# We are IntechOpen,
# the world's leading publisher of
# Open Access books
# Built by scientists, for scientists

## 6,900
Open access books available

## 186,000
International authors and editors

## 200M
Downloads

## 154
Countries delivered to

Our authors are among the

## TOP 1%
most cited scientists

## 12.2%
Contributors from top 500 universities

**BOOK CITATION INDEX**
CLARIVATE ANALYTICS
INDEXED

**WEB OF SCIENCE™**

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

## Interested in publishing with us?
## Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

# Cryopreserving Vegetatively Propagated Tropical Crops – The Case of *Dioscorea* Species and *Solenostemon rotundifolius*

Marian D. Quain[1], Patricia Berjak[2],
Elizabeth Acheampong[3] and Marceline Egnin[4]
*[1]Council for Scientific and Industrial Research Crops Research Institute*
*[2]School of Biological and Conservation Sciences,*
*University of KwaZulu-Natal, Westville Campus, Durban*
*[3]Tissue Culture Laboratory, Department of Botany, University of Ghana, Legon, Accra*
*[4]Plant Biotechnology and Genomics Research Laboratory, Tuskegee University*
*[1,3]Ghana*
*[2]South Africa*
*[4]USA*

## 1. Introduction

Root and tuber crops in the Sub-Saharan African region play a major role in daily diet, accounting for over 50% of the total staple. *Dioscorea* spp. and *Solenostemon rotundifolius* are among the tuber staples in West Africa. *Solenostemon rotundifolius* (Poir) J.K. Morton is an edible starchy tuber crop known to have originated in tropical Africa (Schippers, 2000). It occurs in western, central, eastern and southern Africa. In Ghana, it is popular in the northern part of the country and its common name is Frafra potato (Tetteh and Guo, 1993). In South Africa, it occurs mainly in coastal KwaZulu-Natal, eastern Mpumalanga and northwestern Cape and it is commonly known as Zulu round potato (Schippers, 2000). It is used to combat famine as it has high protein content, and has medicinal and social values (Kay, 1973). It flowers profusely, yet has rare seed production and is therefore propagated vegetatively by means of vine cuttings and tuber sprouts. Storage of the tuber in hot climates is a problem. In Ghana, it is stored in dry places or left on the ground under trees where conditions are cool. The tuber is stored buried in the ground to maintain the good quality for about two months. Otherwise the tuber sprouts within a shorter period. However, in South Africa, the tuber stores well through the winter months (Schippers 2000).

The germplasm is endangered because although field and *in vitro* gene banks are being used for conservation, these serve short to medium term purposes, and are expensive. Efforts to conserve the germplasm in the longer-term under slow growth *in vitro* are hampered by the relatively rapid growth of the cultures. Cryostorage which is recognised as the very safe cost effective option for the long-term conservation of genetic resources, especially vegetatively propagated species and crops with recalcitrant seeds (Engelmann & Engels., 2002) therefore

provide a viable alternative to the long-term storage, and ensure recovery of stable germplasm (Gonzalez-Arnao *et al.*, 1999).

*Dioscorea* species, colloquially known as yams, of family Dioscoreaceae are perennial monocotyledonous climbers with underground tubers which, in some species are edible and serve as major staples in sub-Saharan Africa. Propagation is routinely vegetative, using either the tubers or vine cuttings. Farmers ensure the production of true-to-type crops by using clonal planting material, because of the social and staple importance attached to yams in sub-Saharan Africa. Hence the conservation of clonal germplasm of yam is extremely important. *Dioscorea* spp. has about 700 species within the family, nine of which are medicinal plants that accumulate steroid saponins in their rhizomes. Six species of Dioscorea *D. bulbifera*, *D. cayenensis*, *D. dumentorum*, *D. prahensilis*, *D. alata* and *D. rotundata* contain mealy starch with a good level of vitamin C and other nutritive substances, which serve as major staples in sub-Saharan Africa. *Dioscorea rotundata* is native of West Africa, where it plays important role in the socio cultural life of the people. *Dioscorea alata* is the most widespread worldwide and is most cultivated in Southeast Asia, the Caribbean and West Africa. *Dioscorea rotundata* is now utilised in other parts of the world, and it has become a foreign exchange earner particularly in Ghana.

*In vitro* slow growth tissue culture methods have been used in conserving the germplasm (Ashun 1996; Ng & Daniel 2000; Ng & Ng 1991). Although this method usefully complements the traditional form of conservation, it serves only short- to medium-term storage purposes. Thus cryopreservation, which imposes a stasis on metabolic and deteriorative processes, is a worthwhile option to be explored.

Explant treatment to attain low water content which is critical for cryopreservation has in some protocols been by exposing tissues to stress, which enhance desiccation and cold tolerance (Withers 1985; Jitsuyama *et al.*, 2002). Such stress has been induced by abscisic acid, sugars, mannitol and sorbitol (Mastumoto *et al.*, 1998; Jitsuyama *et al.*, 2002; Veisseire *et al*, 1993; Panis *et al.*, 2002; Walter *et al.*, 2002). The use of cryoprotectants, which exert osmotic stress and lead to loss of free water from tissues and vitrification when frozen has also been induced by using reagents such as sucrose, glycerol, DMSO, ethylene glycol, proline and many others (Engelmann et al., 1994; Harding & Benson, 1994; Matinez-Montero *et al*, 1998; Plessis *et al.*, 1993; Nishizawa *et al.*, 1993). Desiccation of tissues on activated silica gel (Hatanaka *et al.*, 1994; Cho *et al.*, 2002), in laminar air flow cabinets (Gonzalez-Benito & Pezez, 1994; Thammasiri, 1999) and flash driers (Berjak *et al.*, 1999; Pammenter *et al.*, 1991; Wesley-Smith *et al.*, 1992; Walter *et al.*, 2002; Potts & Lumkin 1997) have all been used to appreciably reduce water content to enhance cryotolerance. Although these treatments have all been reported to be successful in enhancing cryopreservation of some tissues, there are differences in response to known protocols which have been mainly attributed to specie and variety specificity (Gonzalez-Benito *et al.*, 2002; Martinez-Montero *et al.*, 1998; Panis *et al.*, 2002; Gonzalez-Arnao *et al.*, 1999).

The prevention of the formation of lethal ice crystals when tissue is exposed to sub-zero temperatures is essential for successful cryopreservation, of vegetatively propagated germplasm. This chapter looks at the various attempts made to cryopreserve germplasm of *Solenostemon rotundifolious* and possible underlying mechanism that might have led to failure of tissues to respond to all methods utilized. Tissue survival, water contents and ultrastructure are used as parameters for analyzing response to various treatments. Also, response of yam *in vitro*-grown explants (shoot tips and axillary buds) to various desiccation

procedures and their ability to survive after exposure to cryogenic temperatures is investigated here, with the ultimate aim of developing a simple protocol for long-term conservation of the germplasm of *Dioscorea* species via cryopreservation. Parameters that need critical investigation are discussed.

## 2. Materials and methods

### 2.1 *Solenostemon rotundifolius*

### 2.1.1 Source of explant

*In vitro* cultures of *Solenostemon rotundifolius* accession number UWR 002 was obtained from the *in vitro* gene bank that had been maintained under slow growth conditions at 18ºC. *In vitro* cultures were multiplied on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 0.7% agar. Subculturing was carried out at four-weeks intervals. Cultures were maintained under a 16 h photoperiod (40 µM /m²/s¹) at 25ºC $\pm$1ºC.

### 2.1.2 Conditioning donor plant material in culture (pregrowth)

Nodal cuttings were cultured on MS medium supplemented with either 0.058 M (2%) or 0.1 M sucrose or 0.1 M mannitol and 0.8% agar. Cultures were incubated for two to three weeks after which uniformly developed plantlets were used for various experiments.

### 2.1.3 Conditioning excised explants in culture (preculture)

*Solenostemon rotundifolius* nodal cuttings consisting two buds (having lateral buds which are microscopically globular and covered by leaf primodia as described by Niino *et al.*, 2000) were obtained from pregrown cultures, positioned on sterile nylon mesh cut side down and placed on fresh pregrowth media overnight. Explants were then transferred on mesh to media with higher sucrose concentrations (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 M) sucrose for one to seven days. Media supplemented with 0.1 and 0.3 M mannitol were also used as pregrowth treatment. Incubation was under a 16 h photoperiod (40µM/m²/s⁻¹), at 25°C$\pm$1°C.

### 2.1.4 Silica gel dehydration

Explants were placed on oven-sterilised aluminium foil and dehydrated over approximately 35 g activated silica gel in covered 90 mm glass Petri dishes for 30 min to 16 h under sterile conditions.

### 2.1.5 Rehydration

Explants were rehydrated following cryoprotection treatment, silica gel dehydration and cooling. This was carried out in cryovials containing liquid MS medium supplemented with 0.1 M sucrose, 1mM $MgCl_2.6H_2O$ and 1 µM $CaCl_2.2H_2O$ for 30 minutes. Re-hydrated buds were cultured on growth medium, incubated under continuous dark conditions till signs of growth and development were observed (at least one week) before they were transferred to a dual photoperiod.

### 2.1.6 Cryoprotection

Liquid medium (MS) supplemented with 0.2, 0.4 M, 0.07 M, 0.14 M, sucrose, 2.0 M, 0.64 M, 1.28 M, 3.23 M glycerol, 2.42 M ethylene glycol, 0.017 M raffinose, 0.64 M, 1.28 M and 1.92 M DMSO, in varied combinations, plant vitrification solution II (PVS2), (Sakai *et al.*, 1990) and half strength PVS2 were used to cryoprotect nodal cutting explants for 5, to 40 minutes. Explants used were obtained from shoots grown on 0.1 M mannitol for 2-3 weeks. Excised explants were cultured on 0.3 M mannitol for 72 hours, and cryoprotected in cryovials, using 1 ml cryoprotectant solution (the 1 ml cryoprotectant was decanted and replace with 0.5 ml during cryoprotection). To enhance explant cryoprotection, dehydration over activated silica gel for 60 minutes either before or after cryoprotection was also investigated. Following cryoprotection, the cryoprotectant solution was decanted and explants were washed three times with rehydration solution (described above). However, explants subjected to cooling, (LN or freezing to -70°C) were immediately on retrieval, rewarmed in water bath at 40°C for two - three minutes. Following rewarming, tissues were then allowed to stay for 30 min in the rehydration solution before blotting dry, and cultured on growth medium. Incubation was in continuous dark conditions till signs of growth and development were observed. Developing cultures were transferred to 16 h photoperiod.

### 2.1.7 Frafra potato assessments

#### 2.1.7.1 Water content determination

Individually weighed explants were oven-dried at 80°C for 48 hours to determine dry mass. Water content was determined individually for 5-10 explants, and expressed on a dry mass (g $H_2O$ g$^{-1}$ dry mass) basis.

#### 2.1.7.2 Survival

Explant survival was assessed weekly for three weeks after culturing. Generally, 8-10 explants were used per treatment and experiments were replicated three times. Surviving explants were those which showed shoots with buds, leaves, and root development.

#### 2.1.7.3 Transmission electron microscopy

A standard glutaraldehyde-osmium fixation method was used, followed by dehydration through an acetone series embedding in a low viscosity epoxy resin (Spurr, 1969). Sections of the meristematic regions of axillary buds and shoot tips were collected on 200 mesh hexagonal copper 3.05 mm grids. Sections were post-stained with uranyl acetate and lead citrate, washed with distilled water, and viewed and photographed with a JEOL 100-S transmission electron microscope.

### 2.2 *Dioscorea rotundata*

### 2.2.1 Source of explant

*In vitro* cultures of *Dioscorea rotundata* ("Pona"), accession number PS 98 013 were obtained from the *in vitro* gene bank of the Department of Botany, University of Ghana, Legon, where the plants were maintained under long-term slow growth conditions at 18°C. Cultures were multiplied and sub-cultured at six-week intervals on Murashige and Skoog (MS) medium

(Murashige and Skoog 1962) with 2.5 µM kinetin, 20 mg l-1 L-cysteine, 2% (0.056 M) sucrose, 0.7% agar, and maintained under a 16 h photoperiod (40 µ mol m-2 s-1) at 25±1°C.

### 2.2.2 Conditioning excised explants in culture (preculture)

Yam shoot tips (~1 - 2 mm) were excised from cultures grown on MS pregrowth medium [as above, except containing 3% (0.09 M) sucrose instead of 2% sucrose] for five weeks, placed on sterilised nylon mesh, which was then positioned, explant cut side down, on fresh medium, overnight. Explants were then transferred on the mesh to semi-solid medium with higher sucrose concentrations (0.3, 0.5, 0.7 and 1.0 M, the control material continuing to be exposed to 0.09 M sucrose) in 90 mm Petri dishes for one, three, five or seven days, each followed by transfer either to growth-enhancing medium (MS complete salts with vitamins, 3% sucrose, 5 µM kinetin, 20 mg l-1 L-cysteine, 0.8% agar, 1% filter-sterilised casein hydrolysate at pH 5.7 ± 0.1), or further conditioned for cryopreservation.

### 2.2.3 Silica gel dehydration and cooling of explants

Yam explants dehydrated using the same methodology as described above for Frafra potato. Dehydrated explants were placed in cryovials, which were plunged into, and maintained in, liquid nitrogen for one hour, or cooled at 1°C min-1 in a Nalgene cryo freezing container (Mr Frosty™), to -70°C, and maintained for at least four hours at this temperature. Rewarming was effected immediately on retrieval from the cryogen, in a water bath at 40°C for two to three minutes for all treatments.

### 2.2.4 Rehydration

The rehydration solutions consisted of MS complete salts with vitamins, 2.5 µM kinetin, 20 mg l-1 L-cysteine, 1mM $MgCl_2.6H_2O$, 1µM $CaCl_2.2H_2O$, 1% casein hydrolysate (filter-sterilised), and 1 M sucrose, at pH 5.7±0.1, magnesium and calcium chlorides having been shown to enhance explant recovery of date palm somatic and pea zygotic embryos (MyCock 1999). Rehydration was for 30 minutes. Rehydrated buds were blotted dry and cultured on growth medium (as above). Cultures were incubated under continuous dark conditions at 24±1°C until signs of growth and development were observed, before they were transferred to 16 h photoperiod (40 µ mol m-2 s-1) at 25±1°C.

### 2.2.5 Cryoprotection with modified plant vitrification solution 2(MPVS2)

Explants that had been pregrown and precultured were exposed to 1 ml plant vitrification solution 2 (PVS2) as designed by Sakai and colleagues (Sakai et al., 1990), but modified as follows (MPVS2): basic MS medium, 30% glycerol, 15% ethylene glycol, 15% DMSO (v/v), 0.4 M sucrose (w/v), 0.1 M $CaCl_2.2H_2O$ and 1% D-raffinose at pH 5.7±0.1. Inclusion of calcium chloride and raffinose has been found to be beneficial in promoting recovery after cryopreservation in other species (Mycock, 1999). Explants were treated for 0, 10, 20, 30 or 40 minutes in cryovials. The vitrification solution was decanted and the explants washed three times in 1 ml rehydration solution (as described above) for 30 minutes, then cultured on growth medium and incubated in the dark. Cultures were transferred to the alternating light/dark conditions once signs of growth and development were observed. Prior to being cooled to -70 or -196°C, cryoprotected explants were suspended in fresh 0.5 ml MPVS2 in

cryovials. The explants to be cryopreserved were then exposed to cryogenic conditions for specified durations, rewarmed, vitrification solution removed, rehydrated, and incubated as described above.

### 2.2.6 Yam assessment

#### 2.2.6.1 Water content determination

Water content of yam explants was determined following the same procedure as described for Frafra potato above.

#### 2.2.6.2 Survival assessment

Yam explant survival was determined following the same procedure as described above of Frafra potato.

#### 2.2.6.3 Tetrazolium test for viability

Shoot tips which were pretreated with high sucrose concentrations; pretreated and cryoprotected with MPVS2; pretreated, cryoprotected, and vitrified, were cultured on growth medium for 5-7 d following which they were transferred to a 0.1% aqueous solution of 2,3,5-triphenyl tetrazolium chloride (TTZ) and incubated in the dark overnight. Control material was obtained from cultures under standard growth room conditions. Patchy red staining, located around the meristematic region as a result of respiratory activity in viable cells was scored as the tissue having survived the various treatments.

## 3. Results and discussion

### 3.1 Frafra potato

### 3.1.1 Pregowth

Water contents of Frafra potato nodal cutting explants following pregrowth on medium supplemented with 0.058 M (2%) sucrose was extremely high (22.25$\pm$1.7 gH$_2$O g$^{-1}$ dry wt) to enhance successful cryopreservation. As explant size and geometry have a marked effect on the success of freezing hydrated material (Wesley-Smith *et al.*, 1995), nodal cuttings (two buds per explant) to be used for cryopreservation experiments were split into two halves with one bud per explant, this adequately lowered water content of explants (8.78$\pm$1.07 gH$_2$O/g dry wt). The water content of the single (well trimmed) bud is similar to that of explants excised from greenhouse established plant (10.16$\pm$0.98 g/g dry wt. data shown in Table 2). Since cultures grown on medium supplemented with 2% sucrose were extremely wet for cryopreservation, higher sucrose or mannitol concentration (0.1 M) was employed to enhance desiccation tolerance, which subsequently improves cryotolerance .

Pregrowth of explants on medium supplemented with 0.1 M mannitol lowered the water content of explants from 19.5 under control condition (medium supplemented with 0.058 M sucrose), to 10.4 gg$^{-1}$, which did not affect survival (Fig. 1). The ultrastructure was as well constituted as that of the control explants (Plate 1a), with ongoing metabolism indicated by abundant cristate mitochondria (Plate 1b), Golgi bodies and profiles of endoplasmic recticulum (insert). Growth on 0.1 M sucrose supplemented medium, also lowered the water

content of explants (11.4 gg$^{-1}$) as shown in Fig. 1, this observation is in agreement with response of oil palm explant water content when treated with sucrose (Dumet *et al.*, 1993) and while this did not reduce survival it resulted in severely distended organelles and evidence of tonoplast disruption and lobed nuclei (Plate 1d and e). Sucrose has been extensively used to treat plant tissues prior to cryopreservation (Panis *et al.*, 2002; Grospietsch *et al.*, 1999; Gonzalez-Benito & Perez, 1994; Santos & Stushnoff 2003), studies have however, not investigated the structural effect of sucrose on tissue. The damage revealed by ultrastructure (Plate 1f) could have predisposed explants negatively to subsequent steps.

### 3.1.2 Preculture

Culturing individual Frafra potato buds on 0.3 M sucrose for 3 d (Table 1), lowered water content from 11.4 g g$^{-1}$ (after growth on 0.1 M sucrose medium) to 7.3 g g$^{-1}$ and explant survival was at 100 %. This level of sucrose has been applied in other crops such as carrots (Dereuddre *et al.*, 1991), wasabi (Mastumoto *et al.*, 1998), and African violet (Shibili *et al.*, 2004). Similarly, explant on medium supplemented with 0.3 M mannitol which were derived from 0.1 M mannitol supplemented medium, water contents reduced further from 10.47 to 7.42 gg$^{-1}$ and survival was still at 100 % (Table 1). Mannitol and its isomer, sorbitol have been used for pre-treatment of plant tissues before cryopreservation (Wang *et al.*, 2001) as well as in long term storage culture media as osmoticums (Ashun, 1996; Egnin et al, 1998). The growth of explants on regrowth medium following preculture varying sucrose media is shown in the Plate 2.
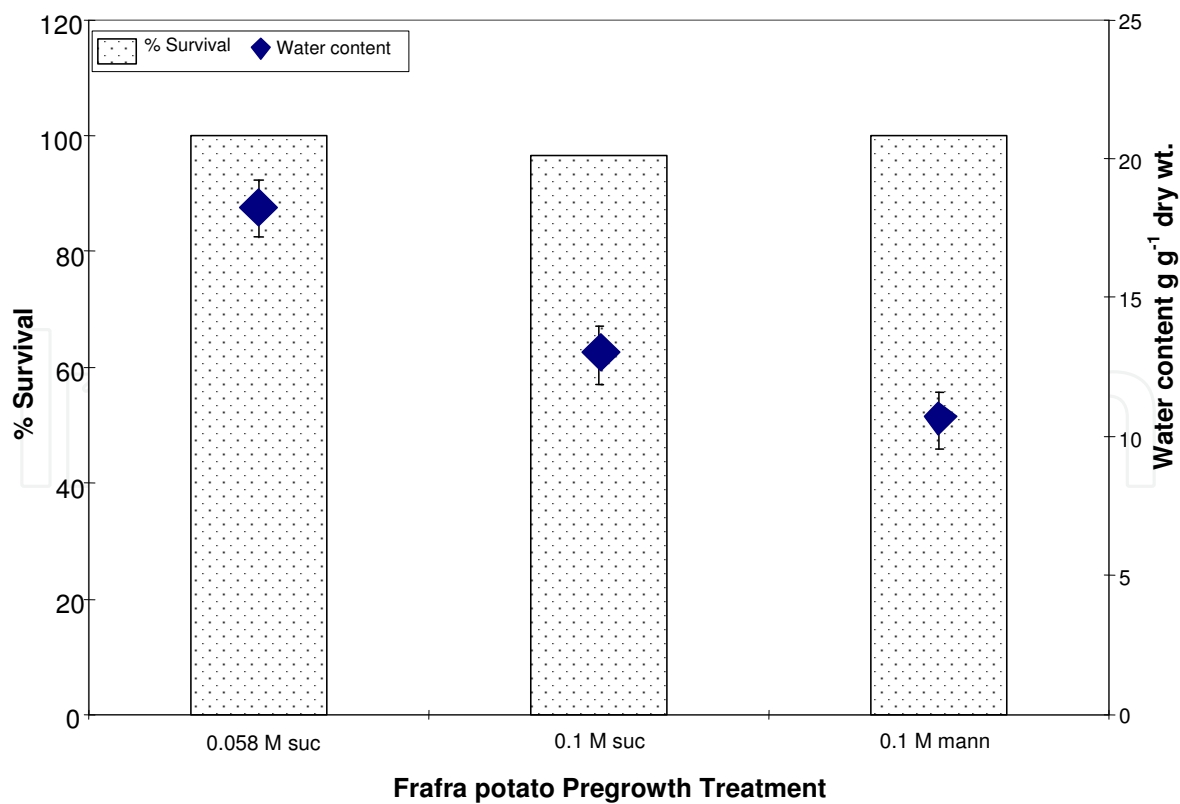


Fig. 1. Survival and water content of Frafra potato cultures on three pregrowth media $\pm$ SD. Survival P $\geq$0.05, n=30, and WC P$\leq$ 0.05, n=15-30
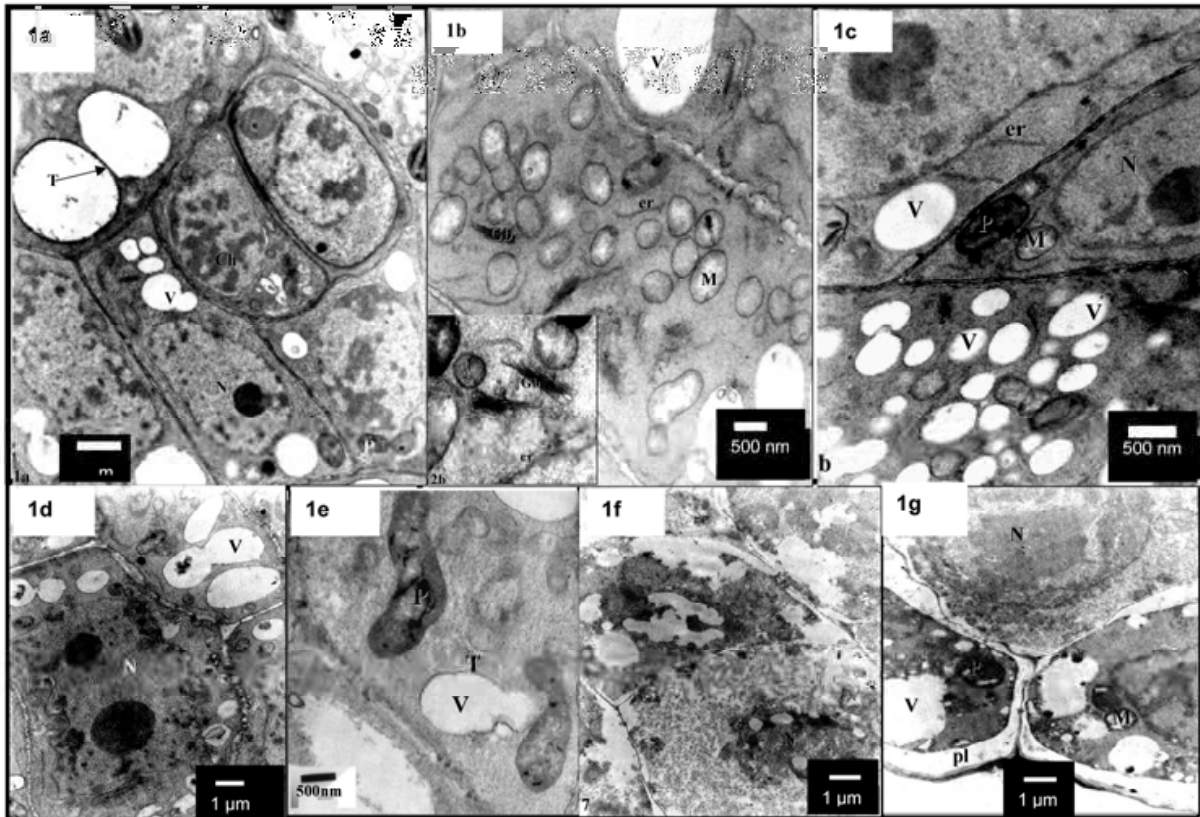
Plate 1. Ultrastructure of *Solenotemon rotundifolius* explant meristematic cells. Legend are as follows: Ch, heterochromatin, V, vacuoles, T, tonoplast, P, plastids, M, mitochondrial, ER, endoplasmic reticulum, N, nucleus Gb, Golgi bodies, pl, plasma membrane.

Plate 1a Control explants cultured on 2% sucrose supplemented medium. Cells show oval nuclei, normally-distributed heterochromatin, vacuoles each with a well-defined tonoplast, small, relatively dense plastids, circular mitochondrial profiles with dense matrices, and profiles of endoplasmic reticulum.

Plates 1b (insert), & c. Explants pregrown on 0.1 M mannitol for three weeks cells (1b). Ongoing metabolic activity indicated by abundance of mitochondria, Golgi bodies and profiles of endoplasmic reticulum (insert), many plasmodesmata are visible. A group of relatively small vacuoles is shown (1c), which appeared typical of mannitol treatment to reduce water content.

Plate 1d After sucrose (0.1 M) pregrowth for three weeks, there was evidence of tonoplast disruption, lobed nuclei with possibility of vacuole fission or fusion. Plate 1(e) shows somewhat distorted plastids and a potentially autolysing cell (lower left) where vacuolar dissolution (tonoplast disruption) appears to have occurred. Such events would have predisposed these explants negatively to subsequent steps.

Plate 1f. Explants pregrown on 0.1 M sucrose, precultured on 0.3 M sucrose for 3 d and then dehydrated over activated silica gel for 120 min. Water content was 0.16 g g$_{-1}$ while survival of the sample was only 2.5%. Most specimens presented this appearance of advances intracellular deterioration, nuclear remains; and plasma membrane.

Plate 1g. Explants pregrown on 0.1 M mannitol and then preculture on 0.3 M mannitol for 3 d, dehydrated over activated silica gel for 120 min, during which water content was lowered to 0.11 g g-1. This was accompanied by 38.1% survival. Nuclear and cytoplasmic derangement had occurred although some cells had few intact organelles, shown in this illustration of what was probably a non-surviving explant.
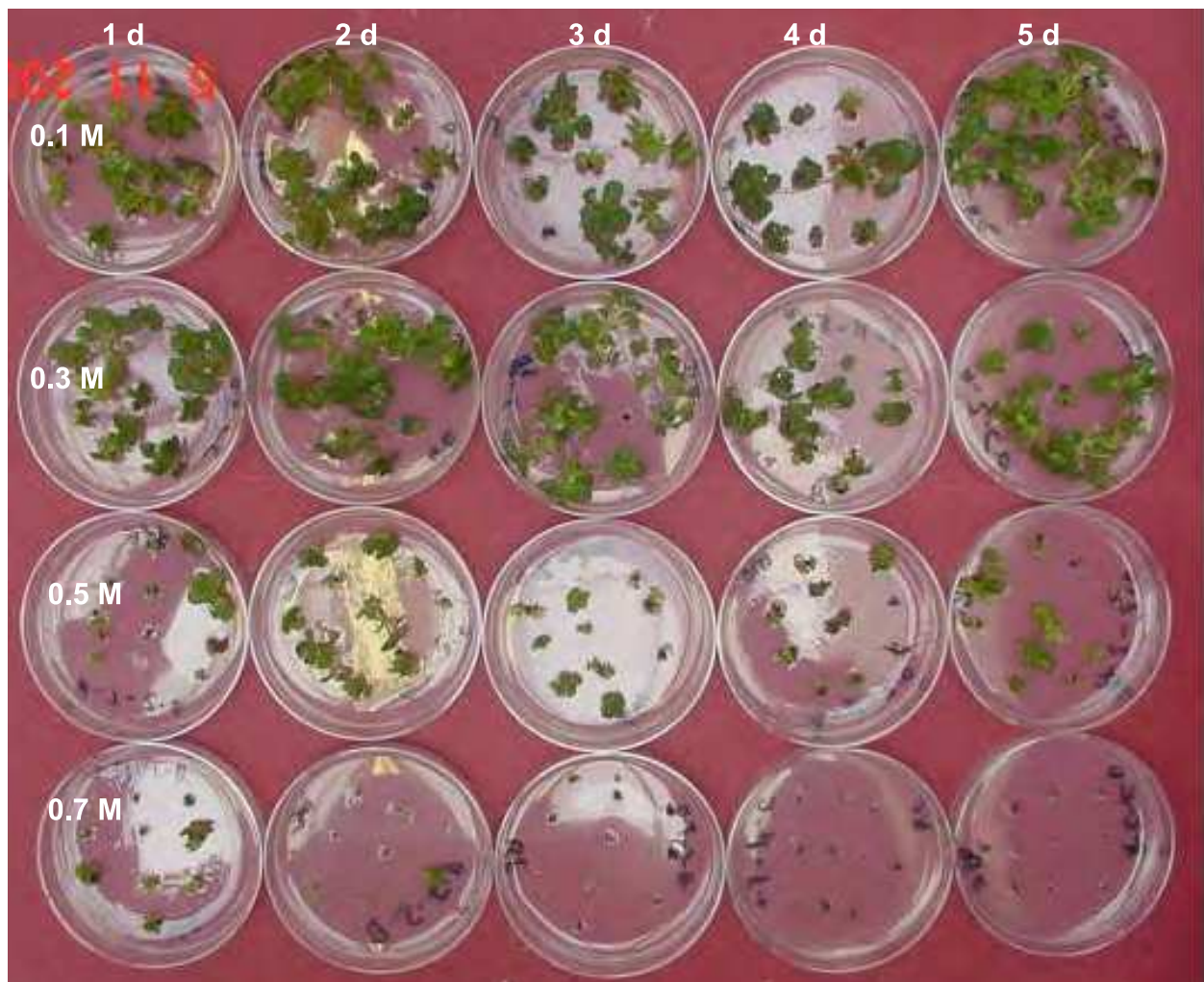
Plate 2. Development of *Solenostemon rotundifolius* explants pregrown and precultured on medium supplemented with increasing sucrose concentrations 0.1 M, 0.3, 0.5 M, and 0.7 M for 1 d, 2 d, 3 d, 4 d, and 5 d and grown on growth medium in 90mm Petri plates. Data taken at 3-weeks.

### 3.1.3 Dehydration

Dehydrating explants over silica gel, considerably lowered water content (Table 1). In experiments by other investigators, this technique has been successfully used to dehydrate and cryopreserve citrus axes, (Santos & Stushnoff, 2003) and encapsulated somatic embryos of *Coffee canephora* (Hatanaka *et al.*, 1994).

Mannitol treated (0.3 M) explants of Frafra potato used in this particular experiment, when dehydrated over activated silica gel, the lowest water content (0.11 g g$^{-1}$) was recorded for 120 min dehydration and survival was 73% (Table 1). However, sucrose treated (0.3 M) explants indicated lowest water content of 0.16 g g$^{-1}$ which although, is higher than in mannitol (stated above), survival was as low as 5% (Table 1). It is possible that damage suffered by tissues as revealed by ultrastructure (Plate 1d&e) during sucrose treatment predisposed them to further damage on dehydration. It was evident here that mannitol treated tissue are more desiccation tolerant than sucrose treated tissues although other

| Treatment | Water Content g/g dry weight | % Survival |
|---|---|---|
| 0.058 M sucrose for 1 d | 18.22 ± 0.96 | 100 ± 0 |
| 0.058 M sucrose for 3 d | 22.37 ± 2.02 | 86.67 ± 4.7 |
| 0.058 M sucrose for 5 d | 16.7 ± 0.65 | 100 ± 0 |
| 0.1 M sucrose for 1 d | 12.93 ± 0.66 | 96.67 ± 0.86 |
| 0.1 M sucrose for 3 d | 14.18 ± 0.03 | 100 |
| 0.1 M sucrose for 5 d | 12.74 ± 0.4 | 100 |
| 0.3 M sucrose for 1 d | 8.82 ± 0.4 | 100 |
| 0.3 M sucrose for 3 d | 7.98 ± 0.73 | 96.67 ± 0.86 |
| 0.3 M sucrose for 5 d | 6.17 ± 0.39 | 100 |
| 0.5 M sucrose for 1 d | 4.13 ± 0.38 | 96.67 ± 0.86 |
| 0.5 M sucrose for 3 d | 4.22 ± 0.41 | 86.67 ± 2.27 |
| 0.5 M sucrose for 5 d | 3.37 ± 0.27 | 96.67 ±0.86 |
| 0.1 M sucrose dehydrated for 0 min | 11.45 ± 0.50 | 100 |
| 0.1 M sucrose dehydrated for 60 min | 0.58 ± 0.15 | 50 ± 1.8 |
| 0.1 M sucrose dehydrated for 90 min | 0.56 ± 0.12 | 30 ± 3.6 |
| 0.1 M sucrose dehydrated for 120 min | 0.14 ± 0..02 | 35 ± 10.6 |
| 0.3 M sucrose dehydrated for 0 min | 6.09 ± 0.16 | 96.67 ± 0.8 |
| 0.3 M sucrose dehydrated for 60 min | 0.79 ± 0.17 | 56.25 ± 1.14 |
| 0.3 M sucrose dehydrated for 90 min | 0.37 ± 0.07 | 33.18 ± 0.58 |
| 0.3 M sucrose dehydrated for 120 min | 0.16 ± 0.61 | 5 ± 2.4 |
| 0.1 M mannitol for 3 d and dehydrated for 0 min | 10.51 ± 0.6 | 80 ± 2.08 |
| 0.1 M mannitol for 3 d and dehydrated for 60 min | 0.42 ± 0.07 | 40 ± 0.11 |
| 0.1 M mannitol for 3 d and dehydrated for 90 min | 0.29 ± 0.05 | 40 ± 0.11 |
| 0.1 M mannitol for 3 d and dehydrated for 120 min | 0.08 ±±0.01 | 20 ± 0.09 |
| 0.3 M mannitol for 3 d and dehydrated for 0 min | 7.42 ± 0.38 | 96.67 ± |
| 0.3 M mannitol for 3 d and dehydrated for 60 min | 0.31 ± 0.05 | 71 ± 0.09 |
| 0.3 M mannitol for 3 d and dehydrated for 90 min | 0.24 ± 0,05 | 70 ± 0.10 |
| 0.3 M mannitol for 3 d and dehydrated for 120 min | 0.11 ± 0.4 | 73 ± 0.10 |

Table 1. Treating FP 002 with different sucrose concentrations during development of Preculture conditions using nodal cuttings with single buds

reports have successfully used sucrose to induce dehydration tolerance (Dumet *et al.*, 1993; Grospietsch *et al.*, 1999; Santos & Stushnoff, 2003).

Sucrose treated tissues had totally been deranged after 120 minutes (Plate 1f) of dehydration compared with mannitol tissues (Plate 1g) which has some intact nuclei and few organelles present. These must be responsible for the survival recorded (Table 1). It is possible the presence of the intact nuclei and organelles in the mannitol treated cells could be reconstituted for normal plant growth and development to occur.

| Crop accession | Water content |
|---|---|
| FP UER 001 | 18 + 5.14 |
| FP UER 002 | 10 + 0.9 |
| FP UWR 003 | 9.6 + 2.7 |
| FP UER 004 | 14.6 + 2.1 |
| 99/053 | 8.4 + 1.1 |
| 99/1033 | 11.2 + 0.6 |
| 99/016 | 11.2 + 1.6 |
| 99/022 | 11.35 + 0.6 |

Table 2. Water content of screenhouse of Frafra potato established in greenhouse for three months

### 3.1.4 Cryoprotection

Explants treated with 0.1 and then 0.3 M sucrose or mannitol, on exposure to PVS2 indicated only about 20% survival (Table 3). This observation was contrary to report by Niino *et al.*, (2000) that *S. rotundifolius* innala recorded high survival on treating with PVS2 and subsequently, 85% survival on exposure to liquid nitrogen. *Solenostemon rotundifolius* used in this study, were extremely sensitive to both the loading solution (0.4 M sucrose + 0.2 M glycerol, data not shown) and PVS2, which in other reported studies, led to successful cryopreservation of other crops including *S. rotundiflius* (Wang *et al.*, 2003; 2001; Turner *et al.*, 2001; Niino *et al.*, 2000). On screening for appropriate vitrification (cryoprotection) solution, the following cryoprotection solutions listed in Table 3 were tested. It was indicative from results that DMSO and Ethylene Glycol at the concentration (15%) that they occur in PVS2 did not have any lethal effect on the explants. However, sucrose and Glycerol at the concentrations that they occur in PVS2 (0.4 M and 30% respectively) were found to be lethal to the tissues (Table 3). The use of PVS2 at half concentration and a combination of 2.5% Glycerol, 5% sucrose, 7.5% DMSO and 7.5% Ethylene glycol (coded PVSB) resulted in survival and growth of explants. The responses confirm indication that cryoprotectants at full-strength are toxic to plant cells (Rheinhoud *et al.*, 1995). These treated explants, however did not survive on exposure to liquid nitrogen. Combining the cryoprotection treatment with dehydration (data not shown) as has been reported by other investigators as enhancing high cryosurvival (Wang *et al.*, 2003; 2001; Turner *et al.*, 2001), did not result in survival after cryopreservation in this study. Encapsulating explants prior to treatment with PVS2 also did not result in explant survival.

Ultrastructural studies indicated that tissues treated with ½PVS2 (Plate 3) and PVSB (Plate 4) for 15 min, which survived had well constituted cells, however, some tonoplasts were not
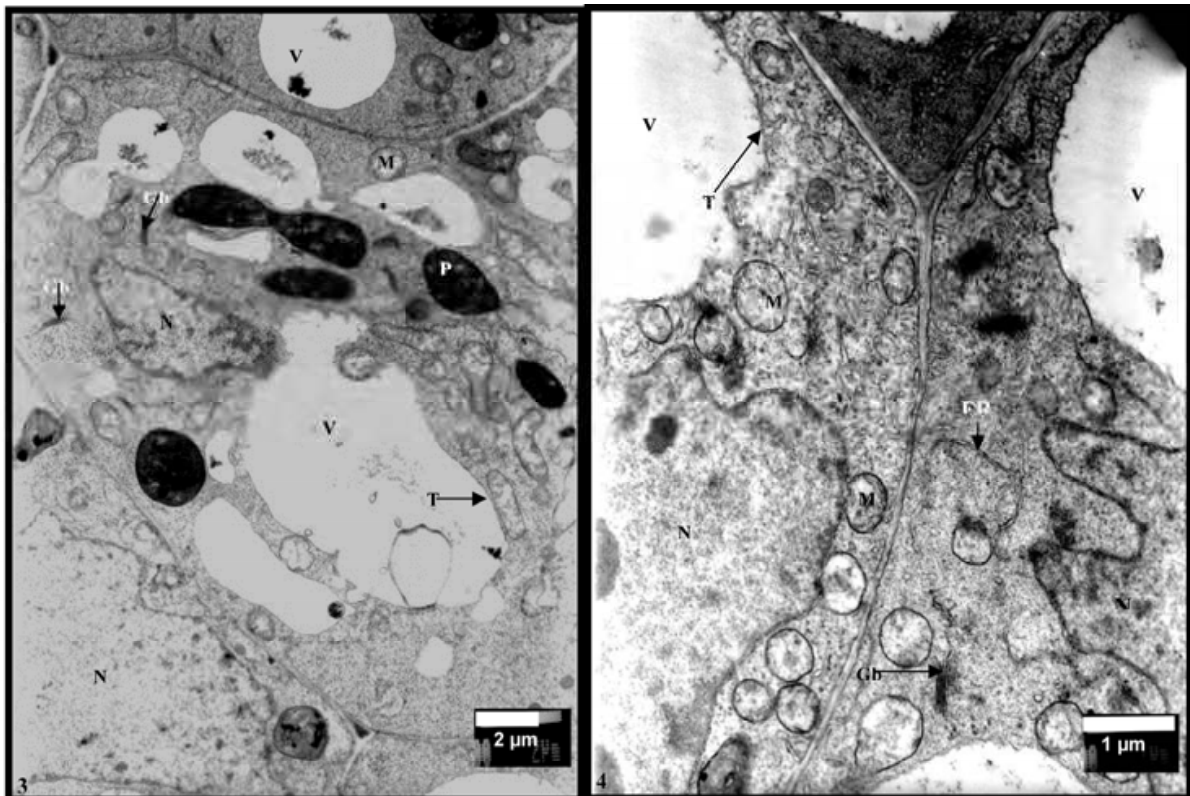
Plate 3 & 4. Ultratructure of surviving Frafra potato after mannitol pregrowth and
preculture treatments, followed by subjecting explants for 15 min to ½PVS2 (Plate 3) and
PVSB (Plate 4). (**3**) Although cells were well organised, some extent of autophagy was
concluded to have occurred, in terms of intravacuolar inclusions in the ½ PVS2-treated
material. (**4**) Cells appeared exceptionally active but showed distinctly lobed nuclei (N).
Other organelles that can be recognized are mitochondria (M), endoplasmic reticulum (ER),
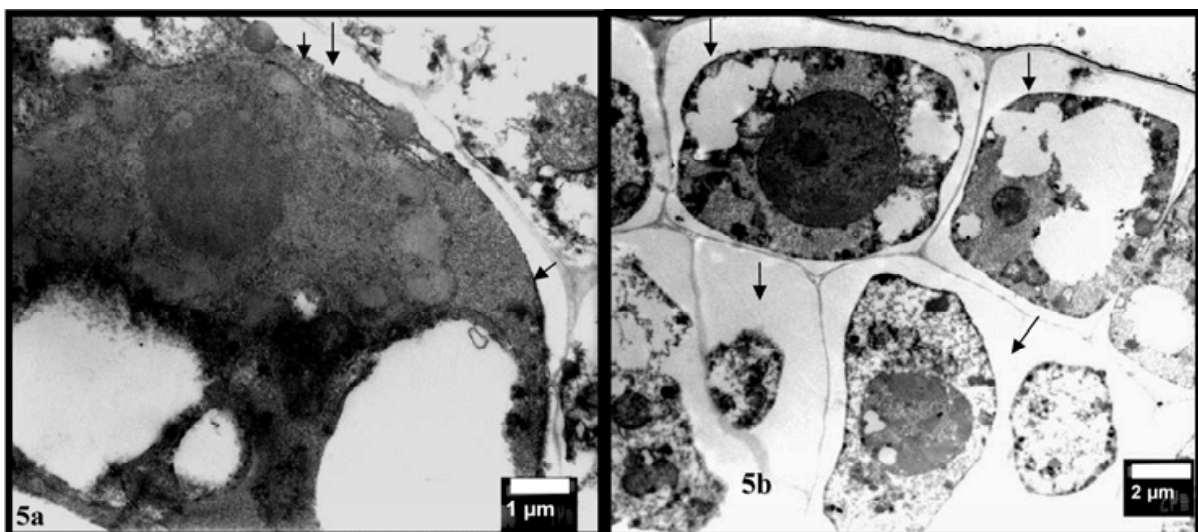Golgi bodies (Gb), plastids (P), and vacuoles (V) with tonoplast (T) intact.



Plate 5a & b. Ultratructure of non-surviving Frafra potato following mannitol pregrowth
and preculture treatments, then subjecting explants to ½PVS2 for 15 (Plate 5a) and 40 min
(Plate 5b) Cells were highly plasmolysed (arrows) and damaged to the extent that organelles
could not be easily recognized.

distinct and some extent of autophagy was observed in ½ PVS2, treated tissues (Plate 3). Slight deposition of starch was observed in plastids of tissues treated with PVSB. Tissues, which did not survive after the 15min treatments (Plate 5a&b), showed highly plasmolysed cells and totally deranged cells, exposing the lethal effect of vitrification solution.

Increasing glycerol and sucrose concentration as well as long exposure duration led to reduced survival and regeneration of encapsulated nodal cuttings treated with the plant vitrification solutions as indicated in (Table 4). This observation is contrary to other crops where encapsulation vitrification has been highly recommended for successful cryopreservation (Charoensub *et al*., 1999; Wang *et al*, 2003) however confirms that cryoprotectants can be damaging to plant tissues and the extent of effect varies according to type and concentration of cryoprotectant as well as plant species (Berjak *et al*., 1996).

| Treatment | % Survival |
|---|---|
| 3.23 M Glycerol + 0.4 M Sucrose + 0.017 Raffinose | 33.64 ± 1.39 |
| 3.23 M Glycerol + 0.4 M Sucrose | 22.71 ± 2.77 |
| PVS2 + 0.017 M raffinose | 8.28 ± 0.20 |
| PVS2 | 20 ± 0 |
| 0.07 M S + 0.3 M G 15 min | 100 |
| 0.07 M S + 0.3 M G+0.14 M S +0.6 M G, 15 min | 100 |
| 0.14 M S + 0.6 M G, 15 min | 100 |
| 0.64 M DMSO for 15 min | 90±0 |
| 1.28 M DMSO for 15 min | 100± 0 |
| 1.92 M DMSO for 15 min | 100 ± 0 |
| 2.42 M EG + 0.017 M Raffinose | 84.24±1.46 |
| 2.42 M EG | 77.28±0.97 |
| ½ PVS2 + 1% Raffinose | 80.63±1.82 |
| 0.3 M G + 0.2 M S + 0.96 M DMSO + 2.42 M EG + 0.017 M Raffinose (PSVB) | 69.17±2.26 |

S: sucrose, G: Glycerol, EG: Ethylene Glycol

Table 3. Survival (± SD) of Frafra potato explants, pregrown (0.1 M mannitol) and precultured (0.3 M mannitol) prior to cryoprotection treatments. n=30 – 40, P<0.05

| Frafra potato Variety | Treatment | Water Content g/g dry wt | % Survival |
|---|---|---|---|
| | PVSB for 15 min | 4.13 ± 0.16 | 55 ± 2.04 |
| | PVSB for 40 min | 4.45 ± 0.25 | 55 ± 2.04 |
| FP 002 | ½ PVS2 for 15 min | 3.36 ± 0.06 | 38.75 ± 0.3 |
| | ½ PVS2 for 40 min | 3.26 ± 0.17 | 0 |
| | PVS2 for 15 min | 2.00 ± 0.10 | 20 |
| | PVSB for 15 min | 2.66 ± 0.16 | 59.01 ± 1.5 |
| FP 003 | ½ PVS2 for 15 min | 2.58 ± 0.23 | 53.76 ± 2.3 |
| | Control | 5.63 ± 0.31 | 100 |
| | PVSB for 15 min | 2.91 ± 0.28 | 54.56 ± 1.4 |
| FP 004 | ½ PVS2 for 15 min | 2.7 ± 0.14 | 66.62 ± 3.47 |
| | Control | 6.59 ± 0.62 | 100 |

½PVS2 and PVSB were always supplemented with 0.017 M Raffinose

Table 4. FP explants treated with Plant Vitrification Solution

### 3.1.5 Cryopreservation

Although explants treated with mannitol and dehydrated over activated silica gel for 90 minutes had water content 0.24 gg$^{-1}$ and survival was 70 % (Table 1), when exposed to liquid nitrogen, survival was nil. Ultrastructure indicated extensively degraded cells with withdrawn and broken plasmalemma, cytoplasm and nucleoplasm were all damaged (Plate not shown). Explants from all dehydration treatments did not survive on exposure to liquid nitrogen as well as ultra-cold liquid nitrogen (slash), although, it has been reported that rapid cooling enhance cryosurvival (Wesley-Smith *et al.*, 1992). Having dehydrated explant to water content of 0.11gg-1, it is obvious from ultrastructure (not shown) that the prolonged stress exerted decreased explant ability to withstand freezing since there is a level below which dehydration stress is increasingly apparent (Wesley-Smith *et al.*, 1992). Unlike the loss of viability in *S. rotundifolius*, explant at higher water contents have been reported to survive on exposure to liquid nitrogen (Berjak *et al.*, 1995; Kioko *et al.*, 1998 and 2000)

During cryopreservation all metabolic processes cease, it is possible that mannitol treated explants were too active metabolically judging from the high number of mitochondria occurring in the cytoplasm (Plate 1b). Hence bringing the systems to a halt caused a breakdown in all the plant metabolic systems causing cytoplasm to lose its viability since following dehydration, only few organelles could be observed in cytoplasm (Plate 1f). It is also possible that with the occurrence of high number of small vacuoles in mannitol treated explant (Plate 1c) which is a characteristic whereby, large vacuoles volumes are reduced by redistributing them into smaller vesicles on exposure to mannitol (Gnanapragasam & Vasil, 1992), being an advantage for survival since water contents are relatively low (Reinhoud *et al.*, 1995). However, it is probably that, the water present in the vacuoles did not have high viscosity, which would prevent the formation of ice crystals during cooling and thawing hence causing degeneration of plant cell integrity.

Sucrose treated explant, ultrastructure indicated cytoplasmic breakdown at all stages of treatment. Although the plant cell were not in a high metabolic state prior to exposure to liquid nitrogen, cellular degeneration had already set in and may have had a major role to play, leading to loss of viability on exposure to liquid nitrogen.

The above and all associated factors need to be investigated further. These will help optimise plant cell structure prior to cryopreservation. Based on the ultrastructural studies carried out, it is obvious that the use of mannitol for pregrowth and preculture treatment, the plant tissues develop capability to tolerate other stress (desiccation). *S. rotundifolius* tissues besides yielding high explant survival, results in stable ultrastructure for further plant growth and development. However the treatment does not necessarily result in survival on exposure to cryopreservation. The use of higher concentration of mannitol may enhance cryotolerance. Other critical factors that have to be investigated include maturation of explant supported by constitution of ultrastructure and related water content which play crucial rôle in cryopreservation (Chandal *et al.*, 1994; Berjak *et al.*, 1993). However, the extremely high water content (18.7 – 9.64 g/g dry wt) of plant even in the greenhouse (graph not shown), may still make it difficult to cryopreserved tissues of local accessions of *S. rotundifolius*. Several attempts were made to adequately harden Frafra potato (Table 5) prior to subjecting explants to various treatments and then cooling however, none of them resulted in explants survival after cooling.

| Treatment | Water content |
| --- | --- |
| Six months in culture | 8.1± 1.2 |
| Shoot grown from tuber under sterile conditions (8 weeks) | 19.5 ± 1.6 |
| Vitrified shoot grown from tuber under sterile conditions (8 weeks) | 24.7 ± 3.9 |
| Normal shoots transferred to vented vessels (3 weeks) | 8.2 ± 1.3 |
| Vitrified shoots transferred to vented vessels (3 weeks) | 12.7 ± 2.5 |
| Normal shoots transferred to dry air-line (3 weeks) | 7.2 ± 1.0 |
| Normal shoots transferred to humid air-line (3 weeks) | 18.4 ± 2.2 |
| Cultures transferred to RITA vessels | 13.0 ± 2.2 |

Table 5. Other attempts to acclimatize the new Frafra potato accession 99/053 to lower water content that might enhance cryosurvival.

### 3.2 *Dioscorea rotundata*

Comparatively, yam explants cultured on medium supplemented with 0.3 M sucrose for 3-5 d considerably reduced tissue water content from about 12.2 g g-1 dry mass to between 4.8 and 5.5 g g-1 dry mass before cryoprotection with modified PVS2 (MPVS2) or silica gel dehydration. Following cryoprotection with MPVS2 the Plate (6) below indicated the growth of nodal explants.
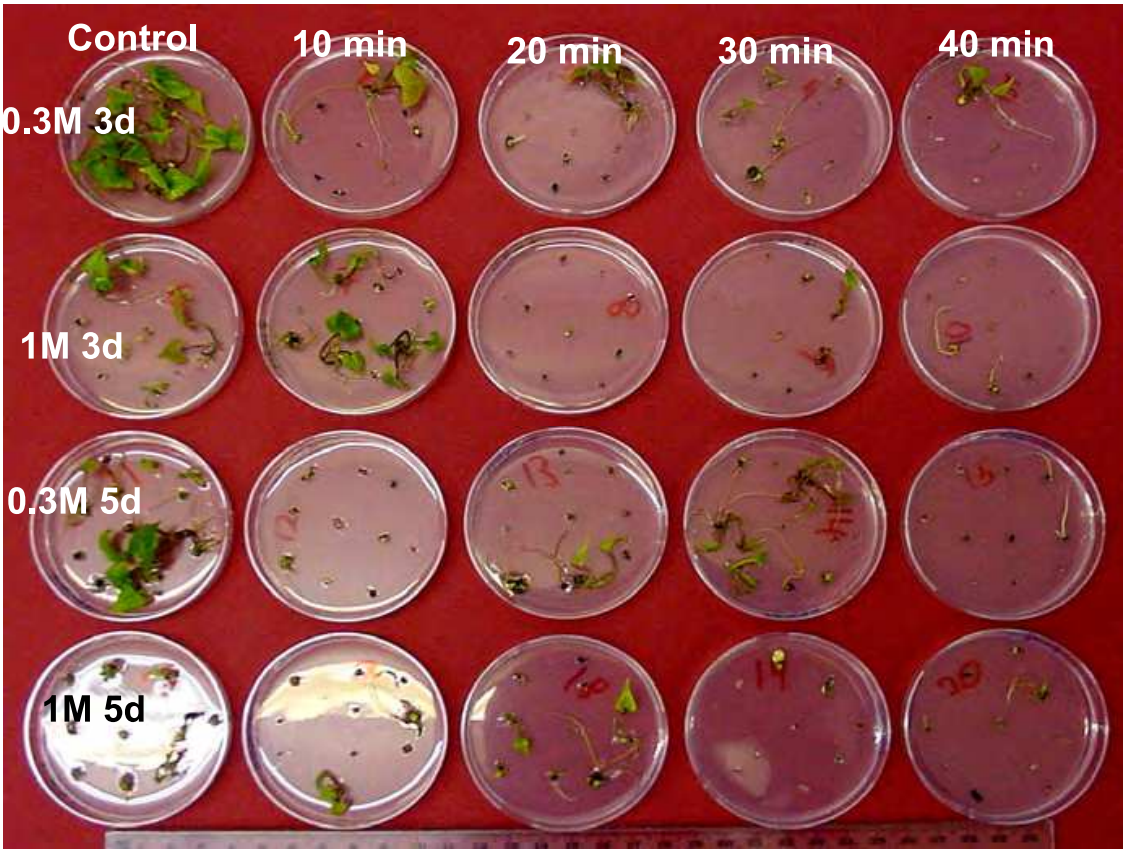


Plate. 6. Growing cultures of yam explant subjected to pregrowth on 0.09 sucrose supplemented medium for 5 weeks, precultured on medium containing 0.3 m sucrose for 3 or 5 d, treated with MPVS2 for varied duration and unloaded with rehydration solution containing 0.3 or 1 M sucrose and cultured on regeneration medium for six week

Ultrastructural studies indicated that cells had deposits of starch in plastids following sucrose treatments. Survival for *D. rotundata* shoot tips treated with MPVS2 vitrification solution, and cooled to -70ºC, was 16% for 15 min treatment and 44% for 40 min. Explant rehydration was in 1.0M sucrose supplemented solution. After the 40 min MPVS2 treatment the TTZ test indicated 88% viability retention of explants cooled to -70ºC, and 44% at -196ºC. Plantlet development was obtained for -70ºC-cooled shoot tips, whereas only callus development occurred from tissues exposed to liquid nitrogen. Explant regeneration was not obtained with silica gel dehydration techniques. It was concluded that vitrification-solution based cryopreservation presently offers the best option for conservation of this *Dioscorea* species.

## 4. Conclusions

Undoubtedly, cryopreservation has high potential for the long-term storage of vegetative explants. It is, however, vital to achieve appropriate tissue water content and the quality of the initial material. This study has shown that vitrification-based cryopreservation protocol is useful for yam explants. However, Frafra potato is extremely sensitive to the vitrification based protocol while explants of the latter easily become hyperhydric and are impossible to dehydrate sufficiently for cryopreservation

The findings are relevant for cryopreservation of a range of yam germplasm (Quain et al., 2009) and also provide a sound basis for further attempts to cryopreserve Frafra potato genetic resources. The technique represents developed simple, cost-effective and potentially reliable methodology that does not require sophisticated equipment. Such procedures should be adapted for germplasm conservation of other species, using limited resources in laboratories in sub-Saharan Africa.

Findings in this present study suggest that in order to achieve an optimal recovery of cryopreserved explants, the donor plants should be adequately conditioned and the recovery media enriched while testing the different cryogenic procedures. Although encapsulation vitrification and encapsulation dehydration procedures have been used in cryopreservation procedure, for the yams used in this study, the use of non-encapsulated explant proved to be a better option. The encapsulation of explants after cryopreservation in the production of e.g. synthetic seeds (Naidoo, 2006; Perán *et al.*, 2006), is, however, worth exploring.

The key to successful cryoprotection has been suggested as inducing tolerance to vitrification solutions and the ability of explants to tolerate dehydration treatment by cryoprotectants has been hypothesised by several researchers as the determining factor for successful cryosurvival (Langis & Steponkus, 1990; Reinhoud *et al.*, 1995). It is still not conclusive whether having been hardened, explants being used in procedures would survive cryopreservation. However, Frafra potato explants were not amenable to conditioning by any of these pretreatments. The requirements for successful cryopreservation differ for different species. There is the possibility that the optimum developmental stage of the explant for successful cryopreservation varies from species to species. Therefore there is the need to ascertain and test many parameters on the basis of each species. However, culture conditions especially those that will obviate hyperhydricity, are of paramount importance, as presently indicated for Frafra potato.

It can be concluded from the experiments that:

- Successful cryopreservation of *Dioscorea rotundata* is possible using a simple vitrification protocol.
- The procedure incorporates
  - pregrowth of the donor plant on 0.09 M sucrose-supplemented medium for five weeks,
  - preculture on 0.3 M sucrose supplemented medium for 5 d
  - MPVS2 solution for 40 min,
  - Rapid cooling in liquid nitrogen or slow cooling to -70°C.
- For the first time successful cryopreservation of *Dioscorea rotundata* accession 'Pona' which is an elite variety in Ghana has been achieved.
- The technique represents developed simple, cost-effective and potentially reliable methodology that does not require sophisticated equipment.
- Procedures can be adapted for germplasm conservation of other species, using limited resources in laboratories in sub-Saharan Africa.
- To achieve an optimal recovery of cryopreserved explants the donor plants should be adequately conditioned.
- Frafra potato is extremely sensitive to the vitrification based protocol.

Frafra potato explants easily becomes hyperhydric, and are impossible to dehydrate sufficiently for cryopreservation, this provide a sound basis for further attempts to cryopreserve Frafra potato genetic resources. These observations therefore make available information for further investigation towards development of cryopreservation protocol.

## 5. Acknowledgements

## 6. References

[1] Ashun MD (1996). *In vitro studies on micropropagation of various yam species (Dioscorea species)* M.Phil. Thesis submitted to University of Ghana – Legon.

[2] Berjak, P., Mycock, DJ, Walker, M, Kioko, JI, Pammenter NW, and Wesley-Smith J (1999). Conservation of Genetic Resources Naturally occurring as recalcitrant seeds. In M. B., K.J. Bradford and J Vazquez-Ramus (eds) *Seed Biology Advances in Applications* pp. 223-228.

[3] Berjak P, Mycock DJ, Wesley-Smith J, Dumet D. & Watt MP (1996) Strategies for *in vitro* conservation of hydrated germplasm. M. N. Normah *et al.*, (eds). In vitro *conservation of plant genetic resources* 19-52.

[4] Berjak, P, Mycock DJ, Watt P, Wesley-Smith J and Hope B (1995). Cryopreservation of Pea (Pisum sativum L.). In Y.P.S. Bajaj (ed) Biotechnology in Agriculture and Forestry (32) Springer: 293-307.

[5] Berjak, P, Vertucci CW and Pammenter N.W. (1993). Effects of developmental status and dehydration rate on the characteristics of water and desiccation-sensitivity in recalcitrant seeds of *Camellia sinensis*. *Seed Science Research* 3, 155-166.

[6] Chandel, KPS, Chaudhury R & Radhamani J (1994), Biological mechanisms determining the recalcitrance in seeds of tea, cocoa and jackfruit. IBPGR-NBPGR Report.

[7] Charoensub R, Phansiri S, Sakai A & Yongmanotchai W (1999). Cryopreservation of cassava *in vitro*-grown shoot tips cooled to –196°C by vitrification. *CryoLetters* 20, pp. 89-94.

[8] Cho EG, Noor NM, Kim HH, Rao VR & Engelmann F (2002) Cryopreservation of *Citrus aurantifolia* seeds and embryonic axes using a desiccation protocol. *CryoLetters* 23, 309-316.

[9] Dereuddre J, Bland S. & Hassen N (1991). Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in Liquid nitrogen: 1. Effect of Preculture. Cryoletters 12 pp.125-134.

[10] Dumet D, Engelmann F, Chardrillange N, Duval Y, & Dereuddre J (1993). Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. *Cryo-Letters* 14: pp. 243-250.

[11] Egnin M, Mora A & Prakash, C.S. (1998). Factors Enhancing *Agrobacterium tumefaciens*-Mediated Gene Transfer in Peanut (*Arachis Hypogaea* L.). In Vitro *Cellular and Developmental Biology-Plants* 34, 310-318.

[12] Engelmann F & Engels JMM, (2002), Technologies and strategies for existing conservation. In JMM Engels, VR Rao, AHD BROWN and MT Jackson (eds), Managing plant Genetic Diversity, CAB International Wallingfield/IPGRI, Rome, pp 89-104.

[13] Engelmann F, Dambier D & Ollitraut P (1994), cryopreservation of cell suspension and embryogenic callus of citrus using a simple freezing process. *Cryo-Letters* 15: pp. 53-58.

[14] González-Arnao MT, Urra C, Engelmann F, Ortiz R, & Delafe C (1999). Cryopreservation of encapsulated sugarcane apices - effect of storage-temperature and storage duration. *CryoLetters* 20, pp. 347-352.

[15] Gonzales-Benito ME & Perez C (1994). Cryopreservation of embryonic axes of two cultivars of hazelnut (*Corylus avellana* L.). *Cryoletters*, 15, pp. 41-46

[16] Harding K & Benson EE (1994). A study of growth, flowering, and tuberisation in plants derived from cryopreserved potato shoot-tips: implications for *in vitro* germplasm collections. *Cryo Letters* 15, 59-66.

[17] Hatanaka T, Yasuda T, Yamaguchi T & Sakai A (1994). Direct regrowth of encapsulated somatic embryos of coffee (*Coffee canephora*) after cooling in liquid nitrogen. *Cryoletters* 15, pp. 47-52.

[18] Jitsuyama Y, Suzuki T, Harada T & Fujikawa S (2002). Sucrose incubation increases freezing tolerance of Asparagus (*Asparagus officianalis* L) embryonic cell suspensions. *Cryoletters* 23, pp. 103-112.

[19] Kay DE (1973) TPI Crop and Product Digest No. 2 Root CROPS. London: Tropical Products Inst.

[20] Kioko J, Berjak P, Pammenter NW, Watt MP & Wesley-Smith J, (1998). Desiccation and cryopreservation of embryonic axes of *Trichilia dregeana* SOND. *Cryo-Letters* 19: pp. 15-26

[21] Kioko, J, Berjak P, Pritchard H & Daws M (2000). Seeds of African pepper bark (Wurdurgia *salutaris*) can be cryopreserved after rapid dehydration in silica gel. In F. Engelmann and H. Takagi (eds.) *Cryopreservation of Tropical Germplasm, Current Research Progress and Application.* IPGRI: 371-377

[22] Gnanaprasam, S and Vasil IK (1992). Cryopreservation of immature embryos, embryogenic callus and cell suspension cultures of gramineous species. *Plant Sciences* 83, pp. 205-215.

[23] González-Benito ME, Prieto RM, Herradón E & Martín C (2002) Cryopreservation Of *Quercus Suber* And *Quercus Ilex* Embryonic Axes: In Vitro Culture, Desiccation And Cooling Factors *CryoLetters* 23, 283-290

[24] Grospietsch M, Stadulkora E & Jiri Z (1999) Effect of osmotic stress on the dehydration tolerance and cryopreservation of *Solanum Tuberosum* shoot tips. *Cryoletters* 20, 339-346.

[25] Hatanaka T, Yasuda T, Yamaguchi T & Sakai A (1994). Direct regrowth of encapsulated somatic embryos of coffee (*Coffee canephora*) after cooling in liquid nitrogen. *Cryoletters* 15, pp. 47-52.

[26] Langis P, & Steponkus PL (1990). Cryopreservation of rye protoplast by vitrification. *Plant Physiology* 92, pp. 666-671.

[27] Matinez-Montero ME, Gonzalez-Arnao MT, Borroso-Nordelo C, Puentes-Diez C & Engelmann F (1998) Cryopreservation of sugarcane embryogenic callus using a simple freezing process. *Cryo-Letters* 17, 171-176.

[28] Matsumoto T, Sakai A & Nako Y (1998). A novel preculturing for enhancing the survival of *in vitro* – grown meristems of wasabi (*wasabi japonica*) cooled to -196oC by vitrification. *Cryoletters* 19, pp. 27-36.

[29] Murashige T & Skoog E (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15, 473 – 497.

[30] Mycock D (1999). Addition of Calcium and Magnesium to a Glycerol and Sucrose cryoprotectant solution improves the quality of plant embryo recovery from cryostorage. Cryoletters 20, pp 77-82.

[31] Naidoo, S, (2006). *Investigations into the Post-harvest behavior and germplasm conservation of the seed of selected* Amaryllid *species*. MSc Thesis University of Kwa-Zulu Natal Durban, South Africa.

[32] Niino T, Hettiarachchi A, Takahashi J &. Samarajeewa PK, (2000). Cryopreservation of lateral buds of *in vitro* grown innala plants (*Solenostemon rotundifolius*) by vitrification. *Cryoletters* 21 pp. 349 – 356.

[33] Nishizawa, S, Sakai A, Amano Y & Matsumoto T (1993), Cryopreservation of Asparagus (*Asparagus officinalis* L.) embryogenic cells and subsequent plant regeneration by simple freezing method. *CryoLetters* 13, 379-388.

[34] Ng SYC & Ng NQ (1991) in *Tissue Culture for Conservation of Plant Genetic Resources*, (ed) JH Dodds, Chapman and Hall, London, pp 11-39.

[35] Ng NQ & Daniel IO (2000) in *Cryopreservation of Tropical Germplasm, Current Research Progress and Application,* (eds) F Engelmann and H Takagi, Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan / International Plant Genetic Resources Institute, Rome, Italy, pp 136-139.

[36] Pammenter NW , Vertucci CW & Berjak P (1991). Homoiohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii*. *Plant Physioplogy* 96, 1093-1098.

[37] Panis B, Strosse H, Van Den Hende S & Swennen R (2002) Sucrose preculture to simplify cryopreservation of banana meristem cultures. *CryoLetters* 23, 375-384 (2002)

[38] Perán R, Berjak P, Pammenter NW & Kioko JI (2006). Cryopreservation, encapsulation and promotion of shoot production of embryonic axes of a recalcitrant species *Ekerbergia capensis*, Sparrm. *Cryoletters* 27 (1), pp. 5-16.

[39] Plessis P, Leddet C, Collas A & Dereuddre J. (1993). Cryopreservation of *Vitis Vinefera* L. Cv Chardonnay shoots tips by encapsulation-dehydration: effects of pretreatment, cooling and postculture conditions. *Cryoletters* 14, pp. 309-320.

[40] Potts SE & Lumpkin TA (1997). Cryopreservation of Wasabi species seeds. Cryoletters. 18, pp.185-190.

[41] Quain, MD, Berjak, P, Acheampong, E, and Kioko, JI, (2009) Sucrose Treatment And Explant Water Content: Critical Factors to Consider in Development of Successful Cryopreservation Protocols for Shoot Tip Explants of the Tropical Species *Dioscorea rotundata* (Yam). *CryoLetters*, 30 (3), 212-223

[42] Reinhoud PJ, Schrijnemakers WM, van Iren F & Kijne JW (1995) vitrification and heat shock treatment improve cryopreservation of tobacco cell suspensions compared to two-step freezing. *Plant Cell Tissue and Organ Culture* 42: pp. 261-267.

[43] Sakai A, Kobayashi S & Oiyama I (1990) Cryopreservation of nuceller cells of navel orange (*Citrus sinensis* Obs, var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Report* 9, 30-33.

[44] Santos IRI & Stushnoff C (2003) Desiccation and freezing tolerance of embryonic axes from *Citrus sinensis* (L) OSB. pretreated with sucrose. *CryoLetters* 24, 281-292.

[45] Schippers, R.R. (2000). African Indigenous Vegetables. An Overview of the Cultivated Species. Chatham. UK: NRI/ACP-EU Technical

[46] Shibli RA, Moges AD, and Karam NS (2004). Cryopreservation Of African Violet (*Saintpaulia Ionantha* Wendl.) Shoot Tips. In vitro *Cellular and Develoment Biology - plant* 40, 4, pp. 389-395(7)

[47] Spurr A R (1969) A low-viscosity epoxy resin embedding medium for electromicroscopy. *Journal of Ultrastructure Research* 26, 31-43

[48] Tetteh JP & Guo JI (1993) *Problems of Frafra Potato Production in Ghana*. A dissertation. School Centre for Agricultural and Rural Cooperation of Agriculture, University of Cape Coast.

[49] Thammasiri K. (1999). Cryopreservation of embryonic axes of jackfruit. *Cryo-letters* 20, pp. 21-28.

[50] Turner SR, Touchell DH, Senarata T, Bunn E, Tan B & Dixon KW (2001) Effect of Plant Growth Regulators on Survival and Recovery Growth Following Cryopreservation. *Cryoletters* 22, 163-174.

[51] Veisseire P, Guerrier J & Coudret A. (1993). Cryopreservation of embryonic suspension of *Hevea brasiliensis*. *CryoLetters* 14, 295-302.

[52] Wang J-H, Bian R-W, Zhang Y-W & Cheng H-P (2001) The dual effect of antifreeze protein on cryopreservation of rice (*Oryza sativa* L.) embryogenic suspension cells. *CryoLetters* 22, 175-182.

[53] Wang Q, Li P, Batuman Ö, Gafny R & Mawassi M., (2003). Effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus *in vitro*. *CryoLetters* 24, 293-302.

[54] Walter C, DH. Touchell, P Power, J Wesley-Smith and MF. Antolin (2002), A Cryopreservation protocol for embryos of the endangered species *Zizania texana*. *CryoLetters* 23, 291-298.

[55] Wesley-Smith J, Vertucci CW, Berjak P, Pammenter NW & Crane J (1992) Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. *Journal of Plant Physiology* 140, 596-604.

[56] Withers, LA (1985). Cryopreservation of cultured cells and protoplasts. In: K.K. Kartha (ed) *Cryopreservation of plant cells and organs*. CRC press Inc., Boca Raton. pp 243-267.
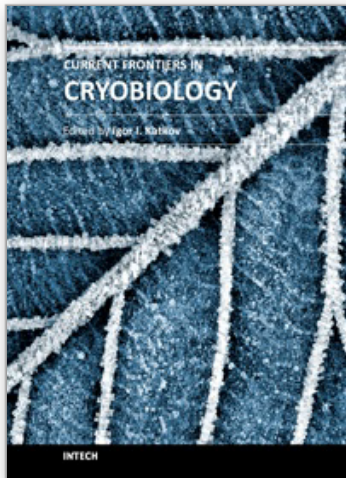
**Current Frontiers in Cryobiology**

Edited by Prof. Igor Katkov

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

**How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Marian D. Quain, Patricia Berjak, Elizabeth Acheampong and Marceline Egnin (2012). Cryopreserving Vegetatively Propagated Tropical Crops - The Case of Dioscorea Species and Solenostemon rotundifolius, Current Frontiers in Cryobiology, Prof. Igor Katkov (Ed.), ISBN: 978-953-51-0191-8, InTech, Available from: http://www.intechopen.com/books/current-frontiers-in-cryobiology/cryopreserving-vegetatively-propagated-tropical-crops-the-case-of-dioscorea-species-and-solenostemon

**INTECH**

open science | open minds