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Cryopreservation of Malayan kumquat (*Fortunella polyandra*) embryonic axes by vitrification

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Abstract

Cryopreservation of embryonic axes of *Fortunella polyandra* was investigated using a vitrification-based technique. Plant vitrification solution 2 (PVS2) was added to cryovials containing embryonic axes (2 mm) and kept for 0, 5, 10, 15, 20, 25 or 30 minutes at 25°C and for 0, 15, 30, 45, 60 or 75 minutes at 0°C before plunging into liquid nitrogen. Another set of embryonic axes with the same treatment but not exposed to liquid nitrogen was used as a control. The highest survival percentage of the embryonic axes was 50.0% with 15 minutes exposure to PVS2 at 25°C and 53.30% with 60 minutes exposure at 0°C. Gradual increase in exposure to PVS2 from 50% to 100% at PVS2 in one to four steps was also studied at both temperatures. The embryonic axis survival rates of 50.0-56.7% obtained for 2- to 4-step PVS2 treatments at both temperatures were significantly better than those of embryonic axes directly exposed to PVS2 (survival of 30%), showing that the period of exposure to PVS2 and sequential applications of PVS2 to the embryonic axes are important for successful cryopreservation of *F. polyandra*.

Keywords: cryopreservation; embryonic axis; *Fortunella polyandra*; vitrification.
Abbreviations: BA-benzyladenine; MS-Murashige and Skoog; PVS2- plant vitrification solution 2.

Introduction

Fortunella polyandra (Ridl.) Tanaka, also known as the Malayan kumquat, is a cultivated species in Malaysia (Jones, 1989). It is a sought-after ornamental plant due to its attractive golden yellow fruits believed, especially among the Chinese, to bring good luck and prosperity. The fruits are also used for the treatment of diabetes. *F. polyandra* seeds have shown sensitivity to the temperatures of 5˚C and lower necessary for cryopreservation (Normah and Yap, 2001). Hence cryopreservation of embryonic axes of *F. polyandra* for long term conservation of this species has continued to be investigated. Al Zoubi and Normah (2012) found that the highest normal seedling percentage of cryopreserved axes was 50% attained at the water content of 0.16 g H_2O g^{-1}dw and 0.15 g H_2O g^{-1}dw when desiccated in a laminar airflow and ultra-rapidly, respectively. To improve the survival percentage, vitrification represents an alternative cryopreservation technique. In vitrification, cell viscosity is increased to a point at which ice formation is inhibited and water becomes vitrified on exposure to cryogenic temperatures. This can be achieved by adding cryoprotective additives at a very high concentration and by removal of water by evaporative desiccation and osmotic dehydration (Benson, 2008). However, high concentrations of cryoprotectants can cause cryoprotectant toxicity and non-penetrating components can result in osmotic injury. Fahy et al. (1986) have suggested the use of a mixture of different additives to reduce toxicity, limit extreme evaporative drying and stabilise the glasses formed. One plant vitrification solution that has been very successful for cryopreservation of a number of plant materials (Sakai et al., 2008) is Plant Vitrification Solution 2 (PVS2), a cryoprotectant mixture of 30% glycerol, 15% ethylene glycol, 15% dimethyl sulphoxide (DMSO) and 0.4M sucrose. Successful use of vitrification for cryopreservation of close relatives of *F. polyandra* has been reported in the work done on *Citrus madurensis* embryonic axes by Cho et al. (2001) and by Malik and Chaudhury (2006) on *Citrus macroptera* Mont and *Citrus latipes* Tanaka. Sakai et al. (1990, 1991, 2008) have noted that effective vitrification requires control of the procedure used for dehydration with special attention to cryoprotectant permeation and prevention of injury due to chemical toxicity or excessive osmotic stress during dehydration. In addition, Engelmann (1997) and Tiau et al. (2009) have stressed the critical factor of the length of contact between vitrification solution and explants, which has been shown to affect the survival percentage of the cryopreserved plant material. Sakai (2000) in his review reported that the range of duration of the treatment that ensures high survival after freezing for several plant species (e.g., navel orange; Sakai et al., 1991) was generally very narrow when vitrification was performed at room temperature but that treatment of the samples with vitrification solutions at 0°C was found to widen that range (Shibli et al., 2006). The use of stepwise application of PVS2 solution to reduce both toxicity and desiccation effects of direct exposure to the solution has been shown to be successful in *Citrus madurensis* embryonic axes (Cho et al., 2001), *Saintpaulia ionantha* shoot tips (Moges et al., 2004) and *Phoenix dactylifera* embryonic callus (Subaih et al., 2007). This study investigated the potential for successful cryopreservation of *F. polyandra* embryonic axes using a vitrification technique, focusing on the effects of different PVS2 exposure durations at 25°C and 0°C and to observing the effects of a varying number of step-wise applications of PVS2 at both temperatures on survival of *F. polyandra* embryonic axes after cryopreservation.

Results

Effects of various periods of exposure to PVS2 on the survival percentage of cryopreserved* F. polyandra *embryonic axes

The survival percentages of *F. polyandra* embryonic axes after the vitrification treatment at 25°C and 0°C with different exposure times to PVS2 are shown in Fig 1 and 2, respectively. The results showed that the percentages of survival of the non-cryopreserved (i.e. not treated with liquid nitrogen, or -LN) embryonic axes of *F. polyandra* at 25°C decreased significantly with the gradual increase in the period of exposure to PVS2. The vitrified embryonic axes exposed for 5 minutes showed complete survival, but the survival decreased to 63% after 30 minutes exposure. For the embryonic axes vitrified with PVS2 and subjected to liquid nitrogen (+LN), a significant difference was found in the values for all durations of PVS2 application. Vitrification up to 15 minutes enabled a moderate survival percentage of 50%. However, when the period of exposure was further increased, the survival percentage decreased until no embryonic axes survived the 30 minutes exposure period. The colour of the vitrified embryonic axes was white when they were placed on the medium, but the viable embryos turned yellow to green within about one week (Fig 3). Germinated embryos showed no callus formation. The survival of non-cryopreserved (-LN) embryonic axes of *F. polyandra* exposed to PVS2 at 0°C decreased with a gradual increase in the duration of exposure, as depicted in Fig 2. After 75 minutes of exposure 63.3% of the non-cryopreserved (-LN) embryonic axes survived. No significant difference was found between the embryonic axes exposed to PVS2 for 30 and 45 minutes but without exposure to LN. Exposure of embryonic axes to LN decreased the survival percentage. The highest survival percentage of 53.3% was obtained for embryonic axes exposed to PVS2 for 60 minutes with LN. A further increase in the period of exposure to PVS2 was found to decrease the survival of the cryopreserved embryonic axes significantly.

Effects of step-wise exposure to PVS2 on the survival of cryopreserved* F. polyandra *embryonic axes

There were no significant differences in the survival of the non-cryopreserved embryonic axes among the different regimes of PVS2 exposure (Fig 4). A high survival percentage of 83.3 - 90.0% was obtained for the non-cryopreserved embryonic axes at 25^{o}C. A maximum survival rate of only 56.7% of the cryopreserved embryonic axes was obtained when the embryonic axes were exposed to PVS2 in four increments. However, no significant difference was found between the two-step, three-step and four-step methods. Similar results were obtained for the 0°C treatments (Fig 5), where the best regime for PVS2 exposure was the four-step method with survival of 56.7%.

Discussion

The effect of different durations, concentrations and exposure temperatures of vitrification solution on* F. polyandra *embryonic axes survival after LN exposure

One of the keys to successful cryopreservation by vitrification is a careful control of dehydration and prevention

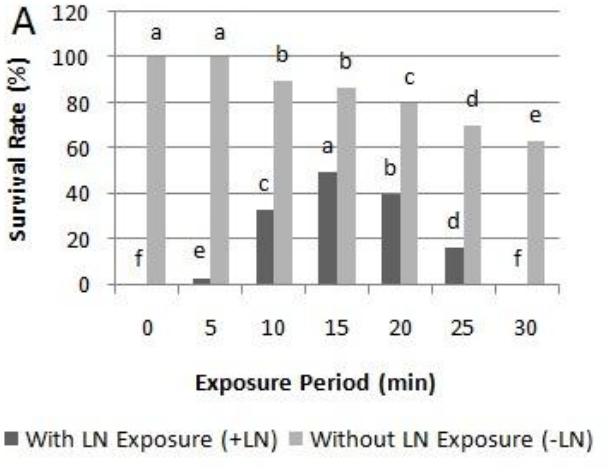

Fig 1. Survival (%) of cryopreserved (+LN) *Fortunella polyandra* embryonic axes with different durations of exposure (min) to PVS2 at 25˚C. Means with the same letters for +LN and –LN are not significantly different at p≤0.05 based on Duncan Multiple Range Test (DMRT).

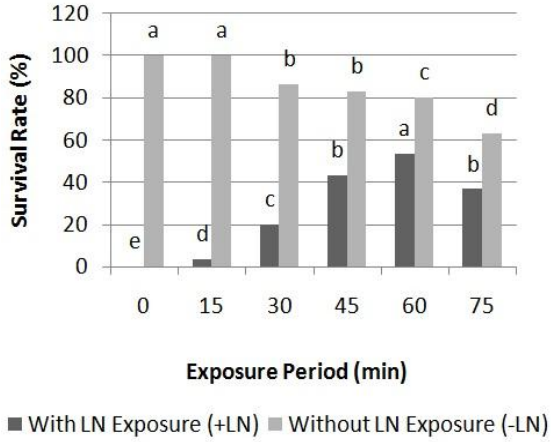

Fig 2. Survival (%) of cryopreserved (+LN) *Fortunella polyandra* embryonic axes with different durations of exposure (min) to PVS2 at 0°C. Means with the same letters for +LN and –LN are not significantly different at p≤0.05 based on Duncan Multiple Range Test (DMRT).

Fig 3. Viable embryonic axis of *Fortunella polyandra* germinating after cryopreservation [3-4 weeks].

of injury by chemical toxicity or excess osmotic stress during treatment with PVS2. Optimisation of the time of exposure and the temperature during exposure to PVS2 are equally important for a high level of regrowth after vitrification. To determine the optimal period of exposure to PVS2, embryonic axes of *F. polyandra* were treated with PVS2 at 25°C or 0°C for different periods of time before they were plunged into the LN. With cryopreserved embryonic axes of *F. polyandra*, the highest survival percentages (50 and 53.3%) were obtained for PVS2 treatments for 15 minutes at 25°C and and 60 minutes at 0°C, respectively. Further increase in exposure time caused a significant decrease in survival rate. Cho et al. (2001) have found that the maximal survival of embryonic axes of *Citrus madurensis* was obtained when PVS2 exposure was 60-80 minutes at 0°C and 20-25 minutes at 25°C. Similarly, the optimal time for exposure of excised Wasabi meristems to PVS2 has been found to be 10 minutes at 25°C or 30-60 minutes at 0°C (Matsumoto et al., 1998). We propose that the duration of contact between the explants and the vitrification solution is a critical factor. PVS2 contains both penetrating and non pentrating cryoprotectants. Penetrating (colligative) cryoprotectants such as glycerol and DMSO increase the initial osmolarity of cells before initiation of the freezing process, considerably reducing the level of water that needs to be frozen out to achieve osmotic equilibrium, and therefore the extent of the dehydration that occurs in the cell is better tolerated (Meryman and Williams, 1985). However, a high concentration of cryoprotectants can cause cryoprotectant toxicity, while the non-penetrating components can cause osmotic injury. The prolonged period of exposure may have caused both toxicity and osmotic injury to the cells of the embryonic axes from which the cells can no longer recover. This proposal is supported by previous reports (Lambardi et al., 2000**;** Cho et al., 2001; Shatnawi et al., 2011) that have suggested that prolonged exposure to a concentrated vitrification solution could injure the plant material, via either chemical toxicity or strong osmotic stress. In embryonic axes treated at 25°C and 0°C, the treated controls (without cooling in LN) maintained high survival rates (> 80%) for exposures of up to 15 and 60 minutes, respectively. At these exposure periods the best survival rates after cryopreservation were observed, but these survival rates were much lower than those of the controls (Fig 1). These results suggest that PVS2 is not highly toxic to the embryonic axes. Nevertheless, during the remaining freezing and recovery steps, additional injury must have occurred to cause an additional reduction in survival beyond the survival rate achieved with dehydration by PVS2. Vitrification of the embryonic axes at 0°C was found to slightly improve the survival of axes after cryopreservation as compared to vitrification at room temperature. Dehydration at 0°C instead of at room temperature allows for the reduction of toxicity of the vitrification solution and thus broadens the window of exposure while ensuring the survival of samples (Shibli et al., 2006). In many vitrified cells and meristems, PVS2 treatment at 0°C was required to reduce injurious effects. Such a treatment produced high recovery, even in tropical plants (Kuranuki and Sakai, 1995; Cho et al., 2001). However, in the present study, the difference in survival rates was not very obvious. The maximum survival rate of 56.7% was achieved when the four-step vitrification protocol was used at both 25 and 0°C (Fig 3). Moges et al. (2004) observed high survival rate of shoot tips of the African violet after treatment with a four-step protocol. The current study found a positive effect on the survival rate when the concentration of the vitrification solution was progressively increased using the

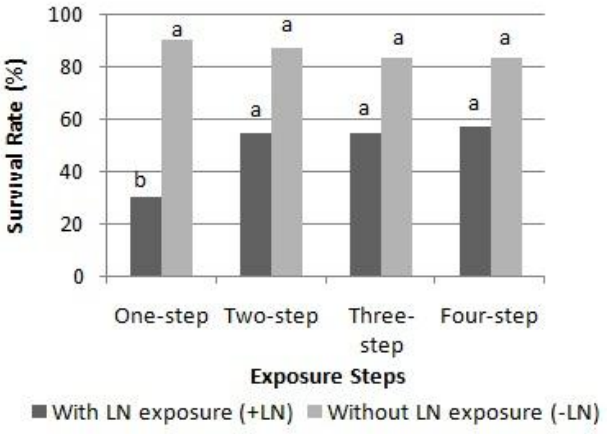

Fig 4. Survival (%) of cryopreserved (+LN) *Fortunella polyandra* embryonic axes with step-wise PVS2 exposure at 25°C. Note: Exposure steps indicate different combinations of PVS2 exposure time and solution concentration: One-step = 50% PVS2 for 15 min; Two-step = 50% PVS2 for 7.5 min, then 100% PVS2 for 7.5 min; Three-step = 25% PVS2 for 5 min, 50% PVS2 for 5 min, then 100% PVS2 for 5 min; and Four-step = 25% PVC2 for 4 min, 50% PVC2 for 4 min, 75% PVS2 for 4 min then 100% PVS2 for 4 min. Means with the same letters for +LN and –LN are not significantly different at p≤0.05 based on Duncan Multiple Range Test (DMRT).

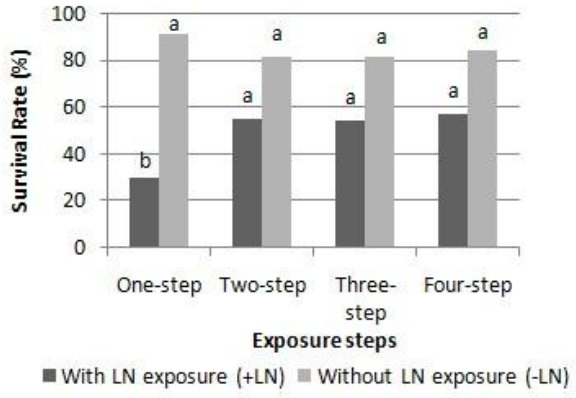

Fig 5. Survival (%) of cryopreserved (+LN) *Fortunella polyandra* embryonic axes with step-wise PVS2 exposure at 0°C. Exposure steps indicate different combinations of PVS2 exposure time and solution concentration: One-step = 50% PVS2 for 15 min; Two-step = 50% PVS2 for 7.5 min, then 100% PVS2 for 7.5 min; Three-step = 25% PVS2 for 5 min, 50% PVS2 for 5 min, then 100% PVS2 for 5 min; and Four-step = 25% PVC2 for 4 min, 50% PVC2 for 4 min, 75% PVS2 for 4 min then 100% PVS2 for 4 min. Means with the same letters for +LN and –LN are not significantly different at p≤0.05 based on Duncan Multiple Range Test (DMRT).

two- to four-step vitrification protocol established by Matsumoto and Sakai (2003) with grape apices. Cho et al. (2001) obtained the maximum survival rate of 85% using the three-step protocol at 0 °C and a 70% survival when using the two-step procedure at 0°C for the embryonic axes of *Citrus madurensis*. It seems that the step-wise vitrification protocol minimised the chemical toxicity of PVS2 or the osmotic stress induced by PVS2. Gonzalez-Arnao et al. (1998) and Shatnawi et al. (1999) reported that survival and regrowth were reduced when directly dehydrated with 100% PVS2 before freezing. This reduction in survival rate might have been caused by the osmotic stress induced by PVS2

(Grospietsch et al., 1999) and by the phytotoxicity effects of PVS2 (Benson et al., 1996). According to Abdelnour-Esquivel and Engelmann (2002), the detrimental effect of incubation of apices in the full-strength vitrification solution could be avoided by a sequential increase in the concentration of PVS2 vitrification solution; this was confirmed to be the only way to achieve survival of chayote apices after freezing. Thus both the period of exposure to PVS2 and sequential applications of PVS2 are important when establishing cryopreservation protocols. Perhaps due to reduced injury caused by osmotic dehydration in the axes, the present technique improved cryopreservation survival over that reported by Al Zoubi and Normah (2012), where the evaporative desiccation method had been used.

Materials and methods

Plant material

F. polyandra mature fruits were obtained from the experimental plot of Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor, Malaysia.

Seed sterilisation and excision of embryonic axes

Seeds of *F. polyandra* were rinsed with 80% ethyl alcohol for 2 min, followed by sterilisation using 20% commercial bleach (Clorox) with two drops of Tween 20 for 20 minutes. The seeds were then rinsed (three times) with sterile distilled water. The testa was removed from the seeds, and the embryonic axes were aseptically excised under a stereomicroscope with the aid of fine-ended forceps and a needle. The axes (2 mm) were separated from the cotyledons with a scalpel blade (leaving a small block of the cotyledon attached to the axes). The excised embryonic axes were then placed on a filter paper moistened with sterile distilled water and kept in a closed Petri dish prior to the vitrification process.

Effects of different periods of exposure to PVS2 on the normal seedling recovery of cryopreserved **F. polyandra** *embryonic axes*

A vitrification technique based on that of Sakai et al. (1990) was used. Excised embryonic axes of *F. polyandra* were cultured on pregrowth medium (basic MS medium with 0.72M sucrose) for three days, followed by preculturing on MS medium with 0.3 M sucrose for one day (based on the method described in Chandrabalan et al., 2011). These embryonic axes were then put into sterile cryovials to which 1 ml of MS liquid medium containing 2M glycerol with 0.4M sucrose (loading solution) was added and held for 20 minutes. The loading solution was then removed and 1 ml PVS2 (30% glycerol, 15% ethylene glycol, 15% DMSO with 0.4 sucrose) was added to the cryovials for 0, 5, 10, 15, 20, 25 or 30 minutes at 25°C or for 0, 15, 30, 45, 60 or 75 minutes at 0°C. The PVS2 was changed every 5 minutes for the treatment at 25°C and every 15 minutes for that at 0°C. Fresh PVS2 was added before plunging the cryovials into liquid nitrogen (LN) for at least 1 hour. Another set of embryonic axes with the same treatment but not exposed to liquid nitrogen was used as a control (-LN). To thaw the cryovials were quickly removed from the liquid nitrogen and put into sterile distilled water at 40°C for 1 minute. PVS2 was aseptically pipetted out, and 1 ml unloading solution (MS + 1.2M sucrose) was added to the sample and held for 20 minutes (Nishizawa et al., 1993). The unloading solution was then pipetted out, and the axes were cultured on MS with 0.3 M sucrose medium lined with sterile filter paper for 16 hours. Then, the embryonic axes were cultured on the recovery medium [(Murashige and Skoog (1962) (MS) medium with 1mg.L^{-1} 6-benzyladenine (BA)] . All cultures were incubated at 24±2°C under fluorescent lamps with light intensity of 22.26 µEcm^{-2} second^{-1} (3000 lux) under a 16-hour photoperiod. Data were collected weekly for two months to determine the survival percentages.

Effects of step-wise exposure to PVS2 on the survival of cryopreserved **F. polyandra** *embryonic axes*

The protocol was carried as above, but after the loading step, the following procedures for PVS2 exposure at 25°C were performed:
One-step: 50% PVS2 fo**r** 15 minutes;
Two-step: 50% PVS2 for 7.5 minutes, then 100% PVS2 for 7.5 minutes;
Three-step: 25% PVS2 for 5 minutes, 50% PVS2 for 5 minutes, then 100% PVS2 for 5 minutes;
Four-step: 25% PVC2 for 4 minutes, 50% PVC2 for 4 minutes, 75% PVS2 for 4 minutes, then 100% PVS2 for 4 minutes.
For exposure of axes to PVS2 at 0°C, the following protocol was followed:
One-step: 50% PVS2 for 60 minutes;
Two-step: 50% PVS2 for 30 minutes, then 100% PVS2 for 30 minutes;
Three-step: 25% PVS2 for 20 minutes, 50% PVS2 for 20 minutes then 100% PVS2 for 20 minutes
Four-step: 25% PVC2 for 15 minutes, 50% PVC2 for 15 minutes, 75% PVS2 for 15 minutes then 100% PVS2 for 15 minutes.
The cryovials with the embryonic axes and the vitrification solution were directly plunged into the LN and stored for at least 1 hour. Thawing, unloading, and culturing were done as described above.

Survival assessment and statistical analysis

The percentage of normal seedlings (i.e., seedlings with roots and shoots with one to two leaves) was calculated to assess the survival of the axes. For each treatment, 10 embryonic axes were used and replicated three times, and each experiment was repeated at least three times. Due to limited materials for explants, the experiments for 25°C and 0°C were carried out separately. Data were subjected to a statistical analysis of variance (one way-ANOVA) using the Statistical Anaysis Software (SAS, USA). The Duncan Multiple Range Test (DMRT) was used for the comparison of each treatment tested.

Conclusion

In conclusion, embryonic axes of *F. polyandra* were moderately successful (50.0-56.7%) in surviving cryopreservation when the vitrification technique was employed. Differences in duration of exposure to PVS2 were significant, but the lower temperature during exposure did not increase the survival of the axes. However, step-wise application of PVS2 significantly improved their survival (56.7%). It is suggested that improvement of the survival of embryonic axes of *F. polyandra* after cryopreservation may be possible with further modification of the vitrification technique.

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References

Abdelnour-Esquivel A, Engelmann F (2002) Cryopreservation of chayote (*Sechium edule* JACQ.SW.) zygotic embryos and shoot-tips from *in vitro* plantlets. CryoLett. 23(5):299-308

Al Zoubi OM, Normah MN (2012) Desiccation sensitivity and cryopreservation of excised embryonic axes of *Citrus suhuiensis* cv. Limau madu, Citrumelo [*Citrus paradisi* Macf. x *Poncirus trifoliate* (L.)] and *Fortunella polyandra.* CryoLett. 33(3):240-250

Benson EE (2008) Cryopreservation theory. In: Reed BM (ed.) Plant cryopreservation: A practical guide. Springer Science Business Media, LLC, New York, USA

Benson EE, Wilkinson M, Todd A, Ekuere U, Lyon J (1996). Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot-tips. CryoLett. 17:119-128

Chandrabalan DC, Clyde MM, Normah MN (2011) Two-step preconditioning - a feasible method for cryopreservation of *Fortunella polyandra* shoot tips using vitrification technique. Acta Hort. 908:289-296

Cho EG, Hor YL, Kim HH, Rao VR, Engelmann F (2001) Cryopreservation of *Citrus madurensis* zygotic embryonic axes by vitrification: Importance of loading and treatment with vitrification solution. CryoLett. 22:391-396

Engelmann F (1997) *In vitro* conservation methods. In: Ford-Lloyd BV, Newbury JH, Callow JA (eds.) Biotechnology and plant genetic resources: conservation and use. Wallingford: CAB International Publishers

Fahy GM, Takahashi T, Meryman HT (1986) Practical aspects of ice-free cryopreservation. In: Smit-Sibinga TH, Das PC (eds) Aspects of ice-free cryopreservation. Martinus-Nijhoff, Boston, Massachusetts

Gonzalez-Arnao MT, Ravelo MM, Villavicencio CU, Montero MM, Engelmann F (1998) Cryopreservation of pineapple (*Ananas comosus*) apices. CryoLett. 19:375-382

Grospietsch M, Stodulkova E, Zamecnik J (1999) Effect of osmotic stress on the dehydration tolerance of cryopreservation of *Solanum tuberosum* shoot tips. CryoLett. 20:339-346

Jones DT (1989) Collection, utilization, and conservation of citrus genetic resources in Malaysia. In A.H. Zakri (ed) Genetic resources of under-utilised plants in Malaysia. Malaysian National Committee on Plant Genetic Resources, Kuala Lumpur

Kuranuki Y, Sakai A (1995) Cropreservation of *in vtro*-grown shoot tips of tea (*Camellia sinensis*) by vitrification. CryoLett. 16: 345-352

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

Malik SK, Chaudhury R (2006) Successful cryopreservation of embryonic axes of two wild and endangered Citrus species of north eastern India for long term conservation. Plant Genetic Resources: Characterization and Utilization 4:204-209

Matsumoto T, Sakai A (2003) Cryopreservation of axillary shoot tips of *in vitro* grown grape (*Vitis*) by a two-step vitrification protocol. Euphytica 131:299-304

Matsumoto T, Sakai A, Nako Y (1998) A novel preculturing for enhancing the survival of *in vitro*-grown meristems of wasabi (*Wasabia japonica*) cooled to -196°C by vitrification. CryoLett. 19:27-36

Meryman HT, Williams RJ (1985) Basic principles of freezing injury to plant cells: Natural tolerance and approaches to cryopreservation. In: Kartha KK (ed) Cryopreservation of plant cells and organs, CRC Press Inc., Boca Raton, Florida

Moges AD, Shibli RA, Karam NS (2004) Cryopreservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. *In Vitro* Cell Dev Bio-Plant. 40:389-395

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

Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Sci. 91: 67-73

Normah MN, Yap LV (2001) *In vitro* conservation of *Citrus* spices. In: Proceeding of the International Conference on *in situ* and *ex situ* biodiversity conservation in the millennium, Kota Kinabalu, Sabah, pp. 365-371

Sakai A (2000) Development of cryopreservation techniques In: Engelmann F, Takagi H (eds) Cryopreservation of tropical plant germplasm: current research progress and application. JIRCAS, Tsukuba/IPGRI, Rome

Sakai A, Hirai D, Niino T (2008) Development of PVS-based vitrification and encapsulation-vitrification protocols In: Reed BM, (ed.) Plant cryopreservation: A practical guide. Springer Science Business Media, LLC, New York, USA, pp 33-57

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

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

Shatnawi MA, Engelmann F, Frattarelli A, Damiano C (1999) Cryopreservation of apices of *in vitro* plantlets of almond (*Prunus dulcis* Mill.). CryoLett. 20:13-20

Shatnawi MA, Shibli RA, Abu-Romman SM, Al-Mazra'awi MS, Al Ajlouni ZI, Shatanawi WA, Odeh WH (2011) Clonal propagation and cryogenic storage of the medicinal plant *Stevia rebaudiana.* Sp J of Agr Res. 9(1):213-220

Shibli RA, Shatnawi MA, Subaaih WS, Ajlouni MM (2006) *In vitro* conservation and cryopreservation of plant resources: A review, World J. of Agr. Sci. 2(4): 372-382

Subaih WS, Shatnawi MA, Shibli RA (2007) Cryopreservation of date palm (*Phoenix dactylifera*) embryogenic callus by encapsulation-dehydration, vitrification and encapsulation-vitrification. Jo J of Agr Sci. 3(2):156-171

Tiau TK, Rathinam X, Keng CL, Subramaniam S (2009) An assessment of early factors influencing the PVS2 vitrification method using protocorm-like bodies of *Dendrobium sonia* 28. Am-Eurasian J Sustain Agric. 3(3):280-289