Regeneration rates of *Dendrobium bobby* messina plbs with ascorbic acid using PVS2 vitrification

Jessica Jeyanthi James Antony, Chan Lai Keng, Sreeramanan Subramaniam*

School of Biological Sciences,Universiti Sains Malaysia (USM), Minden Heights, 11800, Penang, Malaysia.Email : sreeramanan@gmail.com*/ jessicaerina61@gmail.com)

Abstract Cryopreservation techniques using PVS2 vitrification was applied on PLBs of *Dendrobium* Bobby Messina, with survival monitored through observations of growth rate and the 2,3,5-triphenyltetrazolium chloride (TTC) analyses. The parameters optimized were PLBs size, preculture concentration, preculture duration, PVS2 incubation temperature and duration. The optimized parameters obtained were 3-4mm of PLBs precultured in 0.2M sucrose for 1 day, treated with a mixture of 2M glycerol and 0.4M sucrose supplemented with half strength liquid MS media at 25°C for 20 minutes and subsequently dehydrated with plant vitrification solution 2 (PVS2) at 0°C for 20 minutes prior storage in liquid nitrogen. Following rapid warming in a water bath at 40°C for 90 seconds, PLBs were washed with a half strength liquid MS media supplemented with 1.2M sucrose. Subsequently, PLBs were cultured on half strength semi-solid MS media supplemented with 2% (w/v) sucrose in the absence of growth regulator. The optimized vitrification method was successful in preserving this orchid as it produced growth recovery in cryopreserved PLBs up to 40%. Ascorbic acid was added in the media to evaluate the regeneration process of cryopreserved PLBs. However, growth recovery rate was only 10% at 0.6mM ascorbic acid. RAPD analysis using 6 primers indicated that cryopreserved and non-cryopreserved PLBs from vitrification method were genetically faithful to the mother plant. However, 3 primers showed polymorphism and 1 primers indicated partial polymorphism between the cryopreserved and non-cryopreserved PLBs in comparative to the mother plant.
Keywords: *Dendrobium* Bobby Messina, Cryopreservation, PVS2 Vitrification, Ascorbic Acid, RAPD.

Introduction

Cryopreservation is known as storage of biological material at ultra-low temperatures normally at the temperature of liquid nitrogen (-196ºC). At low temperature, all cellular divisions and the metabolic processes of cryopreserved cells are halted. Consequently, the plant materials can be stored theoretically for unlimited period of time (Bajaj 1995; Towill 1996; Engelmann 2000; Burritt 2008). The vitrification method has been successfully applied to seeds of a few orchid species, such as mature seeds of *Bletilla striata* (Ishikawa *et al*. 1997) *and Doritis pulcherrima* (Thammasiri 2000), and immature seeds of *Bletilla striata* (Hirano *et al*. 2005a) and *Ponerorchis graminifolia* var. *suzukiana* (Hirano *et al*. 2005b). Apart from that, droplet vitification method is also reported to be successful in cryopreservation of vanilla (*Vanilla planifolia* 'Andrews') (Gonzalez-Arnao *et al*. 2009).

In order to achieve high growth in cryopreservation protocol, the optimization of several key factors plays a large role. The basic factors for the success in cryopreservation are physiological status of the plant material, preculture and cryoprotective treatments, cooling and rewarming rates and growth recovery conditions such as addition of antioxidants such as ascorbic acid. In addition, the exposure to excessive harsh physical environment such as very low temperatures, high osmotic pressure, dehydration and exposure to certain chemicals such as cryoprotectants may causes physiological stress and in return gives rise to genetic instability (Panis & Lambardi, 2005). This stress is caused by the injury produced by the cryopreservation procedures that produces the formation of free radicals, which leads to genetic alterations (Benson & Bremner, 2004). The examples of free radicals are reactive oxygen species (ROS) including superoxides, hydrogen peroxide and hydroxyl radicals (Mittler 2002).

Thus, there are chances of somaclonal variation in regenerated plants. Consequently, the long-term genetic consequences of dehydration and freezing injury for *in vitro* conservation need to be evaluated (Harding 1996; Gagliardi *et al*. 2007). Plants regenerated following cryogenic treatments should be genetically identical to the starting materials and genetic fidelity needs to be considered in the cryopreservation process (Ashmore 1997). Hence, the aim of this study was to optimize the preculture duration, PVS2 incubation condition and regrowth condition on the cryopreservation of *Dendrobium* Bobby Messina PLBs. Furthermore, the genetic genetic fidelity of the cryopreserved explants of *Dendrobium* Bobby Messina was determined using RAPD marker. Therefore, the objectives of the current

study is to evaluate the genetic fidelity of regenerated cryopreserved and non-cryopreserved PLBs by PVS2 vitrification method of *Dendrobium* Bobby Messina comparing with the PLBs stock culture by RAPD technique using selected primers.

Materials and Methods

Optimization of preculture condition and PVS2 incubation conditions

Protocorm-like bodies (PLBs) 3-4mm of *Dendrobium* Bobby Messina were selected from 4 weeks old culture and precultured in half strength semi-solid MS media (Murashige & Skoog, 1962) supplemented with 0.2M sucrose at 25°C for 0 to 5 days under 16 hours photoperiod. After preculture treatment, PLBs were dehydrated with 1.5 mL of loading solution (2M glycerol supplemented with 0.4M sucrose in half-strength liquid MS media) in 2mL cryotubes at 25ºC for 20 minutes. Subsequently, the PLBs were then dehydrated in 1.5 mL PVS2 solution (Sakai *et al*. 1990) at 0ºC for 20 minutes and/or at 0ºC or 25ºC for 0, 20, 40, 60, 80, 100 minutes. PVS2 solution contains 30 % (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO supplemented with 0.4M sucrose in half-strength liquid MS medium. After dehydration in PVS2 solution, the PLBs were resuspended in fresh 1.5 mL of ice cold PVS2 and were directly plunged into liquid nitrogen for a minimum of 24 hours. Cryopreserved PLBs were thawed at 40°C for 90 to 120 seconds. After thawing, PVS2 solution was drained from the cryotubes and replaced with 1.5 mL of half-strength liquid MS media supplemented with 1.2M sucrose in which the PLBs were washed at 25ºC for 20 minutes. Then, the PLBs were then transferred onto a layer of sterilized filter paper over half strength semi-solid MS media supplemented with 2% sucrose and 2.75 g/L gelrite in the absence of growth regulator at 25°C for 24 hours. After 24 hours, the PLBs were transferred onto half-strength semi-solid MS media supplemented with 2% sucrose and 2.75 g/L gelrite without the presence of any growth regulators. For the 1st week, PLBs were kept in dark condition; 2nd week, PLBs were kept in dim light condition and at the 3rd week, survived PLBs were finally exposed to 16 hours photoperiod.

Viability, Growth Assessment and Statistical Analysis

After 3 weeks in culture, the survival of cryopreserved and non-cryopreserved PLBs were assessed based on growth observation and viability assay via triphenyl tetrazoliumchloride (TTC) spectrophotometrical analysis at 490nm (Verleysen *et al*. 2004). Each experiment consisted of 6 replicates per treatment with 10 samples each. All data were subjected to independent sample t-test.

Cryopreservation of *Dendrobium* Bobby Messina PLBs using optimized PVS2 vitrification conditions

The optimized protocol consisting of 3-4mm PLBs precultured with half strength semi-solid MS media supplemented with 0.2M sucrose at 25°C for 1 day. Then, precultured PLBs were then treated with a mixture of 2 M glycerol and 0.4M sucrose supplemented with half-strength liquid MS media at 25°C for 20 minutes. PLBs were then dehydrated with plant vitrification solution 2 (PVS2) at 0°C for 20 minutes prior to storage in liquid nitrogen. Following rapid warming in a water bath at 40°C for 90 to 120 seconds, the PLBs were washed with half-strength liquid MS media supplemented with 1.2M sucrose. Subsequently, the PLBs were cultured on half strength semi-solid MS media supplemented with 2% sucrose without any growth regulator. After 2 months, PLBs were subcultured on half strength semi-solid MS media supplemented with 2% sucrose and 1mg/L BAP. Growth recovery was recorded as in percentage of regenerated cryopreserved PLBs (green) comparing with non-cryopreserved PLBs.

Cryopreservation of *Dendrobium* Bobby Messina PLBs using optimized PVS2 vitrification with an addition of ascorbic acid

The optimized protocol which gave rise to 40% growth of cryopreserved PLBs recovery after 2 months in culture was chosen for this experiment. Therefore, this experiment was conducted for the evaluation of effect of ascorbic acid on the growth recovery of cryopreserved PLBs in comparison with the non-cryopreserved PLBs. The experimental procedure is the same as been mentioned. However, ascorbic acid was added at a concentration of 0.6mM at the four main steps such as preculture, loading, unloading and

growth recovery. Growth recovery was scored as in percentage of regenerated cryopreserved PLBs (green) comparing with non-cryopreserved PLBs.

RAPD Analysis of *Dendrobium* Bobby Messina

In vitro cultures of regenerated protocorm-like bodies (PLBs) (100mg) of *Dendrobium* Bobby Messina from PLBs stock culture, cryopreserved and non-cryopreserved PLBs regenerated from PVS2 vitrification treatment were selected for RAPD analysis (Khosravi *et al*., 2009).

Results and Discussion

Optimization of preculture condition and PVS2 incubation conditions

In optimization in preculture condition, cryopreserved PLBs that were precultured in 0.2M sucrose for 1 day showed very the highest viability rates (Fig. 1). In optimization in PVS2 exposure conditions, cryopreserved PLBs precultured in 0.2M sucrose for 1 day and exposed to PVS2 solution at 0ºC for 20 minutes appeared to have the highest viability rates (Fig. 2). The key to successful cryopreservation is not dependent upon the induction of freezing tolerance but rather on dehydration tolerance (Panis & Lambardi 2005). Preculture involves the culture of the excised explants on medium containing sucrose or sugar alcohols for few hours to several days. Explants are dehydrated to appropriate water contents prior to immersion into the liquid nitrogen (Engelmann 1997). One of the keys to successful cryopreservation by vitrification is the cautious control of dehydration and avoidance of injury by chemical toxicity or excess osmotic stresses during treatment with PVS2. Optimizing the time of exposure and the temperature during exposure to PVS2 is vital for producing a high level of growth recovery following vitrification (Thinh 1997).

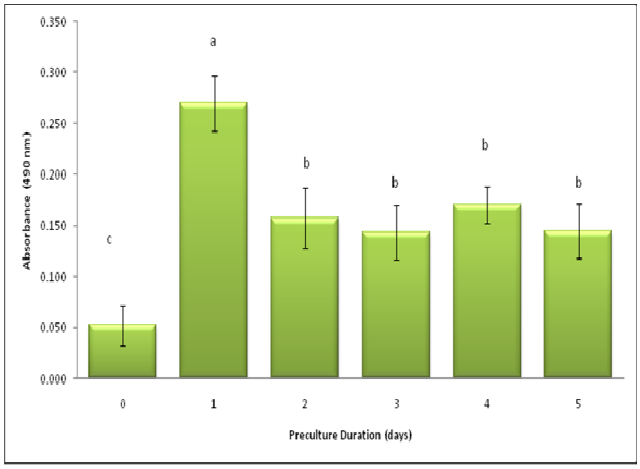

Figure 1. Effect of preculture durations on viability of cryopreserved PLBs precultured in 0.2M sucrose for 0 to 5 days. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent ± SD of means of 6 replicates.

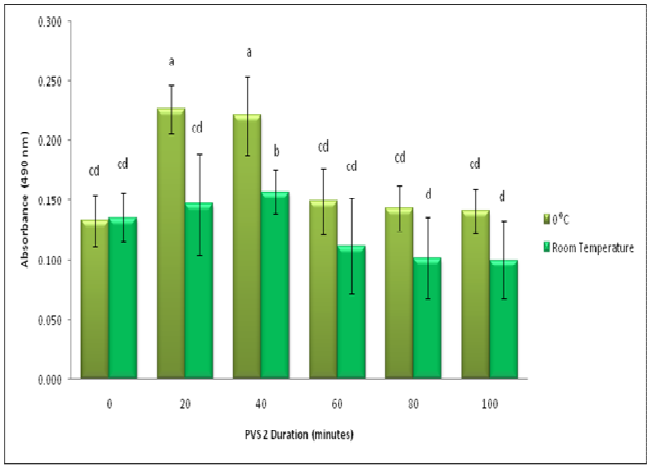

Figure 2. Effect of PVS2 incubation temperatures and durations on viability of cryopreserved PLBs precultured in 0.2M sucrose. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent ± SD of means of 6 replicates.

Growth recovery percentage of cryopreserved and non-cryopreserved PLBs of *Dendrobium* Bobby Messina using optimized vitrification protocol

This optimized protocol gave rise to regeneration percentage in non-cryopreserved and cryopreserved as 100% and 40%, respectively within 2 months (Table 1). The morphological characteristics of regenerated cryopreserved PLBs compared with the non-cryopreserved PLBs appear to be the same such as the appearance of green, healthy and able to regenerate into complete plantlet with normal shoots and leaves. Thus, this indicates that the PLBs precultured in 0.2M sucrose and dehydrated with PVS2 solution at 0°C for 20 minutes will be more vital to be used as a cryopreservation protocol for *Dendrobium* Bobby Messina. A minimum of 40% growth recovery following cryostorage was recognized as satisfactory for most plant germplasm (Dussert *et al*. 2003).

Growth recovery percentage of cryopreserved and non-cryopreserved PLBs of *Dendrobium* Bobby Messina precultured in 0.2 M with the addition of 0.6 mM ascorbic acid

In view to evaluate the effect of addition of ascorbic acid on recovery of cryopreserved PLBs, optimized protocol was used in the cryopreservation of PLBs of *Dendrobium* Bobby Messina with an addition of 0.6 mM ascorbic acid in four main steps in the cryopreservation procedures which are preculture, loading, unloading and growth recovery steps. However, regeneration was lower with percentage in non-cryopreserved and cryopreserved as 30% and 10%, respectively within 2 months after thawing (Table 2).

Table 1. Percentage of growth in cryopreserved and non-cryopreserved PLBs precultured in 0.2M sucrose. Growth recovery of cryopreserved and non-cryopreserved PLBs within 3 Months.

Percentage of growth (%)	1 week	2 week	3 week	4 week	2 months	3months
Non-cryopreserved	100% [a]	100% [a]	100% [a]	100% [a]	100% [a]	100% [a]
Cryopreserved	100% [a]	30% [c] ±0.063	30% [c] ±0.063	38% [b] ±0.041	40% [b]	40% [b]

Note*Results were analysed by one way ANOVA and means were compared b y Tukey test. SD represents of means of 6 replicates

Table 2. Percentage of growth in cryopreserved and non-cryopreserved PLBs precultured in 0.2M sucrose with the addition of 0.6 mM ascorbic acid.

Treatment of cryopreservation	Percentage of growth (%)
Cryopreserved (+ LN)	10 ± 0.087
Non-cryopreserved (-LN)	30 ± 0.056

Note*Results were analysed based on Independent sample t-test. Significant differences observed between both LN and Non LN. Vertical bars represent ± SD of means of 6 replicates.

RAPD Analysis of *Dendrobium* Bobby Messina

RAPD analysis using 6 primers indicated that cryopreserved and non-cryopreserved PLBs from vitrification method were genetically faithful to the mother plant. However, 3 primers showed polymorphism and 1 primers indicated partial polymorphism between the cryopreserved and non-cryopreserved PLBs in comparative to the mother plant (Table 3; 4). RAPD molecular method is necessary to verify cryopreserved plants genetic stability. No genomic variations were detected in the regenerated material from most cryopreservation experiments such as embryogenic cultures of Scots pine (*Pinus sylvestris* L.) precultured using three different mixtures as cryoprotectants (Haggman *et al*. 1998), potato plants (*Solanum tuberosum* L.) derived from meristems cryopreservation by encapsulation vitrification (Hirai & Sakai, 2000), *Prunus* Ferlenain plants (Helliot *et al*. 2002), *Arachis* species (Gagliardi *et al*. 2003), *Diascorea Vitis vinefera* (Zhai *et al*. 2003) and *Indigofera tinctoria* (Nair & Reghunath, 2009).

Table 3. RAPD band paterns on cryopreserved PLBs comparing with the PLBs stock culture.

Primers	Total number of bands in PLBs stock culture	Total number of bands in cryopreserved PLBs	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragments	SI Index
OPA04	8	0	0	0	100-1500	0
OPAW17	12	*0*	*0*	*0*	300-2000	*0*
OPB11	2	*2*	*2*	*0*	1000-1500	*1*
OPB12	2	*2*	*2*	*0*	900-1200	*1*
OPB18	8	*2*	*2*	*0*	200-2000	*0.4*
OPD01	1	*1*	*1*	*0*	3000	*1*
OPG13	6	*6*	*6*	*0*	500-1500	*1*
OPG14	4	*4*	*4*	*0*	600-1000	*1*
OPG15	0	*2*	*0*	*0*	900-1200	*0*
OPB 14	1	*1*	*1*	*2*	1000	*1*
Total number of bands	*44*	*20*	*18*	*2*	-	-

Note: SI index mean similarity index.

Table 4. RAPD band paterns on non-cryopreserved PLBs comparing with the PLBs stock culture.

Primers	Total number of bands in PLBs stock culture	Total number of bands in cryopreserved PLBs	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragments	SI Index
OPA04	8	0	0	0	100-1500	0
OPAW17	12	*0*	*0*	*0*	300-2000	*0*
OPB11	2	*2*	*2*	*0*	1000-1500	*1*
OPB12	2	*2*	*2*	*0*	900-1200	*1*
OPB18	8	*2*	*2*	*0*	200-2000	*0.4*
OPD01	1	*1*	*1*	*0*	3000	*1*
OPG13	6	*6*	*6*	*0*	500-1500	*1*
OPG14	4	*4*	*4*	*0*	600-1000	*1*
OPG15	0	*1*	*0*	*1*	900	*0*
OPB 14	1	*1*	*1*	*0*	1000	*1*
Total number of bands	*44*	*19*	*18*	*1*	-	-

Note: SI index mean similarity index.

Conclusions

The optimized protocol for *Dendrobium* Bobby Messina based on TTC spectrophotometrical analysis and growth recovery were 3-4mm PLBs precultured in 0.2M sucrose for 1 day, treated with a mixture of 2M glycerol and 0.4M sucrose supplemented with half strength liquid MS media at 25°C for 20 minutes and subsequently dehydrated with plant vitrification solution 2 (PVS2) at 0°C for 20 minutes prior to storage in liquid nitrogen. Following rapid warming in a water bath at 40°C for 90 seconds, the PLBs were washed with half strength liquid MS media supplemented with 1.2M sucrose. Subsequently, the PLBs were cultured on half strength semi-solid MS media supplemented with 2% (w/v) sucrose without any growth regulators. The RAPD results from 6 selected primers obtained from cryopreserved and non-cryopreserved PLBs by vitrification method indicated that the genetic stability of PLBs following cryopreservation was maintained. Thus, this indicates that this method can be utilised as cryopreservation protocol for the germplasm conservation of *Dendrobium* Bobby Messina.

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References

Ashmore S.E. 1997. Current *in vitro* conservation techniques. In: Engelmann, F (ed), Status Report on the Development and Application of *in vitro* Techniques for the Conservation and Use of Plant Genetic Resources, pp 5–18, IPGRI, Rome, Italy.

Bajaj Y.P.S. 1995. Cryopreservation of plant cell, tissue and organ culture for the conservation of germplasm and biodiversity. In: Bajaj, Y.P.S. (ed), Biotechnology in agriculture and forestry cryopreservation of plant germplasm I, pp 3–18, Springer, New York.

Benson E.E., Bremner D.H. 2004. Oxidative stress in the frozen plant: a free radical point of view. In: Fuller, B., Lane, N., Benson, E.E. (eds), Life in the frozen state, pp 205–242, CRC, FL.

Burritt D.J. 2008. Efficient cryopreservation of adventitious shoots of *Begonia* x *erythrophyllausing* encapsulation–dehydration requires pretreatment with both ABA and proline. Plant Cell Tissue Organ and Culture, 95: 209–215.

Dussert S., Engelmann F., Noirot M. 2003. Development of probabilistic tools to assist in the establishment and management of cryopreserved plant germplasm collections. Cryo-Letters, 24: 149-160.

Engelmann F. 1997. Importance of desiccation for the cryopreservation of recalcitrant seed and vegetatively propagated species. Plant Genetic Resources Newsletter, 112: 9–18.

Engelmann F. 2000. Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann, F., Takagi, H. (eds), Cryopreservation of tropical germplasm Current Research Progress and Application, pp 8–20, Japan International Research Center for Agricultural Sciences and International Plant Genetic Resources Institute, Rome.

Gagliardi R.F., Pacheco G.P., Carneiro L.A., Valls J.F.M., Vieira M.L.C., Mansur E. 2003. Cryopreservation of *Arachis* species by vitrification of *in vitro* grown shoot apices and genetic stability of recovered plants. Cryo-Letters, 24: 103–110.

Gagliardi R.F., Hanai L.R., Pacheco G., Oliveira C.A., Carneiro L.A., Valls J.F.M., Mansur E., Vieira M.L.C. 2007. Assessment of genetic stability among *in vitro* plants of *Arachis retusa* using RAPD and AFLP markers for germplasm preservation. Journal of Integrative Plant Biology, 29: 307–312.

Gonzalez-Arnao M.T., Lazara-Vallejo C.E., Engelmann F., Gamez-Pastrana R., Martinez-Ocampo Y.M., Pastelin-Solano M.C., Diaz-Ramos C. 2009. Multiplication and cryopreservation of vanilla (*Vanilla planifolia* 'Andrews'). *In Vitro* Cellular & Development Biology-Plant, 45: 574-582.

Harding, K. 1996. *In vitro* conservation of plant genetic resources. In: Normah, M.N., Narimah, M.K., Clyde, M.M. (eds), Approaches to assess the genetic stability of plants recovered from *in vitro* culture, pp 135–168, Plant Biotechnology Laboratory, Malaysia.

Helliot B., Madur D., Dirlewanger E., de Boucaud M.T. 2002. Evaluation of genetic stability in cryopreserved *prunus*. *In Vitro* Cellular & Development Biology-Plant, 38: 493-500.

Hirai D., Sakai A. 2000. Cryopreservation techniques. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation–vitrification. JIRCAS Japan International Research Center for Agricultural Science, 8: 205–211.

Hirano T., Ishikawa K., Mii M. 2005b. Cryopreservation of immature seeds of *Ponerorchis graminifolia* var. *suzukiana* by vitrification. Cryo-Letters, 26: 139–146.

Ishikawa K., Harata K., Mii M., Sakai A., Yoshimatsu K., Shimomura K. 1997. Cryopreservation of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. Plant Cell Reports, 16: 754–757.

Khosravi A.R., Kadir M.A., Kadzemin S.B., Zaman F.Q., De Silva A.E. 2009. RAPD analysis of colchicine induced variation of the *Dendrobium* Serdang beauty. African Journal of Biotechnology, 8: 1455-1465.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science, 7: 405–410.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology, 15: 473–497.

Nair D.S., Reghunath B.R. 2009. Cryoconservation and regeneration of axillary shoot meristems of *Indigofera tinctoria* (L.) by encapsulation-dehydration technique. *In Vitro* Cellular & Development Biology-Plant, 45: 565-573.

Panis B., Lambardi M. 2005. Status of cryopreservation technologies in plants (crops and forest trees). In: Ruane, J., Sonnino, A. (eds), The role of biotechnology for the characterization and conservation of crop, forest, animal and fishery genetic resources in developing countries, pp 61–78, Turin, FAO.

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

Thammasiri K. 2000. Cryopreservation of seeds of a Thai orchid (*Doritis pulcherrima* Lindl.) by vitrification. Cryo-Letters, 21: 237–244.

Thinh N.T. 1997. Cryopreservation of germplasm of vegetatively propagated tropical monocots by vitrification. Doctoral Paper, Faculty of Agriculture, Kobe University, Japan.

Towill L.E. 1996. Vitrification as a method to cryopreserve shoot tips. In: Trigiano R.S., Gray D.J. (eds), Plant tissue culture concepts and laboratory exercises, pp 297–304, CRC Press, Boca Raton.

Uchendu E.E., Leonard S.W., Traber M.G., Reed B.M. 2009. Vitamin C and E improve regrowth and reduce lipid peroxidation of blackberry shoot tips following cryopreservation. Plant Cell Reports, 29: 25-35.

Verleysen H., Samyn G., Bockstaele E.V., Debergh P. 2004. Evaluation of analytical techniques to predict viability after cryopreservation. Plant Cell Tissue and Organ Culture, 77: 11-21.

Zhai Z., Wu Y., Engelmann F., Chen R., Zhao Y. 2003. Genetic stability assessments of plantlets regenerated from cryopreserved *in vitro* cultured grape and kiwi shoot tips using RAPD. Cryo-Letters, 24: 315–322.