Emir. J. Food Agric. 2014. 26 (6): 565-576
doi: 10.9755/ejfa.v26i6.18024
http://www.ejfa.info/

Regular Article

PLANT TISSUE CULTURE

Establishment of encapsulation-dehydration technique for *in vitro* fragmented explants of *Rosa hybrida* L. cv. Helmut Schmidt

Safiah Ahmad Mubbarakh, Nurul Ashikin Izhar, Arulvilee Rajasegar, Sreeramanan Subramaniam*

School of Biological Sciences, Universiti Sains Malaysia, 11800, Gelugor, Penang, Malaysia

Abstract

This study was carried out to evaluate the encapsulation-dehydration technique on IFEs of *Rosa hybrida* L. cv. Helmut Schmidt. The survival of IFEs was first assessed based on the effects of four sucrose concentrations (0, 0.25, 0.5 and 0.75 M) at different durations (0, 24 and 48 hours). The IFEs were then encapsulated and osmoprotected on shaker at 110 rpm with various sucrose concentrations (0, 0.1, 0.5 and 0.75 M). Subsequently, encapsulated IFEs were dehydrated under laminar air flow during three different time periods (0, 3 and 6 hours) on oven sterilized 50 g silica gel. The encapsulated IFEs were plunged into liquid nitrogen for a minimum duration of 24 hour. Encapsulated IFEs were thawed at 40ºC for 90 seconds and cultured on full-strength MS medium supplemented with 3% sucrose for a week in dark, a week in semi-light and an additional 1 week under full light exposure. IFEs were then evaluated for survival by 2,3,5-Triphenyltetrazolium Chloride assay at 490 nm. The best conditions for the encapsulation-dehydration of IFEs of *Rosa hybrida* L. cv. Helmut Schmidt was obtained when IFEs were precultured in 0.25 M sucrose for 24 hours, osmoprotected in 0.75 M sucrose and dehydrated for 3 hours. Histological analysis showed cryopreserved IFEs with intensely stained nucleus but with severely damaged membranes.

Key words: Cryopreservation, *In vitro* fragmented explants, 2,3,5-Triphenyltetrazolium Chloride assay, Histological analysis

Introduction

Roses are among the important flowers worldwide that are widely grown as cut flowers, potted plants and as specimens (Wang et al., 2002). It contains more than 100 species and comes in variety of colours and fragrance (Lynch, 2002). Roses have well significant commercial value to the horticulture society (Short and Roberts, 1991). Therefore it is a must for rose breeders to maintain their cultivars before they face extinction (Luza and Polito, 1988).

Traditionally roses were propagated mostly by vegetative methods such as stem cutting, layering, budding, grafting, and tissue culture (Pati et al, 2006). The asexual propagation method of roses is pricey, as requires space and has high risk of loss due to diseases and environmental stress. Therefore, propagation of plants using tissue culture procedures has grown as an established technique to reproduce crops that are complicated if were propagated traditionally (Razavizadeh and Ehsanpour, 2008). Since *in vitro* tissue culture is vulnerable to contamination, somaclonal variation and human blunders (Goncalves et al., 2010) hence cryopreservation is a promising alternative for conservation of vegetatively propagated plants due to its repeatability, safety, long-term storage possibility, stability of phenotypic and genotypic characters, minimal storage space and maintenance requirements (Chmielarz, 2013).

There are a few types of cryopreservation methods that have been applied to roses namely vitrification, encapsulation-dehydration, encapsulation-vitrification and droplet vitrification contributing to the number of cryopreserved species to increase sharply (Hua and Hong, 2010). Encapsulation-dehydration protocols are much easier to handle compared to other protocols (Zainuddin et al., 2011). It is a synthetic seed technology which was first developed for cryopreservation of pear and potato shoot tips (Fabre and Dreuddre, 1990). Benson et al. (1996)

Received 13 February 2014; Revised 08 March 2014; Accepted 10 March 2014; Published Online 25 March 2014

*Corresponding Author

Sreeramanan Subramaniam
School of Biological Sciences, Universiti Sains Malaysia, 11800 Gelugor, Penang, Malaysia

Emails: sreeramanan@usm.my; sreeramanan@gmail.com

stated that encapsulation dehydration improved the recovery after cryostorage and produced higher rates of shoot formation compared to the slow freezing method. Similarly, Maneerattanarungroj et al. (2007) reported that encapsulation-dehydration method is the best as it resulted in higher growth recovery post liquid nitrogen treatment for *Cleisostoma areitinum* (Rchb.f.). This could be due to the protection capacity by the calcium alginate beads from a direct damage through dehydration mechanism (Srivastava et al., 2009). Beads act as a protective layer for the explants as it is immersed in LN and toxic cryoprotectants. In addition, this technique involved sucrose as the only cryoprotectant and it is easier to handle as compared to the costly programmable freezers (Martinez and Revilla, 1999). Many plants were successfully cryopreserved by encapsulation-dehydration namely Melia azadarah (Scocchi et al., 2004), *Oncidium bifolium* seeds and protocorms (Flachsland et al., 2006), *Vitis vinifera* L. somatic embryos (Miaja et al., 2004) and *Fragaria x ananassa* (Clavero-Ramirez et al., 2005).

Source of plant, plant physiological status, culture conditions, pretreatment conditions, cryopreservation methods, cryogenic facilities, regimes and post-thawing are the factors that also contribute to the successful cryopreservation system (Reed et al., 2004). Generally in cryostorage study, the most suitable plant part used is a meristem culture as it allows the production of virus free lines under *in vitro* condition (Reed, 2008). The explants have to be in a suitable growth stage to allow dissection and freezing tolerance, with the ability to regenerate into plantlets after cryogenic storage (Normah and Makeen, 2008). Different plants response differently to different treatments. Therefore, optimizing each step is crucial for explants to recover after cryopreservation. To date no report has been published on *Rosa hybrida* L. cv. Helmut Schmidt using IFEs as starting material for encapsulation-dehydration. The aim of this study is to optimize and establish an encapsulation-dehydration technique to cryopreserve *Rosa hybrida* L. cv. Helmut Schmidt.

Materials and Methods

Plant material

Surface sterilization of internodal segment

Rosa hybrida L. cv. Helmut Schmidt was chosen for plant regeneration and was obtained from Penang Hill, Malaysia. The internodal segments were cut to 3 cm in length and washed under running tap water for 45 minutes with 3 drops of Tween-20 emulsifier. Next, the sterilization of the internodal segments was carried out in laminar air flow with 70% ethanol for 2 minutes followed by 40% sodium hypochlorite (NaClO, Chlorox 5.2%) for 20 minutes. Internodal segments were rinsed seven times with sterile distilled water to remove traces of clorox completely. Internodal segments were dissected into 1.5 cm in length prior to transfer in full strength semi-solid Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 2.0 $mg.l^{-1}$ BAP and 30 $g.l^{-1}$ sucrose. The pH was adjusted to 5.7 prior to autoclaving. The internodal segments of *Rosa hybrida* L. cv. Helmut Schmidt cultures were grown at 25ºC under 16 hours photoperiod (Philips TLD, 36W, 150$\mu mol.m^{-2}.s^{-1}$). The fully grown *in vitro* explants were harvested after 4 weeks of culture. Explants were then proliferated by incubation in the tissue culture room at 25±2ºC and were subcultured every 4 weeks on full strength semi-solid Murashige and Skoog medium supplemented with 3% sucrose, 2.75 $g.l^{-1}$ GelriteTM and 1 $mg.ml^{-1}$ BAP.

IFEs for cryopreservation experiment

IFEs were used as a starting material for cryopreservation study. *In vitro* fragmented explant (IFE) is a new target tissue reported by Gonzalez-Arnao (Gonzalez-Arnao et al., 2009) who worked on *Vanilla planifolia*. IFEs with size range of 3-4mm in length were dissected carefully from *in vitro* culture guided by an autoclaved graph paper underneath the sterile Petri dish. The base of *in vitro* grown planlets was dissected and partially cleaned using scalpel to eliminate oxidized tissues. The cleaned base was then used in cryopreservation study. Through microcuttings, a large number of shoots would be produced.

Media preparation

The preculture medium contained 0, 0.25, 0.5 and 0.75 M of sucrose in full-strength MS semi-solid medium. Sodium alginate (3%) was prepared by dissolving slowly in 0.4 M sucrose in full-strength MS media with the absence of calcium chloride, $CaCl_2$. Calcium chloride solution (0.1M) was then supplemented with 0.4 M sucrose. The osmoprotection media contained 0.75 sucrose supplemented with full strength MS media. The pH was adjusted to 5.8 before autoclaving.

Preculture

For the optimization of preculture conditions, 3-4 mm sized IFEs were selected from four weeks old cultures and precultured on full-strength semi-

solid MS medium supplemented with different concentrations of sucrose (0, 0.25, 0.5 and 0.75 M) for 24, 48 and 72 h with the temperature set at 25±2ºC.

Encapsulation-dehydration

The precultured IFEs were then suspended in 3% sodium alginate medium. IFEs that were dispersed in 0.1 M $CaCl_2.2H_2O$ solution were allowed to harden for 30 minutes to form beads approximately of 4 mm in diameter. Beads were allowed to polymerize and were taken out aseptically one by one gently using sterilised micropipette and were placed into a conical flask which contained osmoprotection medium with different concentrations ranging from 0, 0.25, 0.5 and 0.75 M. The conical flasks were then placed on a shaker (110 rpm) overnight under plant tissue culture room conditions (25 ± 2°C, 16 h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W, 150 $\mu mol.m^{-2}.s^{-1}$).

Dehydration

Beads from the conical flasks containing osmoprotection medium were poured into a waste beaker aseptically using a sieve. The beads were collected using forceps and transferred to culture jars contained 50 g oven-sterilized silica gel (dried in oven at 110ºC overnight) with a filter paper placed in it. The beads were on the sterilized silica gel for different durations ranging from 0, 3 and 6 hours.

Freezing, Thawing and growth recovery

Dehydrated beads were placed in cryovials and directly immersed in LN for 24 hours. Cryovials were warmed in a water bath at 40ºC for 90 seconds. The beads from the cryovial were taken out and transferred aseptically into Petri dishes containing growth recovery medium and were put in dark in culture room condition for a week followed by semi-light and light condition for another 1 week each. For the control (non-cryopreserved) samples, the cryostorage and thawing step was omitted and were transferred to growth recovery medium. The beads were stored in culture room at 25±2°C with 16 hours photoperiod under cool white fluorescent lamp at 150 $\mu mol^{-2}s^{-1}$.

Determination of viability 2,3,5-Triphenyltetrazolium Chloride (TTC) assay

The viability of living cells in both cryopreserved and non-cryopreserved of IFEs was determined through spectrophotometrical analysis which is based on the metabolic activity of living cells. Colourless triphenyltetrazolium chloride was reduced by dehydrogenases in living plant cells to yield triphenylformazon, a reduced TTC which coloured red. The procedure in this study was adapted from Verleysen et al. (2004).

IFEs were taken out from each bead and rinsed three times with 5 mL distilled water. After discarding the distilled water, IFEs were soaked in 5 mL TTC solution (0.6% [w/v]) TTC, 0.05% Tween 80 in 0.05 M Na_2HPO_4/KH_2PO_4 buffer at pH 7.4) overnight in dark condition. After 18 hours, TTC solution was discarded and IFEs were rinsed with 7 mL distilled water thrice. Then, IFEs were resuspended in universal bottle containing 7 mL 95% ethanol and triphenyl formazon was extracted from IFEs by boiling in 80ºC water bath for an hour. The formazan extracts were cooled to room temperature, topped up to 7 ml with 95% ethanol. Analysis was then carried out using spectrophotometer (Hitachi U-1900) where absorbance was taken at 490 nm.

Experimental designs

Each treatment consists of four replicates which were exposed to LN, four replicates without exposure to LN and each replicate contains five IFEs. Experiments were conducted in two conditions for all the optimization tested; (i) cryopreserved and (ii) non-cryopreserved. For cryopreserved experiment (+LN), beads containing IFEs were immersed in LN before growth recovery process. In non-cryopreserved experiment (-LN), beads contained IFEs were subjected directly to growth recovery medium after dehydration steps. Survival was estimated three weeks after treatment by 2,3,5-triphenyl tetrazoliumchloride (TTC) spectrophotometrical analysis at 490 nm. Means were analysed through independent sample t-test and one-way ANOVA and differentiated using Tukey test $p \leq 0.05$.

Histological analysis

Histology analyses were carried out on IFEs of cryopreserved, non-cryopreserved and stock culture. IFEs were fixed in FAA (95% ethyl alcohol: glacial acetic acid: formaldehyde: water, 10:1:2:7) for a week. The IFEs were then rinsed with distilled water for three times in duration of half an hour each. Series of IFEs transferred into alcohol TBA were carried out with 50% TBA for two hours, 70% TBA for two hours, 85% TBA for two hours, 98% TBA for two hours and 100% TBA for three hours. IFEs were then treated with TBA I and II (100% TBA) for three hours respectively for overnight. IFEs were then exposed to xylene for ten minutes followed by treatment with xylene and wax for 30 minutes. Samples were then treated with wax

I, II and III (pure wax) for an hour respectively. Then, specimens were blocked and sliced using six micron microtome (Leica RM 2135). Safranin and fast green were used to stain the specimens. Permanent slides were observed under Light microscope (Olympus BX41).

Results

Effects of various preculture concentrations on the viability of IFEs

Effect of various preculture concentrations on the viability of cryopreserved and non-cryopreserved IFEs were investigated using spectrophotometric-TTC assay. Cryopreserved IFEs which were precultured in full-strength MS medium supplemented with 0, 0.25, 0.5 and 0.75 M sucrose showed no significant difference analyzed using ANOVA. For cryopreserved IFEs which were pretreated in full strength MS media without the presence of sucrose prior to cryostorage showed the lowest viability rate when compared to other sucrose concentrations. However, cryopreserved and non-cryopreserved IFEs precultured in full strength MS media supplemented with 0.25 M sucrose gave the best viability rate based on spectrophotometric-TTC assay (Figure 1). When high concentrations of the sucrose were involved (0.5 and 0.7 M), IFEs were damaged although preculture seem to be an essential step in cryopreservation. The non-cryopreserved IFEs also showed the same pattern of viability respectively on each concentration (Figure 1). Thus, for the following optimization, 0.25 M of sucrose was chosen as the preculture treatment.

Effects of different preculture durations on the viability of IFEs

IFEs that were not precultured prior to LN storage resulted in low viability based on spectrophotometric-TTC assay. Since the best sucrose concentration was 0.25 M (Figure 1), so it was further applied in subsequent experiments. Based on the TTC analysis, the viability rate of 0 and 24 h did not show any significant difference. Cryopreserved IFEs which were precultured in full strength MS media supplemented with 0.25 M sucrose showed highest viability rate when precultured for 1 day comparative to other durations tested based on spectrophotometric-TTC assay. In non-cryopreserved IFEs, all treatments duration showed no significant difference. All non-cryopreserved IFEs showed decreasing viability rate when were precultured in full strength MS media supplemented with 0.25 M sucrose for 0-48 hours (Figure 2). Similarly, both +LN and –LN treatment gave best viability rate when IFEs were precultured for 24 h in 0.25M preculture media and showed a decreased in viability when were preculture for more than 24 hours.

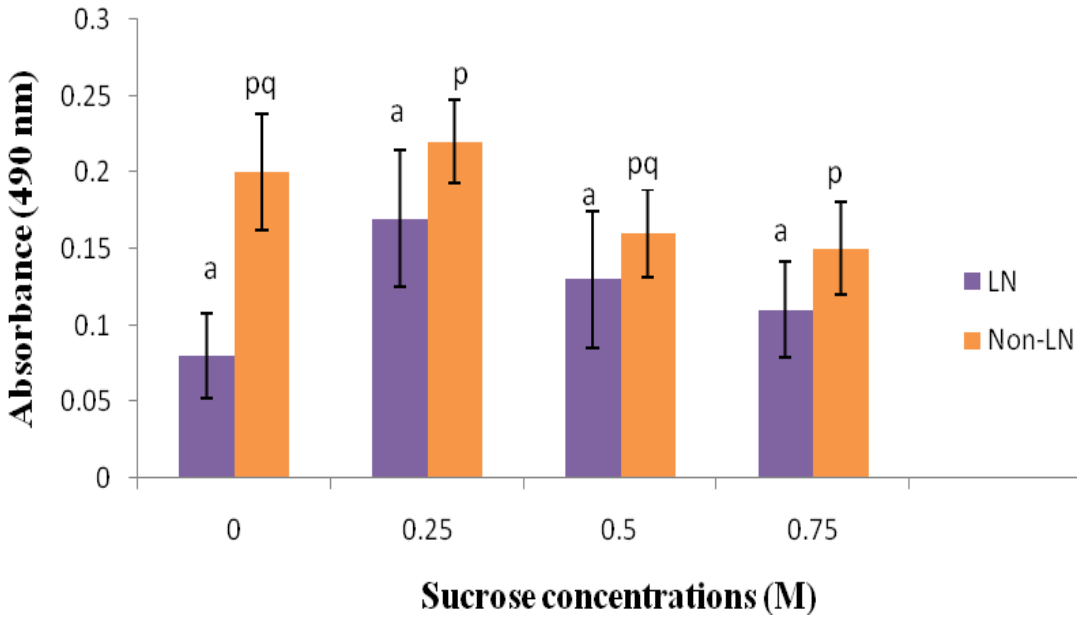

Figure 1. Effects of different preculture sucrose concentrations on viability of both cryopreserved (+LN) and non-cryopreserved (-LN) IFEs as obtained from the spectrophotometric-TTC assay. Cryopreserved and non-cryopreserved treatments were analyzed separately. Bars marked by different letters are significantly different according to Tukey's multiple range test at p=0.05. Vertical bars represent ±SD means of four replicates.

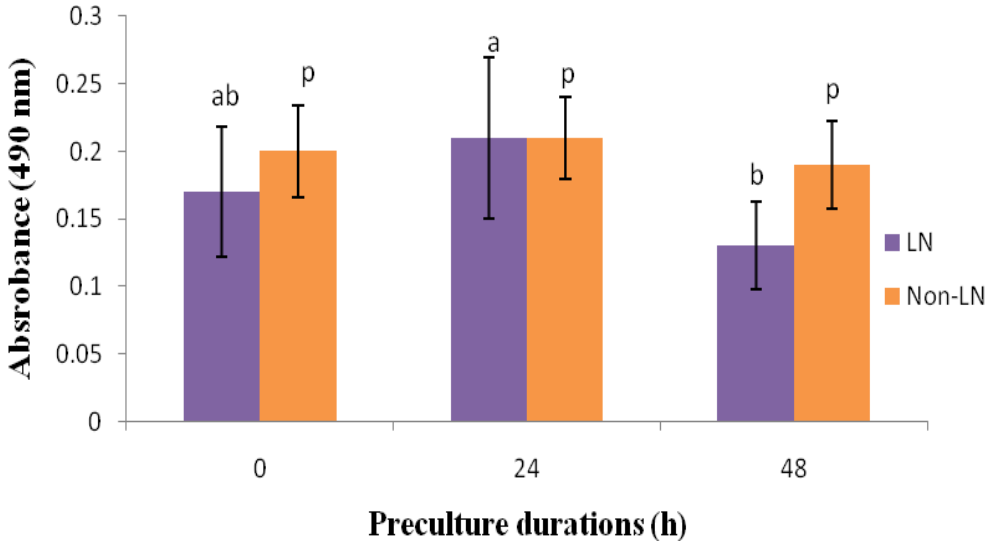

Figure 2. Effects of different preculture durations on viability of both cryopreserved (+LN) and non-cryopreserved (-LN) IFEs as obtained from the spectrophotometric-TTC assay. Cryopreserved and non-cryopreserved treatments were analyzed separately. Bars marked by different letters are significantly different according to Tukey's multiple range test at p=0.05. Vertical bars represent ±SD of means of four replicates.

Effects of different osmoprotection concentrations on the viability of IFEs

Based on TTC analysis, the best viability rate achieved when encapsulated IFEs were osmoprotected in 0.5 M sucrose (Figure 3). In non-cryopreserved IFEs, 0, 0.1 and 0.75 M sucrose concentrations showed no significant differences. However, there was a significant differences between 0.5 and 0.75 M of sucrose for non cryopreserved IFEs. Therefore, for the fourth parameter, 0.75 M of sucrose was chosen as optimized osmoprotection concentration (Figure 3).

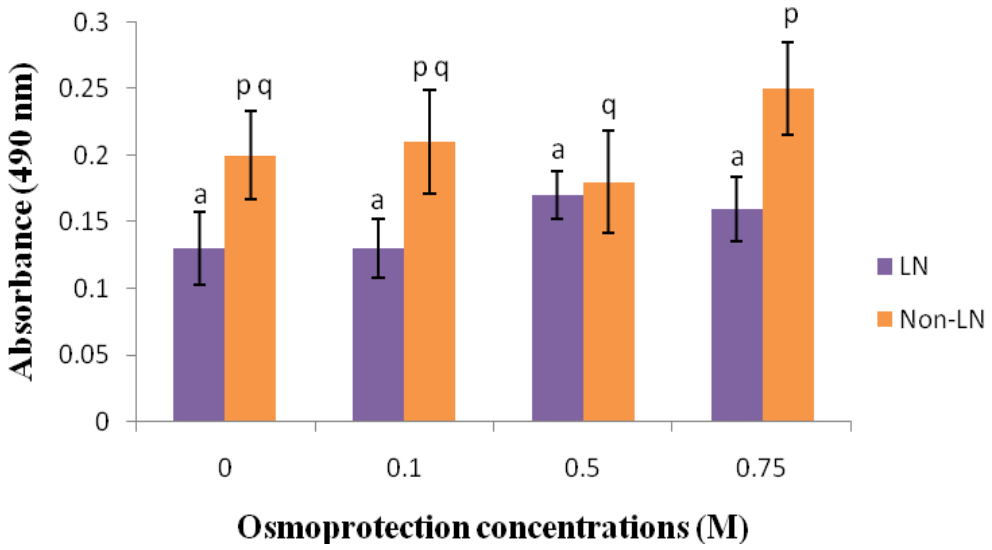

Figure 3. Effects of different osmoprotection concentration on viability of both cryopreserved (+LN) and non-cryopreserved (-LN) IFEs as obtained from the spectrophotometric-TTC assay. Cryopreserved and non-cryopreserved treatments were analyzed separately. Bars marked by different letters are significantly different according to Tukey's multiple range test at p=0.05. Vertical bars represent ±SD of means of four replicates.

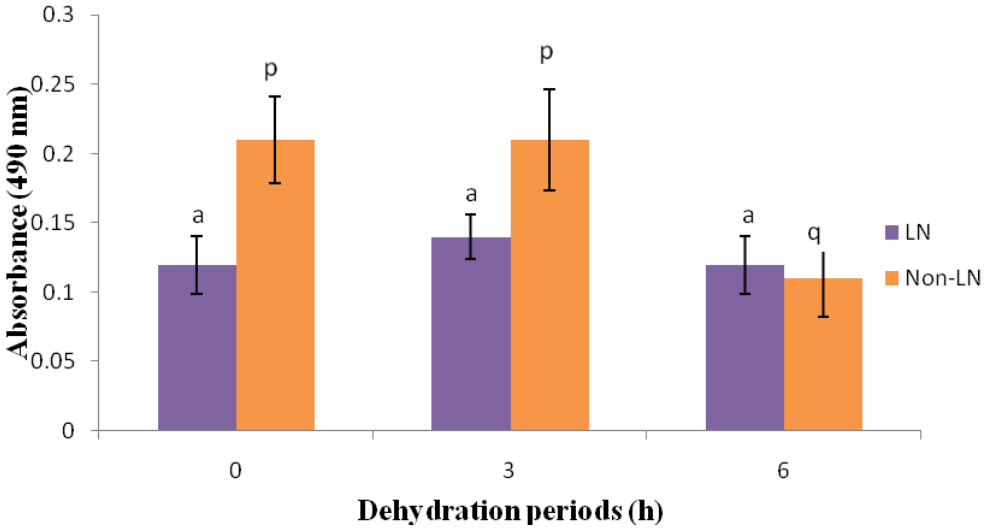

Figure 4. Effects of different dehydration periods on viability of both cryopreserved (+LN) and non-cryopreserved (-LN) IFEs as obtained from the spectrophotometric-TTC assay. Cryopreserved and non-cryopreserved treatments were analyzed separately. Bars marked by different letters are significantly different according to Tukey's multiple range test at p=0.05. Vertical bars represent ±SD of means of four replicates.

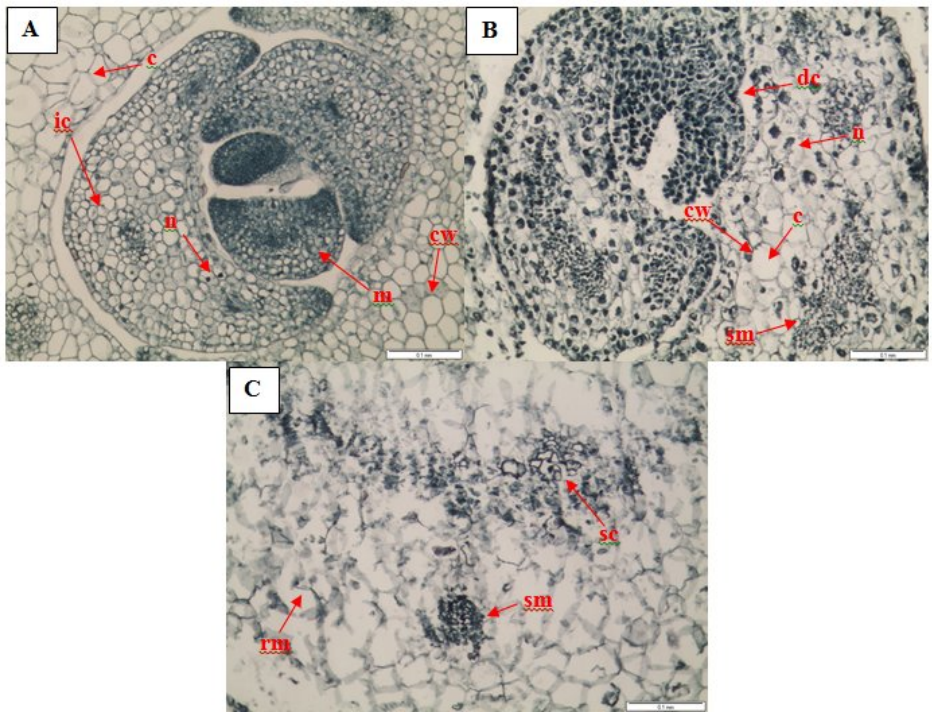

Figure 5. Histological cross section of IFEs (100 µm). (A) The cross section of stock culture (B) The cross section of non-cryopreserved IFEs (C) The cross section of cryopreserved IFEs. (n: nucleus; c: cytoplasm; cw: cell wall; ic: intact cells, vn; voluminous nucleus, um; unruptured membrane, rm; ruptured membrane, m; dc: dense cytoplasm; meristematic region, sc: shrinked cells; sm: storage materials).

Effect of dehydration periods on the viability of IFEs

Cryopreserved and non cryopreserved IFEs were dehydrated for 0 to 6 hours in silica gel prior to a plugged into LN. Cryopreserved IFEs showed no significant difference among the three treatments. However, dehydration for three hours showed the best viability for both cryopreserved and noncryopreserved IFEs (Figure 4). When IFEs were dehydrated for more than 3 hours, there was a decrease in viability.

Histological Analysis

Histological observation was evaluated for stock culture, non-cryopreserved and cryopreserved IFEs. Stock culture IFEs act as the control treatment to compare between cryopreserved and non-cryopreserved IFEs. Based on Figure 5, the cross section of stock culture IFEs (Figure 5A) displayed large amount of volume packed cells and voluminous nucleus with high meristematic region with intact cell walls (Figure 5A). Non-cryopreserved IFEs displayed more packed cells and visible nucleus (Figure 5B) compared to cryopreserved IFEs (Figure 5C). Nevertheless, non-cryopreserved IFEs showed conserved cell structures and more homogenous cell population compared to cryopreserved IFEs (Figure 5B). High number of ruptured membrane and shrinked cells can be observed in cryopreserved IFEs (Figure 5C).

Discussion

Cryopreservation is the conservation technique used to store materials in liquid nitrogen (LN) at ultra low temperature (-196˚C). It is a long term storage procedure used to preserve germplasm using minimum space and maintenance (Short and Roberts, 1991). It has been applied to a wide range of plant species and tissues (Reed, 2001). It involves the removal of all freezable water from tissue by physical or osmotic dehydration, followed by ultra-rapid freezing (Kaviani, 2011). The advantages of cryostorage are that normal cell division and growth are halted thus biochemical, metabolic processes and normal cell divisions are arrested (Harding, 2004). Thus, any plant materials can be stored without alterations for unlimited periods (Lambardi et al., 2000). Moreover, storing in LN gives low risk of contamination and genetic stability is preserved (Harding, 20004).

Encapsulation-dehydration is a protocol developed by Fabre and Dereuddre (1990) that consists of seven (7) main stages: (i) preculture, (ii) formation of beads, (iii) osmoprotection, (iv) dehydration, (v) immersion in LN, (vi) thawing and (vii) culture in growth recovery (Kaczmarczyk et al., 2012). In this study, a simple and efficient method of encapsulation-dehydration has been established to cryopreserve IFEs for long term storage.

In order to withstand cryoinjury, the plant material for cryopreservation should be totipotent and meristematic. This type of tissue is most likely to withstand freezing since it contains only small vacuoles with minute amount of water and a dense cytoplasm with high nucleo-cytoplasmic balance. Explants that are young and possess rapid growing tissues such as shoot tips and nodal segments are good targets as starting materials for cryopreservation study (Kaviani, 2011). They are easily propagated *in vitro* which provide plenty of materials to work with and proved to be a reliable source of potentially regenerable tissues.

According to Reed et al. (2005), the success of a cryopreservation protocol also depends on tolerance and sensitivity of germplasm to overcome stress due to ultra low temperature. The tolerance of plants to harsh cryopreservation procedures depends on the ability of that particular plant to withstand cryoinjury. Ice formation and colligative damage are the two main factors that cause stress to plants stored in LN. Ice formation causes structural and osmotic damages in cells and led to mechanical injury. The manipulation of liquid, glassy and solid (ice) states of water is the main goal in order to avoid intracellular ice formation (Day et al., 2008). Hence, the main approach in cryopreservation is to control freezing rate which means the control of extracellular ice crystallization (Day et al., 2008). Extracellular ice crystallization is a process defined as nucleation, a point at which ice crystals are initiated.

Cryoprotectant is a substance either chemical or a mixture of chemical that acts as a protector of tissue from freezing temperatures. According to Ishikawa et al. (Ishikawa et al., 1997), it is essential for cryoprotectant to have low molecular weight, easily miscible with solvent, non-toxic at low concentration and easily permeable into cells. Sucrose is the most recommended cryoprotectant as it posses zero toxicity and act as carbon source for plant culture (Dumet et al., 1993). Sucrose could induce cytoplasmic vitrification in order to avoid the formation of intracellular ice crystal during the rapid cooling in LN (Lurswijidjarus and Thammasiri, 2004).

Different roses withstand different sucrose concentrations therefore optimization of preculture concentration is vital. Pretreatment prepares the explants for the freezing process and is carried out using various cryoprotective substances like sorbitol, mannitol, sucrose and polyethyleneglycol. They have an osmotic role of dehydrating the cells and also protecting membranes and enzymatic binding sites from freezing injuries. Preculture of protocorm-like body on solid medium with 0.3-0.7M sucrose improved the regrowth of cryopreserved specimens after LN exposure (Niino et al., 1992). Preculture is the foremost important step as it prepares the cells to being immersed in LN. It also helps in the accumulation of sugar inside the cells with increased solute concentration that maintained integrity of cell membranes especially during dehydration and freezing thus reduces the crystallization of ice during dehydration and freezing (Plessis et al., 1993). The benefit of using sucrose is that it offers osmotic dehydration during treatment and reduces water content in the tissue (Tanaka et al., 2004). Besides that, sucrose molecules penetrate cells and replace the water content (Dumet et al., 1993). Preculture at high sucrose concentrations was important for the survival of *Rosa hybrida* L. cv. Helmut Schmidt IFEs after cryopreservation. Shoot tips of *Dianthus caryophyllus* L. that were precultured in MS medium supplemented with 0.25 M and *Dendrobium* sonia-17 precultured in MS medium supplemented with 0.5 M of sucrose showed similar result in this study (Subramaniam et al., 2011).

Preculture duration is required for reduction of cell injuries during encapsulation-dehydration (Touchell et al., 2002). *Saintpaulia ionantha* Wendl that were precultured in semi-solid medium supplemented with 0.3 M sucrose and were subjected to 48 h preculture duration showed high viability rate (Moges et al., 2004) while cryopreservation of *Dendrobium* Bobby Messina by encapsulation-dehydration, 0.4 M sucrose for 3 days gave the higest TTC absorbance (Antony et al., 2013). Preculture duration played an important role for viability and survival of cryopreserved plant tissue (Ishikawa et al., 1997). Positive effects obtained in *Dendrobium* sonia-28 PLBs with 0.5M sucrose for six days in both cryopreserved and non-cryopreserved explants (Ching et al., 2012). In this study, cryopreserved IFEs precultured in full-strength MS medium supplemented with 0.25 M sucrose showed the best viability rate at 24 h based on TTC analysis. These results propose that different plants respond to different preculture condition due to genotype differences.

Cellular freezing can cause detrimental effects due to intracellular crystallization of water. Osmoprotection regulates the osmotic dehydration of tissues which increases the growth recovery (Matsumoto et al., 1994). Through osmosis, cryoprotectant replaces vacant spaces available in cells when free moving water particles left the cytoplasm. Optimization that based on osmoprotection concentration was conducted to obtain the best sucrose concentration that able to prevent intracellular ice formation during cooling in LN (Pandey et al., 2008). The optimum concentration of *Ipomoea batatas* was 40 ml in basal medium supplemented with 30 $g.l^{-1}$ sucrose and one $g.l^{-1}$ casamino acid with 24 h on rotary shaker at 90 rpm (Hirai and Sakai, 2003). Meanwhile, 0.75 M sucrose showed a high viability rate in non-cryopreserved IFEs and 0.5 M sucrose in cryopreserved IFEs based on TTC analysis. A similar result was observed by *Morus alba* L. at 0.75 M which produced the highest viability after post-cryopreservation (Padro et al., 2012). Generally, sugar as an osmoprotectant helps to stabilise the plasma membrane by forming hydrogen bonds with the membranous phospholipid and also increase production of endogenous cryoprotective compound such as sugar (sucrose) and sugar alcohols in target explants (Lambardi et al., 2008).

Dehydration of encapsulated tissue greatly reduce cell's water but not to the extent of reaching wilting point thus making tissues immersed in LN to survive (10). Dehydration for long periods leads to low viability after cryopreservation (Al-Ababneh et al., 2002). The best viability rate was shown at 3 h and lowest viability rate at 6 h of dehydration period for both treatments. In contrast, *Rosa multiflora* was desiccated for 2h (Lynch et al., 1996), Saintpaulia *ionantha* Wendl for 1h (Moges et al., 2004) and *Morus alba* L. was desiccated for 9h (Padro et al., 2012) reflected in high regrowth rate after cryopreservation. The dissimilar dehydration tolerance in different plant species was mainly due to optimal water content of each explant within the encapsulated beads (Wang et al., 2000).

Histological observation was carried out after three weeks for cryopreserved and non-cryopreserved IFEs of *Rosa hybrida* L. cv. Helmut Schmidt in comparison with the stock culture IFE. Cross section of cryopreserved, non-cryopreserved and IFEs stock culture showed clear presence of nucleus, cell wall and cytoplasm. Cryopreserved IFEs showed damaged cells and symptoms of

plasmolysis, shrinkage and rupture of cell wall. Plasmolysed cells were isolated from surrounding cells through rupture of plasmodesmata (Georget et al., 2009). Plasmodesma integrates cell-to-cell interactions by trafficking molecules that pass through it. This result was similar for *Gentiana* spp. that showed high structural damage after cryopreservation (Mikula et al., 2006). This signifies that sucrose pretreatment was unable to protect the cells of cryopreserved IFEs from freezing damage. Barraco et al. (2013) observed obvious changes in cell structures including plasmolysis, intensely stained nucleus and severe cell shrinkage. The reason behind the shrinkage and intense staining of nuclei might be due to chromatin contraction and enhanced protein content (Barraco et al., 2013). Some nucleoli were no longer noticeable because of nucleus shrinkage and chromatin condensation. In addition, the accurate thawing duration is important as the vast temperature difference between LN storage (-196ºC) and room temperature may cause ice crystallization and this causes cells to become damage. However, in comparison, cross section of stock culture IFEs displayed an undamaged, perfect polyhedral shape with highly dense cytoplasm. Accumulation of starch was seen in cryopreserved and non-cryopreserved IFEs. Similarly in coffee apices and *Brassidium* Shooting Star orchid, starch grains were seen in the cells (Grapin et al., 2011; Mubbarakh et al., 2013). This build-up of starch in cells is due to the uptake of sucrose which was metabolised to form starch (Bachiri et al., 2000).

Conclusion

As a conclusion, to protect cells from low temperature, cell dehydration is an essential step in cryopreservation. All the dehydration durations were not able to induce tolerance to immersion in liquid nitrogen as there was no growth after thawing. However the best optimized parameters based on spectrophotometric-TTC assay were 0.25 M of sucrose for precultured concentration, 24 h of precultured duration, 0.75 M of sucrose from osmoprotection concentration and 3 h of dehydration period. Histological analysis revealed that cryopreserved IFEs showed severely ruptured membranes while non-cryopreserved IFEs conserve their cell structures. Therefore, the current research with *Rosa hybrida* L. cv. Helmut Schmidt will be continued along this way.

Acknowledgements

The authors wish to thank MOHE for FRGS 2011 grant and Universiti Sains Malaysia for supporting this study.

References

Al-Ababneh, S. S., N. S. Karam and R. A. Shibli. 2002. Cryopreservation of sour orange (*Citrus aurantium* L.) shoot tips. In Vitro Cell Dev. Plant 38(6):602-607.

Antony, J. J. J., S. A. Mubbarakh and S. Subramaniam. 2013. Effect of Plasmolysis on Protocorm-Like Bodies of *Dendrobium* Bobby Messina Orchid Following Cryopreservation with Encapsulation– Dehydration Method. Appl. Biochem. Biotechnol. 1-12.

Bachiri, Y., C. Bajon, A. Sauvanet, C. Gazeau and C. Morisset. 2000. Effect of osmotic stress on tolerance of air-drying and cryopreservation of *Arabidopsis thaliana* suspension cells. Protoplasma 214:227-243.

Barraco, G., I. Sylvestre and M. Collin. 2013. Histocytological analysis of yam (*Dioscorea alata*) shoot tips cryopreserved by encapsulation–dehydration. Protoplasma 251(1):177-89.

Benson, E. E., B. M. Reed, R. Brennan, K. A. Clacher and D. A. Ross. 1996. Use of thermal analysis in the evaluation of cryopreservation protocols for *Ribes nigrum* L. germplasm. Cryo. Lett. 17:347-362.

Ching, L., J. Antony, R. Poobathy and S. Subramaniam. 2012. Encapsulation-vitrification of *Dendrobium* sonia-28 supported by histology. Plant Omics 5(4):345-350.

Clavero-Ramirez, I., J. Galvez-Farfan, J. M. Lopez-Aranda and M. E. Gonzalez-Benito. 2005. Apex cryopreservation of several strawberry genotypes by two encapsulation-dehydration methods. Cryo. Lett. 26(1):17-24.

Day, J. G., K. C. Harding, J. Nadarajan and E. E. Benson. 2008. Cryopreservation Molecular Biomethods Handbook, pp. 917-947, Springer.

Dumet, D., F. Engelmann, N. Chabrillange and Y. Duval. 1993. Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step. Plant Cell Rep. 12(6):352-355.

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

Flachsland, E., G. Terada, A. Scocchi, H. Rey, L. Mroginski and F. Engelmann. 2006.

Cryopreservation of seeds and *in vitro*-cultured protocorms of *Oncidium bifolium* Sims. (Orchidaceae) by encapsulation-dehydration. Cryo Lett. 27:235–242.

Georget, F., F. Engelmann, R. Domergue and F. Côte. 2009. Morpho-histological study of banana (*Musa* spp. cv. Grande Naine [AAA]) cell suspensions during cryopreservation and regeneration. Cryo Lett. 30(6):398-407.

Goncalves, S., L. Fernandes, and A. Romano. 2010. High-frequency in vitro propagation of the endangered species *Tuberaria major*. Plant Cell Tiss. Org. 101:359–363.

Gonzalez-Arnao, M. T., C. E. Lazaro-Vallejo, F. Engelmann, R. Gamez-Pastrana, Y. M. Martinez-Ocampo, M. C. Pastelin-Solano and C. Diaz-Ramos. 2009. Multiplication and cryopreservation of vanilla (*Vanilla planifolia* 'Andrews'). In Vitro Cell Dev-Pl. 45(5):574-582.

Grapin, A., A. Gallard and M. Chevalier. 2011. Cryopreservation of Pelargonium species: a comparative study of encapsulation-dehydration and droplet-vitrification protocols. Acta Hort. 918:131–137.

Harding, K. 2004. Genetic integrity of cryopreserved plant cells: a review. Cryo Lett. 25(1):3-22.

Hirai, D. and A. Sakai. 2003. Simplified cryopreservation of sweet potato (*Ipomoea batatas* L.) by optimizing conditions for osmoprotection. Plant Cell Rep. 21(10):961-966.

Hua, Y. M. and S. R. Hong. 2010. A simple cryopreservation protocol of *Dioscorea bulbifera* L. embryogenic calli by encapsulation vitrification. Plant Cell Tiss. Org. 101:349–358.

Ishikawa, K., K. Harata, M. Mii, A. Sakai, K. Yoshimatsu and K. Shimomura. 1997. Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. Plant Cell Rep. 16(11):754-757.

Kaczmarczyk, A., B. Funnekotter, A. Menon, P. Y. Phang, A. Al-Hanbali, E. Bunn and R. L. Mancera. 2012. Current Issues in Plant Cryopreservation. Curr. Front. Cryobiol. 417-429.

Kaviani, B. 2011. Conservation of plant genetic resources by cryopreservation. Aust. J. Crop Sci. 5(6):778-800.

Lambardi, M., A. Fabbri and A. Caccavale. 2000. Cryopreservation of white poplar (*Populus alba* L.) by vitrification of *in vitro* grown shoot tips. Plant Cell Rep. 19:213-218.

Lambardi, M., E. A. Ozudogru and C. Benelli. 2008. Cryopreservation of embryogenic cultures Plant Cryopreservation: A Practical Guide, pp. 177-210, Springer.

Lurswijidjarus, W. and K. Thammasiri. 2004. Cryopreservation of shoot tips of *Dendrobium* Walter Oumae by encapsulation/dehydration. Sci. Asia. 30:293-299.

Luza, J. and V. Polito. 1988. Cryopreservation of English walnut (*Juglans regia* L.) pollen. Euphytica 37(2):141-148.

Lynch, P. 2002. Cryopreservation of *Rosa* (Rose) Cryopreservation of Plant Germplasm II, pp. 344-353, Springer.

Lynch, P., Harris, W. and J. Chartier-Hollis. 1996. The cryopreservation of shoot tips of Rosa multiflora. Plant Growth Regul. 20(1):43-45.

Maneerattanarungroj, P., S. Bunnag and M. Monthatong. 2007. *In vitro* conservationof *Cleisostoma areitinum* (Rchb. f.) Garay, rare Thaiorchid species by an encapsulation–dehydration method. Asian J. Plant Sci. 6:1235–1240.

Martinez, D., S. R. Tames and A. M. Revilla. 1999. Cryopreservation of *in vitro* grown shoot-tips of hop (*Humulus lupulus* L.) using encapsulation-dehydration. Plant Cell Rep. 19:59–63.

Matsumoto, T., A. Sakai and K. Yamada. 1994. Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. Plant Cell Rep. 13(8):442-446.

Miaja, M. L., G. Gambino, R. Vallania and I. Gribaudo. 2004. Cryopreservation of *Vitis vinifera* L. somatic embryos by vitrification or encapsulation-dehydration. Acta Hort. 663:599–603.

Mikuła, A., M. Niedzielski and J. J. Rybczyski. 2006. The use of TTC reduction assay for assessment of *Gentiana* spp. cell suspension

viability after cryopreservation. Acta Physiol. Plant. 28(4):315-324.

Moges, A. D., R. A. Shibli and N. S. Karam. 2004. Cryopreservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. In Vitro Cell Dev-Pl. 40(4):389-395.

Mubbarakh, S. A., S. Rahmah, Z. A. Rahman, N. N. M. Sah and S. Subramaniam. 2013. Cryopreservation of *Brassidium* Shooting Star orchid using the PVS3 method supported with preliminary histological analysis. Appl. Biochem. Biotechnol. 172:1131–1145.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Acta Physiol. Plant. 15(3):473-497.

Niino, T., A. Sakai, H. Yakuwa and K. Nojiri. 1992. Cryopreservation of *in vitro*-grown shoot tips of apple and pear by vitrification. Plant Cell Tiss. Org. 28(3):261-266.

Normah, M. N. and A. M. Makeen. 2008. Cryopreservation of Excised Embryos and Embryonic Axes. In: B. M. Reed (Ed.), pp. 211-240, Plant Cryopreservation: A Practical Guide, Springer Science+Business Media, LLC.

Padrò, M. D. A., A. Frattarelli, A. Sgueglia, E. Condello, C. Damiano and E. Caboni. 2012. Cryopreservation of white mulberry (*Morus alba* L.) by encapsulation-dehydration and vitrification. Plant Cell Tiss. Org. 108(1):167-172.

Pandey, R., N. Sharma and R. Chamola. 2008. Cryoprotectant solutions and pretreatment media for cryopreservation. In: Laboratory manual for *In vitro* conservation of plant genetic resources 3rd edition. S. K. Sharma, A. Agrawal, R. Chaudhury, R. K. Tyagi, R. Pandey, N. Shanna, N. Gupta, S. Malik and Z. Hussain (Eds.), pp. 20-21, National Bureau of Plant Genetics Resources, New Delhi, India.

Pati, P. K., S. P. Rath, M. Sharma, A. Sood and P. S. Ahuja. 2006. *In vitro* propagation of rose—a review. Biotech. adv. 24(1):94-114.

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

Razavizadeh, R. and A. A. Ehsanpour. 2008. Optimization of *in vitro* propagation of *Rosa hybrida* L. cultivar black red. Am. Eurasian J. Agric. Env. Sci. 3(1):96-99.

Reed, B. M. 2001. Implementing cryogenic storage of clonally propagated plants. Cryo Lett. 22(2):97-104.

Reed, B. M. 2008. Plant cryopreservation: a practical guide. In: B. M. Reed (ed.), p. 513, Springer Science + Business Media LLc, New York.

Reed, B. M., I. Kovalchuk, S. Kushnarenko, A. Meier-Dinkel, K. Schoenweiss, S. Pluta and E. E. Benson. 2004. Evaluation of critical points in technology transfer of cryopreservation protocols to international plant conservation laboratories. Cryo Lett. 25(5):341-352.

Reed, B. M., L. Schumacher, D. Dumet and E. E. Benson. 2005. Evaluation of a modified encapsulation-dehydration procedure incorporating sucrose pretreatments for the cryopreservation of Ribes germplasm. In Vitro Cell Dev-Pl. 41(4):431-436.

Scocchi, A., M. Faloci, R. Medina, S. Olmos and L. Mroginski. 2004. Plant recovery of cryopreserved apical meristem-tips of *Melia azedarach* L. using encapsulation/dehydration and assessment of their genetic stability. Euphytica 135:29-38.

Short, K. and A. Roberts. 1991. *Rosa* spp.(roses): *In vitro* culture, micropropagation, and the production of secondary products Medicinal and Aromatic Plants III, pp. 376-397, Springer.

Srivastava, V., S. A. Khan and S. Banerjee. 2009. An evaluation of geneticfidelity of encapsulated microshoots of the medicinal plant: *Cineraria maritima* following six months of storage. Plant Cell Tiss. Org. 99:193–198.

Subramaniam, S., U. R. Sinniah, A. A. Khoddamzadeh, S. Periasamy and J. J. J. Antony. 2011. Fundamental concept of cryopreservation using *Dendrobium* sonia-17 protocorm-like bodies by encapsulation-dehydration technique. Afr. J. Biotechnol. 10(19):3902-3907.

Tanaka, D., T. Niino, K. Isuzugawa, T. Hikage and M. Uemura. 2004. Cryopreservation of shoot apices of *in-vitro* grown gentian plants:

Comparison of vitrification and encapsulation-vitrification protocols. Cryo Lett. 25(3):167-176.

Touchell, D., V. Chiang and C. J. Tsai. 2002. Cryopreservation of embryogenic cultures of *Picea mariana* (black spruce) using vitrification. Plant Cell Rep. 21(2):118-124.

Verleysen, H., G. Samyn, E. Van Bockstaele and P. Debergh. 2004. Evaluation of analytical techniques to predict viability after cryopreservation. Plant Cell Tiss. Org. 77(1):11-21.

Wang, G., M. Yuan and Y. Hong. 2002. *In vitro* flower induction in roses. In Vitro Cell Dev-Pl. 38(5):513-518.

Zainuddin, M., A. L. Julkifle, R. Pobathy, U. R. Sinniah, A. Khoddamzadeh, J. J. J. Antony, Pavallekoodi and S. Subramaniam. 2011. Preliminary analysis of cryopreservation of *Dendrobium* Bobby Messina orchid using an encapsulation-dehydration technique with Evans blue assay. Afr. J. Biotechnol. 10(56):11870-11878.