Kor. J. Hort. Sci. Technol. 30(1):79-84, 2012 DOI http://dx.doi.org/10.7235/hort.2012.11087

Improved Cryopreservation Using Droplet-vitrification and Histological Changes Associated with Cryopreservation of Madder (*Rubia akane* Nakai)

Jung-Yoon Yi[1*], Isabelle Sylvestre[2], Myriam Colin[2], Mohammad Salma[2], Sok-Young Lee[1], Haeng-Hoon Kim[3], Hong-Jae Park[1], and Florent Engelmann[2,4]

[1]*National Academy of Agricultural Science, Suwon 441-853, Korea*
[2]*Institut de Recherche pour le Développement, Montpellier, BP64501, France*
[3]*Technology Cooperation Bureau, Rural Development Administration, Suwon 441-853, Korea*
[4]*Bioversity International, Maccarese, Rome, Italy*

Abstract. An efficient protocol for cryopreservation of madder hairy root cultures has been developed using droplet-vitrification. In previous study, combining loading solution C4 (35% PVS3) and vitrification solution B5 (80% PVS3) was the most effective method. In this study, we tried three types of vitrification solution, B5, A3 (90% PVS2, on ice), and A5 (70% PVS2, on ice). Combining loading solution C4 and vitrification solution A5 (on ice) showed the best regeneration rate in this study. Histological changes of the cells within the hairy root of madder were also observed in different steps. The cells from the hairy roots of the control treatment were full and intact with different size of vacuoles and obvious cell nucleus having a dark nucleolus. After the stage of preparing for cryopreservation (after preculturing, loading, followed by dehydration solution A5 or B5), intercellular spaces had become distinct, and within cells, the cytoplasms had become denser and week plasmolyses had appeared. The cell plasmolyses were much more apparent and we measured the degree of plasmolysis by calculating, the area of cell/the area of cytoplasm. The value of plasmolysis degree was the highest in the combination of preculture, loading solution C4, and dehydration solution A5, 1.97. Because the highest regeneration rates appeared in the treatment of A5 for 20 min, we could assume that the optimal degree of plasmolysis for cryopreservation might be around 1.97. The changes in cell structure during cryopreservation might be a useful basis for the development of a proper long-term preservation method for madder germplasms.

***Additional key words*:** hairy root, loading solution, plasmolysis, regeneration rate, vitrification solution

Introduction

Plants belonging to the Rubiaceae family, also known as madder, have substantial amounts of anthraquinones, especially in the roots, which are used for the mass production of natural red dyes (Gilbert and Cooke, 2001). The advantages of propagation for hairy roots in vitro are their genetic and biosynthetic stability, rapid growth and much of branching on phytohormone-free medium (Georgiev et al., 2007; Hu and Du, 2006). But, at the same time, in vitro maintenance of hairy root cultures is labor-intensive and time-consuming. To resolve these problems, cold storage (at 4℃) and cryopreservation (at -196℃) have been applied, with varying degrees of success, to preserve stock cultures of hairy roots. The first report appeared that Benson and Hammill (1991) have succeeded in cryopreserving *Beta vulgaris* and *Nicotiana rustica* hairy roots using slow and ultra-rapid freezing. Afterwards, cryopreservation has been reported for isolated root cultures of various medicinal plants using slow freezing (Teoh et al., 1996), vitrification (Jung et al., 2001), encapsulation-dehydration (Hirata et al., 2002; Lambert et al., 2009) and encapsulation-vitrification (Xue et al., 2008) techniques.

In our previous study, we have established an efficient and reliable protocol for cryopreservation of hairy roots of

***Corresponding author:** naaeskr@korea.kr

※ Received 15 August 2011; Revised 1 December 2011; Accepted 3 December 2011. This study was carried out with the support of 'Research Program for Agricultural Science & Technology Development (Project No. PJ007386)', National Academy of Agricultural Science, Rural Development Administration, Republic of Korea.

Table 1. Composition of loading and vitrification solutions used in this study.

Solution	Composition (%, w/v)	Duration (min)	Temperature of treatment (℃)	Note
Loading				
C4	17.5 glycerol + 17.5 sucrose	30	25	35% PVS3[z]
Vitrification				
B5	40.0 glycerol + 40.0 sucrose	15	25	80% PVS3
A3	37.5 glycerol + 15.0 DMSO[y]+ 15.0 EG[x] + 22.5 sucrose	20	0	90% PVS2[w]
A5	29.2 glycerol + 11.7 DMSO + 11.7 EG + 17.4 sucrose	20	0	70% PVS2

[z]PVS3 contained 50% glycerol and 50% sucrose in MS.
[y]DMSO: dimethylsulfoxide.
[x]EG: ethylene glycol.
[w]PVS2 contained 30% glycerol, 15% DMSO, 15% ethylene glycol, and 13.7% sucrose in MS.

R. akane using the droplet-vitrification technique. Post-cryo regeneration of percentages up to 83-86% were achieved through optimization of preculture, loading and dehydration steps (Kim et al., 2010) when we combined the loading solution C4 (35% PVS3) and vitrification solution B5 (80% PVS3). In this study, an improved protocol for cryopreservation of madder hairy root cultures has been developed using droplet-vitrification method. We tried three types of vitrification solution, B5, A3 (90% PVS2, on ice), A5 (70% PVS2, on ice) with the same preculture and loading treatment to the previous one (Table 1).

The key component for successful cryopreservation is the degree of injury to cell microstructure. Understanding and minimizing the damage to cell microstructure during cryopreservation improves the technique of plant cryopreservation. The changes in cell micro- or ultrastructure during cryopreservation have been observed on date palm (Bagniol et al., 1992), coffee (Mari et al., 1995), papaya (Zeng et al., 2005), banana (Helliot et al., 2003), and rice (Wang et al., 1998). During this work, an histo-cytological study was carried out each successive step of the cryopreservation protocol, in order to observe the modifications at the cellular level, to understand the meristems of adaptation to low temperatures and also to identify precisely those steps which may be considered as the 'key steps' on which the successful regeneration depends.

In our present study, the observation on the changes of cell structure of in vitro cultured hairy roots of *R. akane* during cryopreservation, as a basis for the development of an appropriate long-term preservation method has been researched.

Materials and Methods

Plant Materials

Hairy roots of *Rubia akane* Nakai, induced by the leaf-disc method using *Agrobacterium rhizogens*, were obtained from Chungnam National University. They were maintained on hormone-free half-strength MS medium (Murashige and Skoog, 1962) supplemented with 30 $g \cdot L^{-1}$ sucrose and 2.2 $g \cdot L^{-1}$ phytagel (Sigma-Aldrich, USA) at 24℃ with 3-5 weeks subculture intervals. Several months before cryopreservation experiments, the subculture intervals were shortened to 1-2 weeks.

Droplet-vitrification Procedure

Root apices, 7-10 mm in length, were dissected from 5-7 day old root cultures during their exponential phase using sharp micro-scissors. Immediately after excision, 15-20 explants were suspended in 12 mL liquid half-strength MS medium supplemented with 0.3 M sucrose and precultured for 54 h (on a rotary shaker at 90 rpm during the first 2-3 h) followed by the same medium supplemented with 0.5M sucrose for 16 h. Precultured explants were cryoprotected with C4 loading solution (17.5% glycerol + 17.5% sucrose, w/v) for 30 min at room temperature, if not indicated otherwise. After loading, the root explants were dehydrated in B5 (80% PVS3), A3 (90% PVS2, on ice), A5 (70% PVS2, on ice). Table 1 shows the composition of loading solutions and vitrification solutions, duration time, and temperature of treatment used in the study. A few minutes before the end of the dehydration period, explants were placed on aluminum foil strips (7 × 20 mm, five explants per strip) and covered with a drop of fresh dehydration solution. Foil strips were rapidly immersed in liquid nitrogen for at least 1 h. For rewarming, samples were transferred to 12 mL pre-heated (40℃) unloading solution (liquid MS medium with 0.8 M sucrose) and gently shaken to separate explants from the foils. After 30 s, half of the solution and the foils were discarded and 6 mL fresh unloading solution at 25℃ were added. Explants were unloaded at 25℃ for 30 min, then

blotted dry on sterile filter paper. Cultures were recovered on half-strength MS medium without growth regulators, solidified with 2.2 $g \cdot L^{-1}$ phytagel, at 25℃ in the dark.

Regrowth Assessment and Statistical Analysis

Regeneration was determined as the number of explants which produced normal roots above 10 mm long 30 days after rewarming and were expressed in percentage of the total number of treated hairy roots. All experiments were performed in three independent replicates, with 15-20 explants per replicate. Data were processed by ANOVA using SAS program Version 9.1 (SAS, Raleigh, NC). Significance of differences between treatments was analyzed using Duncan's multiple range test at $P < 0.05$.

Histological Studies and Assessment of Cell Area

For histological studies, samples were fixed for 48 h at 4℃ in phosphate buffer (pH 7.2) containing 2% paraformaldehyde, 1% glutaraldehyde and 1% caffeine. They were then dehydrated by successive transfers in alcohol baths with progressively increasing alcohol grades (30° to 100°) and inclusion was carried out in Kulser 7100 resin. Three μm thick sections were cut using an automatic microtome (Historange 2218, LKB), and then the samples were treated with periodic acid-Schiff reaction and naphthol blue black. This double staining technique allowed specific characterization of the polysaccharide compounds (starch reserves and walls) which stain red and the soluble and non-soluble proteins which stain blue black (Fisher, 1968). To assess the area of cell, we used Image-J program, Java-based image processing program, which is public domain and developed National Institute of Health, USA.

Results and Discussion

Effect of Vitrification Solution

In previous our study, the highest post-freeze regrowth was observed for the explants precultured step-wise in liquid MS medium supplemented with 0.3 M and 0.5 M sucrose for 54 h and 16 h, then loaded for 30 min in alternative C4 (35% PVS3) loading solution followed by B5 (80% PVS3) dehydration solution for 15 min. In this study, we tested three kinds of dehydration solution, two kinds of modified PVS2 (A3 and A5) and modified PVS3 (B5) with the same preculture and loading treatment to that of previous study. These dehydration solutions contain various concentration of glycerol, sucrose, ethylene glycol, and DMSO (Table 1). As a result, we observed the highest regeneration rate in the treatment of A5 (70% PVS2) for 20 min on ice (Table 2). Because PVS2 solution contains much of toxic chemicals like glycerol, DMSO, and ethylene glycol and can be osmotically stressful to plants. These kinds of cryoprotectants are permeable both to cell wall and to cytoplasmic membrane, so they're assumed more effective for dehydration and cryopreservation. To lessen cytological toxicity, we tested modified PVS2 dehydration solutions, A3 and A5, on ice instead of those in room temperature.

Table 2. Effects of dehydration solution and duration on regeneration of dehydrated (-LN) and cryopreserved (+LN) *Rubia akane* hairy roots after preculture in liquid MS medium supplemented with 0.3 M and 0.5 M sucrose for 54 h and 16 h followed by loading treatment with C4 loading solution.

Dehydration	Duration (min)	Regeneration (%)	
		-LN	+LN
A3[z]	20	63.5 bc[y]	39.2 cd
	10	31.3 cd	13.1 d
A5	20	98.6 a	87.9 a
	10	64.7 bc	44.3 bc
B5	15	74.3 b	54.3 b
	30	38.4 c	12.3 d

[z]A3 and A5 treatments were undergone at 0℃ and B5 at 25℃.
[y]Mean separation within columns by Duncan's multiple range test at 5% level.

Droplet-vitrification method is a compound of droplet-freezing and vitrification and is considered very promising for large-scale implementation (Sakai and Engelmann, 2007), it has the advantages of preliminary osmotic dehydration and high velocities of cooling and re-warming. Samples are dehydrated and cryopreserved in drops of highly concentrated vitrification solution put on aluminum foil strips. Among the cryopreservation methods, droplet-vitrification showed a higher survival than droplet-freezing or vitrification (Kim et al., 2006).

Loading is very critical factor for hairy roots, which have been shown to be highly susceptible to the toxic effects of vitrification solutions (Lambert et al., 2009). Loading implies a short (20-60 min) exposure of samples to cryoprotectant mixture with moderate concentration prior to dehydration with highly concentrated vitrification solutions (Nishizawa et al., 1993). The procedure aims at preparing cells to further osmotic stress, thus mitigating toxicity of vitrification solutions and minimizing sample injury during the dehydration step (Sakai and Engelmann, 2007). We tried to test four kinds of loading solutions, C3, C4, C6, and C7 loading solutions (Kim et al., 2010) and could select C4 loading solution.

The vitrification solutions tested in this study significantly affected the regeneration rates of cryopreserved hairy roots in madder. Cryopreserved hairy roots dehydrated with A5 solution for 20 min on ice showed significantly higher

regeneration rates than A3 or B5 possibly due to optimal permeability of A5 to cell wall or cytoplasmic membrane. PVS2 is highly concentrated vitrification solution and is thus highly toxic to plant tissues (Kim et al., 2006). As shown in Table 2, the highest regeneration rates were present in A5 for 20 min on ice. Twenty minutes is relatively short dehydration duration because sensitivity to dehydration time of madder to modified PVS2 solution was very high. Sensitivity to dehydration time varies widely among different species, eg. from 20 min for persimmon (Matsumoto et al., 2003) to 120 min for *Prunus* shoot tips (Niino et al., 1997).

This study demonstrated the higher regeneration rates of hairy roots after cryopreservation in comparison with our previous study (Kim et al., 2010) by using droplet-vitrification.

Histological Characteristics of Hairy Roots and Assessment of Cell Area

The changes of the cell structure in the in vitro cultured *R. akane* hairy roots during cryopreservation were observed. After the first step of preparation for cryopreservation (preculturing in half-strength MS medium with progressively increased sucrose concentration), the root tips had little changes in comparison with those of the control treatment. After the preculture, hairy roots were cryoprotected in C4 loading solution (second step of cryopreservation) after that, they were dehydrated in A5 or B5 dehydration solution (third step of cryopreservation). At this stage, the intercellular spaces had become denser and slight plasmolyses were apparent. Wilkinson et al. (2003) observed the same results for shoot apices of *Cosmos atrosanguineus*.

Further significant changes in the cell structure occurred after the root tips were stored in liquid nitrogen for 1 h (Figs. 1A and 1B). The cell plasmolyses were much apparent and some cells had got the walls that were broken and ruptured as a result of freezing. In the case of Fig. 1C, the cells seemed to recover 10 days after preculture followed by C4 loading, A5 dehydration, and no liquid nitrogen control. The cells of madder hairy roots that survived were subjected to a 10 day recovery culture after liquid nitrogen and the cells similar to those of the Fig. 1C were observed (Fig. 1D). Some cells in both cases have starch reserves in the form of small starch granules (Fig. 1C, pink dots) around the nucleus. From the tenth day onwards, cell mitotic activity was very intense and regrowth of meristem became active. Starch enrichment of cells indicated the beginning of the polarization process and the first procambial bundles were put in place and the results of this study were very similar to Stewart et al. (2001).

A close relationship appeared between the cell microstructure and the processes of preculturing, loading, dehydration in

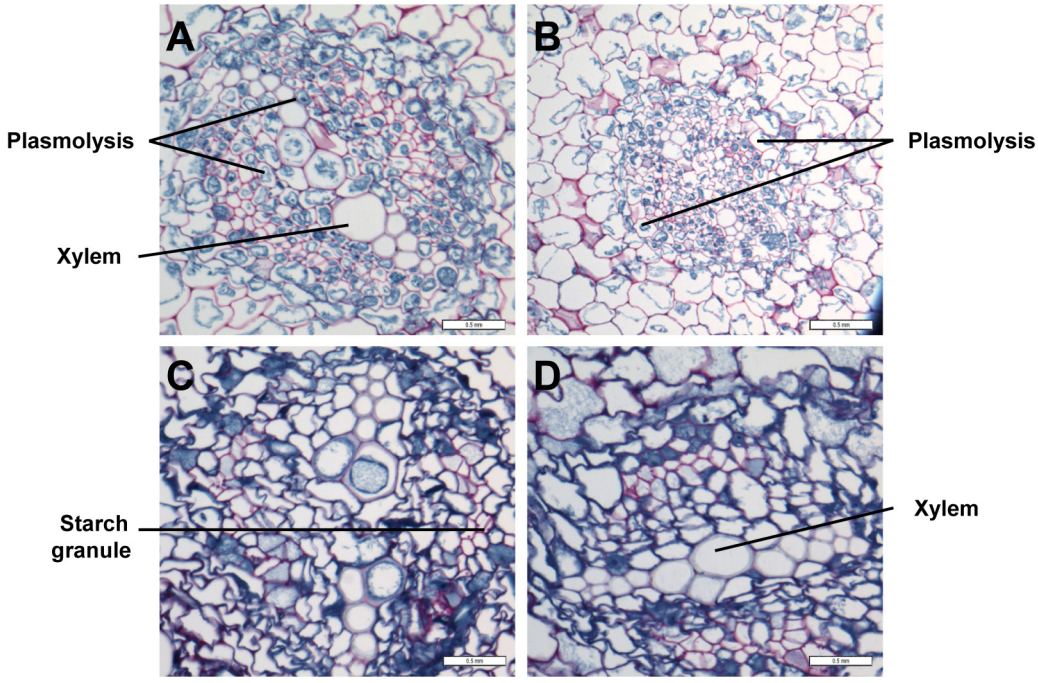

Fig. 1. Histological comparison of hairy root cells originating from an untreated control and combination treatments for cryopreservation (40x magnification). A, severe plasmolysis of hairy roots after preculture, C4 loading, B5 dehydration, and preservation in liquid nitrogen; B, the cells injured due to severe plasmolysis of hairy roots after preculture, C4 loading, A5 dehydration, and preservation in liquid nitrogen; C, the cells of hairy roots that survived after 10 days recovery culture after preculture, C4 loading, and A5 dehydration; D, the cells of hairy roots that regenerated 10 days after preservation in liquid nitrogen; Scale bar indicates 0.5 mm.

Table 3. Differences of plasmolysis according to cryopreservation steps.

Steps	Aver. of cell area	Aver. of cytoplasm area	Degree of plasmolysis (cell/cytoplasm)
Untreated	213.2 ± 12.3	199.8 ± 2.6	1.07 c[z]
PC[y]	208.8 ± 11.2	182.3 ± 12.1	1.15 bc
PC + Loading (C4[x])	203.9 ± 9.8	150.3 ± 5.8	1.35 b
PC + C4 + Vitrification (B5[w])	211.3 ± 10.1	118.8 ± 8.9	1.78 ab
PC + C4 + Vitrification (A5[v])	215.3 ± 7.7	109.4 ± 11.3	1.97 a
10 days after thawing	209.9 ± 9.4	190.7 ± 9.4	1.10 bc

[z]Mean separation within columns by Duncan's multiple range test at 5% level.
[y]PC: preculture.
[x]C4: C4 loading solution.
[w]B5: 80% PVS3 dehydration solution.
[v]A5: 70% PVS2 dehydration solution.

PVS, and regeneration culture after thawing. PVS2 solution has been used for protecting cells from the effects of freezing while it could do harm to the cells and result in their plasmolysis after dehydration (Helliot et al., 2003; Zeng et al., 2005). After dehydration, the majority of meristematic cells were plasmolysed; retracted cytoplasm appeared more dense.

Plasmolysis is the process in plant cells where the plasma membrane pulls away from the cell wall due to the loss of water through osmosis, we could assess the degree of plasmolysis like the value of total cell area/the area of cytoplasm. To optimize the degree of plamolyses for cryopreservation, we assessed the area of cells by using image J, Java-based image program. As shown in Table 3, the degree of plasmolysis is the minimum in control treatment, 1.07 and the maximum in treatment of Preculture + C4 loading + vitrification with A5, 1.97. Because the highest regeneration rates appeared in the treatment of A5 for 20 min, we could assume that the optimal degree of plasmolysis might be around 1.97. Gnanapragasam and Vasil (1992) also reported that when frozen by the optimized procedure, although some damage was apparent the membranous structures were still intact. Any damage was repaired in a short period of time and normal growth was resumed.

The investigation of changes in cell microstructure and assessment of cell area during cryopreservation might be a critical basis for the development of an appropriate long-term conservation method for specific hairy root germplasm. In addition, in order to really understand the recognization and the recovery growth pattern of the meristems, it would be necessary to precisely follow the histo-cytological evolution of explants during a longer period after thawing.

Literature Cited

Bagniol, S., F. Engelmann, and F. Michauxe. 1992. Histo-cytological study of apices from in vitro plantlets of date palm (*Phoenix dactylifera* L.) during a cryopreservation process. CryoLetters 13:405-412.

Benson, E.E. and J.D. Hamill. 1991. Cryopreservation and post freeze molecular and biosynthetic stability in transformed roots of *Beta vulgaris* and *Nicotiana rustica*. Plant Cell Tiss. Org. Cult. 24:163-172.

Fisher, D.B. 1968. Protein sustaining of ribboned epon sections for light microscopy. Histochemie 16:92-96.

Georgiev, M.L., A.I. Pavlov, and T. Bley. 2007. Hairy root type plant in vitro systems as sources of bioactive substances. Applied Microbio. Biotech. 74:1175-1185.

Gilbert, K.G. and G.T. Cooke. 2001. Dyes from plants: Past usage, present understanding and potential. Plant Growth Reg. 34:57-69.

Gnanapragasam, S. and I.K. Vasil. 1992. Ultrastructural changes in suspension culture cells of *Panicum maximum* during cryopreservation. Plant Cell Rep. 11:169-174.

Helliot, B., R. Swennen, Y. Poumay, E. Frison, P. Lepoivre, and B. Panis. 2003. Ultrastructural changes associated with cryopreservation of banana (*Musa* spp.) highly proliferating meristems. Plant Cell Rep. 21:690-698.

Hirata, K., M. Mukai, S. Goda, M. Ishio-Kinugasa, K. Yoshida, A. Sakai, and K. Miyamoto. 2002. Cryopreservation of hairy root cultures of *Vinca minor* (L.) by encapsulation-dehydration. Biotech. Lett. 24:371-376.

Hu, Z.B. and M. Du. 2006. Hairy root and its application in plant genetic engineering. J. Integr. Plant Biol. 48:21-127.

Jung, D.W., C.K. Sung, K. Touno, K. Yoshmatsu, and K. Shimomura. 2001. Cryopreservation of *Hyoscyamus niger* adventitious roots by vitrification. J. Plant Physiol. 158:801-805.

Kim, H.H., E.V. Popova, J.Y. Yi, G.T. Cho, S.U. Park, S.C. Lee, and F. Engelmann. 2010. Cryopreservation of hairy roots of *Rubia akane* (Nakai) using a droplet-vitrification procedure. CryoLetters 31:473-484.

Kim, H.H., J.W. Yoon, Y.E. Park, E.G. Cho, J.K. Sohn, T.S. Kim, and F. Engelmann. 2006. Cryopreservation of potato cultivated varieties and wild species. CryoLetters 27:223-234.

Lambert, E., A. Goossens, B. Panis, M.C. Van Labeke, and D. Geelen. 2009. Cryopreservation of hairy root cultures of

Maesa lanceolata and *Medicago truncatula*. Plant Cell Tiss. Org. Cult. 96:289-296.

Mari, S., F. Engelmann, N. Chabrillange, C. Huet C, and N. Michaux-Ferriere. 1995. Histo-cytological study of apices coffee (*Coffea racemosa* and *C. sessiliflora*) in vitro plantlets during their cryopreservation using the encapsulation-dehydration technique. CryoLetters 16:289-298.

Matsumoto, T., K. Mochida, H. Itamura, and A. Sakai. 2003. Cryopreservation of persimmon (Diospyros *kaki* Thunb.) by vitrification of dormant shoot tips. Plant Cell Rep. 20:398-402.

Murashige, T. and F. Skoog. 1962. A revised medium for a rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:437-497.

Niino, T., K. Tashiro, M. Suzuki, S. Ohuchi, J. Magoshi, and T. Akihama. 1997. Cryopreservation of in vitro grown shoot tips of cherry and sweet cherry by one-step vitrification. Sci. Hort. 70:155-163.

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

Sakai, A. and F. Engelmann. 2007. Vitrification and encapsulation-vitrification and droplet-vitrification: a review. CryoLetters 28:151-172.

Stewart, P., M. Taylor, and D. Mycock. 2001. The sequence of the preparative procedures affects the success of cryostorage of cassava somatic embryos. CryoLetters 22:35-42.

Teoh, K.H., P.J. Weathers, R.D. Cheetham, and D.B. Walcerz. 1996. Cryopreservation of transformed (hairy) roots of *Artemisia annua*. Cryobiology 33:106-117.

Wang, J.H., J.G. Ge, F. Liu, and C.N. Huang. 1998. Ultrastructural changes during cryopreservation of rice (*Oryza sativa* L.) embryogenic suspension cells by vitrification. CryoLetters 19:49-54.

Wilkinson, T., A. Wetten, C. Prychid, and M.F. Fay. 2003. Suitability of cryopreservation for the long-term storage of rare and endangered plant species: A case study for *Cosmos atrosanguineus*. Ann. Bot. 91:65-74.

Xue, S.H., X.J. Luo, Z.H. Wu, H.L. Zhang, and X.Y. Wang. 2008. Cold storage and cryopreservation of hairy root cultures of medicinal plant *Eruca sativa* Mill., *Astragalus membranaceus* and *Gentiana macrophylla* Pall. Plant Cell Tiss. Org. Cult. 92:251-260.

Zeng, J.W., G.J. Yi, and Q.M. Zhang. 2005. Cell ultrastructure of papaya shoot-tip during cryopreservation. Acta Hort. Sinica 32:15-19.