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Comparison of Cryoprotectants and Cryopreservation Protocols for *Eleutherococcus senticosus* via Somatic Embryogenesis

Chang Ho Ahn[1], Jung Won Shin[1], Ha Na Lee[1], Hyun Won Yoon[1], Jeong Min Seo[1], Yeoung Ryul Kim[1], Saeng Geul Baek[1], Jae Ik Nam[1] and Yong Eui Choi[2,*]

[1]*Division of Garden Material Research, Korea Institute of Arboretum Management, Sejong 30106, Republic of Korea*
[2]*Division of Forest Sciences, College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea*

Abstract

A cryopreservation is an essential tool for preservation of germplasm. In this study, the possibility for cryopreservation of embryogenic cells of Siberian ginseng (*Eleutherococcus senticosus*) in liquid nitrogen (-196°C) was evaluated. The effects of glycerol and dimethyl sulfoxide (DMSO) at different concentrations (5%, 10% and 20%) as cryoprotectants on regrowth of cryopreserved *E. senticosus* embryogenic cells were tested. There was significant effect of cryoprotectants on regrowth of embryogenic cells (p=0.0019). The highest and lowest fresh mass gain were achieved when embryogenic cells were frozen with 10% DMSO and 5% glycerol (138.2±5.9 and 61.3±14.6, respectively). The effect of the cryoprotectants on the frequency embryo germination was tested. There was no significant difference between glycerol and DMSO (p=0.846). Three different concentrations of cryoprotectants did not significantly affect the frequency embryo germination (p=0.534). Finally, the genetic fidelity of the plantlets regenerated from non-cryopreserved and cryopreserved embryogenic cells was tested by random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis. RAPD and ISSR analysises showed that there was no genetic variation among regenerants.

Key Words: Siberian ginseng, cryopreservation, cryoprotectant, genetic fidelity, somatic embryogenesis

Introduction

Eleutherococcus senticosus is a woody shrub and high-altitude species distributed in northeast Asia and southeast Russia (Lee 1979). *E. senticosus* is well-known not only for a woody medicinal plant, but also as 'Siberian ginseng'. Since, the cortical tissues of its roots and shoots contain many useful components for medicinal purposes, the demand for *E. senticosus* has recently increased worldwide. However, its populations in South Korea have declined due to over-harvesting, pathogens, and climate change. In particular, it has currently been protected as an extinct species of special concern in South Korea. Unfortunately, it is difficult to regenerate *E. senticosus* naturally due to low seed germination rates and a long germination period. Thus, propagation methods for *E. senticosus* require to overcome these problems.

Plant tissue culture techniques are used as alternative methods for the propagation of plant species that are difficult to propagate by traditional methods (An 2019).

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Corresponding author: Yong Eui Choi

Division of Forest Sciences, College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea
Tel: +82-33-250-8316, Fax: +82-33-252-8310, E-mail: yechoi@kangwon.ac.kr

Somatic embryogenesis (SE) is a powerful alternative technique for conservation and mass propagation of plants, which has become an important technique in the past few decades (Cheong 2019). In particular, SE is widely used for propagation of important medicinal plants, particularly for rare or nearly extinct plant species. Since SE in *E. senticosus* was first reported by Gui et al. (1991), propagation of *E. senticosus* via SE has been reported by Yu et al. (1997), Choi et al. (1999), Choi et al. (2002a, 2002b), Han et al. (2003), Li et al. (2004, 2005), Jeong et al. (2009). Although SE offers many advantages for rapid large-scale propagation and genetic improvement of plants, long-term maintenance of embryogenic cells may be labor-intensive and susceptible to genic variation and contamination during repetition of *in vitro* culture. Chakrabarty et al. (2003) reported DNA methylation during maintenance and subculture of embryogenic callus in *E. senticosus*. To overcome these problems, we need to attempt to consider a method for long-term storage of embryogenic cells of *E. senticosus*.

Cryopreservation in liquid nitrogen (LN) at ultra-low temperature (-196°C) offers several advantages (Gantait et al. 2016). Cryopreservation technique is available to reduce a risk of a contamination for long-term maintenance of biological materials. In addition, it also provides a continuous source of genetically stable cells and tissues (Seol et al. 2020). One of the most essential agents in successful cryopreservation system is the cryoprotectant (CP). Although glycerol and dimethyl sulfoxide (DMSO) has been open used in cryopreservation of various plant species, both glycerol and DMSO can cause osmotic stress to cells. In particular, DMSO is also known to be toxic for some cells due to its high osmolarity (Arakawa et al. 1990; Panis and Lambardi 2005). For successful cryopreservation, it is important not only to consider the choice of the CP but also to determine optimum concentration of CP.

The usage of the CP allows the successful cryopreservation, but chemical and physiological stresses can cause genetic variation in plant cells. Richards et al. (2004) reported allele frequencies in Texas wild rice (*Zizania texana*) although there were no changes between the genotypic frequencies before and after cryogenic exposure. In yellow gentian (*Gentiana cruciata*), Mikuła et al. (2011) showed genetic uniformity of regenerants obtained from cryopreserved proembryogenic mass. Using DNA-marker analyses (AFLPs and SSR), on the other hand, there were no differences in cork oak (*Quercus suber*) somatic embryos between non-cryopreserved and cryopreserved treatments at the DNA-sequence level (Fernandes et al. 2008).

Somatic embryos of *E. senticosus* were successfully conservated for 36 months at 4°C (Li et al. 2004). Choi and Jeong (2002a) reported that the artificial induction of dormancy by a high sucrose treatment strongly enhanced the conservation of encapsulated artificial seeds and enhanced the resistance of somatic embryos after dehydration treatment. To date, there is yet no report on cryopreservation of embryogenic cells of *E. senticosus* in LN at -196°C. In this paper, we tested the effect of CPs on regrowth of embryogenic cells in *E. senticosus*. We also established a successful regeneration system from cryopreserved embryogenic cells and investigated the genetic variation among the plantlets regenerated from cryopreserved embryogenic cells lines using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis.

Materials and Methods

Plant material and embryogenic callus induction

Using a modified protocol of Choi et al. (2002b), seeds of *E. senticosus* were immersed in 70% ethanol for 1 min and rinsed twice with sterile distilled water. Then, the seeds were disinfected for 1 h in 1% sodium hypochlorite and rinsed twice with sterile distilled water. Seed coats were removed with tweezers and a surgical knife and zygotic embryos were cultured *in vitro* on half-strength MS (Murashige and Skoog 1962) medium containing 10 g L^{-1} sucrose and 3 g L^{-1} Phytagel (Sigma) to induce germination. All germinants were cut into 2-mm segments and placed onto MS medium supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g L^{-1} sucrose and 3 g L^{-1} Phytagel in 100×20 mm plastic petri dishes. The pH was adjusted to 5.7 before autoclaving at 121°C for 20 min and the culture room was maintained at 25°C with a 16-h photoperiod of 24 μmol m^{-2} s^{-1} (cool white fluorescent tubes). After 2 months of culture, embryogenic cells were produced and maintained in MS liquid medium with 4.5 μM 2,4-D, 30 g L^{-1} sucrose. The cell suspension was sub-cultured at two week intervals.

Cryopreservation procedure (Freezing, thawing and recovery)

At the start with the cryopreservation procedure, one embryogenic cell line was used in this experiment with three replications. Embryogenic cells were placed onto two layers of sterile filter paper in 100×20 mm plastic Petri dishes for a few seconds and weighted (FW_1, approximately 0.5 g) under sterile condition. Then, they were suspended in 25 ml of MS liquid medium with 4.5 μM 2,4-D in 50 ml flasks and cultured in the dark on a rotary shaker at 110 rpm for 1 week (Fig. 1a, b). The vitrification procedure for cryopreservation was carried out using a modified protocol described in detail by Ahn et al. (2017). Cultures were pretreated by incubating in liquid MS medium including 0.4 M sorbitol for 1 day. Then, they were inoculated into cooled (4°C) MS medium with 0.4 M sorbitol and six CPs at different concentrations of glycerol and DMSO (5%, 10%, and 20%, respectively) and without CPs. Aliquots of 3 ml of embryogenic suspension from each pretreatment were pipetted into 5 ml CoolCell® Filler Vial (BioCision) and placed into a pre-chilled (4°C) CoolCell® 5 ml LX freezing container (BioCision) (Fig. 1c). The container was moved to an ultra-cold freezer (-70°C) for 24 h. Cryovials were stored in LN at -196°C for 6 months.

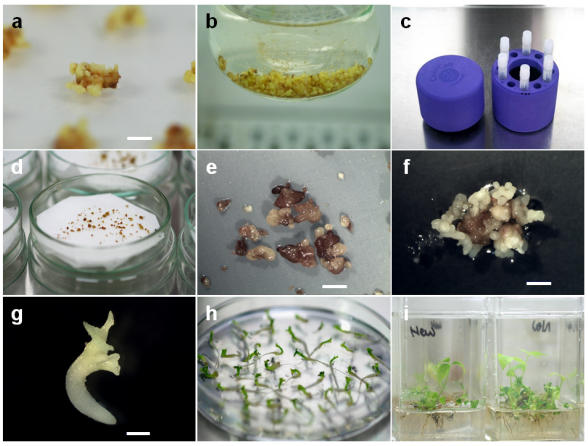

Fig. 1. Re-growth of *E. senticosus* embryogenic cells following cryostorage and plant regeneration via somatic embryogenesis from the cultures of cryopreserved cells of *E. senticosus*. (a) Embryogenic cells of *E. senticosus*, (b) Embryogenic cell suspension in liquid medium, (c) CoolCell 5 ml LX freezing container (BioCision), (d, e) Thawing procedures, (f) Recovered embryogenic cells after from cryopreservation, (g) *E. senticosus* cotyledonary somatic embryo, (h) *E. senticosus* germinants, (i) *E. senticosus* plantlets in polycarbonate Magenta culture vessels. Bar=5 mm (a), 2 mm (d, e, f), 0.1 cm (g).

All cryovials with cryopreserved cells were thawed on the same day. For thawing, the cryovials were transferred to 40°C water for 2-3 minutes. After thawing, immediately vials including embryogenic cells were taken from each vial with a wide mouthed pipette and spread onto a sterile 30-μm nylon mesh (Membrane Solutions), over stacked filter paper discs for approximately 5 minutes (Fig. 1d). Then, the nylon mesh with embryogenic cells was placed onto MS medium gelled with 8 g L^{-1} Plant agar (Duchefa) and incubated in the dark at 24°C (Fig. 1e). Nylon mesh with embryogenic cells was moved to MS medium with 4.5 μM 2,4-D at 2 h, 24 h, and 1 week, to facilitate dilution of residual CPs. Embryogenic cells were transferred directly to MS medium with 4.5 μM 2,4-D (Fig. 1f). The regrowth was followed over 6 weeks. Embryogenic cells were weighted (described above) as the second fresh weight (FW2) and calculated the relative fresh mass gain (%) computed as $(FW_2-FW_1)/FW_1\times100$.

Somatic embryos production and germination

Recovered embryogenic cells were transferred onto semisolid MS medium lacking 2,4-D. For germination experiment, cotyledonary embryos were transferred onto MS medium with 30 g L^{-1} sucrose and 5 μM GA_3 in 10×2 cm Petri dishes. Each Petri dish contained twenty cotyledonary embryos of similar size and stage. After 2 weeks, germinated embryos were counted. Then, the germinated embryos were transferred onto 1/3 strength MS medium with 10 g L^{-1} sucrose, as described by Choi et al. (1999).

DNA extraction and RAPD and ISSR analysis

To identify the effect of CP on genetic variation of *E. senticosus*, RAPD and ISSR analysis were conducted using the University of British Columbia, Canada (UBC) primers (Table 1). Genomic DNA extraction and PCR analysis were conducted using the procedure of Ahn et al. (2011). Briefly, DNA was isolated young leaves from recovered plantlets (Fig. 1i) from non-cryopreserved and cryopreserved embryogenic cells using a Genomic DNA Extraction Kit [Mini] (Real Biotech Corporation). For the RAPD analysis, the PCR amplification was carried out as follows: an initial denaturation step of 7 min at 94°C fol-

Table 1. Primers used and polymorphisms shown by them in RAPD and ISSR analysis of *E. senticosus*

RAPD primer	Sequence (5'-3')	ISSR primer	Sequence (5'-3')
UBC 515	GGG GGC CTC A	UBC 809	AGA GAG AGA GAG AGA GC
UBC 518	ACC GGA CAC T	UBC 811	GAG AGA GAG AGA GAG AC
UBC 534	CAC CCC CTG C	UBC 815	CTC TCT CTC TCT CTC TG
UBC 540	CGG ACC GCG T	UBC 820	GTG TGT GTG TGT GTG TC
UBC 584	GCG GGC AGG A	UBC 824	TCT CTC TCT CTC TCT CG
UBC 594	AGG AGC TGG C	UBC 829	TGT GTG TGT GTG TGT GC

Table 2. Mean relative fresh mass gain (%) for *E. senticosus* embryogenic cells subjected to six cryoprotectant treatments, after 6 weeks of culture

Glycerol			DMSO		
5%	10%	20%	5%	10%	20%
61.3 ± 14.6^{c}	72.5 ± 7.5^{bc}	75.5 ± 13.9^{bc}	115.7 ± 10.5^{ab}	138.2 ± 5.9^{a}	123.1 ± 13.5^{a}

Values are menas and standard error for 3 replications and means followed by the same letter are not significantly different according to the Tukey's test at $p \leq 0.05$.

lowed by 45 cycles consisting of a denaturation step of 1 min at 94°C, an annealing step of 1 min at 37°C, polymerization step of 2 min at 72°C and final extension at 72°C for 10 min. For ISSR analysis, the PCR amplification was carried out as follows: an initial denaturation step of 5 min at 94°C followed by 45 cycles consisting of a denaturation step of 30 s at 94°C, an annealing step of 1 min at 52°C, polymerization step of 1 min at 72°C and final extension at 72°C for 10 min. The RAPD and ISSR reaction products were subjected to electrophoresis on a 1.5% agarose gel in 0.5× TBE (Tris-borate EDTA) buffer and detected by ethidium bromide staining under UV-lights (GelDoc-It^{TS2} Imaging System).

Statistical analysis

All data for mean relative fresh mass gain and the frequency of germination were statistically analysed by analysis of variance (ANOVA) and means were compared using Tukey's multiple range test using IBM SPSS Statistics 23. The significance level was set to $\alpha = 0.05$.

Results and Discussion

The effect of cryoprotectants on regrowth of embryogenic cells and somatic embryo germination

Plant organs and tissues are venerable to freezing injury during freeze drying process. Thus, osmotic pressure, pH, and concentration of CP have to be maintained as elevation can cause cytoinjuries (Bhattacharya 2018). CPs help to maintain proper osmotic pressure in the cells and prevent cell death during thawing and freezing. In general, the correct type and optimal concentration of CP are required for successful cryopreservation. DMSO and glycerol among CPs are most commonly used for the cell survival after freezing and thawing of -196°C.

As shown in Fig. 1, embryogenic cells recovered from cryopreservation were regenerated to plantlets. The fresh mass of embryogenic cells was calculated to test the effect of different CPs on regrowth of *E. senticosus* embryogenic cells. There was significant effect of CPs on the regrowth of embryogenic cells ($F=7.669$, $df=5$, $p=0.0019$). The highest fresh mass gain was achieved when embryogenic cells were frozen with 10% DMSO at 6 weeks of culture (138.2 ± 5.9) while the regrowth of *E. senticosus* embryogenic cells cryopreserved with 5% glycerol showed the lowest fresh mass gain (61.3 ± 14.6) (Table 2). In this experi-

ment, *E. senticosus* embryogenic cells were more susceptible to glycerol than DMSO. According to Al-Bahrany et al. (2012), DMSO appeared to be more appropriate than glycerol for the cryopreservation of date palm (*Phoenix dactylifera*) cell suspension in relation to colony number formation.

We also tested the effect of CPs on embryo germination in *E. senticosus*. The mean number of germination rates was highest in 10% DMSO (72%) and the lowest mean number of germination rates was in 5% glycerol (63%) (Fig 2). Overall, germination rates were not significantly different not only among the different concentrations of CPs but also between DMSO and glycerol ($p=0.534$ and $p=0.846$, respectively). Valladares et al. (2004) reported that the germination rates were similar between non-cryopreserved and cryopreserved embryos in *Q. suber* although they did not test the effect of different concentrations and types of CP. They also showed that development and morphology of plantlets from cryopreserved embryos were not different in comparison with those from non-cryopreserved embryos. In the present study, we show that cryopreservation of embryogenic cells did not affect the somatic embryo formation and their germination into plantlets in spite of different concentrations and types of CP. Therefore, the cryopreservation of *E. senticosus* embryogenic cells may not affect the regeneration capacity via SE.

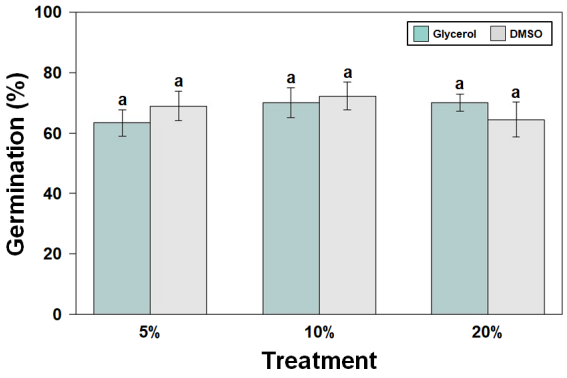

Fig. 2. Effect of cryoprotectants on the frequency embryo germination in *E. senticosus*. Bars represent standard errors and same letters indicate no significant differences at $p \leq 0.05$ by Tukey's test.

Genetic fidelity by RAPD and ISSR markers

Totally, twelve UBC primers for RAPD and ISSR analysis were used to evaluate genetic stability of recovered plantlets derived from both non-cryopreserved and cryopreserved embryogenic cells at DNA level. Six primers were selected for each RAPD and ISSR analysis based on the reproducibility and banding pattern. In RAPD analysis, a total of 22 bands were generated from six RAPD primers and the fragment size ranged from 300 to 1500 bp (Fig. 3a). There was no RAPD fragment pattern variation among regenerants. In the case of ISSR analysis, a total of 16 bands were generated from ISSR primers (Fig. 3b). No polymorphic bands were also detected in ISSR analysis among regenerants. In this experiment, our results showed that no genetic variation was observed in any of all the samples tested. Genetic stability of plant materials in long-term cryostorage is the important issue in terms of preservation of plant germplasm (Benson 2008). Although RAPD and ISSR analysis are still criticized because of reproducibility, both RAPD and ISSR have been widely used to test genet-

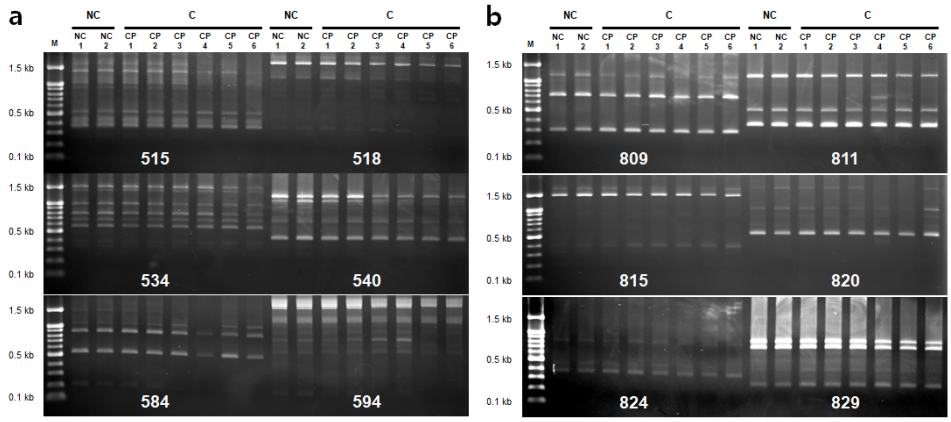

Fig. 3. Profile of PCR products obtained from (a) RAPD and (b) ISSR analysis using the 12 different primers. *Lane M*; 0.1 kb DNA ladder, *Lane 2, 3*; non-cryopreserved cell-lines, *Lane 4-9*; cryopreserved cells with six different cryoprotectants, *Lane 10-11*; non-cryopreserved cell-lines, *Lane 12-17* cryopreserved cells with six different cryoprotectants. NC, non-cryopreserved cells; C, cryopreserved cells; CP, cryoprotectant; M, molecular marker.

ic variation of plants recovered from cryopreservation (Harding 2004). Choudhary et al. (2013) reported that no difference was observed in dormant buds of mulberry before and after cryopreservation of plants using RAPD and ISSR primers. Moreover, Fernandes et al. (2008) reported that there was no significant difference between non-cryopreserved and cryopreserved somatic embryos of *Q. suber* using amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs). Similar result was also seen for apple shoot cultures (Liu et al. 2008). These results proved that the genetic instability in this cryopreservation system was low. Our results also showed that no reproducible variation of the RAPD and ISSR profiles was detected between both non-cryopreserved and cryopreserved embryogenic cell lines in *E. senticosus*.

In conclusion, the optimal condition for cryopreservation was described for embryogenic cells in *E. senticosus*. In addition, no genetic variation in leaves of plantlets regenerated from both non-cryopreserved and cryopreserved embryogenic cells was observed by both RAPD and ISSR analysis. This stable cryopreservation system for *E. senticosus* via SE could not only provide a technical support for establishment of cryo-banking of *E. senticosus* germplasm, but also have obvious advantages over *in vitro* long-term storage of embryogenic cells to overcome genetic variation or mutation during prolonged maintenance of cultures.

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