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Cryopreservation of *Schisandra chinensis* (Turcz.) Baill callus and subsequent plant regeneration

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ABSTRACT. Cryopreservation has been proven significance as a technique for promising the long-term conservation of plant germplasms. This study aimed to establish a cryopreservation protocol for calli of *Schisandra chinensis* (Turcz.) Baill, and to explore the effects of different process parameters on callus viability. Effects of desiccation duration, cryoprotectants and cryopreservation methods, thawing temperature, and post-culture conditions on the viability of cryopreserved calli were assessed. Among different cryoprotectants and freezing procedures, the highest survival was recorded when the water content of callus after 30 min desiccation was 57.3%, were loaded into a cryoprotectant containing 10% ethylene glycol, 8% glucose, and 10% DMSO, and frozen slowly (-1°C/min). Rapid thawing at 40°C for 2 min demonstrated the best recovery of cryopreserved *S. chinensis*

calli. Post-culturing in darkness for one week before transfer to light conditions (under 16 h photoperiod at 36 μmol·m^{-2}·s^{-1}) was beneficial to callus regeneration. Plants regenerated through somatic embryogenesis from cryopreserved calli remained ploidy stable after cryopreservation. The callus cryopreservation procedure established in this study is a promising tool for the conservation of *S. chinensis* resources.

Key words: Resource conservation; Survival; Somatic embryogenesis; Stability

INTRODUCTION

Schisandra chinensis (Turcz.) Baill. is a type of woody, deciduous vine that grows mainly in northeastern Asia (China, Japan, and Korea) and eastern of Russia (Min et al., 2008). The fruits and stems of *S. chinensis* are listed by the Chinese Pharmacopoeia Commission as both food and herbal medicine, and have been widely used to treat various diseases, and as additions to functional foods (Chinese Pharmacopoeia Commission, 2010). According to the results of advanced pharmacological research, lignans, as the bioactive compounds in *S. chinensis*, could prevent conditions such as breast and thyroid cancers, heart disease, and hepatotoxicity (Ip and Ko, 1996; Hancke et al., 1999; Ohsugi et al., 1999; Chang et al., 2005; Li et al., 2005). However, owing to overexploitation, and a lack of organized cultivation, genuine populations have declined rapidly in their natural habitat (Zhang et al., 2007). Thus, development of *S. chinensis* germplasm resources for conservation is of ever-increasing necessity.

Traditionally, field genebanks have been used for the ex situ storage of endangered materials (Engelmann and Engels, 2002). However, there are some drawbacks, such as limits of function and threats to plant security (Withers and Engels, 1990; Engelmann, 1997). These storage resources are subject to pests and diseases, and any other natural disasters, such as flood, weather damage, vandalism, and human error. Besides, they are not beneficial for germplasm exchange, due to the great risks of disease cross-infection between plant species (Lambardi et al., 2001; Engelmann and Engels, 2002). Recently, efficient cryopreservation techniques have been developed and applied to a great number of plant species. To allow vitrification of water inside cells during sample cooling, freezable tissue water was often eliminated by osmotic dehydration during pre-treatment (Engelmann, 2012). For cryopreservation, the callus was usually used, because its cells are relatively undifferentiated (Popova et al. 2009). Although cryopreservation retains the genetic and phenotypic performance of materials, and permits long-term storage of plant tissues (Reed, 2002; Menges and Murray, 2004), it's necessary to evaluate the genetic integrity of plant cells after cryopreservation (Harding, 2004). Flow cytometric analysis of nuclear DNA content indicated genomic instability of some plant species, such as *Taxus* x *media* and *Taxus floridana* (Baebler et al., 2005; Škrlep et al., 2008).

Basic research on the somatic embryogenesis of *S. chinensis* has been performed in our lab, but cryopreservation for tissue culture has not been reported to date. In this study, we are the first to describe the successful cryopreservation and storage of *S. chinensis* calli. To select the most useful cryopreservation method, we investigated the effects of desiccation duration, cryoprotectants and cryopreservation methods, thawing temperature, and post-culture conditions on the viability of cryopreserved calli from *S. chinensis*. Furthermore, relative nuclear DNA content of the regenerated plant was determined.

MATERIAL AND METHODS

Plant material and calli induction

Tender stems of *S. chinensis* were collected from a base at the Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Jilin, China, in July 2014. The explants were washed in running water for 30 min, soaked in 75% (v/v) ethanol for 30 s, sterilized with 0.1% (w/v) $HgCl_2$ solution for 5 min, and then rinsed four to five times with sterile water. The stems were cut into 5 mm pieces and inoculated on MS (Murashige and Skoog) basal medium (Murashige and Skoog, 1962), supplemented with 3.0 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 $mg \cdot L^{-1}$ thidiazuron (TDZ), and 3% (w/v) sucrose for callus induction.

Desiccation procedure

After 2 months' incubation, calli were cut into 2-3 mm squares, and placed on sterile filter paper to remove residual water. Desiccation was initiated by placing calli clumps on open Petri dishes inside a clean bench with a flow of sterile air for 0-60 min. Temperature during desiccation was controlled to 25° ± 1°C, and remained stable among treatments. To find the suitable desiccation time, the level of water loss was determined. Five clumps were sampled and weighed after every 30 min of desiccation. To ensure survival with the residual water content at each stage, desiccated calli were put in 1.8 mL cryotubes (Nunc, Denmark) with 10% ethylene glycol, 8% glucose, and 10% DMSO for 20 min at room temperature, and then transferred to liquid nitrogen (LN). After 24-h cryopreservation, calli were rewarmed in 40°C water and then washed twice with liquid MS containing 3% sucrose. The calli were cultured on solid MS with 3.0 mg/L 2,4-D and 30 g/L sucrose, maintained at 25° ± 1°C in darkness for 1 week, and then moved to light conditions (under 16-h photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Survival rate was determined as the number of new tissues formed around calli clumps after 5-week treatment, and expressed as a percentage.

Cryoprotectants and freezing procedures for callus

Four types of cryoprotectants (S1: plant vitrification solution 2 [PVS2; Sakai et al., 1990]; S2: 10% DMSO and 10% sorbitol; S3: 12.5% DMSO and 0.25% casein hydrolysate [CH]; S4: 10% ethylene glycol, 8% glucose, and 10% DMSO) and three freezing methods were used for cryopreservation. Cryoprotectants were prepared and autoclaved at 121°C for 15 min. Two callus clumps were placed in 1.8 ml cryotubes (Nunc, Denmark) with different cryoprotectants for 20 min at room temperature, and then cryopreserved using different processes. For fast freezing, the cryotubes were directly placed in LN. For step-wise freezing, the cryotubes were first placed in a refrigerator at 4°C for 30 min, and then transferred to -20°C for 30 min, and finally placed in LN. For slow freezing, the cryotubes were put in a Mr. Frosty device (Nalgene), containing isopropyl alcohol as a coolant, and a cooling rate of 1°C/min down to -80°C was reached. Cryotubes with the samples were then placed in LN.

Callus recovery and viability

Rewarming was performed at 4°C in a refrigerator or 25°C (room temperature) or

at 40°C in a water bath. Thawed calli were then rinsed twice with liquid MS containing 3% sucrose. After rewarming, the calli were cultured on MS with 3.0 mg/L 2,4-D and 3% (w/v) sucrose for proliferation, in one of the following four ways: a) cultured under a 16-h photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ without a dark period; b) cultured for 3 days in dark, then under a continuing 16-h photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$; c) cultured for 1 week in darkness, then under a continuing 16 h photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$; d) cultured for 2 weeks in darkness, then under a continuing 16 h photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Somatic embryogenesis and plant regeneration

Calli were subcultured after 2 months, and after successive subculturing twice more, calli were cut into 0.5 x 0.5 cm squares and moved to embryogenic callus induction medium, which consisted of MS with 1.0 mg/L TDZ, 0.2 mg/L Zeatin (Zt) and 3% (w/v) sucrose. Induction of somatic embryos was performed on 1/2 MS. Somatic embryos were cultured on 1/2 MS after 1 month for maturation, and cotyledon embryos were then transferred to 1/2 MS with 1 g/L activated carbon and 3% (w/v) sucrose for germination.

Determination of relative nuclear DNA content

Relative nuclear DNA content was analyzed with a PAS III flow cytometer (Partec, Munster, Germany). Plants regenerated from cryopreserved calli, and plants regenerated from calli without cryopreservation were ground with 300 µL extraction buffer (CyStain UV Precise P, Partec GmbH), and placed at 25°C for 10 min. The nuclear extracts were filtered (30 µL paste, CellTrics, Partec GmbH) into 5-mL plastic tubes, and then 1.2 mL staining buffer (CyStain UV Precise P) was added. After 60 s, the samples were analyzed with the PAS III flow cytometer.

Statistical evaluation

In this study, at least 20 calli clumps were used in each of three replicates. The data variance (ANOVA) was analyzed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA) for Windows. Significantly different means were compared using Duncan's multiple-range test at 5% probability level.

RESULTS

Effect of desiccation on callus survival

After two-week culture, new white or yellowish tissues formed around the callus indicated survival, while unviable calli turned brown after cryopreservation and thawing. With cryopreserved calli, regrowth changed with respect to desiccation duration and water content (Figure 1). Generally, to ensure cell survival after a freezing procedure, water content should be decreased to 20-25% of fresh weight (Bian et al., 2002). In this study, callus water content decreased dramatically after 20-min desiccation (Figure 1). The viability of dehydrated callus was 38.33% at the initial stage of desiccation (0 min); it increased to 52.72% as the water content declined from 92.3 to 74.2% after 20-min desiccation, and the highest survival

(68.89%) was achieved with 57.3% water content after 30-min desiccation. The viability then fell sharply to 40%, at the water content of 40.7%, after 40-min desiccation, and the callus was not able to revive with a water content lower than 17% (60 min desiccation). In the first 30 min, the viability of cryopreserved cells increased as the water content of the callus decreased, which suggested that a certain level of water loss could improve callus viability after cryopreservation. In contrast, no survival was found when the water content was too low. For most plant cells and organ cultures, damage during freezing is associated with intracellular ice formation in highly vacuolated cells, therefore, survival of cryopreserved organs could be rinsed by dropping the water content in cells (Popova et al., 2009). The water content of plant organs for optimal survival varies among plant species because of their differences in dehydration tolerance (Hitmi et al., 1999). Popova et al. (2009) reported that the highest post-cryopreservation survival rate (23%) was obtained when calli were desiccated for 150 min. Paulet et al. (1993) found that survival was similar for dehydration either by air drying or with silica gel, only if the optimum water content was achieved. We completely agree with this view, which can also be applied to calli during cryopreservation, as our study recorded the highest viability (68.89%) with 57.3% water content after 30 min desiccation. In this study we favored air drying, since this method saves steps and can reduce pollution caused by additional components.

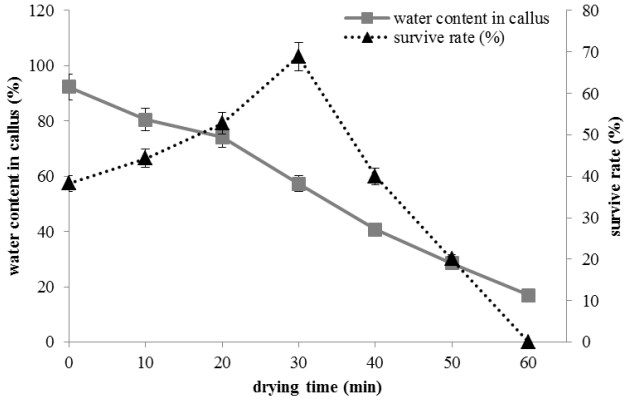

Figure 1. Changes in water content and viability of *Schisandra chinensis* calli after different desiccation durations.

Effect of cryoprotectants and cryopreservation methods on callus viability

In the case of vitrification, suitable cryoprotectants and cryopreservation method were very effective in enhancing callus survival. Dipping into S4 before slow freezing was conducive to survival (Table 1). In contrast, the survival rates of calli in S2 and S3 were much lower. With all cryoprotectants tested, slow freezing achieved the best survival, which was the most efficient method for callus survival. In contrast, step-wise freezing, including two cooling steps before placed the cryotubes into -196°C LN, produced relatively low survival rates, ranging from 5.26 to 40%. Our research highlighted the significance of precisely selecting cryoprotectants and freezing methods to optimize survival of *S. chinensis* calli after freezing. It has been shown previously that overexposure of cells to the vitrification solution could bring chemical toxicity (Rall, 1987). Mixed cryoprotectants are widely used in cryopreservation, and are able to neutralize cytotoxicity by decreasing the total concentration of cryoprotectants

(Withers, 1985). The suitable time of exposure to cryoprotectants varies with plant species, and depends on the temperature during the exposure process (Niino et al., 1992; Nishizawa et al., 1993). Slow freezing allows a stepwise reduction to achieve low temperatures, and prevents a cooling-shock. Pérez et al. (1997) used embryogenic cells of *Citrus* spp, and found that dipping into liquid medium with 10% (v/v) DMSO, freezing by slow cooling, and then quick warming was beneficial to recovering viability. However, fast freezing by direct dipping into LN is widely used because of its simplicity (Grout, 2007). Shoot tips of *Limonium serotinum* treated with vitrification solution containing 30.0% glycerol, 20.0% DMSO, 20.0% ethylene glycol, and 15.0% sucrose (w/v), or 40.0% glycerol and 40.0% sucrose, achieved superior recovery, which reached 76% after cryopreservation by fast freezing (Barraco et al., 2011). Ben-Amar et al. (2013) obtained the highest recovery of embryogenic grapevine cells with a loading solution containing 0.4 M sucrose and 2 M glycerol, and rapid freezing. Among four cryoprotectants and three freezing methods in our study, the highest *S. chinensis* callus survival of 70.0% was achieved when loaded in 10% ethylene glycol, 8% glucose, and 10% DMSO wirh the slow freezing method.

Table 1. Effect of cryoprotectants and cryopreservation method on viability of *Schisandra chinensis* calli.

Cryoprotectants	Cryopreservation method	Survival (%)
S1 (PVS2)	Slow freezing	25.56^{f}
	Step-wise freezing	10.00^{i}
	Fast freezing	37.16^{e}
S2 (10% DMSO+10% sorbitol)	Slow freezing	23.81^{g}
	Step-wise freezing	9.52^{i}
	Fast freezing	38.89^{d}
S3 (12.5% DMSO+0.25% CH)	Slow freezing	15.00^{h}
	Step-wise freezing	5.26^{j}
	Fast freezing	26.32^{f}
S4 (10% ethylene glycol+8% glucose+10% DMSO)	Slow freezing	70.48^{a}
	Step-wise freezing	40.00^{c}
	Fast freezing	48.18^{b}

Different letters within a column indicate a significant difference ($p < 0.05$) according to Duncan's multiple range test.

Effect of thawing temperature on callus viability

To further increase recovery after cryopreservation, the effect of rewarming on regeneration was examined (Figure 2A). Thawing methods markedly affected the recovery of cryopreserved calli. The regeneration rate of calli rapidly thawed in a water bath at 40°C for 2 min was significantly higher than that of calli maintained at 25°C or at 4°C in a refrigerator. Thawing at 4°C for 2 h and 25°C for 10 min gave 5 and 22.22% survival rates, respectively, compared with 70% survival for calli thawed at 40°C for 2 min. In the reverse of the freezing process, transfer from a vitreous to a crystalline solution during thawing could cause a degree of damage to cells, and threaten the survival of meristems. Vitrified material from different species has its own optimal thaw temperature, such as 25°C for apple, pear, cherry, and sweet cherry (Niino et al., 1992), 30°C for tea (Kuranuki and Sakai, 1995), 35°C for mint (Towill, 1990), 40°C for white poplar (Lambardi et al., 2001), and 45°C for gooseberry and currant (Reed, 1995). The optimum thaw temperature for *S. chinensis* calli in our study was 40°C, and this led to the production of new white or yellowish tissues. Therefore, rapidly thawing at 40°C for 2 min proved to be the best method for the recovery of *S. chinensis* calli.

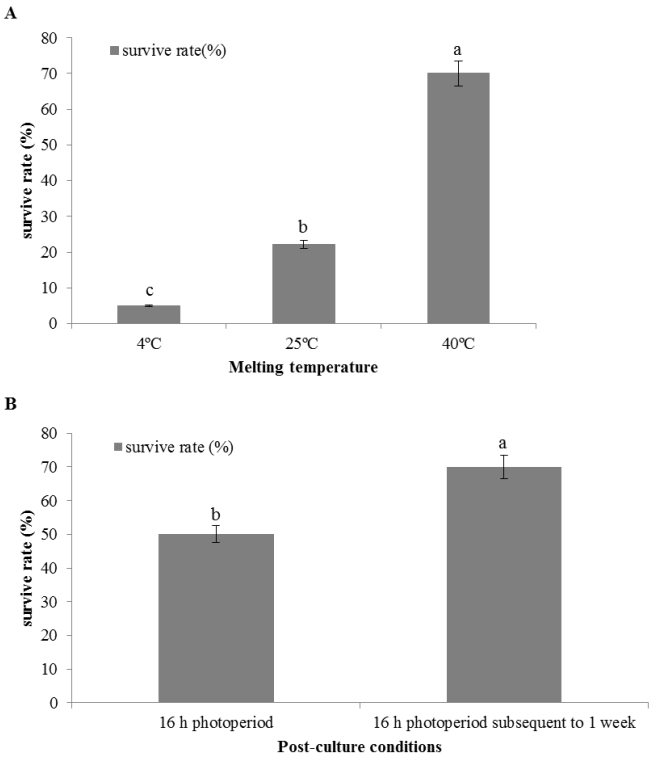

Figure 2. Effect of thawing temperature (**A**) and post-culture conditions (**B**) on *Schisandra chinensis* callus viability

Effect of post-culture conditions on callus viability

We studied the effect of dark culture after freezing on viability, and two different post-culture methods were assessed (Figure 2B). The best survival (70%) was obtained by post-culturing in dark for 1 week, and then moving to light conditions (under 16-h photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$). However, directly post-culturing under a 16-h photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ caused a considerable reduction in callus survival, at only 50%. Post-culture also influenced regeneration of cryopreserved *S. chinensis* calli. Especially, darkness culture for a short time after thawing enhanced survival. An appropriate post-culture condition is necessary for high survival and fast regrowth of the cryopreserved callus. Benson et al. (1989) reported that recovery could eventually occur in darkness, since dark incubation contributed to the avoidance of photooxidation phenomena, which could be harmful to the tissue. In this study, by testing the effects of two different post-culture conditions on the recovery of cryopreserved *S. chinensis* calli, we found that the regeneration rate was markedly decreased without 1 week of dark culture. However, post-culturing in dark for 1 week and then moving to light conditions greatly enhanced callus regeneration. Similar results have been found on protocorm-like bodies of *D. candidum* (Hong and Yin, 2009) and *D. opposita* (Li et al., 2009) after freezing, and this may have been due to damage repair of materials when cultured in darkness. Therefore, we

confirmed that a period of dark culture, prior to light culture, is essential for the recovery of callus following cryopreservation.

Somatic embryos induction and plantlet regeneration

Embryogenic callus was formed from the callus samples after 4-week culture on MS with 1.0 mg/L TDZ and 0.2 mg/L Zt (Figure 3A). Embryogenic calli were then moved to medium for somatic embryo induction. Global somatic embryos were formed after 1 month on 1/2 MS, and then developed into heart embryos, torpedo embryos, and cotyledon embryos in variable numbers (Figure 3B). Embryos were germinated (Figure 3C) and developed into whole plantlets (Figure 3D) within 2 months of culture.

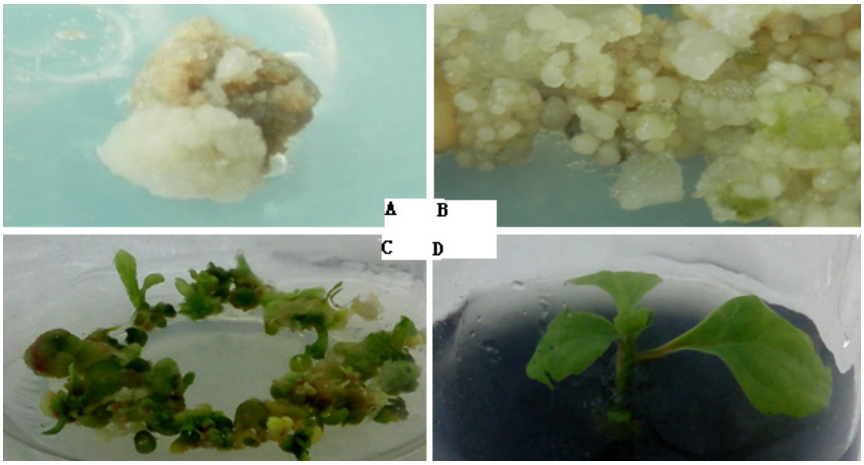

Figure 3. *Schisandra chinensis* plant regeneration. **A.** Embryogenic callus. **B.** Somatic embryos during different stages. **C.** Geminated embryos. **D.** Regenerated plantlet. (All bars = 1 cm).

Analysis of relative nuclear DNA content

Relative nuclear DNA content of *S. chinensis* was detected by flow cytometry. The results indicated that the relative nuclear DNA content was not altered after cryopreservation (Figure 4). Plants regenerated through freezing can be evaluated at morphological, biochemical, chromosomal, and molecular levels (reviewed by Harding, 2004). In our study, all tested plants from both the untreated and the cryopreserved calli showed the same one peak of relative DNA content, which indicated the stability in genome size after cryopreservation. Recently, DNA flow cytometry analysis has been recognized as a convenient technique, due to its simplicity and reliability (Mikula et al., 2008). Similar studies showed that cryopreservation did not change the nuclear DNA content of proembryogenic cells and regenerated plants of *G. tibetica* and *G. cruciata* (Mikula et al., 2008). Consistence of relative DNA content had also been reported in *Ginkgo biloba* calli regenerated after cryopreservation (Popova et al., 2009).

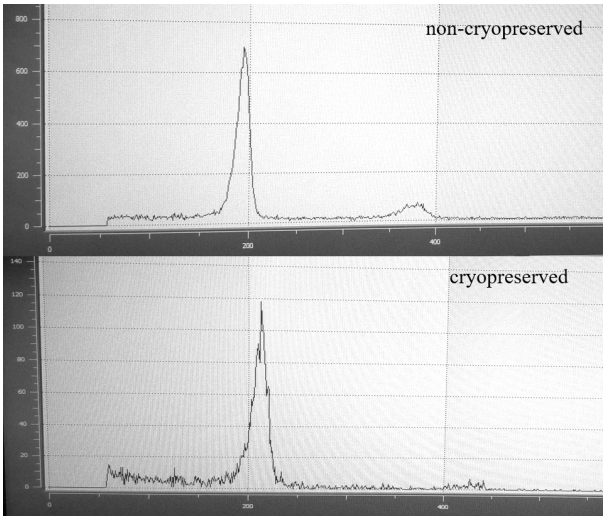

Figure 4. Relative DNA content of nuclei from plantlets regenerated from non-cryopreserved and cryopreserved calli of *Schisandra chinensis*.

CONCLUSIONS

In conclusion, a simple protocol for the cryopreservation of *S. chinensis* calli is described for the first time, focusing mainly on the most important aspects of this procedure: the survival of calli, and subsequent somatic embryogenesis and plant regeneration from cryopreserved calli. We also noted that the regenerated plants remained ploidy-stable after cryopreservation. However, further studies are necessary, with an emphasis on confirming the genetic stability of regenerated plantlets by biochemical and molecular analyses, to make sure that the procedure is applicable to *S. chinensis* germplasm conservations.

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