorado is the only long term plant germplasm storage facility within the US National Plant Germplasm System.

The depletion of germplasm pools and the reduction of naturally occurring genetic resources have attracted international attention and caused great concern. Thus, unconventional methods are being developed for the storage, maintenance, conservation and international

exchange of plant germplasm. The storage of *in vitro* cultures of excised meristems enabled only short and medium term maintenance of germplasm (Bajaj, 1993). However, cryopreservation has the distinct advantage of allowing long-term conservation (Towill, 1990b).

Basically, cryopreservation involves bringing the culture to a state of nondivision and zero metabolism, which is achieved by subjecting it to superlow temperatures (-196 °C). Shoot culture systems have generally been favored for cryopreservation studies because of their greater genetic stability compared with the more disorganized callus and suspension systems. Conventional (two step) cooling techniques have also been used to cryopreserved cells, protoplast and meristems of selected species (Withers, 1987 ; Towill, 1990b). Shoot cultures of sweet potato species have not survived with this technique (Blakesley et al., 1995; Towill, unpublished). Vitrification is an alternative method for attaining cryopreservation (Fahy et al., 1987) and has been applied to sweet potato shoot tips (Towill and Jarret, 1992) and somatic embryos (Blakesley et al., 1995) with some success.

In a vitrification process the material is cooled ultra rapidly, in order that the aqueous solution and also cellular contents vitrify, i.e. form an amorphous glassy structure, thus avoiding the problems caused by ice formation within the tissue (Fahy et al., 1987). In order to achieve vitrification, a rapid and very precisely timed treatment in the presence of very high cryoprotectant concentrations is usually needed. Dilution of the cryoprotective medium after warming is also very precise (Engelmann, 1991). Results of sweet potato shoot tips surviving vitrification and subsequently forming a shoot have been very variable (Towill and Jarret, 1992).

Encapsulation / dehydration is another type of vitrification technique for cryopreservation and is a combination of alginate encapsulation, sucrose exposure and dehydration prior to immersion in liquid nitrogen (LN). This technique has been successful with a number of species (Dereuddre et al., 1990 ; Fabre and Dereuddre, 1990 ; Plessis et al., 1993 ; Mandal et al., 1996) but has never been reported for the shoot tips of sweet potato. Meristems or somatic embryos are encapsulated in alginate and treated with high levels of sucrose prior to dehydration and cooling (Dereuddre et al., 1990; Fabre and Dereuddre, 1990). The encapsulation / dehydration procedure is a combination of the 'synthetic seed' and cryopreservation techniques. The alginate beads protect the apices during handling and probably tend to reduce the fluctuations of environmental parameters such as temperature, moisture content, osmolality and nutrient condition (Fabre and Dereuddre, 1990).

Vitrification and encapsulation /dehydration techniques present two main advantages. Firstly, they offer additional possibilities for cryopreserving plant materials such as sweet potato which appears to be recalcitrant to conventional cooling procedures. Secondly, cooling rate devices are not needed, thus making it less costly.

Our goal was, therefore, to examine the various steps of the cryopreservation procedure (see below) in order to generate a reproducible shoot tip cryopreservation protocol for sweet potato. Specific objectives were to:

1) establish a basic vitrification protocol useful for comparing certain factors.
2) evaluate the effects of sucrose or ABA pretreatment of excised shoot tips on "cryopreservability".

3) evaluate the effects of plant growth conditions such as age, photoperiod and propagation method *(in vitro* vs. growth chamber) on "cryopreservability".

4) evaluate the effect of post-thaw treatment on survival of cryo-treated shoot tips with particular reference to the recovery medium to enhance survival and to initiate shoots from cryo-treated shoot tips.

# CHAPTER I

## REVIEW OF LITERATURE

### Need for Germplasm Conservation / Cryopreservation

Plenty literature is available on the need to conserve plant germplasm for future breeding purposes ( Clark and Roath, 1989 ; Eberhart et al., 1991). Maintenance and preservation of germplasm collections representing a wide array of genetic diversity of cultivated plants and their wild relatives are considered to be essential for plant breeding programs.

Germplasm of many crops is stored in the form of seed. Seeds which can be stored in a manner whereby longevity is increased in a predictable means through a decrease in temperature and moisture content have been described as showing "orthodox' viability characteristics (Roos, 1989). In contrast, there are a number of species which produce short lived seeds that cannot be dried without immediate injury and viability loss. Such seeds are classified as "recalcitrant" (Chin and Roberts, 1980). Orthodox seeds, also termed "desiccation tolerant", are capable of retaining viability after being dried to less than about 5-10% moisture content, and recalcitrant seeds, also termed "desiccation sensitive" lose viability after being dried below a critical limit, usually about 12-30% moisture content (Chin and Roberts, 1980).

Some germplasm can be maintained only vegetatively, usually for reasons related to inability to form seeds, recalcitrant seeds, large seed size, long juvenile periods and extreme heterozygosity where continued crossing might disrupt useful gene combinations (Towill, 1988). Such germplasm is conventionally maintained as clones in greenhouses or field plantings and is propagated by divisions, tubers, cuttings etc. which possess only a limited life span. Thus the

preservation of these clones in greenhouses or in the field is not only time-consuming, labor-intensive and expensive, but above all, the material is exposed to the hazards of pest and diseases. This may result in a loss of germplasm.

The problem of the conservation of genetic resources of vegetatively propagated plants has encouraged attempts to develop unconventional methods. As an alternative, *in vitro* (meristem cultures, suspension cells and somatic embryos) systems have been stored (Dodds et al., 1992). For genetic conservation , plant species are not usually stored as callus cultures as the problem of lack of regeneration occurs in most cases and the callus cultures may undergo mutation and somaclonal variation and therefore the clones cannot be maintained. Although this difficulty can be surmounted by culturing excised meristems, or dividing *in vitro* plants, they still need to be routinely transferred to the fresh medium. They require extra space and manpower, and there is still the risk of contamination during frequent transfers. Thus, the problem of conserving germplasm of vegetatively propagated crops has encouraged attempts to look for other methods. In this regard cryopreservation of excised meristems is a promising approach (Bajaj, 1985 ; Towill, 1990b).

**Cryopreservation**

The notion of cryopreservation has been around since the 1930's. Luyet's (1937) concept was that, living things could be cooled so quickly that ice would not have time to form. Earlier work on rapid cooling of aqueous gelatin gels and other dilute aqueous solutions apparently inspired him.

Cryopreservation is based on the reduction and subsequent arrest of metabolic functions of biological materials, while maintaining viability (Bajaj, 1991). At low temperature, below -130 ° C, almost all the metabolic activities of the cells are at a standstill and they can be preserved in such a state for extended periods.

The essence of cryopreservation is to effect cell dehydration and concentration of the cytosol in a controlled and minimally injurious manner so that ice crystallization in the cytosol is precluded or minimized during quenching in LN. In conventional cryopreservation procedures, cell dehydration is affected by freeze-concentration of the suspending medium. Operationally, the specimens are equilibrated in a solution containing a permeating cryoprotective agent; the suspension is cooled and seeded with an ice crystal at a temperature slightly below its freezing point; the suspension is cooled at a relatively slow rate to an intermediate subzero temperature (eg. -30 °C to -40 °C) prior to quenching in LN. Seeding is the artificial induction of ice formation in the external medium.

During freeze-induced dehydration, the specimens are vulnerable to intracellular ice formation, which in most instances is lethal. The probability of intracellular ice formation is influenced by many intrinsic parameters characteristic of the specimen (the presence and efficacy of intracellular nucleating agents, the stability of the plasma membrane and its ability to preclude seeding of the supercooled cytosol by extracellular ice, the water permeability of the plasma membrane and the surface area to volume ratio of the cell) and the cryopreservation protocol. The general cryopreservation process comprises the following successive steps, which often have to be defined for every species and in some cases for every genotype (Fig. 1) : (1) Plant

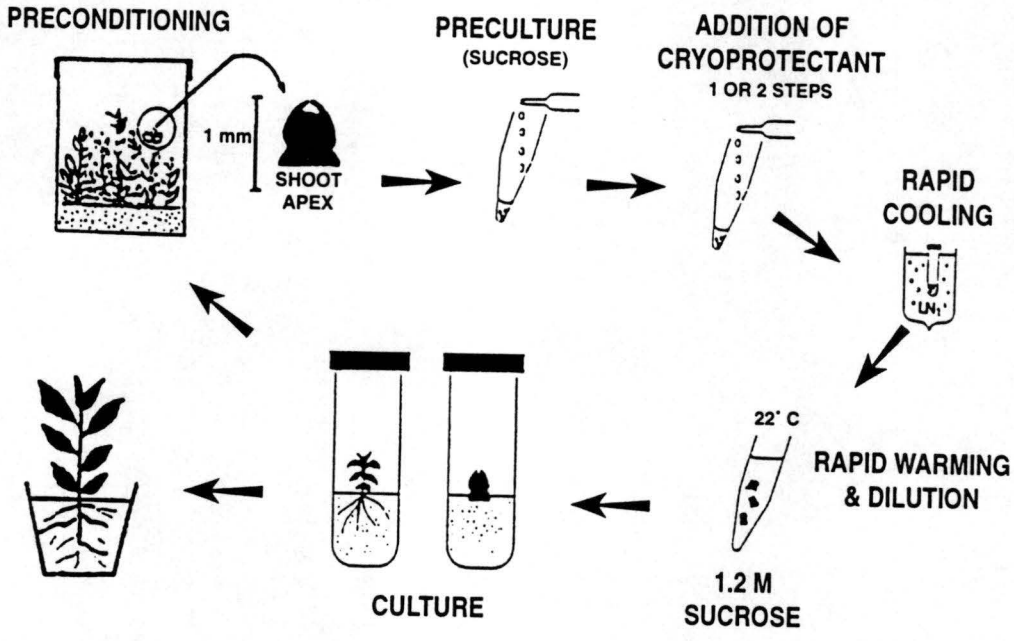



**Fig. 1 General Rapid Cooling Method for Apical Meristems**
(Sakai et al 1990 modified)

environmental conditions, (2) choice of material (explant), (3) pretreatment, (4) cryoprotectant "loading", (5) dehydration, (6) cooling, (7) warming and dilution, (8) post-warming treatment and (9) viability assay. Within each of the above parameters there are many factors and combination of factors that are responsible for the success or failure of a cryopreservation protocol. Cryoprotectant temperature, concentration and exposure duration are examples of such factors affecting the pretreatment and cryoprotective loading and dehydration processes. So too, the rate of cooling and rewarming must be taken into account.

Freezing injury and theories of cryopreservation have been dealt with by several authors (Mazur, 1984; Steponkus, 1984; Morris and Clarke, 1987; Singh and Larouche, 1988). The evidence seems to indicate that membrane damage, particularly to the plasma membrane, is a primary component of freezing injury (Steponkus, 1984). Quinn (1985) has shown that some lipid components convert from a liquid-crystalline to a gel phase during cooling and may not reconstruct during warming. Cryoprotectants apparently protect membranes from damage during cooling and aid in protecting proteins and nucleic acids from inactivation. Freezing injury is apparently not just a single event (Mazur, 1984; Steponkus, 1984). During the cryopreservation process, there are at least five potential causes of injury: osmotic excursions incurred during cryoprotective loading, dehydration and unloading steps; chemical toxicity of the cryoprotectants used; extreme dehydration from exposure to the concentrated cryoprotectants; ice formation during cooling and warming; and mechanical disruption of the specimens because of fracturing of the glass (see below). However, an important requirement prior to cryopreservation is the availability or development of efficient and reliable protocols for faithful regeneration of plants from cryopreserved cells or meristems.

Though slow cooling methods form the basis for cryopreservation, the following modifications and refinements have helped to simplify the protocol and to increase the viability. Moreso, in some cases, the sophisticated and expensive cryostats for controlled freezing rates are not needed as the material is immersed directly into LN (Bajaj, 1991). Recently, two novel cryogenic protocols, the vitrification method (Sakai et al., 1990 ; 1991b) and the encapsulation / dehydration technique (Dereuddre et al., 1990) have been developed for plant systems, and appear effective for a number of plant species (Benson et al., 1989; Towill, 1990; Sakai et al., 1990; Bajaj, 1993; Blaskesley et al., 1995).

**Principles of Vitrification**

Recent work has focussed on the procedures that would eliminate the need for controlled freezing and enable cells and meristems to be cryopreserved by direct transfer from about 0 °C - 25 °C into LN. For successful cryopreservation, it is necessary to avoid lethal intracellular freezing, which occurs during rapid cooling in LN. One approach is to create conditions such that with cooling no ice forms and the whole aqueous environment solidifies as a glass. This process in physical terms is called vitrification. Vitrification reduces the risks for the potentially damaging effects of intra- and extracellular crystallization. At sufficiently low temperatures, a highly concentrative cryoprotective solution becomes so viscous that it solidifies into a metastable glass. The goal is to achieve a sufficiently high concentration of solutes within the cell and to use rapid cooling rates to prevent the solution from freezing into crystalline ice when cooled, thus ensuring its transition into the amorphous or glassy state (Fahy et al., 1984).

The physical properties of vitrification is best described by referring to a supplemented phase diagram such as the one presented by Fahy et al. (1984) (Fig.2). The temperature at which the liquid begins to spontaneously form ice crystals at a significant rate is termed the homogeneous nucleation temperature, $T_h$. If suitable foreign surfaces are present such that the interfacial tension between the new crystal and the surface is lower, then the crystal will form preferentially on this site, a process known as heterogeneous nucleation (Fahy et al., 1984) . It is the heterogeneous nucleation that is responsible for freezing in most everyday situations. Thus a liquid can be supercooled to its melting point, at most, as far as $T_h$. If $T_h$ is sufficiently low then it is possible that a different fate can befall the liquid during cooling. As the liquid is cooled it will generally become more viscous, eventually to the point that it can no longer flow on a measurable timescale. Such a liquid has become a glass and has the structural properties of a liquid but has the mechanical properties of a solid. The temperatures ($T_g$) in which the liquid can no longer retain its liquid-like properties during cooling is called the glass transition, under a define set of conditions . Thus glass formation is expected in a liquid whenever $T_h < T_g$. The glass obtained by continuous cooling is likely to contain an array of crystal nuclei that begin to grow during cooling. However, because the sample was cooled into the glassy state, the growth of these nuclei is arrested along with all other motional processes in the sample. The most common manifestation of the existence of these nuclei is the crystallization of the liquid close to $T_g$ during warming. The ice crystallization on slow warming a glassy solution to temperatures in the vicinity of $T_g$ is often referred to as devitrification ($T_d$) (MacFarlane, 1986). It has been shown that devitrification can even take place in aqueous solutions at temperatures below $T_g$ (Angell and MacFarlane, 1982). For this reason, the glasses that exhibit devitrification behavior

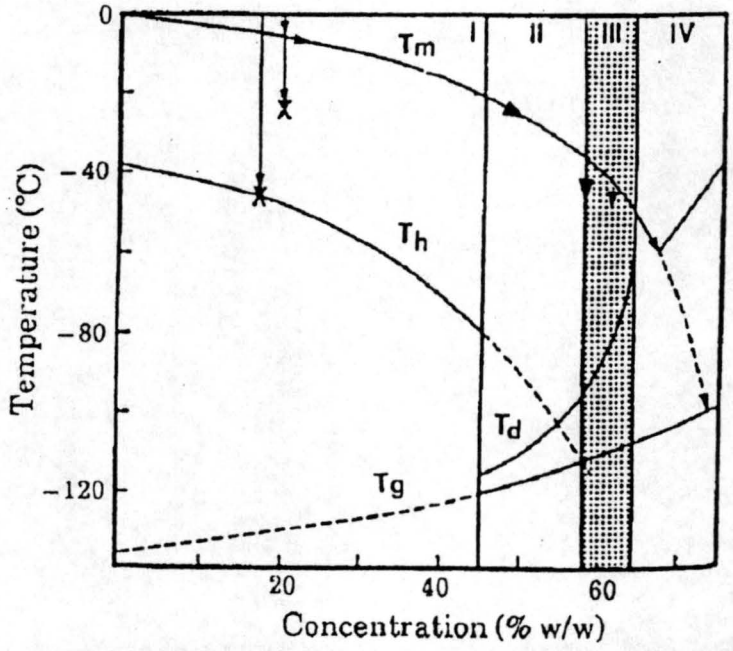



**Fig. 2 Supplemental phase diagram of a hypothetical cryoprotectant.**
**Tm: equilibrium freezing or melting curve; Th: homogeneous nucleation temperature; Tg: glass transition curve; Td: devitrification curve (Fahy et al., 1984)**

have been described as 'doubly unstable' - since the glass is simultaneously unstable with respect to both the liquid and solid states (Angell et al., 1981).

There are certain rather well defined regions in the phase diagram in which different types of vitrification behavior appear. In the relatively dilute region I, vitrification cannot occur because both homogeneous and heterogeneous nucleation are unavoidable (unless extremely rapid cooling is possible). In the more concentrated region II, both types of nucleation are inhibited. However, glass formed in this range is unstable. At still higher concentration, region III, $T_h$ becomes equal to $T_g$ and the value actually falls below that of $T_g$. In this region it is possible to slowly cool even bulk liquids directly to reach the $T_g$ value without experiencing any detectable freezing events. The intersection between the $T_h$ and $T_g$ curve indicates therefore the threshold or lowest possible concentration of cryoprotectant that might be used for vitrification. In other words, this is the optimum concentration which produces a situation where $T_h$ for the solution has dropped well below (as indicated by extrapolation) $T_g$ and therefore the liquid should exhibit no tendency to generate crystals during cooling. Finally in region IV, devitrification is prevented, and the system is virtually stable (Fahy et al., 1984). The glass that is obtained is stable with respect to homogeneous nucleation and should not exhibit a devitrification phenomenom during warming.

Glass fills extracellular spaces in a tissue, and during dehydration may contribute to stop the prevention of additional tissue collapse, solute concentration, and pH alterations. As glass is exceedingly viscous and all chemical reactions that require molecular diffusion are impeded, its formation may lead to molecular/cell/tissue inactivity and stability over time (Burke, 1986).

Vitrification offers considerable promise for the development of procedures with broad applicability, especially for the cryopreservation of complex organs such as shoot tips. The problem of cryopreservation of organized tissues lies in the fact that these tissues contain different cell types, each of which may require different optimum conditions to minimize the problem of intracellular ice formation. Nevertheless the use of this procedure for plant materials is just emerging and there are several aspects that must be refined before it can be implemented for the routine preservation of plant germplasm. Operationally, vitrification procedures of biological specimens consist of five major steps: (1) "loading step", which is the equilibration of the specimen in a solution containing permeating cryoprotectants; (2) dehydration of the specimen in a concentrated solution that will vitrify (the cellular contents are concentrated by dehydration such that they can vitrify); (3) cooling; (4) warming and (5) dilution of the vitrification solution and removal of the cryoprotectant from the cytosol, which is referred to as "unloading" (see below).

**Encapsulation / Dehydration Technique**

This technique was developed by a French research team using meristems of *Solanum* species (Fabre and Dereuddre, 1990). In this technique, prefreezing dehydration is replaced by dehydration at room temperature (Fig. 3) . Shoot tips (Plessis et al., 1993; Mandal et al., 1996), suspension cells (Bachiri et al., 1995) or somatic embryos (Blakesley et al., 1995) are trapped in alginate beads, precultured in sucrose enriched medium, air dehydrated and cooled in LN. Encapsulation / dehydration is indeed a vitrification procedure whereby high sucrose pretreatment eliminates the need for elaborate cryoprotective mixtures, thus making it a major

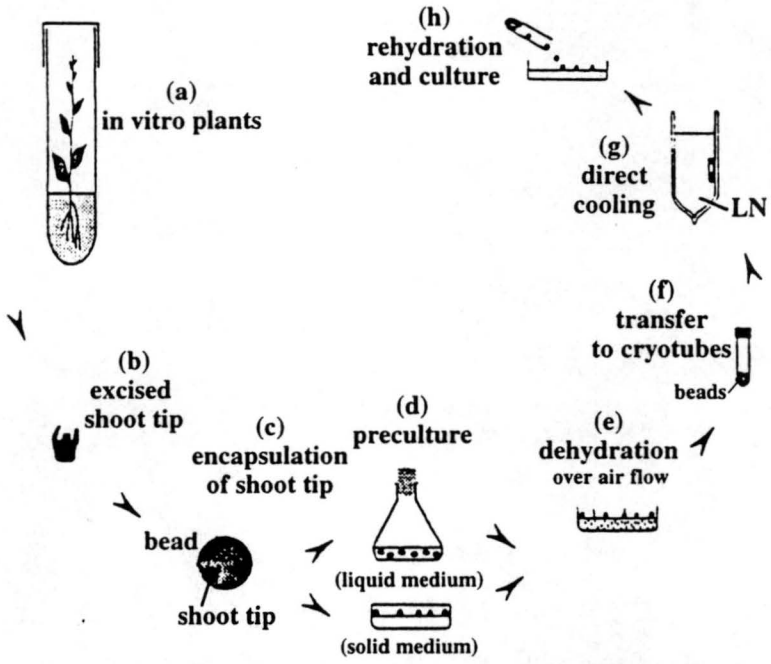



**Fig. 3 General encapsulation/dehydration procedure**
**(Bouafia et al. 1995 modified)**

advantage over the solution-based vitrification cocktail technique. Thermal analysis by differential scanning calometry has confirmed that vitrification occurred during cooling and rewarming in both the beads and explants of encapsulated somatic embryos of carrot (Dereuddre et al., 1991). Increasing sucrose exposure in a stepwise fashion works effectively, allowing sufficient time for permeation into the cells. Pleisis et al. (1993) showed that best results of cryopreservation of *Vitis vinifera* L. cv. Chardonnay (40-60% survival and 30% shoot recovery without callus formation) were obtained after a 2-day stepwise preculture to 1 M sucrose . However, Bouafia et al. (1996) showed that direct preculture with lower sucrose concentrations (0.5 - 0.75M) led to better survival than progressive increase in sucrose concentration to 1 M for every potato cultivar tested. Sucrose seems to be essential during dehydration to replace vital water molecules from cellular macromolecule, thus avoiding denaturation (Crowe et al., 1987 ; Carpenter et al., 1990) (Fig 4). It is believed that sucrose is taken up by the cells or tissues where it may contribute to the maintenance of plasma and inner membrane integrity during dehydration and cooling processes (Plessis et al., 1993). Sucrose can interact with phospholipids reducing Van der Walls interactions among hydrocarbon chains and thus maintain the liquid crystalline structure of the membrane lipids (Crowe et al., 1984; 1988; 1990).

In recent years a number of reports describe successful cryopreservation by encapsulation / dehydration - sucrose preculture followed by direct plunge in LN without any additional cryoprotection. They involved somatic embryos of sweet potato (*Ipomoea batatas*) (Blakesley et al. 1995), meristem cultures of banana (*Musa* spp.) (Panis et al., 1996) and shoot tips of potato (*Solanum* spp.) (Bouafia et al., 1996). Survival after dehydration was enhanced up to 74% by

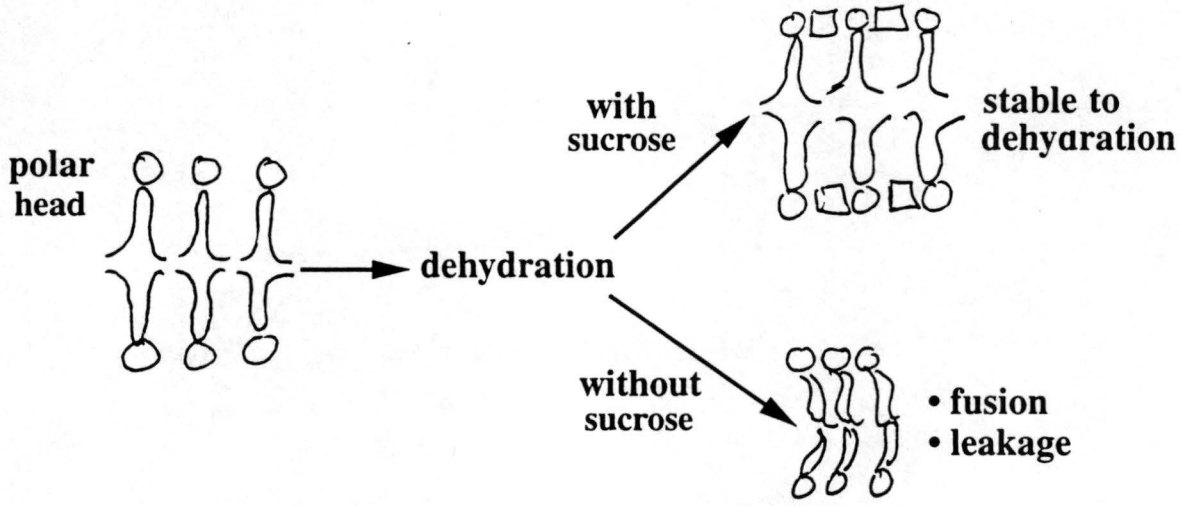



**Fig. 4 Role of sucrose in dehydration.**

**Disaccharides substitute for water molecules between the exposed polar head groups of the lipid bilayer, thus preventing them from coming too close together during dehydration and changing conformation.**

incorporating an initial slow cooling step, i.e. from ambient to 0 °C at -10 °C $min^{-1}$ and then to -40 °C at -0.5 °C $min^{-1}$ prior to plunging the sweet potato somatic embryos into LN (Blakesley et al., 1995). The implication is that most of the water would have left the cell and freeze externally thus preventing intracellular ice formation during the rapid cooling step.

**Cryopreservation Research of Sweet Potato**

One of the first reports of the successful freezing of a higher plant *in vitro* system involved the slow cooling of a suspension culture of sweet potato (Latta, 1971). However, little research was done on cryopreservation of sweet potato until the early 1990's. Conventional (two-step) cooling protocols have been used to cryopreserved suspension cells and embryogenic tissue of selected genotypes (Latta, 1971; Blakesley et al., 1995). Subsequent work has shown that shoot tips of sweet potato are recalcitrant with these techniques (Towill, unpublished ; Blakesley et al., 1995). Some success was also reported for shoot tips of sweet potato using a vitrification technique (Towill and Jarret, 1992; Schnabel-Preikstas et al., 1992; Plessis and Steponkus, 1996b). Results of sweet potato shoot tips surviving vitrification and those subsequently forming a shoot have been very variable (Towill and Jarret, 1992). Schnabel-Preikstas et al. (1992) found that survival of sweet potato shoot tips after vitrification was limited to callus formation. A callus phase prior to shoot formation is undesirable since callusing potentially increases the frequency of genetic variants and then regeneration of the plant must occur. Vitrified and warmed sweet potato shoot tips resulted in growth and development of shoot tips without excessive callus formation (Plessis and Steponkus, 1996a). However, subsequent growth was

slowed considerably. The encapsulation / dehydration technique has been reported with some success for sweet potato somatic embryos proliferated from shoot tips (Blakesley et al., 1995) but to our knowledge this technique has not been reported for sweet potato shoot tips directly.

**Plant Environmental Conditions**

Species exhibiting the ability to develop extensive cold-hardiness are best cryopreserved by a method using the natural ability of the plant to tolerate freezing stress. It is essential that twigs to be cryopreserved be gathered in a cold-acclimated state (Towill, 1990b). Endogenous cryoprotectants are induced in nature and thus enable some species to cold acclimate to very low temperatures (Stushnoff et al., 1993). Species with some ability to cold acclimate show better survival if the plant or plantlet is given a cold treatment *in vivo* (Yelenosky and Guy, 1977) or *in vitro* (Reed, 1988; Matsumoto et al., 1995; Kuranuki and Sakai, 1995). However, many species are not cold-hardy or do not acclimate sufficiently and in these cases cryoprotectants must be supplied.

Little is known about the influence of non-cryogenic factors such as the physiological and morphological status of plants from which the shoot tips are excised. Light may be an important determinant of shoot tip development before and after cooling and may contribute to the variable responses to cryopreservation often observed in most species (Benson et al., 1989), but the reasons for these responses are unknown.

Undefined endogenous compounds are produced during other environmental factors such as water stress (Sharp and Davies, 1989; Tarczynski et al., 1993). These often influence cryoability and probably cryoprotectants that are artificially added during the cryogenic protocol.

The evidence clearly shows that there is a positive correlation between cold acclimation and sugar levels (Sakai and Yoshida, 1968; Sakai, 1986; Stushnoff et al., 1993). Starch is often converted to sucrose during the dark period (Salisbury and Ross, 1992). High levels of endogenous sucrose are expected to be produced in tissues excised immediately after the dark. It is these endogenous cryoprotectants which may be the high temperature glass forming component of living cells (Hirsh, 1987).

**Choice of Explant**

As a general rule, material for cryopreservation is best chosen as young and as meristematic as possible. Shoot tips are a preferred form for low temperature storage of clonal lines because cells in this type of material are the most likely to withstand cooling; they are small, contain only a few vacuoles and are highly cytoplasmic (Engelmann, 1991). Also, shoot culture systems have generally been favored for cryopreservation studies because of their greater genetic stability as compared with the more disorganized callus and suspension systems (Roca et al., 1979). Somatic embryos or embryogenic tissues might provide alternative systems for some species for this purpose if shoot cultures are not readily available or if they have not survived with this technique. Somatic embryo systems are likely to be more stable genetically than callus and suspension systems (Blakesley et al., 1995). It is also important, however, to investigate cryopreservation techniques with embryogenic tissues because they are frequently used in genetic transformation protocols and their long term maintenance in a suitable stable state can be very labor intensive (Blakesley et al., 1995).

Shoot tips can be harvested from *in vitro* or *in vivo* grown plants. It is possible that shoot tips derived from both sources show different responses when exposed to cryogenic conditions since plants from these two sources differ physiologically and morphologically. Early cryopreservation protocols for some crops such as potato were originally developed for glasshouse grown material. However, it is frequently more desirable to cool shoot tips derived from *in vitro* plant cultures (Towill, 1981 ; Harding et al., 1991). *In vitro* maintenance reduces the risks of contamination during the culture of the shoot tips and may offer the possibility of easier handling during the cryopreservation process by eliminating the surface sterilization step of the explants.

The physiological stage of the material is also important. In the case of cell suspensions, material harvested at the exponential growth stage is most likely to be able to successfully withstand cooling probably because cell size and vacuoles are small (Sugawara and Sakai, 1974; Withers, 1985; Yoshida et al., 1993; Reinhoud et al., 1995). Reports for cryopreservation of carnation meristems showed that survival decreased progressively with their rank on the shoot axis, starting from the terminal meristem (Dereuddre et al., 1988). Potato meristems taken from long-term *in vitro* cultures showed less "cryopreservability" than those taken from recently initiated *in vitro* plantlets (Harding et al., 1991).

**Pretreatment of Excised Shoot Tips**

Pretreatment corresponds to a culture of the explant for a certain period of time under conditions which prepare it for the cooling procedures. In order for cells and meristems to be capable of vitrifying upon rapid cooling into LN, cells and meristems need to be preconditioned

to enhance the tolerance for dehydration and subsequent cooling. Pretreatment is often carried out from 1 to 3 days using various sugars or low levels of cryoprotectants within a solid or liquid culture medium (Haskins and Kartha, 1980; Towill, 1984; Matsumoto et al., 1994). Towill reported the effectiveness of this technique for several potato clones and related species (Towill, 1981a; b; 1984). A high level of sugar or sorbitol during preculture was reported to be essential in achieving high survival rates of cryopreserved wasabi meristems (*Wasabia japonica*) ( Matsumoto et al., 1994). Kuranuki and Sakai (1996) showed that not only is the concentration of the preculture medium important but also the duration of preculture. The greatest levels of shoot formation of tea (*Camelia sinensis*) cooled to -196 °C was obtained in the shoot tips precultured with 0.2 M sucrose for 2 days. Pretreatment, therefore, may increase the applicability of cryopreservation to less resistant materials.

**Application of Cryoprotectants**

Addition of cryoprotectants to the suspension medium is essential for good survival rates of hydrated tissues after exposure to low temperatures. The most common cryoprotectants used are dimethyl sulfoxide (DMSO), ethylene glycol and propylene glycol. The most effective cryoprotectants must permeate the cell, and the above three do so fairly rapidly in plant cells. Some low molecular weight compounds, such as sucrose, glucose, glycerol, mannitol and proline do not permeate, or do so very slowly, but are effective if used in combination with permeating cryoprotectants (Finkle et al., 1985; Towill, 1990b).

The type and concentration of a cryoprotectant as a function of the amount of ice that can be tolerated by a biological system differs for vitrification and slow cooling procedures. Basically, the ultimate amount of ice formed can be controlled in an essentially known way by selecting the initial concentration of the cryoprotectant (Fahy and Hirsh, 1982). Fahy et al. (1987) discusses these principles and their relevance to vitrification. "It was determined that in the presence of 6% polyvinylpyrolidone, glycerol will vitrify at a concentration of only about 42% by volume instead of the 68% by volume encountered as a result of slow cooling. This shows therefore, that the concentration of glycerol required to vitrify the system is less than the concentration of glycerol required to prevent the system from succumbing to mechanical injury during slow cooling. Consequently, for such a system vitrification would seem to be the method of choice."

The mechanism of action of these cryoprotective additives is unclear. One undisputed effect is to lower the freezing point of the medium to which it is added (MacFarlane and Forsyth, 1990). These cryoprotectants do have an osmotic role and act thus by dehydrating the cells but they may act also by direct interaction to protect membranes and enzymatic binding sites from freezing injury (Crowe et al., 1987; Fahy et al., 1987). There are suggestions that the accumulation of sucrose and glycerol maintains the stability of membranes in the dehydrated state by replacing the water molecules at the charged exterior surface of the membranes (Crowe et al., 1987; Crowe et al., 1988). The glass formed upon cooling protects the interior of the cells.

To effect vitrification of the cytosol during quenching in LN, it is necessary to increase the solute concentration of the cytosol. This can be facilitated by first introducing (loading)

moderate concentrations (1 - 2M) of permeating cryoprotectants into the cytosol (Steponkus et al., 1992; Nishizawa et al., 1992 ; Matsumoto et al., 1994). To minimize the time for equilibration, the loading step is usually done at room temperature. However, the cryoprotectants may be added at lower temperatures for a longer time if toxicity is a problem . In some instances, the vitrification solution was diluted and used for the loading step (Sakai et al., 1990; Towill and Jarret, 1992). Following loading, dehydration is achieved by exposing the specimens to extremely concentrated solutions (vitrification solutions). Typically, solutions in excess of 8M are used and more than 90% of the osmotically active water is removed from the cell (Steponkus et al., 1992). Many different solutions ranging from extremely complex to rather simple formulations have been successfully used for vitrification of animal (Fahy and Hirsh, 1982) and plant (Sakai et al., 1990; Plessis and Steponkus, 1996) specimens. The exposure to the cryoprotectant solutions must be regulated according to the size of the specimen, cryoprotectant toxicity and temperature (Finkle et al., 1985).

**Cooling and Rewarming Procedures**

Rates at which the sample is cooled and then rewarmed are also critical factors determining survival after cryopreservation (Mazur, 1984; Towill, 1990; Reinhoud et al., 1995). Different types of cooling processes can be carried out : slow, rapid and ultra-rapid cooling. In the first case, a programmable cooling apparatus can be used to obtain precise and reproducible cooling conditions, although more simple systems have been devised. Damage to cells during cooling can be divided into two categories : intracellular ice formation and "solution effects" (Mazur, 1984). The function of slow cooling is to allow most of the water to leave the cells such

that intracellular freezing does not occur. If adequate dehydration is not achieved, large intracellular ice crystals form that can severely damage cell membranes (Mazur, 1984; Steponkus et al., 1985). If cells are allowed to dehydrate too much, the second major cause of cellular damage during cooling, solution effects, becomes dominant. Solution effects are thought to occur when cooling rate is too slow (Farrant et al., 1982). The exact nature of these solution effects has not been defined. Mazur (1984) reported that they could be due to high concentrations of electrolytes inside and outside the cell. Injury due to concentration of electrolytes is probably of a biochemical nature, involving destabilization of cell membranes and may be due to certain solutes reaching toxic levels. Solution effects may also include pH alterations, macromolecular interactions and membrane effects. If cooling is too rapid, vapor pressure equilibrium between the cell and the extracellular environment is not maintained and intracellular ice forms (Mazur, 1984).

Using low levels of cryoprotectants in cells, the cooling rates that give optimum survival for plant cells, protoplasts and small tissues such as shoot tips, are usually about 0.25- 1 °C $min^{-1}$ (Towill, 1990). Two step cooling methods are used for cryopreservation. Samples are slowly cooled to between about -30 °C and -40 °C and are then immersed in LN. However, using higher levels of cryoprotectants and rapid cooling rates can also preserve viability. At higher cryoprotectant concentrations, cooling rates accessible by ordinary quenching techniques for small samples allow apparent vitrification of the sample (Fahy et al., 1984). The amount of ice that forms on cooling can be affected drastically by cooling rate and the concentration of the cryoprotectant used. With reference to Fig. 2, there are disadvantages to using high cooling rates

in combination with concentrations of cryoprotectant found in region II. First, cooling rates required are not applicable to large objects such as mammalian organs and secondly, the small ice nuclei formed during cooling manifest themselves during warming along the curve labeled $T_d$ by recrystalizing into large disruptive crystals (Fig. 2). $T_d$ is the devitrification curve, i.e. ice formation from the previously vitreous liquid.

Typically, during vitrification the samples are cooled by quenching in LN. Plunging tobacco cell suspensions directly into LN yielded higher survival rates than two-step cooling; 55% and 36% respectively (Reinhoud et al., 1995). Shoot tips plunged directly into LN gave about 60%, 72%, 75% and 80% survival with tea, (*Camellia sinensis*) (Kuranuki and Sakai, 1996), banana (*Musa* spp.) (Panis et al., 1996), mint (*Mentha* spp.) (Towill, 1990) and lily (*Lilium japonicum*Thunb) (Matsumoto et al., 1995) respectively. It is important to note that cooling / warming systems are very dependent on the cryoprotectant type, and concentration used.

LN is commonly used for cooling the samples. It is cheap and usually readily available. Ultra rapid rates may be obtained by placing samples on copper grids or strips of aluminum foil and plunging into LN. An alternative to obtain faster cooling rates, is to use partially solidify nitrogen ('nitrogen slush') as the cryogenic medium, which results in a cooling rate of 400 °C $s^{-1}$ over the range of 0 to -60 °C (Steponkus et al., 1992). Nitrogen slush is prepared by placing LN in a vacuum chamber to increase the rate of evaporation so that the temperature is decreased below the boiling point (-196 °C) to about -209 °C (the freezing point).

**Post-Warming Treatments**

Post-warming treatment consists of culturing the sample under conditions that ensure the optimal recovery. Typically, cryoprotectant addition and dilution as well as the cooling and warming steps are stressful and damaging events in any cryopreservation protocol (Steponkus et al., 1992) . Cryoprotectant toxicity can be arguably considered the central problem of cryobiology (Fahy, 1987). Dilution of the vitrification solution and removal of the cryoprotectants from the samples is referred to as the unloading step. The cryoprotectants are toxic if kept too long in contact with the samples. Rapid dilution may be detrimental to some cells ( Withers, 1979). Therefore, dilution of the suspending medium and efflux of the cryoprotectants from within the samples are usually accomplished by dilution in a hypertonic medium or a step-wise dilution (Steponkus et al., 1992).

The composition of the culture medium can be transitorily modified (mineral composition, antioxidants and hormonal contents). The post-warm culture medium may influence the viability of vitrified cells. The elimination of ammonium from the post-warm culture medium increased the survival of cryopreserved rice cells (Kuriyama et al., 1989) and *Lavandula vera* cells (Kuriyama et al., 1996) after which ammonium ions were necessary for optimum cell growth. Anthony et al., (1996) reported that adding a surfactant, Pluronic F-68, to post-warm culture medium had beneficial effects on post-warm growth following cryopreservation of rice cells, but the reasons for the improved growth are unknown.

Growth regulator concentration also may require adjustment in the recovery medium in order to enhance survival and minimize callus formation prior to shoot initiation of LN-treated shoot tips (Towill and Jarret, 1992; Schnabel-Preikstas et al., 1992). Callus formation must be minimized or eliminated to reduce the risks of somaclonal variants after shoot initiation (Withers et al., 1988).

Currently regrowth is the only definitive assessment of viability after warming. However, other viability assays may be used, but are often found wanting or difficult to apply. The most common methods used include : visual evaluation of tissue browning of buds (Quamme and Stushnoff, 1983), conductivity tests (Quamme and Stushnoff , 1983), fluorescein diacetate (FDA) (Reinhoud et al., 1995) and triphenyl tetrazolium chloride (TTC) (Towill and Mazur, 1974). The limitation of these viability assays, with the possible exception of conductivity tests is that they are all destructive tests. Seufferheld (1995) showed that the visual evaluation of tissue browning of buds gave a positive response of survival of vitrified apple buds, however, these same buds when grafted by patch budding did not grow. Viability assessment by FDA-staining only tests whether enzymes in the cell, deacetylase fluorescein-diacetate, are still functional and whether the plasma membrane is still intact, but it can not predict whether the cells resume growth. Therefore, survival rates should be checked by regrowth experiments. Also, the other viability assays are deemed unsuitable for shoot tips because of the small size of the shoot tips.

Finally, recovery usually occurs in the dark or dim light in order to avoid photooxidation which can be harmful to the sample (Benson et al., 1989). There exists the possibility that disturbances in oxidative metabolism can lead to free radical production in plant tissues exposed to low and cryogenic temperatures. Free radicals and activated oxygen species are themselves

highly toxic, however, their damaging effects are further enhanced by secondary oxidative reactions.

# CHAPTER II

# MATERIALS AND METHODS

Micropropagated sweet potato plants (*Ipomoea batatas* (L.) Lam) PI 290657 were used in the present study. Stock cultures of three other genotypes (PI 573324, PI 508515 and PI W235) were also tested. Growth chamber plants of PI 290657 were used in only one study. All lines were obtained from the Southern Regional Plant Introduction Station in Griffin, Georgia as *in vitro* plants.

## *In Vitro* Maintenance

*In vitro* grown sweet potato plants were multiplied from nodal segments and routinely cultured on solid Murashige and Skoog (MS), 1962 modified basal medium (half strength of ammonium nitrate and potassium nitrate, termed ½ MS medium) containing 2% sucrose and 0.65% agar at pH 5.7. All cultures were incubated at 25 °C with a 16h photoperiod under cool white fluorescent light at 60μE $m^{-2} s^{-1}$.

## Ex - Plant Preparation

Shoot tips consisting of 2-3 leaf primordia plus the apical dome, 0.5 mm - 1.0 mm in length, were excised from axillary buds from 4 - 13 week old *in vitro* plants. In one experiment shoot tips were excised from different positions along the stems of 8 week old *in vitro* grown

source plants. Each stem was divided into four sections to include shoot tips excised from the terminal apical bud, just below the terminal apical bud, the center of the stem and the base of the stem (Fig. 5). Stems varied in total number of buds, therefore, care was taken to select those stems consisting of the terminal bud plus 8 axillary buds.

Shoot tips were excised under sterile conditions with the aid of a dissecting microscope either immediately after the 8 hr dark period, 3 or 10 hours in light after the dark period.

**Growth Chamber Plant Conditions**

Growth chamber plants were established from 8 week old *in vitro* plants. Plants were maintained in a soil : peat : vermiculite (2 : 2 : 1) mixture in 900 mm pots and incubated under cool white fluorescent light at a 16/8 hour photoperiod. Plants were fertilized using Peters Compound (20 : 20 : 20) as required.

**Aseptic Preparation of Shoot Tips from Growth Chamber Plants**

Nodal segments, 5 mm in length each with one bud, were taken from growth chamber plants. The 5 mm segments were placed in a 100 ml Erlenmeyer flask containing a small concentration of detergent, and shaken for 15 min. This was followed by placing the segments in a tygon flexible plastic tube with one end sealed with a wire mesh and the other end attached to a tap. The segments were washed in running tap water for 30 min. Following this, the segments were placed in a 100 ml Erlenmeyer flask, containing 70% ethanol and shaken (manually) for 3 min. The ethanol was drained from the flask, replaced with 0.52 % sodium hypochlorite

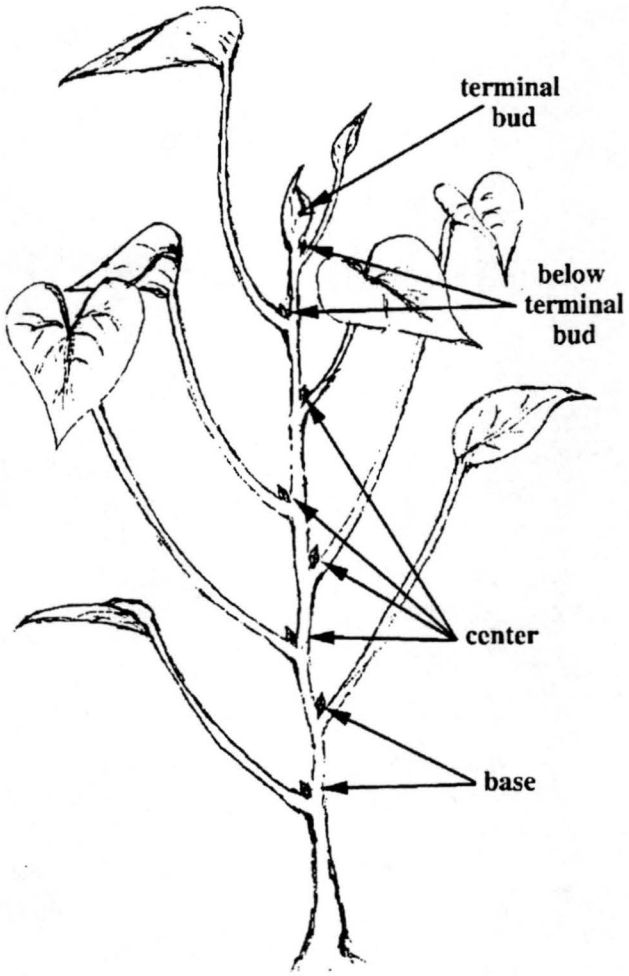



**Fig. 5 Classification of buds with reference to their location along the sweet potato stem**

solution (20% commercial bleach) and shaken for an additional 20 min. Then the hypochlorite solution was drained and the nodal segments rinsed three times with sterile distilled water, shaking the flask manually for approximately 1 min for each rinse. The axillary shoot tips were excised, as described above for *in vitro* grown plantlets.

**Pretreatments**

Shoot tips were precultured in a liquid MS medium containing 2% sucrose at 25 °C for 24 hr with a 16/8 hr photoperiod, after which they were transferred to MS medium containing 0.3, 0.5 or 0.75 M sucrose for an additional 24 hr at 25 °C.

ABA preculture was also tested. Following a 24 hour preculture in 2% sucrose in liquid MS, shoot tips were exposed to liquid MS containing 0.3M sucrose, supplemented with 0.1 or 1.0 mg/l ABA for 24 or 48 hr at 25 °C. ABA was filter sterilized and was added to the MS medium after autoclaving.

**Solution-Based Vitrification Procedure**

Precultured shoot tips were placed in a 12 x 75 mm test tube and then loaded with cryoprotectants composed of 20 % PVS2 (see below) or 2 M glycerol + 0.4 M sucrose for either 20 or 60 min at 22 °C before dehydration with a vitrification solution. The loading solution was removed and the shoot tips were dehydrated by exposing to a concentrated vitrification solution at 22 °C for 10, 16 or 26 min. The vitrification solution, referred to as PVS2, consisted of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol (EG) and 15 % (w/v) dimethylsulfoxide (DMSO) and

was prepared in MS and 0.4 M sucrose (Sakai et al., 1990). The 20% PVS2 solution was prepared by adding 1 ml of 100% PVS2 to 4 ml of 2% liquid MS medium.

Following exposure to PVS2, shoot tips were removed from the test tubes with a Pasteur pipette and placed along with a small amount (1/4 drop) of vitrification solution on thin strips of sterile aluminum foil. The foil strips, 40 x 2 mm in size, were folded to enclose the shoot tips and were then immersed into a nitrogen slush and held for 15- 30 min. The storage temperature was always maintained between -209 °C and - 196 °C. The nitrogen slush, which gave a cooling rate of about 400 °C $s^{-1}$, was obtained by placing the LN in a vacuum chamber to increase the rate of evaporation so that the temperature is decreased below the boiling point of LN (-196 °C). Other cooling rates were also tested whereby shoot tips were contained in 0.2 ml PVS2 in polypropylene straws or in 1.0 ml PVS2 in cryo-vials and plunged in nitrogen slush.

Foil strips containing the shoot tips were rapidly warmed by immersing in 3 ml of 1.2 M sucrose in MS for 20 min at 22 °C and then dislodging the shoot tips from the foil. This step also dilutes the PVS2 from the shoot tips. Polypropylene straws and cryo-vials were warmed by placing in a 37 °C water bath for approximately 5 and 30 seconds respectively or until the solid had just liquefied. The PVS2 was removed and shoot tips were placed in 3 ml of 1.2 M sucrose in MS for 20 min at 22 °C.

**Encapsulation / Dehydration Vitrification Procedure**

Shoot tips precultured in 2 % sucrose in MS for 24 hr were encapsulated in alginate according to the technique of Dereuddre et al. (1990) with some modifications (Fig 3). Shoot tips were suspended in calcium- and ammonium-free MS medium supplemented with 3 % (w/v) Na-alginate solution (medium viscosity) and 0.3 M sucrose. The mixture was dispensed as a drop from a sterile plastic transfer pipette (cut to adjust tip size) into 50 ml of 100 mM calcium chloride which solidifies the drop as a bead. Beads were held in the calcium chloride solution for 45 min at 22 °C. Beads of about 4-5 mm in diameter and containing one shoot tip each were precultured in sucrose-enriched liquid medium in Erlenmeyer flasks on a rotary shaker (85rpm). Stepwise increase in sucrose in the preculture medium was achieved in 6 days : 0.3 M, 1 day; 0.5 M, 1 day; 0.75 M, 1 day and 1.0 M, 3 days.

After preculture, shoot tips containing beads were rapidly surface-dried on sterile tissue paper and were subjected to dehydration in a laminar flow hood for different lengths of time. The moisture contents of the shoot tips were estimated by taking the fresh weight of 10 blank beads at specific intervals. Dry weight was determined by drying the 10 beads devoid of shoot tips in an oven (85 °C) to constant weight for approximately 48 hr. For cooling, dehydrated beads were plunged directly into nitrogen slush and stored for 15-30 min. Storage temperature was always maintained between -209 °C and -196 °C. Encapsulated shoot tips were removed from the nitrogen slush individually and rapidly warmed by placing in liquid 2 % sucrose in MS at 30 °C

for approximately 5 sec. Encapsulated shoot tips were then plated on ammonium-free MS solid medium supplemented with 1 μM NAA, 0.5 μM BAP, 0.1 μM Kinetin and 3 % sucrose for 2 days in complete darkness. On the 3rd day of culture the shoot tips were aseptically removed from the beads, kept on the same medium and incubated in dim light for 3 days. On the 5th day the shoot tips were transferred to ammonium containing MS medium with the same hormone composition as mentioned above.

**Recovery Media for Shoot Tips**

Regrowth on culture medium is one of the most definitive means of testing viability after cryopreservation. The recovery medium may provide a chance for repairing damage to cryopreserved plant cells. Enhanced survival after culture on different media means that viable uninjured cells as well as reversibly damaged cells, given appropriate conditions (such as growth-regulator, antioxidants and mineral content), can now divide.

Non-treated shoot tips were cultured on MS basal medium supplemented with 3 % sucrose and different concentrations of NAA, BAP and Kinetin respectively. Cultures were observed for growth characteristics for 8-12 weeks. Based on results of the above experiment, media compositions were selected and a second trial was conducted using LN-treated shoot tips.

Shoot tips exposed to the solution-based vitrification technique were cultured on recovery media composed of solid MS medium supplemented with 0.045 mg/ml BAP, 0.01 mg/ml IBA and 3% sucrose without various ions ($NH_4^+$, $NO_3^-$, $Fe^{2+}$). Cultures were incubated in darkness for

2 days, dim light for 3 days and subsequently transferred to MS media containing the respective ions and incubated in the usual light intensity (60 $\mu M^{-2} s^{-1}$) at 25 °C.

Another recovery medium tested was the addition of Pluronic F-68 at 0.01% and 0.1 % respectively to MS medium supplemented with 0.045 mg/ml BAP, 0.01 mg/ml IBA and 3 % sucrose. Cultures were incubated under the same conditions as described above.

Recovering shoot tips were recorded as a percentage of the total number of shoot tips forming normal shoots 8 weeks after plating. Subsequently these were transferred to a hormone-free regeneration medium as described above (see *in vitro* maintenance).

**Statistical Analyses**

Results were expressed as survival in independent experiments. On average, experimental treatments comprised 20-35 shoot tips which were distributed onto 4 - 7 replicate 35 x 10 mm recovery plates (5/plate). One foil strip (4-5 shoot tips) represented 1 replicate. Percent recovery was determined for each plate. Survival was calculated as the percentage of shoot tips forming shoots 8 weeks after rewarming and culture. It is important to note that researchers distinguish between percent survival (green/growing), i.e. either callus formation or shoot, and percentage showing shoot growth. The analysis of variance (ANOVA) statistical test was applied to the data using SAS computer software. Statistical significance was calculated at 5 % level. Chi-square analysis was used to determine statistical significance in one of the recovery media studies (Table 9).

# CHAPTER III

# RESULTS AND DISCUSSION

The development of cryopreservation using vitrification techniques should be an adjunct to the existing maintenance strategies and, thus enhance the availability of sweet potato germplasm.

Previous attempts in this laboratory to cryopreserve sweet potato shoot tips were partially successful. One of the major limitations of the vitrification protocol developed by Towill and Jarret (1992) included high levels of within and between treatment variability for explant survival. Also, untreated control, vitrification treated control and LN exposed shoot tips all exhibited callus formation. The regeneration of shoot from controls and vitrified shoot tips was very variable.

This study was therefore to improve the variable response with regard to overall survival and percent shoot formation of a cryopreservation protocol .

## Solution - Based Vitrification Procedure

### Pretreatment

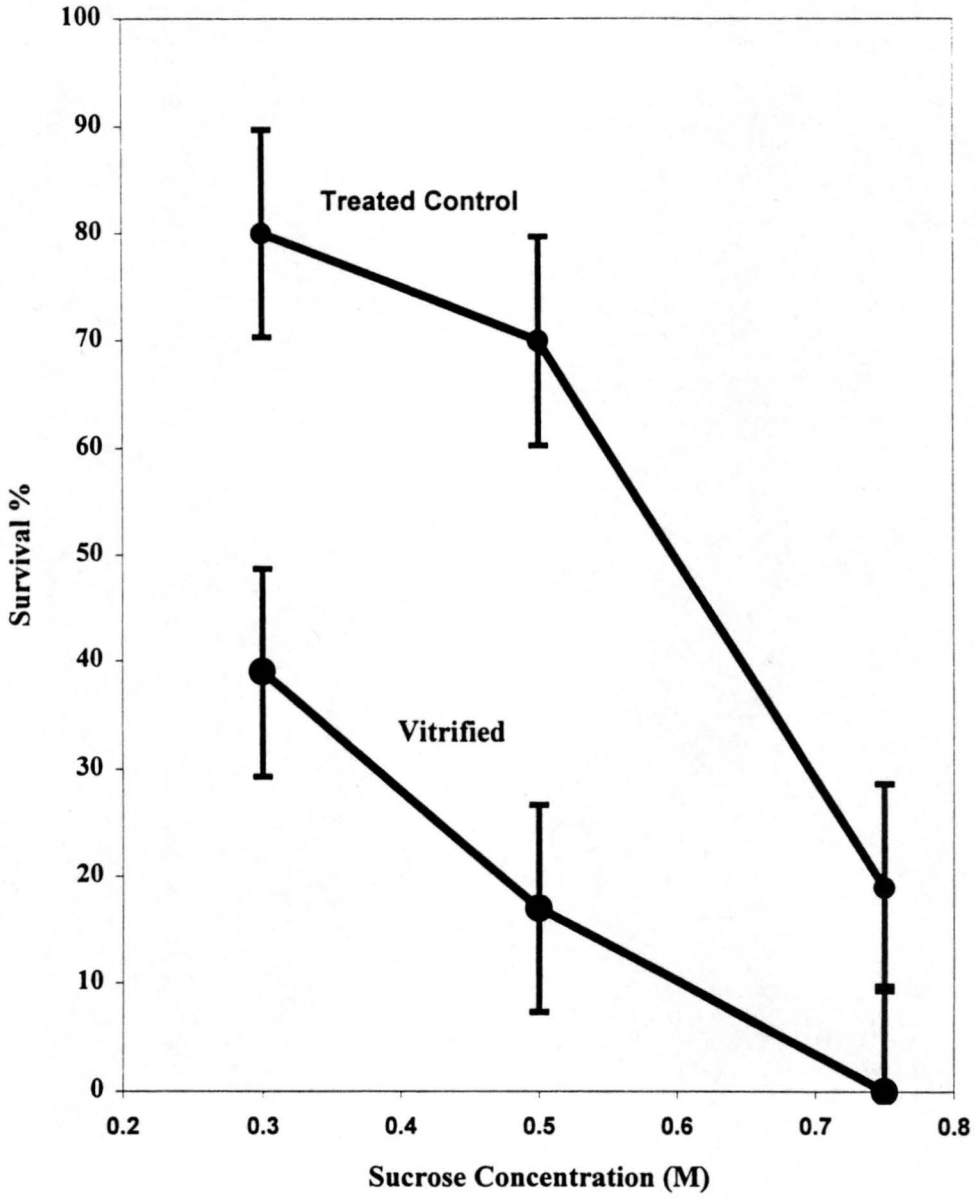
Preculturing with sugar was reported to be very important in improving survival of cryopreserved meristems in several species (Nino and Sakai, 1992; Matsumoto et al., 1994; Kuranuki and Sakai, 1995). Sucrose may enhance the tolerance for dehydration and contributes to the glass formed by subsequent cooling in LN. Although the complexities of its effects are unknown, it is believed to be absorbed slowly into the cells, and may act as an energy source in

increasing cell survival or may protect the proteins in the protoplasm under conditions of severe dehydration (Crowe et al., 1990). Sucrose concentration and preculture duration are important factors (Matsumoto et al., 1994; 1995; Plessis and Steponkus, 1996a).

Shoot tips were precultured in a liquid MS medium containing 2% sucrose at 25 °C for 24 hr, and were then transferred to MS medium containing 0.3M, 0.5M or 0.75M sucrose for an additional 24 hr at 25 °C. The time in culture in 2% sucrose is believed to allow repair of any damage due to the excision process and was not varied in the experiments. The highest survival was obtained in shoot tips precultured with 0.3 M sucrose (Fig. 6). As the sucrose concentration increased, from 0.3M to 0.75M, survival of shoot tips decreased significantly (P=0.0001) for both treated control (cryoprotectant-exposed) and shoot tips cooled to ca. -200 °C. Results of sucrose preculture of wasabi (*Wasabia japonica*) and lily (*Lilium japonica Thumb*) showed that 0.3M sucrose was also optimum for survival after vitrification (Matsumoto et al., 1994; 1995). As a result, in the following experiments, 0.3M sucrose was chosen as the solute for preculturing.

From preliminary experiments examining duration of sucrose exposure, shoot tips were precultured in MS medium supplemented with sucrose at various concentrations and exposure times and then plated on culture medium. These shoot tips were not exposed to any cryoprotectants, nor cooled. Shoot tips precultured in sucrose, added in a step-wise manner from 0.3M to 1.0M (1 day incubation in 0.3M, 0.5M, and 0.75M sucrose respectively) and held in 1.0M sucrose for 6 days subsequently gave rise to callus formation on growth culture medium (100% survival) (data not shown). These results seem to indicate that sweet potato shoot tips (PI

**Fig. 6 Effect of Sucrose Preculture on % Survival of Sweet Potato Shoot Tips ( PI 290657) Cooled to ca. -200 C**




cryo-treated and vitrified shoot tips were cultured on regular MS medium

290657) can tolerate up to 1.0M sucrose precultured for 6 days in sucrose alone when gradually exposed but preculture for 1 day in 0.75M sucrose in addition to subsequent cryoprotectant exposure is detrimental, probably due to osmotic stress imposed from a combination of factors. Although some of these interactions are intriguing, the small size of the shoot tips precludes any chemical analyses. A one day pretreated exposure to 0.3M sucrose was used for all subsequent experiments.

Sucrose is not likely to be the only substance involved in dehydration tolerance; other sugars, proteins and ABA may also be partially involved (Meurs et al., 1992; Na and Kondo, 1996). ABA is thought to play an important role in accelerating adaptation to various stresses such as desiccation (Bartels et al., 1988). Freezing tolerance can be induced in some plant species by exposure to low temperatures, changes in photoperiod or by a combination thereof (Levitt, 1980). It was observed that ABA was able to circumvent a low temperature requirement in the induction of freezing tolerance in an embryogenic microspore-derived cell suspension of winter rape (Orr et al., 1986). The effect of ABA as a promoter of freezing tolerance of cells and organs was reported by various workers (Tanino et al., 1990; Shimonishi et al., 1991; Kendall et al., 1993). ABA is thought to stimulate the synthesis of proteins that are involved in the protection from desiccation damage (Ooms et al., 1993). Dehydration after ABA-preculture has enhanced solute accumulation, consequently promising to increase freezing tolerance in plant cells (Kim and Janick, 1989; Shimonishi et al., 1991; Kendall et al., 1993).

In our study, a pretreatment using ABA preculture was also tested. Shoot tips were first precultured in MS medium supplemented with 2% sucrose for 24 hr at 25 °C after which they

were transferred to MS medium containing 0.3M sucrose, supplemented with ABA at 0.1 or 1.0 mg/L for 24 or 48 hr at 25 °C. Survival rates of sweet potato shoot tips cooled to ca. -200 °C varied with ABA concentration and pretreatment time (Table 1). With 24 hr preculture, the addition of ABA did not influence survival after cooling at either concentrations compared to cooled samples with no ABA. With 48 hr preculture, survival of ABA treated and cooled samples were about the same or slightly higher than non-ABA, cooled samples. The high levels of viability found in these tests precludes any firm conclusion, but shows that ABA itself was not toxic, nor did it impede shoot development. Note also, that the recovery medium used was MS ($NH_4^+$ -free) (to be discussed later).

Further investigations are necessary to tests different ABA concentrations and extended ABA preculture periods. Also, to determine if there is any correlation between ABA preculture and accumulation of carbohydrates or proteins. As mentioned earlier, the small size of the shoot tips makes chemical analysis difficult.

Addition of Cryoprotectants

Precultured shoot tips were loaded with two different cryoprotective solutions for 20 or 60 min at 22 °C before being dehydrated with PVS2 for 10, 16, or 26 min at 22 °C ( Tables 2 & 3) . These exposure times for the cryoprotective loading solutions and PVS2 were selected based on results of several preliminary trials conducted by the author. Less than 10 min and more than 30 min exposure of PVS2 at 22 °C seemed ineffective for successful vitrification of sweet potato (PI 290657) shoot tips (data not shown). As shown in Table 2, the loading solutions tested resulted in moderate to high rates of survival of shoot tips cooled to ca. -200 °C; however,

**Table 1. Effect of ABA conc. and preculture duration on the survival of sweet potato (PI 290657) shoot tips exposed to PVS2 (controls) and then cooled to ca. -200 °C.**

| Preculture Duration (hr) | LN | Survival (% ± SE) ABA Conc. (mg/L) | | | |
|---|---|---|---|---|---|
| | | Exp 1. | | Exp 2. | |
| | | 0.0[1] | 0.1 | 0.0[1] | 1.0 |
| 24 | - | 100 | 95 ± 5 | 95 ± 5 | 95 ± 5 |
| | + | 70 ±12[a] | 64 ±16[a] | 100[a] | 81 ± 11[a] |
| 48 | - | 95 ± 5 | 90 ± 6 | 85 ± 9 | 95 ± 5 |
| | + | 95 ± 5[a] | 96 ± 4[a] | 80 ± 11[a] | 100[a] |

[1] preculture medium was MS salts + 0.3M sucrose (no ABA)

Following exposure to cryoprotectant and cooling, shoot tips were cultured on MS medium without $NH_4NO_3$ for 5 days prior to transfer to regular MS medium.

[a] Numbers within rows for each experiment at different preculture periods followed by the same letter are not significantly different ($p<0.05$)

**Table 2. Effect of sucrose preculture and loading solutions on the survival of vitrified sweet potato (PI 290657) shoot tips following dehydration with PVS2 for 16 min**

| Sucrose Preculture | Loading Solution | Survival (% ± SE) PVS2 Exposed | Vitrified |
|---|---|---|---|
| + | No Loading | 76 ± 5 | 55 ± 11[b] |
| + | 20 % PVS2 | 96 ± 3 | 36 ± 7[b] |
| + | 2M Gly. + 0.4M Suc. | 90 ± 4 | 90 ± 4[a] |
| - | 2M Gly. + 0.4M Suc | 46 ± 7 | 0 [c] |

Following exposure to cryoprotectant and cooling, shoot tips were cultured on MS medium without $NH_4NO_3$ for 5 days prior to transfer to regular MS medium.

[a] Numbers within column followed by the same letter are not significantly different ($p<0.05$)

**Table 3. Survival of vitrified sweet potato (PI 290657) shoot tips following exposure to loading solution and PVS2.**

| Exposure Time (min) | | Survival (% ± SE) | |
|---|---|---|---|
| 2M Gly. + 0.4M Suc. | PVS2[1] | Cryoprotectant Exposed | Vitrified |
| 20 | 10 | 100 | 50 [ab] |
| 20 | 16 | 100 | 23 ± 6[c] |
| 20 | 26 | 100 | 35 ± 4[bc] |
| 60 | 10 | 80 ± 12 | 10 ± 10[d] |
| 60 | 16 | 80 ± 12 | 66 ± 9[a] |
| 60 | 26 | 60 ± 10 | 20 ± 12[cd] |

[1] PVS2 composed of 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% DMSO (w/v)

Following exposure to cryoprotectant and cooling, shoot tips were cultured on regular MS medium (ie. containing $NH_4NO_3$).

[a] Numbers within column followed by the same letter are not significantly different ($p<0.05$)

highest level of survival was observed in the vitrified shoot tips loaded with 2M glycerol + 0.4M sucrose (90% ± 4). The effects of preculturing and loading on survival of vitrified shoot tips are also summarized in Table 2. Elimination of the preculture step resulted in no survival after cooling to ca. -200 °C, even when cultured on an improved recovery medium (see below). In this species, sucrose preculture seems to be essential for the survival of cryopreserved shoot tips by vitrification. Statistical analysis showed that survival levels from the no loading treatment was not significantly higher than survival from the 20% PVS2 treatment (P=0.07). Also, exposure to PVS2 for 16 min was more detrimental to shoot tips that were not loaded (76%) compared to those loaded (90%, 96%). Sucrose concentration is critical when the shoot tips are precultured in sucrose followed by cryoprotectant exposure (Fig. 3). From results in preliminary trials, shoot tips precultured in sucrose, in the absence of cryoprotectants (non-treated controls) tolerated higher levels of sucrose than those that were exposed to cryoprotectants . This is presumably due to dehydration or chemical toxicity from the interaction of cryoprotectants with sucrose preculture.

In the vitrification method, cells and shoot tips are dehydrated by a concentrated vitrification solution (PVS2). However, several studies show that the direct exposure of less tolerant cells and shoot tips to the vitrification solution results in harmful effects due to osmotic stress and chemical toxicity ( Fahy et al., 1982; 1984; 1987) . The harmful effects due to dehydration or toxicity often can be alleviated or eliminated by adequate preconditionings such as preculture with sucrose or sorbitol (Table 2) (Yamada et al., 1991; Nino et al., 1992) and cryoprotective loading (Langis and Steponkus, 1990; Nishizawa et al., 1992; 1993; Matsumoto et

al., 1994; 1995). In this study, 2M glycerol + 0.4M sucrose was more effective than 20% PVS2 in obtaining survival after cooling. The role of the loading solutions is to allow permeation of individual solutes so that the subsequent concentrated vitrification solution may provide the desired degree of dehydration.

In the original procedure of Sakai et al. (1990), the vitrification solution was diluted and used for the loading step. This procedure has been followed for other plant species (Towill and Jarret, 1992). However, there is no reason why the loading solution needs to be of the same composition as the vitrification solution (Steponkus et al., 1992). In fact, in our study, the combination of glycerol and sucrose was a more effective loading solution than the diluted vitrification solution. Of all the solutes that have been proposed as cryoprotectants, glycerol is the most waterlike in its ability to maintain the hydrophobic forces that are essential to the tertiary and quaternary conformation of macromolecules (Tanford, 1973) and is often fairly permeable in animal cells. The surface energy of glycerol solutions is also compatible to that of water, unlike most other solutes that markedly alter solution surface energies and, thereby, the interfacial forces that are important for membrane integrity. An additional proposed asset of glycerol is its relatively large molar volume so that during freezing it retains intracellular water colligatively, thus further forestalling cell volume reduction (Meryman and Williams, 1985).

Most cells do not possess a glycerol facilitated transport mechanism, and for them the transmembrane diffusion of glycerol is relatively slow with a half-time of the order of 3 to 4 min or more (Meryman and Williams, 1985). Because of the care that must be taken in the introduction and removal of glycerol, the times required can be of the order of 30 to 60 min to

achieve a concentration sufficiently high to result in good protection from freezing injury. Glycerol permeates plant cells poorly and this may explain why 60 min exposure of 2M glycerol + 0.4M sucrose was a more effective loading treatment than 20 min exposure in our study. In addition, multicellular organs such as shoot tips are more problematic to be cryopreserved than unorganized cells because they contain different cell types, each of which have a unique optimum set of conditions required to minimize the probability of intracellular ice formation and also because permeation is required across an array of cells.

Loading with cryoprotectants is not always required for the successful vitrification of plant materials. Langis et al. (1990), demonstrated that loading was not necessary for the successful vitrification of carnation shoot tips, while Steponkus et al. (1992) showed that loading was actually detrimental to potato shoot tips.

In summary, precultured shoot tips exposed to 2M glycerol + 0.4M sucrose (loading solution) and PVS2 (dehydrating solution) for 60 min and 16 min, respectively, gave the highest survival after vitrification (Table 3). Treated shoot tips without cooling retained high levels of survival (between 60 - 100%). However, survival levels of vitrified shoot tips were relatively poor to moderate (10 - 66%). As to be discussed later, the recovery medium was regular MS medium (containing $NH_4^+$). Compare results in Table 2, where survival following preculture and 2M glycerol + 0.4M sucrose (60 min) and PVS2 (16 min) was higher (90%). In this case, the recovery medium was MS ($NH_4^+$ - free).

Preliminary experiments included testing of other loading solutions such as combinations of ethylene glycol and sucrose. Also, the Steponkus Vitrification Solution (SVS) composed of 40% ethylene glycol, 15% sorbitol, 6% Bovine Serum Albumin and 29% water was tested.

However, the concentrations and exposure times tested were toxic (data not shown). These observations further suggest that shoot tips of sweet potato (PI 290657) are sensitive to osmotic stress. Extended exposure times of PVS2 at 0 °C, as contrasted to 25 °C used in all the other studies, did not enhance survival after cooling (data not shown). Lowering the temperature of the cryoprotectant and extending the exposure time potentially minimizes chemical toxicity (Fahy et al., 1987) but also lowers permeation kinetics of solutes and of water. Note, also at the time these preliminary trials were conducted, other steps (cooling, post-warm treatments) in the cryopreservation protocol were not yet optimized by the author. Hence, the results of the trials were preliminary and better survivals may be obtained with different cooling and preculture temperature regimes.

Cooling

The method and rate of cooling are important in any cryopreservation protocol (Mazur, 1984 ; Bajaj, 1995). Meristems of a number of plant species have survived sudden immersion in LN. Bajaj (1977b) reported survival of frozen potato meristems using an ultra-fast method of freezing. Later, similar observations were made by Grout and Henshaw (1978) with *Solanum gonicalyx.* These findings were further confirmed by Benson et al. (1989) and Harding et al. (1991), who reported that ultra-rapid cooling improved total survival and conserved the ability of potato shoot tips to develop into plants (as contrasted to callus formation).

Initial experiments in this study consisted of the use of filter paper strips for cooling (as were used by Towill and Jarret, 1992) . Following exposure to PVS2, shoot tips were placed on a small strip (ca. 5mm x 10mm) of sterile tissue paper saturated with PVS2. The paper strip was

folded to enclose the shoot tips, quenched in LN (cooling rate estimated at about 4000 °C $min^{-1}$ and held for about 15-30 min). Warming was achieved by rapidly transferring the paper strips from LN into 3 ml of 1.2M sucrose for 30min at 30 °C (warming rate estimated at about 9000 °C $min^{-1}$ ). However, I obtained no survival using this cooling method (data not shown)(culture was on regular MS medium). Cooling using copper grids[4] (same procedure as for paper strips) did result in some survival (data not shown), suggesting that a faster cooling rate was required for successful vitrification of shoot tips treated as above. Considerable variation in survival among repeated experiments was observed using the copper grids. No explanation is apparent but the small grids are difficult to manipulate. The following investigations concentrated on acquiring an ultra-rapid cooling rate.

Following exposure to PVS2, shoot tips were packaged in three different ways prior to plunging in solidified LN (slush) which was obtained by placing the LN in a vacuum chamber to increase the rate of evaporation so that the temperature is decreased below the boiling point of LN (-196 °C) to about -209 °C. Shoot tips were placed along with a small amount of PVS2 either on thin strips of sterile aluminum foil, in 0.2ml PVS2 in polypropylene straws, or in 1ml PVS2 in cryo-vials. These were then plunged into nitrogen slush. The exact cooling rate for the respective packaging systems was not measured. However, aluminum, coupled with the very small volume of PVS2 used would give the fastest cooling rate. Straws should cool faster than the cryo-vials. The faster cooling rates in LN slush compared to LN occur because immersion of a holder into slush does not produce the vapor barrier around the holder that occurs with immersion in LN.

Survival after cooling was relatively high for all packaging systems (Table 4). However, the highest survival was obtained in the shoot tips packaged in aluminum foil prior to LN slush exposure (P=0.0001). Survival of shoot tips packaged in vials (70%) was higher (not significantly) than those packaged in straws (56%); this latter result was not expected, as shoot tips packaged in the straws should potentially survive better because of the faster cooling and warming rates. This discrepancy may be due to the difficulty in removing the PVS2 from the straws immediately after warming and thus a slightly extended time of PVS2 exposure to shoot tips packaged in the straws than shoot tips packaged in the cryo-vials.

The added advantage of the use of the aluminum foil for packaging is in agreement with the hypothesis that fast cooling is commensurate with fast rewarming (MacFarlane, 1986). Also, the foil strips facilitated the handling of many shoot tips. Although both straws and cryo-vials were warmed in a 37 °C water bath, the PVS2 in the straws was observed to liquefy faster than in the cryo-vials, approximately 8 and 40 seconds, respectively. The exact rewarming rate in each straw and cryo-vial was not measured. A very rapid cooling rate will permit low, stabilizing temperatures to be reached before intracellular ice forms. A combination of high cryoprotectant concentration and hydrostatic pressure can permit vitrification of the entire specimen, thus circumventing all of the effects of ice formation (Meryman and Williams, 1985). And finally, rapid warming is necessary to prevent intracellular recrystallization (devitrification) i.e., the growth of small ice crystals into damaging large crystals (MacFarlane, 1986). Since under the usual conditions of cryoprotectant addition, the cellular contents are far from ideal with regard to

**Table 4. Effect of packaging system (cooling rate) on survival of vitrified sweet potato (PI 290657) shoot tips.**

| Packaging System[1] | Survival (% ± SE) | |
|---|---|---|
| | Cryoprotectant Exposed | Vitrified |
| Foil Strips | 100 | 100[a] |
| Straws | 100 | 55 ± 11[b] |
| Cryo-vials | 100 | 70 ± 15[b] |

[1] Following preculture and PVS2 exposure, shoot tips were placed :
(1) on thin strips of foil along with small drops of PVS2 and the foil strips were folded to enclose the shoot tips before cooling,

(2) in straws containing 0.2 ml PVS2 before cooling or

(3) in cryo-vials containing 1 ml PVS2 before cooling

Cryoprotectant exposed and cooled shoot tips were cultured on MS medium without $NH_4NO_3$ for 5 days prior to transfer to regular MS medium.

[a] Numbers within column followed by the same letter are not significantly different ($p<0.05$)

glass formation, even rapid cooling probably leads to the initiation of very small, and infrequent ice nuclei.

The observation that high survival occurred in vials suggests, however, that fast rates of cooling and warming are not the sole reason for better survival in foil as opposed to paper strips. The recovery here was on $NH_4^+$ - free MS medium (see below).

**Plant Environmental Conditions**

Another set of factors was examined to see their effect on viability after vitrification, with the intent of selecting the best conditions to give reproducible levels of survival amongst experiments.

Age of *In Vitro* Plants

Shoot tips excised from *in vitro* plants of various ages were compared for survival levels. There was no significant difference in survival after vitrification from shoot tips excised from 4, 8, 10 or 13 week old *in vitro* plants (Table 5). All treatments resulted in relatively high survival after vitrification (between 88-93%). There are sparse data for other species to corroborate this observation. Some data suggests that shoot tips for older *in vitro* plants of *Mentha* sp. and *Arachis glabrata* may survive vitrification protocols in higher levels (Towill, unpublished). The exponential growth stage of cell suspensions of various species shows the best survival after cryogenic treatment. (Sugawara and Sakai, 1974; Withers, 1985; Yoshida et al., 1993; Reinhoud et al., 1995).

**Table 5. Effect of age of *in vitro* grown sweet potato (PI 290657) source plants on survival after vitrification.**

| Age (weeks) | Survival (% ± SE) | |
|---|---|---|
| | Cryoprotectant Exposed | Vitrified |
| 4 | 100 | 93 ± 3[a] |
| 8 | 93 ± 3 | 90 ± 6[a] |
| 10 | 100 | 88 ± 8[a] |
| 13 | 88 ± 8 | 93 ± 3[a] |

Following exposure to cryoprotectant and vitrification, shoot tips were cultured on MS medium without $NH_4NO_3$ for 5 days prior to transfer to regular MS medium.

[a] Numbers within column followed by the same letter are not significant different ($p<0.05$)

Location of Shoot Tips Along Stem Axis

Since very little is known about the effect of the physiological and morphological state of the source plants on "cryopreservability", we decided to excise shoot tips located at different positions along the stems of 8 week old *in vitro* grown source plants. Each stem was divided into four sections to include shoot tips excised from (1) the apical bud, (2) just below the apical bud, (3) the center of the stem and (4) the base of the stem (Fig. 5). Stems varied in total number of buds, therefore, care was taken to select those stems consisting of the terminal bud plus 8 axillary buds. Survival of shoot tips decreased progressively from the terminal bud to the base of the plant where no survival was observed after vitrification (Table 6). Results are consistent with previous research on carnations (Dereuddre et al., 1988) but differ from mint, where there was no position effect (Towill, unpublished). Note, however, that survival was relatively poor to moderate for all treatments and this can be attributed to the growth recovery medium which contained $NH_4NO_3$ . For all the experiments, in order to obtain enough material, all buds, apical and axiallary were pooled for experiments and were randomized in a flask. Experiments with $NH_4^+$ - free media showed a high level of survival (Tables 1, 2, 4, 5 & see below). Thus these results are intriguing and may be further tested, but from a practical viewpoint, high levels of viability are reproducibly obtained from pooled buds when appropriately recovered.

Light Conditions

The effect of light conditions on *in vitro* source plants was also tested. Light may be an important determinant of shoot tip development before and after freezing and may contribute to the variable responses to cryopreservation often observed in sweet potato. Benson et al. (1989)

**Table 6. Survival of vitrified sweet potato (PI 290657) shoot tips excised from various locations along the stem axis.**

| Shoot Tip Location | Survival (% ± SE) | |
|---|---|---|
| | Cryoprotectant Exposed | Vitrified |
| Terminal bud | 95 ± 5 | 62 ± 13[a] |
| Below terminal bud | 40 | 45 ± 26[ab] |
| Center | 80 ± 11 | 28 ± 16[b] |
| Base | 80 ± 14 | 0[c] |

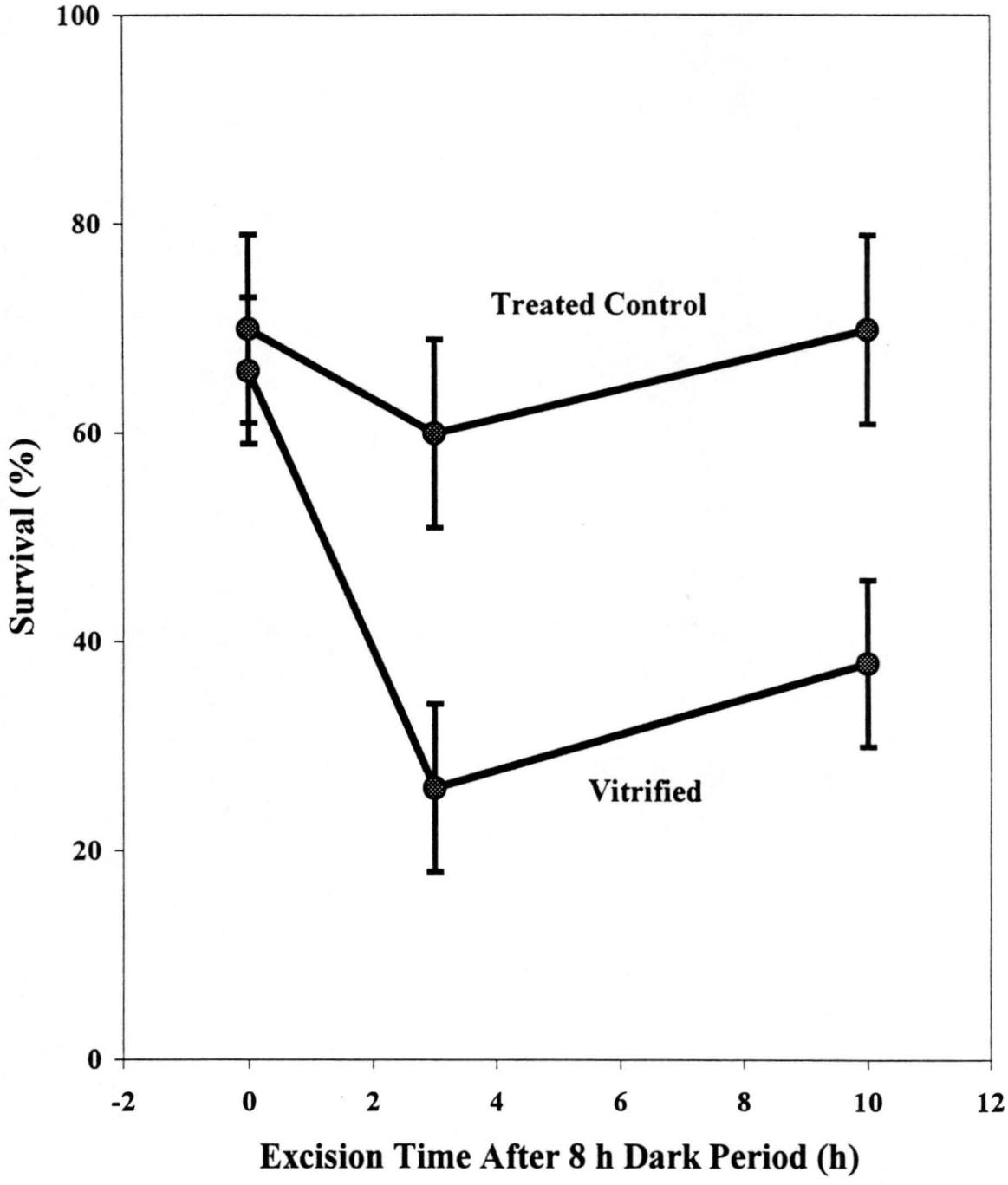
Following exposure to cryoprotectant and vitrification, shoot tips were cultured on regular MS medium (ie. containing $NH_4NO_3$).

[a] Numbers within column followed by the same letter are not significantly different ($p<0.05$)

showed that pre- and post -freeze light conditions can influence survival in two cultivars of *S. tuberosum*. Here, source plants were incubated under a 16/8 hr photoperiod and shoot tips were excised immediately after the 8 hr dark period, 3 or 10 hr in light after the dark period. Statistical analysis of these three variables showed that survival of shoot tips excised immediately after the 8 hr dark period (in darkness) was significantly better than the 3 or 10 hr light period (Fig. 7). These results seem to support our hypothesis that starch produced during the light photoperiod is converted to sucrose during the dark period. An accumulation of sucrose during the dark period may enhance survival of vitrified shoot tips. The argument for this is that sucrose can serve both to contribute to glass formation and to protect the functional and structural integrity of membranes and proteins upon severe dehydration (Crowe et al., 1990) (Fig.4). Many angiosperm seeds have been shown to adapt to desiccation by accumulating sucrose and larger oligosaccharides (Leopold, 1990). In fact, endogenous sucrose has been known to play a significant role in the tolerance of biological materials to desiccation for a long time (Crowe et al., 1987). However, further studies need to be done to determine the actual sucrose levels in the shoot tips at the various excisions times (in darkness and in light) and to determine the correlation between endogenous sucrose levels and survival of vitrified sweet potato shoot tips.

Other environmental conditions such as cold acclimation of cold hardy species (Nino et al., 1992; Matsumoto et al., 1995; Kuranuki and Sakai, 1995), heat shock treatment (Reinhoud et al., 1995) etc. prior to cryopreservation are in fact advantageous. Cold acclimation results in the accumulation of endogenous solutes that decreases the extent of cell dehydration at any given osmotic potential, contributes to the stabilization of proteins and membranes during extreme dehydration, and increases the glass-forming tendency of the cytoplasm (Steponkus et al., 1992).

**Fig. 7 Effect of Light Conditions on Survival of Sweet Potato Shoot Tips (PI 290657) Cryopreserved by Vitrification**




cryo-treated and vitrified shoot tips were cultured on regular MS medium

But the treatments are only beneficial for plants possessing the ability to cold acclimate. Sweet potato is very cold-sensitive and, hence, was not expected to benefit from cold treatments, but the potential for use of heat shock treatment is not known.

In Vitro vs. Growth Chamber Grown Source Plants

The vitrification protocol was tested for *in vitro* and growth chamber grown plants. Statistical analysis showed that survival after vitrification from *in vitro* grown plants was significantly higher (P=0.0009) than that of growth chamber grown plants (Table 7). The surface sterilization technique (see above) that was developed for the growth chamber plants was successful in controlling contaminants. However, both treated controls (shoot tips exposed to cryoprotectants but not cooled) and vitrified shoot tips of the growth chamber plants grew considerably slower than that of *in vitro* grown plants. This could be attributed to the added stress on the shoot tips from the sterilants used during the sterilization process. The problems of using shoot tips derived from growth chamber plants as compared with shoot tips of *in vitro* grown plants of this particular sweet potato line can be summarized as : the lower survival level, the slower recovery growth and the additional surface sterilization step. Thus these results show that our protocol does give survival for shoot tips from *in vivo* plants (which are somewhat different in size) albeit that application to these shoot tips probably will require some alteration of the steps of the protocol.

**Recovery Media for Shoot Tips**

These studies served a dual purpose, in that, regrowth, the most definitive viability assay, was used but most intriguingly, the recovery medium offers the means of repairing sublethal

**Table 7. Survival of vitrified shoot tips from *in vitro* and growth chamber grown sweet potato (PI 290657) source plants.**

| Source Plant | Survival (% ± SE) | | Time for Vitri. Process (hr) |
|---|---|---|---|
| | Cryoprotectant Exposed | Vitrified | |
| *In vitro* | 100 | 93 ± 4[a] | 3.5 |
| Growth chamber | 86 ± 5 | 62 ± 6[b] | 5.0 |

Following exposure to cryoprotectant and vitrification, shoot tips were cultured on MS medium without $NH_4NO_3$ for 5 days prior to transfer to regular MS medium.

[a] Numbers within column followed by different letters are significantly different ($p<0.05$)

injury. The concept of maximizing the post-warm repair of damaged sites has, thus far, hardly been studied. There are indications that recovery from damage does take place (Kuriyama et al., 1989; 1996; Anthony et al., 1996). Studying how the degraded molecules and structures of stressed and damaged tissues are reorganized and reassembled and how the repair process could be enhanced is an open field of future research.

Mineral Composition

Following the vitrification process, shoot tips were cultured on solid MS recovery media with various modifications. MS medium was supplemented with 0.045mg/ml BAP, 0.01mg/ml IBA and 3% sucrose with or without various ions ($NH_4^+$, $NO_3^-$, $Fe^{2+}$). After 5 days of culture on the modified media, shoot tips were transferred to regular MS (containing all salts) medium. Survival of shoot tips cultured on MS ($NH_4NO_3$-free) was threefold over those cultured on regular MS (Table 8). It is unknown why the survival level of cryo-exposed (but not cooled) shoot tips on the MS media was much lower than other experiments but it shows that exposure to the regime is, itself, injurious, and may be a component of experiment-to-experiment variation. $KNO_3$ was added to increase the $NO_3^-$ level to that in regular MS, however, extra $KNO_3$ did not make any difference in survival to that of MS ($NH_4NO_3$ - free), suggesting that the detrimental ion is $NH^+_4$ and not $NO^-_3$. These results are similar to that of previous research on rice cells (Kuriyama et al 1989) and *Lavandula vera* cells (Kuriyama et al., 1996) and are consistent with their observation of increased survival from shoot tips recovered on MS ($NH_4NO_3$ - free) as compared to recovery on regular MS. However, shoot tips that were left on MS ($NH_4NO_3$ - free) for a prolonged period (4 weeks) grew very slowly and were purplish in color. These observations indicate that ammonium ions inhibited recovery from cooling injury, but that

**Table 8. Effect of various recovery media on the viability of vitrified sweet potato (PI 290657) shoot tips.**

| Medium | Survival (% ± SE) | |
|---|---|---|
| | Cryoprotectant Exposed | Vitrified |
| MS (control) | 39 ± 1 | 28 ± 4[c] |
| MS ($Fe^{2+}$ - free) | 72 ± 5 | 60 ± 6[b] |
| MS ($NH_4NO_3$ - free) | 96 ± 4 | 85 ± 6[a] |
| MS ($NH_4NO_3$ - free + $KNO^1_3$) | 100 | 85 ± 6[a] |

[1] $KNO_3$ was added to increase the $NO_3$ level to that in MS

[a] Numbers within column followed by the same letter are not significantly different ($p<0.05$)

ammonium ions were eventually needed for normal growth of both non-cooled shoot tips and shoot tips that had recovered from cooling injury. These results suggest that ammonium ions, one of the major components of MS medium, have a deleterious effect on cryopreserved sweet potato shoot tips and that at least some part of the cooling injury is not lethal but is repairable in the absence of ammonium ions. Ethylene, ethane and other volatiles are produced during stress and cell death and could be measured to assess viability and stability during culture (Benson and Withers, 1988). Survival on MS ($Fe^{2+}$ - free) medium showed a similar trend, (but to a lesser degree) to that of MS ($NH_4NO_3$ - free) treatment. This was the case for all repeated experiments in this particular study. Probably, like $NH_4^+$, $Fe^{2+}$, inhibits recovery from cooling injury. Transition metals especially $Fe^{2+}$ have a major role in free radical mediated oxidative stress (Fenton reaction). The Fenton reaction involves the breakdown of lipid peroxides giving rise to toxic aldehydes. Further research should include gas chromatography studies to determine measurements of volatiles over MS ($NH_4^+$ & $Fe^{2+}$ - free) cultures versus regular MS cultures.

Hormonal Content

Towill and Jarret (1992) and Schnabel-Preikstas et al. (1992) both reported some success of vitrification of sweet potato shoot tips. However, a major limitation in both cases was the formation of a high percentage of callus from vitrified shoot tips. Callus formation is usually unwelcome as it potentially increases the risk of somaclonal variation if shoots develop from adventitious buds.

Following warming, shoot tips were cultured on MS ($NH_4NO_3$-free) for 5 days and subsequently transferred to regular MS medium for initiation of shoots directly from cryo-treated shoot tips. Shoot tips were cultured on MS medium supplemented with 3% sucrose and different

concentrations of NAA, BAP and kinetin respectively. Shoot tips cooled to ca. -200 °C and subsequently cultured on MS medium supplemented with 1 μM NAA, 0.5 μM BAP and 0.1 μM kinetin initiated shoots without intermediary callus formation ($\chi^2 = 80.00$) (Table 9). However, subsequent growth was slowed considerably. Shoots were kept on this medium for 4 weeks after which they were transferred to MS basal medium without hormones. Non- treated shoot tips gave similar results but growth was more vigorous than PVS2-treated and cryo-treated shoot tips.

Surfactant/Antioxidant

Pluronic F-68 is a nonionic polyoxyethylene (POE)-polyoxypropylene (POP) surfactant that has been employed extensively as a cell protecting agent in both animal (Handa-Corrigan et al., 1992; Papoutsakis et al., 1991) and plant culture systems (Anthony et al., 1994; 1996; Elibol, 1996). Pluronic F-68 acts as a foam-stabilizing agent in agitated/aerated cultures and is believed to protect cells against fluid-mechanical damage. The effect of Pluronic F-68 in the recovery medium was tested. Following warming, shoot tips were cultured on MS medium supplemented with 0.045 mg/ml BAP, 0.01 mg/ml IBA, 3% sucrose and Pluronic F-68 at 0.01 or 0.1 %. Pluronic F-68 was filter sterilized and added to the medium after autoclaving. Both concentrations of Pluronic F-68 in the recovery medium resulted in relatively high survival rates (84% and 78% respectively) of shoot tips cooled to ca. -200 °C over vitrified shoot tips cultured on regular MS (28% and 50% respectively) (Table 10). It is believed that Pluronic F-68 promotes the increased uptake of nutrients, growth regulators, or oxygen into cells during the post-warm period. This would be expected to alter metabolic flux, allowing biochemical pathways to operate more efficiently, particularly under the stress conditions of early post-warm recovery

**Table 9. Initiation of shoots from vitrified sweet potato (PI 290657) shoot tips cultured on various media**

| Media | Growth pattern | |
|---|---|---|
| | Callus | Shoots |
| I[1] | + | - |
| II[2] | - | + |
| III[3] | +++ | - |

$\chi^2 = 80.00$

[1] MS medium supplemented with 0.54$\mu$M NAA, 4.4$\mu$M BAP & 4.5$\mu$M Kinetin

[2] MS medium supplemented with 1$\mu$M NAA, 0.5$\mu$M BAP & 0.1 $\mu$M Kinetin

[3] MS medium supplemented with 1$\mu$M NAA, 5 $\mu$M BAP & 1$\mu$M Kinetin

+ moderate growth

+++ very heavy callus growth

**Table 10. Survival of vitrified sweet potato (PI 290657) shoot tips recovered on two conc. of pluronic F-68 in the culture medium[1].**

| | Pluronic F-68 conc. (%) | Survival (% ± SE) | |
|---|---|---|---|
| | | Cryoprotectant Exposed | Vitrified |
| Exp. 1. | 0.0 (control) | 80 ± 6 | 28 ± 8[b] |
| | 0.01 | 100 | 84 ± 7[a] |
| Exp. 2. | 0.0 (control) | 73 ± 12 | 50 ± 10[a] |
| | 0.10 | 73 ± 6 | 78 ± 8[a] |

[1] Culture medium was the regular MS medium (containing $NH_4NO_3$) with or without Pluronic F-68.

[a] Numbers within column in each experiment followed by the same letter are not significantly different ($p<0.05$)

(Anthony et al., 1996). Further studies are required to determine the mechanism(s) by which surfactants, such as Pluronic F-68, can facilitate post-warm survival and growth of plant cells. Since these data were obtained on a complete MS (with $NH_4NO_3$) medium, it is obvious that several treatments may have the ability to alleviate injury from the injuring parts of the cryogenic protocol.

In preliminary experiments, ascorbic acid at 1 mg/l and 10 mg/l was also tested in the recovery medium. However, the concentrations tested in combination with others factors did not improve survival (data not shown). Studies on mammalian tissues have shown that the addition of antioxidants and compounds which reduce the production of free radicals can improve survival and function on return to normal temperatures ( Gower et al., 1987). Similar practices have proved useful in the recovery from low temperature stored seeds (Benson, 1990).

**Other Sweet Potato Lines**

After having looked at the various factors that affect cryopreservation, and using only the optimum conditions that resulted in best survival for each factor, we tested 3 other sweet potato lines using the same vitrification protocol. As shown in Table 11, survival was relatively high, ranging from 62% - 97%. This therefore implies that the cryopreservation protocol developed in this study is applicable to other sweet potato lines and proves once more that vitrification does offer a greater potential for developing procedures with broad applicability or, in the least, requiring only minor modifications for different genotypes. However, extended studies using additional sweet potato lines need to be investigated.

**Table 11. Survival of shoot tips from four sweet potato lines after cooling to ca. -200 °C.**

| Line | Survival (% ± SE) | |
|---|---|---|
| | Cryoprotectant Exposed | Vitrified |
| PI 290657 | 100 | 97 ± 3 |
| PI 573324 | 100 | 83 ± 6 |
| PI W-235 | 77 ± 6 | 63 ± 8 |
| PI 508515 | 64 ± 4 | 62 ± 8 |

Based on results of previous experiments by the author, optimum conditions were utilized :

- 4 week old *in vitro* plants excised immediately after the 8 hr dark period
- shoot tips precultured in MS containing 2% sucrose for 24hr and 0.3M sucrose for another 24 hr
- shoot tips loaded with 2M Gly + 0.4M suc. for 1hr and dehydrated with PVS2 for 16 min at 22 °C
- shoot tips placed in aluminum foil and quenched in solidified LN (slush)
- warming / dilution in 1.2 M sucrose for 20 min at 22 °C
- shoot tips cultured on MS without $NH_4NO_3$ for 5 days and then transferred to regular MS.

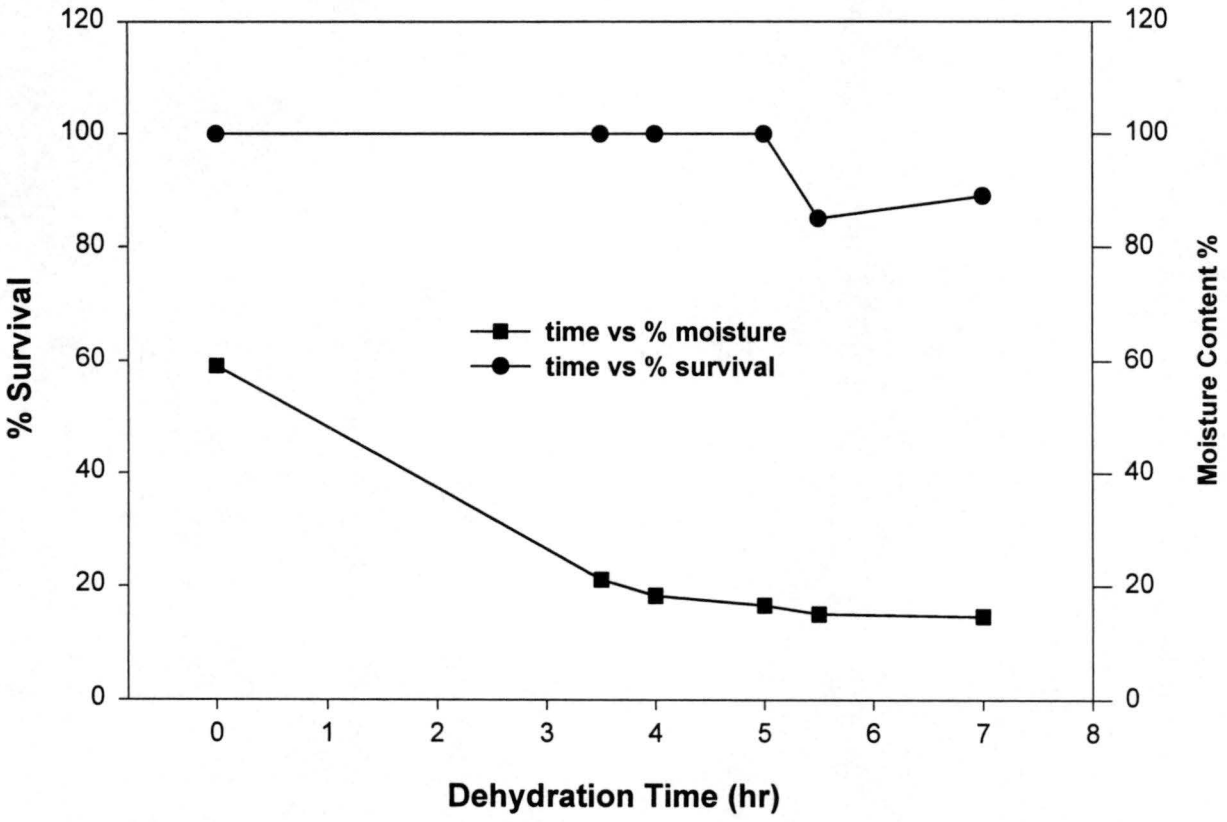
**Encapsulation-Dehydration Vitrification Procedure**

This technique has been successful with a number of species and is applicable to several different culture systems (Dereuddre et al., 1990; Plessis et al., 1993; Blakesley et al., 1995; Mandal et al., 1996). Like the solution-based vitrification technique, encapsulation / dehydration offer additional possibilities for plant materials that do not survive using the conventional two-step cooling procedures. However, this procedure simplifies the cryopreservation protocol by easier handling of cells, simplification of the cryoprotective agents, with sucrose alone as cryoprotectant and elimination of slow cooling devices. However, survival levels of cryopreserved sweet potato (PI 290657) shoot tips were greater for shoot tips vitrified by the solution-based vitrification method than the encapsulation /dehydration vitrification method, but the success observed suggests that more detailed study might yield a more useful, efficient procedure.

Dehydration Studies

Encapsulated shoot tips were precultured in sucrose-enriched medium for 6 days. Sucrose concentration was increased in a stepwise manner : 0.3M, 1 day; 0.5M, 1 day; 0.75M 1 day and 1.0M 3 days. At the end of the preculture period, shoot tip-containing beads were rapidly surfaced-dried by blotting with sterile tissue paper and were subjected to dehydration in a laminar flow hood for up to 7hours. At the end of 1M sucrose preculture, 100% survival was observed (0 hr air-drying) (Fig.8). Thus progressive increase in sucrose concentration to 1M as proposed by Plessis et al. (1991; 1993) for cryopreservation of grapevine shoot tips gave good survival for sweet potato (PI 290657) shoot tips. After increased air drying periods, survival slowly decreased to 89% (7 hr air-drying). This lower survival level seemed unrelated to the

**Fig. 8 Changes in moisture content and subsequent survival of encapsulated sweet potato (P1290657) shoot tips after dehydration as a function of dehydration duration (0 to 7 hr)**




Following dehydration, shoot tips were cultured on MS medium without $NH_4NO_3$ for 5 days prior to transfer to regular MS medium.

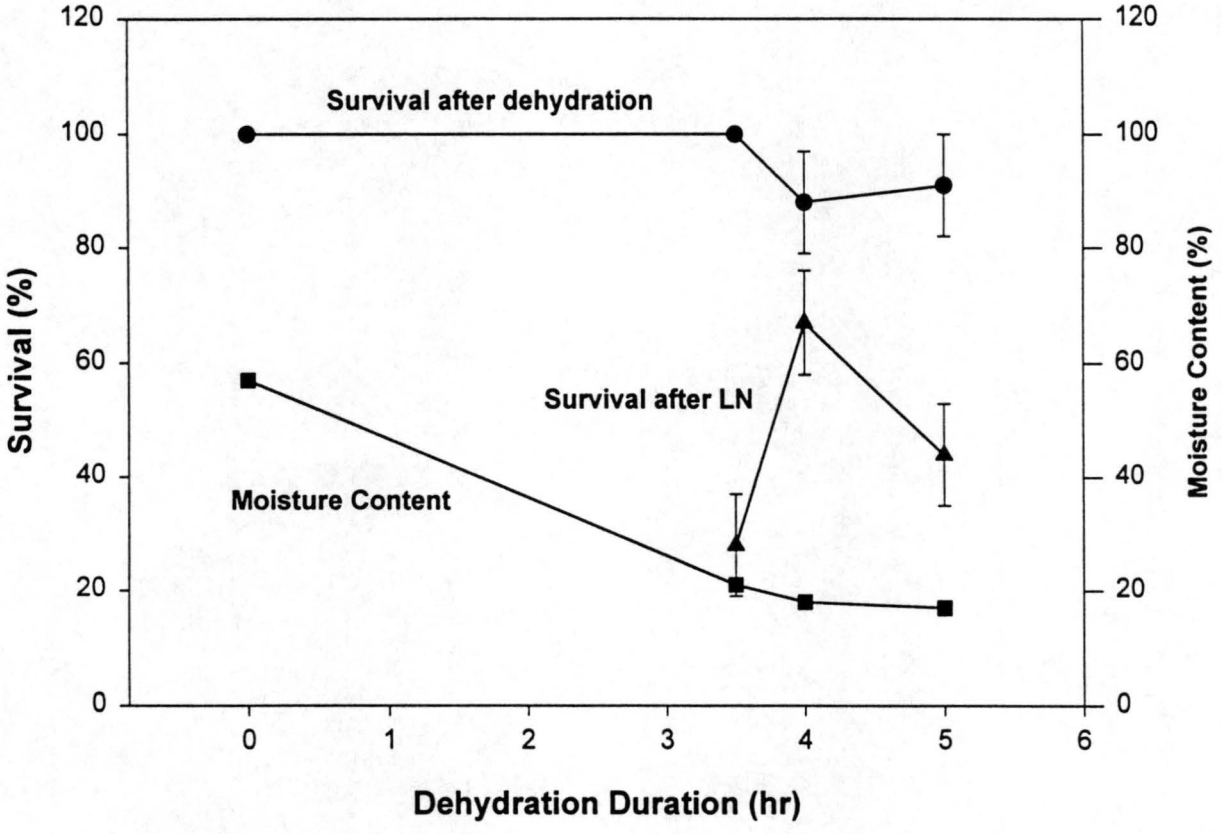
sensitivity of the shoot tips to preculture at high sucrose concentration since all shoot tips survived preculture up to 1M. During air-drying of 0 to7 hours the moisture content (MC) of the beads decreased from 59% to 14.7%.

Several preliminary experiments were conducted which included the comparison of a sucrose preculture directly and exposure in a step-wise manner. Observations showed that shoot tips precultured in 0.75M sucrose in a stepwise increase gave greater survival after 4 hr dehydration than shoot tips precultured in 0.75M sucrose directly (data not shown). Dehydration over silica gel and under the laminar flow hood was compared. Dehydration over silica gel was achieved much faster than under the laminar flow hood for the same dehydration duration (data not shown). The implication of the method of dehydration is important as rapid removal of water from the beads (as in the case of dehydration over silica gel) may eventually lead to a cork formation of the beads thus preventing further dehydration of the interior of the bead and subsequently the encapsulated shoot tip.

Dehydration and Cooling

Survival after dehydration and cooling in solidified LN, was also studied in relation to dehydration duration (0 to 5 hr air-drying) (Fig. 9). Encapsulated shoot tips were precultured in the same manner as above (Dehydration Studies). Percent moisture content of the beads over a 5 hour dehydration duration produced a similar trend as observed in the previous experiment. However, after 5 hours air-drying time, survival after dehydration was lower (91%). After cooling to ca. -200 °C, following 4 hr dehydration, survival was 67%. Further drying, up to 5 hr decreased the survival rate after cooling (44%). The highest survival (67%) of vitrified

**Fig. 9 Survival of vitrified encapsulated sweet potato (PI 290657) shoot tips after 5 hr dehydration**



encapsulated shoot tips occurred after 4 hr dehydration corresponding to 18.1% MC (Fig. 9). For encapsulated sweet potato shoot tips (PI 290657), a moisture content of about 18% seemed to be optimum for survival of shoot tips precultured in 1M sucrose after a stepwise increase following rapid cooling and rewarming. However, getting the same moisture content to coincide with a given dehydration duration for encapsulation shoot tips precultured in the same manner was problematic. In order to alleviate this problem, further studies are required to dehydrate encapsulated shoot tips over certain relative humidities. Note also, that several preliminary experiments were conducted looking at preculture concentration, duration, direct preculture vs. stepwise increase but all seemed inconclusive. It seemed that following a faster cooling rate (use of LN slush) and the use of MS ($NH_4^+$-free) medium, survival was eventually achieved using the encapsulation / dehydration technique.

In preliminary experiments, three cooling procedures were tested for encapsulated shoot tips precultured in sucrose (stepwise increase up to 0.7M) and held for 2 days. The encapsulated shoot tips were subsequently dehydrated under the laminar flow hood for up to 3.5 hr. Cooling was done by : (1) using the two-step cooling method, where samples were progressively cooled to (0.5 °C $min^{-1}$) from -5 °C to -40 °C before immersion in LN, (2) placing the encapsulated shoot tips in cryo-vials followed by immersion in LN and (3) direct quenching of the encapsulated shoot tips in LN. No survival was obtained after any of the cooling procedures tested (data not shown), but note, culture was on $NH_4^+$ containing MS medium.

This study constitutes the first reported attempt at using the encapsulation / dehydration technique to cryopreserve shoot tips of sweet potato. Although the results obtained in this study

are encouraging, improving the conditions for dehydration and cryoprotection could reduce their variability. More precise relative humidity control would allow a closer correlation of the dehydration period and bead moisture content in order to standardize experimental conditions and improve reproducibility. These findings, indicate that sweet potato (PI 290657) can tolerate dehydration to as low as 14.7 % MC (Fig. 8). It would be interesting to apply this encapsulation / dehydration technique to other sweet potato lines.

# CONCLUSIONS

The vitrification procedure, as described in this study, was successful for the cryopreservation of sweet potato shoot tips. The potential problems associated with the vitrification solution (osmotic stress or chemical toxicity and cooling as well as possible injury caused by the mechanical damage of the cells due to fracturing of the glass) were almost completely overcome by preconditioning, sucrose preculture, cryoprotectant loading with glycerol + sucrose, rapid cooling and warming and post-warm treatments.

Shoot tips excised from *in vitro* plants soon after the 8 hr dark period showed increased survival levels over those excised from *in vitro* plants incubated in the light prior to excision. Sucrose preculture treatment had a profound effect on survival during vitrification of sweet potato shoot tips. Sucrose concentration was critical whereby 0.3M resulted in optimum survival while 0.75M sucrose was in fact detrimental when used with the cryoprotectant regime. An ABA preculture treatment, (0.1 mg/l and 1.0 mg/l ABA supplemented to MS + 0.3M sucrose) showed an increase in survival over the non ABA treatments only after 48 hr in preculture while 24 hr in preculture appeared insufficient time to induce dehydration tolerance.

Highest survival was obtained using 2 M glycerol + 0.4 M sucrose as a 'loading' solution for 1 hr and dehydrating with concentrated PVS2 for 16 at 22 °C. Loading with 20% PVS2 resulted in lower survival levels than both the glycerol / sucrose combination and no loading at all.

Failure to obtain any survival using cryo-vials or paper strips plunged in LN suggested that a faster cooling rate was essential. Highest survival was obtained by placing the shoot tips in strips of aluminum foil and plunging in solidified LN (slush). But relatively good survival (70%) was also obtained using the cryo-vials plunged in LN slush, providing a suitable recovery medium was used.

The most important finding in this study was the fact that survival was always greater (sometimes up to three-fold) for vitrified shoot tips cultured on MS ($NH_4^+$ - free) medium over the regular MS medium. The use of other modified recovery medium such as the addition of Pluronic F-68 at 0.1% and 1.0% also showed an increase in survival level over the controls. These results show that the cryopreservation protocol is injurious but that the injury is repairable if materials are suitably treated in the recovery phase. There are, of course, numerous interactions. Thus, for example, even sub-optimally cooled shoot tips (in a cryo-vial) may recover if appropriately treated post - warming.

Previous attempts to develop a vitrification procedure for the cryopreservation of sweet potato shoot tips of PI 290657 and PI 508515 as reported by Jarret and Towill (1992) were only partially successful in that survival after cooling was variable and often limited to callus formation. This improved procedure gave the resumption of shoots without excessive callus formation. However, subsequent growth was slowed considerably. Additional research is needed to modify the growth medium to obtain a better growth rate.

The highest survival following encapsulation / dehydration vitrification procedure was 67%, lower than that obtained following the solution-based vitrification technique (93% -100%). Since sweet potato shoot tips were found to be recalcitrant to the conventional cryopreservation

procedures, it is encouraging to find out that relatively high survival is possible using two other cryopreservation techniques.

Two major limitations of the cryopreservation of sweet potato shoot tips previously reported were: (1) the variation among response of a given sweet potato line and (2) the variation amongst sweet potato lines. Generally, cryopreservation of plant cells is often very species and genotype specific. The use of a modified vitrification protocol for sweet potato shoot tips may be applicable to a wider range of genotypes. However, our goal to generate a reproducible shoot tip cryopreservation protocol for sweet potato was somewhat fulfilled since relatively high levels of survival were achieved for four sweet potato lines tested.

## REFERENCES

Anthony, P., Davey, M. R., Power, J. B., Washington, C. and Lowe, K. C. (1994) Synergistic enhancement of protoplast growth by oxygenated perfluorocarbon and pluronic F-68. *Plant Cell reports* 13 : 251-255.

Anthony, P., Jelodar, N. B., Lowe, K. C., Power, J. B., Davey, M. R. and Washington, C. (1996) Pluronic F-68 increases the post-thaw growth of cryopreserved plant cells. *Cryobiology* 33 : 508-514.

Angell, C. A. and MacFarlane, D. R. (1982) Conductimetric and calometric methods for the study of homogeneous nucleation below both $T_h$ and $T_g$. *Advances in Ceramics IV*, Ohio Univ. Press, pp 66-76.

Angell, C. A., Sare, E. J., Donnella, J. and MacFarlane, D. R. (1981) Homogeneous nucleation and glass transition temperatures in solutions of Li salts in $H_2O$ : A doubly unstable glass region. *J. Phys. Chem.* 85 : 1461-1464.

Austin, D. F. (1988) The taxonomy, evolution and genetic diversity of sweet potato and related wild species. In *Exploration and maintenance and utilization of sweet potato genetic resources*, first planning conference, Lima, Peru. International Potato center (CIP), pp 27-59.

Bachiri, Y., Gazeau, C., Hansz, J. Morisset, C. and Dereuddre, J. (1995) Successful cryopreservation of suspension cells by encapsulation-dehydration. *Plant Cell Tissue and Organ Culture* 43 : 241-248.

Bajaj, Y. P. S. (1977b) Initiation of shoots and callus from potato tuber-sprouts and axillary buds

frozen at -196 °C. *Crop Improv.* 4 : 48-53.

Bajaj, Y. P. S. (1985a) Cryopreservation of germplasm of potato (*Solanum tuberosum* L.) and Cassava (*Manihot esculentum* Crantz): viability of excised meristems cryopreserved up to four years. *Indian J Exp Biol* 23: 285-287.

Bajaj, Y. P. S. (1991) Storage and cryopreservation of *in vitro* cultures. In *Biotechnology in Agriculture and forestry,* 17. High-tech and micropropagation, I. ed Y. P. S. Bjaj, Spring-Verlag, Berlin, Heidelberg New York, pp 361-381.

Bajaj, Y. P. S. (1993) A suggested method for *in vitro* long-term storage at 4 C of *Chrysanthemum* and *Petunia* germplasm. *Plant Tissue Culture* 3: 57-58

Bartels, D., Singh, M. and Salamini, F. (1988) Onset of desiccation tolerance during development of the barley embryo. *Planta* 175 : 485-492

Benson, E. E. and Withers, L. A. (1988) Gas chromatographic analysis of volatile production by cryopreserved plant tissue cultures : a non destructive method for assessing stability . *Cryo-Letters* 8 : 35-46.

Benson, E. E., Harding, K. and Smith, H. (1989) Variation in recovery of cryopreserved shoot-tips of *Solanum tuberosum* exposed to different pre- and post-freeze light regimes. *Cryo-Letters* 10 : 323-344.

Benson, E. E. (1990) Free radical damage in stored plant germplasm, International Board for Plant Genetic Resources (publishers), Rome, Italy.

Blakesley, D., Al-Mazrooei, S. and Henshaw, G. G. (1995) Cryopreservation of embryogenic tissue of sweet potato (*Ipomoea batatas*) : use of sucrose and dehydration for

cyroprotection. *Plant Cell Reports* 15:259-263

Bouafia, S., Jelti, N., Lairy, G., Blanc, A., Bonnel, E. and Dereuddre, J. (1996) Cryopreservation of potato shoot tips by encapsulation-dehydration. *Potato Research* 39 : 69-78.

Burke, M. J. (1986) The glassy state and survival of anhydrous biological systems. *In*: Leopold, A. C. (Ed.), *Membrane, Metabolism and Dry Organisms*, Cornell Univ. Press, Ithaca, N.Y. pp. 358-364.

Carpenter, J. F., Crowe, J. H. and Arakawa, T. (1990) Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states. *J Diary Sci.* 73 : 3627 - 3636.

Chin, H. F. and Roberts, E. H. (1980) *Recalcitrant Crop Seeds*, Tropical Press, Kuala Lumpur.

Clark, R. L. and Roath, W. W. (eds) (1989) *U. S. Plant Germplasm System Science and Technology* (*Plant Breeding Reviews*, Vol. 7) Timber Press, Portland, Oregon.

Crowe, J. H., Crowe, J. F., Carpenter, L. M. and Wistrom, C. A. (1987) Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochem.* J. 242 : 1-10.

Crowe, J. H., Crowe, J. F., Carpenter, L. M., Rudolph, A. S., Wistrom, C. A., Spargo, B. J. and Anchordoguy, T. J. ( 1988) Interaction of sugars with membranes. *Bioche. et Biophy. Acta* 947 : 367-384.

Crowe, J. H., Whittam, M. A., Chapman, D. and Crowe, L. M. (1984) Interactions of phospholipids monolayer with carbohydrates. *Biochemica et Biophysica Acta.* 769 : 151-159

Crowe, J. H., Carpenter, J. F., Crowe, L. M. and Anchordoguy, T. J. ( 1990) Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. *Cryobiology* 27 : 219-231.

Dereuddre, J., Fabre, J. and Bassaglia, C. (1988) Resistance to freezing in liquid nitrogen of carnation (*Dianthus caryophyllus* L. Var Eolo) axillary shoot tips excised from different aged *in vitro* plantlets. *Plant Cell Reports* 7 : 170-173.

Dereuddre, J., Hassen, N., Blandin, S. and kaminski, M. (1991) Resistance of alginate coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen : 2 Thermal analysis. *Cryo-Letters* 12 : 135-148.

Dereuddre, J., Scottez, C., Arnaud, Y. and Duron, M. (1990) Resistance d'apex caulinaries de vitroplants de poirer (*Pyrus cummunis* L. cv. Beurre Hardy), enrobes dans l'alginate, a une deshydratation puis une congelation dans l'azote liquide: effet d'un endurcissement prealable au froid. C. R. Acad. Sci. Paris, serie III. 310 : 317-321.

Dodds J.H., Silva-Rodriquez, D. and Tovar, P. (1992) Micropropagation of potato (*Solanum tuberosum L.*). In Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 19. High-tech and micropropagation III. Springer, Berlin Heidelberg New York, pp 91-106

Eberhart, S. A., Roos, E. E. and Towill, L. E. (1991) Strategies for long-term management of germplasm collections. In *Genetics and Conservation of Rare Plants*, ed. D. A. Falk and K. E. Holsinger, Oxford University Press, New York, pp. 135-152.

Elibol, M. (1996) Use of perfluorocarbon in the culture of *Saccharomyces cerevisiae. Biotechnology techniques* 10 : 987-990.

Englemann, F. (1991) *In vitro* conservation of tropical plant germplasm-a review.

*Euphytica* 57: 227-243.

FAO (1978) Production Yearbook, Vol. 41 Rome, Italy.

Fabre, J. and Dereuddre, J. (1990) Encapsulation dehydration : a new approach to cryopreservation of *Solanum* shoot tips. *Cryo-lett.* 11 : 413-426.

Fahy, G. M. and Hirsh, A. (1982) Prospects for organ preservation by vitrification. In *Organn Preservation, Basic and Applied Aspects* eds. D. E. Pegg, I. A. Jacobsen , N. A. Halasz, pp 399-404 MTP Press, Lancaster.

Fahy, G. M., MacFarlane, D. R., Angell, A. C. and Meryman, H. T. (1984) Vitrification as an approach to cryopreservation. *Cryobiol.* 21, 407-426.

Fahy, G. M., Levy, D. I. and Alli, S .E. (1987) Some emerging principles underlying the physical properties, biological actions and the unity of vitrification solutions. *Cryobiology* 24: 196-213.

Farrant, G. D., Elsden, R. P. and Seidel, G. E. Jr. (1982) Effect of slow cooling of bovine embryos prior to plunging in liquid nitrogen. *Theriogenology* 17 : 88 (Abstr.).

Finkle, B. J., Zavala, M. E. and Ulrich, J. M. (1985) Cryoprotective compounds in the viable freezing of plant tissues. In *Cryopreservation of Plant Cells and Organs*, ed K. K. Kartha, CRC Press, Boca Raton, FL. pp 75-113.

Gower, J. D., Fuller, B. J. and Green, C. J. (1987) Lipid peroxidation in the cortex and medulla of rabbit kidneys subjected to cold ischaemia and the value of protective agents. *Free Rad. Res. Commun.* 3 : 107-115.

Grout, B. W. W. and Henshaw, G. G. (1978) Freeze preservation of potato shoot tip culture. *Ann. Bot.* 42 : 1227-1229

Handa-Corrigan, A., Zhang, S. and Brydges, R. (1992) Surface-active agents in animal cell cultures. In *Animal Cell Biotechnology,* eds R. E. Spier, J. B. Griffiths, Academic Press, London, Vol 5, pp 279-301.

Harding, K., Benson, E. E. and Smith, H. (1991) The effects of pre-freeze *in vitro* culture period on the recovery of cryopreserved shoot tips of *Solanum tuberosum* *Cryo-Letters* 12 : 17-22.

Haskins, R. H. and Kartha, A. A. (1980) Freeze preservation of pea meristem :cell survival. *Can J. Bot.* 58 : 833-840.

Hirsh, A. G. (1987) Vitrification in plants as a natural form of cryopreservation. *Cryobiology* 24 : 214-228.

Jarret, R. L. And Gawel, N. (1991) Chemical and environmental growth regulation of sweet potato (*Ipomoea batatas* (L.) Lam.) *in vitro*. *Plant Cell Tissue Org. Cult.* 25 : 153-159.

Jones, A. (1990) Unreduced pollen in a wild tetraploid relative of sweet potato. *J. Amer. Soc. Hort. Sci.* 115 : 512-516.

Kendall, E. J., Kartha, K. K., Qureshi, J. A. and Chermak, P. (1993) Cryopreservation of immature spring wheat zygotic embryos using abscisic acid pretreatment. *Plant Cell Rep.* 12 : 89- 94.

Kim, Y. H. and Janick, J. (1989) ABA and polyox-encapsulation or high humidity increases survival of desiccated somatic embryos of celery. *HortScience* 24 : 674-676.

Kuranuki, Y. and Sakai, A. (1995) Cryopreservation of *in vitro* grown shoot tips of tea (*Camelia sinensis*) by vitrification. *Cryo-Letters* 16 : 345-352.

Kuriyama, A., Watanabe, K., Ueno, S. and Mitsuda, H. (1989) Inibitory effect of ammonium ion on recovery of cryopreserved rice cells. *Plant Science* 64 : 231-235.

Kuriyama, A., Watanabe, K., Kawata, K., Kawi, F. and Kanamori, M. (1996) Sensitivity of cryopreserved *Lavadula vera* cells to ammonium ion. *J. Plant Physiol.* 148 : 693-695.

Langis, R. and Steponkus, P. L. (1990) Cryopreservation of rye protoplasts by vitrification. *Plant Physiol.* 92 : 666-671.

Langis, R., Schnabel-Preikstas, B., Earle, E. D. and Steponkus, P. L. (1990) Cryopreservation of carnation shoot tips by vitrification. *Cryobiology* 27 : 657-658.

Latta R (1971) Preservation of suspension cultures of plant cells by freezing. *Can. J. Bot.* 49: 1253-1254

Leibo, S. P. and Mazur, P. (1978) Methods for the preservation of mammalian embryos by freezing. In *Methods in mammalian Reproduction*, ed J. C. Daniel Jr., Academic press, New York, pp 179-201.

Leopold, A. C. (1990) Coping with desiccation. In *Stress Responses in Plants : Adaptation and Acclimation Mechanisms*, eds R. G. Alsher, J. R. Gummings, Wiley-Liss Inc., New York, pp 37-56.

Levitt, J. (1980) Responses of plants to environmental stresses: chilling, freezing and high temperature stresses, vol. 1. New York and London Acad. Press.

Luyet, B. (1937) The vitrification of organic colloids and of protoplasm. *Biodynamyca*, Vol. 1 (No. 29) 1-14.

MacFarlane, D. R. (1986) Devitrification in glass forming aqueous solutions. *Cryobiology* 23 : 230-244.

MacFarlane, D. K. and Forsyth, M. (1990) Recent insights on the role of cryoprotective agents in vitrification. *Cryobiology* 27 : 345-358.

MacFarlane, D., Forsyth, A. and Barton, C. A. (1992) Vitrification and devitrification in cryopreservation. In *Advances in Low Temperature Biology,* ed. P. Steponkus, Vol. 1 JAI Press, London, pp 221-277.

Mandal, B. B., Chandel, K. P. S. and Dwivedi, S. (1996) Cryopreservation of yam (*Dioscorea* spp.) shoot apices by encapsulation-dehydration. *Cryo-Letters* 17 : 165-174.

Matsumoto, T., Sakai, A. and Yamada, K. (1995) Cryopreservation of *in vitro* grown apical meristems of lily by vitrification. *Plant Cell, Tissue and Organ Culture* 41 : 237-241.

Matsumoto, T., Sakai, A. and Yamada, K. (1994) Cryopreservation of *in vitro* grown apical meristems of Wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. *Plant Cell Rep.* 13 : 442-446

Mazur, P. (1984) Freezing of living cells : mechanisms and implications. *Am. J. Physiol.* 247 : 125-142.

Meryman, H. T. and Williams, R. J. (1985) Basic principles of freezing injury to plant cells; natural tolerance and approaches to cryopreservation. In *Cryopreservation of Plant Cells and Organs,* ed. K. K. Kartha, CRC, Press, Florida

Meurs, C., Basra, A. S., Karssen, C. M. and Loon, L. C. (1992) Role of abscisic acid in the induction of desiccation tolerance in developing seeds of *Arabidopsis thaliana. Plant Physiol.* 98 : 1484-1493.

Morris, G. J. and Clarke, A. (1987) In *The Effects of Low Temperatures on Biological Systems*, eds B. W. W. Grout, G. J. Morris, Arnold Press, Baltimore, Maryland, pp 72-119.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15 : 473-497.

Na, H. and Kondo, K. (1996) Cryopreservation of tissue-cultured shoot primordia from shoot apices of cultured protocorms in *Vanda pumila* following ABA preculture and desiccation. *Plant Science* 118 : 195-201.

Nino, T. and Sakai, A. (1992) Cryopreservation of alginate coated *in vitro* grown shoot tips of apple, pear and mulberry. *Plant Sci.* 87 : 199-206.

Nino, T., Sakai, A., Magoshi, J. and Kato, S. (1992) Cryopreservation of *in vitro* grown shoot tips of apple and pear by vitrification. *Plant Cell Tissue Organ Cult.* 28 : 261-266.

Nishiyama, I. (1971) Evolution and domestication of sweet potato. *Bot. Mag. Tokyo*, 84 : 377-387.

Nishizawa, S., Sakai, A., Amano, Y. and Matsuzawa, T. (1992) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic cells and subsequent plant regeneration by a simple freezing method. *Cryo-Letters* 13 : 379-388.

Ooms, J. J. J., Leon-Kloosterziel, K. M., Bartels, D., Koornneei, M. and Karssen, C. M. (1993) Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana. Plant*

*Physiol.* 102 : 1185-1191.

Orjeda, G., Freyre, R. and Iwanaga, M. (1990) Production of 2n pollen in diploid *I. trifida*, a wild putative wild ancestor of sweet potato. *J Herd.* 81 : 462-467.

Orr, W., Keller, W. A. and Singh, J. (1986) Induction of freezing tolerance in an embryogenic cell suspension culture of *Brassica napus* by abscisic acid at room temperature. *J. Plant Physiol.* 126 : 23-32

Panis, B., Totte, N., Van Nimmen, K., Withers, L. A. and Swennen, R. (1996) Cryopreservation of banana (Musa spp) meristem cultures after preculture on sucrose. *Plant science* 121 : 95-106.

Papoutsakis, E. T. (1991) Media additives for protecting fully suspended animal cells against agitation and aeration damage. *Trends Biotecnol.* 9 : 427-437.

Plessis, P., Leddet, C., Collas A. and Dereuddre, J. (1993) Cryopreservation of *Vitis vinifera* L. cv. Chardonnay shoot tips by encapsulation-dehydration : effects of pretreatments, cooling and postculture conditions. *Cryo-Letters* 14 : 309-320.

Plessis, P. and Steponkus, P. (1996a) Effect of preculture in a sucrose-containing medium on the sugar content of potato shoot tips : relation to survival after vitrification. *Cryobiology* 33 : 654 (Abstr.)

Plessis, P. and Steponkus, P. (1996b) Cryopreservation of sweet potato shoot tips by vitrification. *Cryobiolgy* 33 : 655 (Abstr.)

Purseglove, J. W. (1988) Tropical Crops: dicotyledons. New York, Wiley

Quamme, H. A. and Stushnoff, C. (1993) Resistance to environmental stress. In *Methods in Fruit Breeding*, eds, J. N. Moore, J. Janick, Purdue University Press, West Lafayette, IN, pp 242-266.

Quinn, P. J. (1985) A lipid-phase separation model of low-temperature damage to biological membranes. *Cryobiology* 24 : 128-146.

Rall, W. F. and Fahy, G. M. (1985) Ice-free Cryopreservation of mouse embryos by vitrification. Nature, London. 313 : 573-575.

Reed, B. M. (1988) Cold acclimation as a method to improve survival of cryopreserved *Rubus* meristems. *Cryo-Letters*, 9 : 166-171

Reinhoud, P. J., Uragami, A., Sakai, A. and Iren, F. van (1995) Vitrification of plant cell suspensions. In *Methods of Molecular Biology: Cryopreservation and Freeze-Drying Protocols*, Vol 38, eds, J. G. Day, M. R. McLellan Humana Press Inc. Totowa, N. J.

Roca, W. M., Bryan, J. E. and Roca, M. R. (1979) Tissue culture for the international transfer of potato genetic resources. *American Potato Journal* 56 : 1-10.

Roos, E. E. (1989) Long- term seed storage. *Plant Breeding Reviews* 7: 129-158.

Sakai, A. (1986) Cryopreservation of germplasm of woody plants. In *Biotechnology in Agriculture and forestry I.* ed Y. P. S. Bjaj, Spring-Verlag, Berlin, Heidelberg New York Tokyo, pp 113-129.

Sakai, A. Kobayashi, S. and Oiyama, I. (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* var. *brasiliensis* Tanaka) by vitrification . *Plant Cell Rep.* 9 : 30-33.

Sakai, A., Kobayashi, S. and Oiyama, I. (1991b) Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* var *brasiliensis* Tanaka) cooled to -196 °C. *J. Plant Physiol.* 137 : 465-470.

Sakai, A. (1993) Cryogenic strategies for survival of plant cultured cells and meristems cooled to -196 °C. JICA, GRP, Ref. No. 6.

Sakai, A. and Yoshida, S. (1968) The role of the sugar and related compounds in variation of freezing resistance. *Cryobiology* 5 : 160-174.

Salisbury, F. B. and Ross, C. W. (1992) *Plant Physiology.* Wadsworth Publishing Company, Belmont California.

Schnabel-Preikstas, B., Earle, E. D. and Steponkus, P. (1992) Cryopreservation of sweet potato shoot tips by vitrification. *Cryobiology* 29 : 739 (Abstr.).

Scowcroft, W. R. (1984) Genetic variability in tissue culture : impact on germplasm conservation and utilization. IBPGR report, Rome, Italy.

Seufferheld, M. J. (1995) Cryopreservation of cold tender genetic resources. Phd dissertation, Colorado state University, Fort Collins, CO.

Sharp, R. E. and Davies, W. F. (1989) Regulation of growth and development of plants growing with a restricted supply of water. In *Plants Under Stress. Biochemistry, Physiology, and Ecology and Their Application to Plant Improvement.*, eds H. G. Jones, T. J. Flowers, M. B. Jones. Cambridge University Press, Cambridge, UK, pp 131-157

Shimonishi, K., Ishikawa, M., Suzuki, S. and Osawa, K. (1991) Cryopreservation of melon somatic embryos by desiccation method. *Jap. J. Bred.* 41 : 347-351.

Shiotani, I. (1988) Genomic structure and the gene flow in sweet potato and related species. In *Exploration and Maintenance and Utilization of Sweet Potato Genetic Resources*. First Planning Conference, Lima, Peru, International Potato Center (CIP), pp 61-73.

Singh, J. and Laroche, A. (1988) Freezing tolerance in plants : a biochemical overview. *Biochem. Cell Biol.* 66 : 650-657.

Steponkus, P. L. (1984) Role of the plasma membrane in freezing injury and cold acclimation. *Annu. Rev. Plant Physiol.* 35 : 543-584.

Steponkus, P. L. (1985) Fundamental aspects of cryoinjury as related to cryopreservation of plant cells and organs. In *Biotechnology in Plant Science : Relevance to Agriculture in the Eighties*, eds M. Zaitlin, P. Day, A. Hollaender, Academic Press, New York, pp 145-159.

Steponkus, P. L., Stout, D. G., Wolfe, J. and Lovelace, R. V. E. (1985) Possible role of transient electric fields in freezing induced membrane destabilization. *J. Membr. Biol.* 85 : 191-198.

Steponkus, P. L., Langis, R. and Fujikawa, S. (1992) Cryopreservation of plant tissues by vitrification. In *Advances in low temperature biology*, ed .P. L. Steponkus Vol. 1 JAI Press, London, pp 1-61.

Stushnoff, C., Remmele, R. L., Esensee, V. and Mc. Neil, M. (1993) Low temperature induced biochemical mechanisms: Implications for cold acclimation and deacclimation. In *Interacting stress on plants in a changing climate* eds M. B. Jackson, C. R. Black , Nato ASI Series I : Global Environment Change, Vol. 16 Springer-Verlag, Berlin, pp 647-657.

Sugawara, Y. and Sakai, A. (1974) Survival of suspension-cultured sycamore cells cooled to the temperature of liquid nitrogen. *Plant Physiology* 54 : 772-774.

Tanico K., Weiser, C. J., Fuchigami L. and Chen, T. H. H. (1990) Water content during abscisic acid induced freezing tolerance in bromegrass cells. *Plant Physiol.* 93 : 460-464

Tanford, C. (1973) The hydrophobic effect : Formation of micelles and biological membranes. John Wiley and Sons, New York, 10.

Tarczynski M. C., Jensen, R. G. and Bohnert, H. J. (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science*, 259 : 508-510.

Towill, L. E. (1984) Survival at ultra-low temperatures of shoot tips from *Solanum tuberosum* groups andigena, phureja, stenotomum, tuberosum, and other tuber-bearing *Solanum* species. *Cryo-Letters* 5 : 319-326.

Towill, L. E. (1981a) *Solanum etuberosum* : a model for studying the cryobiolgy of shoot tips in the tuber bearing *Solanum* species. *Plant Sci. Lett* 20 : 315-324

Towill, L. E. (1981b) Survival at low temperatures of shoot tips from *cultivars of Solanum tuberosum* group tuberosum. *Cryo-Letters* 2 : 373-382.

Towill, L. E. (1990a) Cryopreservation of isolated mint shoot tips by vitrification. *Plant Cell Reports* 9 : 178-180.

Towill, L. E. (1990b) Cryopreservation. In: *In Vitro Methods for Conservation of Plant Genetic Resources*. (ED) J.H. Dodds, Chapman and Hall, London pp.41-69.

Towill, L. E. (1988) Genetic consideration for germplasm preservation of clonal materials. *HortScience*, 23, 91-97.

Towill, L. E. and Jarret, R. L. (1992) Cryopreservation of sweet potato (*Ipomoea batatas* (L)

Lam.) Shoot tips by vitrification. *Plant Cell Reports* 11: 175-178.

Towill, L. E. and Mazur, P. (1974) Effect of cooling and warming rates on survival of frozen *Acer saccharum* tissue cultures. *Plant physiol.* 54 : 10.

Withers, L. A. (1979) Freeze preservation of somatic embryos and clonal plantlets of carrots *(Dacus Carota* L.) *Plant Physiol.* 63 : 460-467.

Withers, L. A. (1985) In *Cell Culture and Somatic Cell Genetics of Plants* Vol. 2, ed. I. K. Vasil, Academic Press, New York, pp 253-316.

Withers, L. A. (1987) The effects of low temperature on biological systems. (ED) B.W.W. Grout and G. J. Morris, Arnold Press London, pp. 389-409.

Withers, L. A., Benson, E. E. and Martin, M. (1988) Cooling rate / culture medium interactions in the survival and structural stability of cryopreserved shoot tips of *Brassica napus*. *Cryo-Letters* 9 : 114-119

Yamada, T., Sakai, A., Matsumura, T. and Huguchi, S. (1991) Cryopreservation of apical meristems of white clover (*Trifolium repens* L) by vitrification. *Plant Sci.* 78 : 81-87.

Yen, D. E. (1976) Sweet potato *Ipomoea batatas* (Convolvulaceae). In *Evolution of Crop Plants*, ed N. W. Simmonds, London, pp 42-45.

Yelenosky, G. and Guy, L. (1977) Carbohydrate accumulation in leaves and stems of "Valencia" orange at progressively colder temperatures. *Bot. Gaz.* 138 (1) : 13-17

Yoshida, S., Hattanda, Y. and Suyama, T. (1993) Variations in chilling sensitivity of suspension cultured cells of mung bean (*Vigna radiata* [L.] Wilczek) during the growth cycle.

*Plant Cell Physiology* 34 : 673-679.

## APPENDIX

**General protocol developed for cryopreservation of sweet potato shoot tips by vitrification**

**in vitro plantlet**

↓ - ½ strength MS multiplication media without hormones, 16/8 photoperiod, 25 °C

**conditioned plantlet**

↓ - 4 week old plant excised immediately after the 8 hr dark period

**isolated shoot tip**

↓ **sucrose preculture**

↓ -MS + 2% sucrose & MS +0.3M sucrose for 24 hr respectively

↓ **application of cryoprotectants**

↓ -loading : 2M glycerol + 0.4M sucrose for 1 hr at 22 °C

↓ -dehydration : PVS2 for 16 min at 22 °C

↓ **cooling**

↓ -ultra rapid **:** shoot tips placed in aluminum foil and quenched in LN 'slush'

↓ **warming / dilution**

↓ - rapid : foil containing shoot tips placed in 1.2M sucrose for 20 min at 22 °C

**post warm treatments**

↓ -shoot tips cultured on MS ($NH_4NO_3$ – free) medium for 5 days ( 2 days in darkness and 3 days in dim light) before transfer regular MS

↓ **culture media**

↓ - MS supplemented with 1μM NAA, 0.5 BAP and 0.1 Kinetin

↓ - after 4 weeks shoots transferred to ½ strength MS multiplication media without hormones