

Cryopreservation of *Panax ginseng* Adventitious Roots

Suk Young Oh · Chun Hua Wu · Elena Popova ·
Eun Joo Hahn · Kee Yoeup Paek

Received: 10 April 2009 / Revised: 19 May 2009 / Accepted: 19 May 2009 / Published online: 16 June 2009
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Abstract We tested desiccation and/or vitrification procedures to cryopreserve the adventitious roots of *Panax ginseng*, the source of commercially produced ginsenosides. When only desiccation was applied, the post-freeze survival of 3- to 4-mm root tips was <14% regardless of the composition of the preculture medium or the explant origin. Callus formation was frequently observed after cryopreservation. In contrast, 90% survival and 32.5% root formation efficiency were achieved after cryopreservation when a vitrification protocol was followed. Adventitious root cultures in flasks and bioreactors were reestablished from root tips cryopreserved by vitrification. A prolonged lag-phase and lower biomass production were recorded in post-freeze-regenerated cultures compared with control roots that were subcultured four times in flasks. However, biomass accumulations did not differ between control and regenerated roots at the end of the sixth subculturing period. After 40 days of culture in bioreactors, a mean value of 12.5 g dw L⁻¹ was recorded for post-freeze-regenerated cultures versus 9.1 g dw L⁻¹ for the control roots. Production of triol and diol ginsenosides in our bioreactor cultures also was enhanced after cryopreservation, by 41.0%

and 89.8%, respectively. These results suggest that the vitrification method is successful for cryopreservation of *P. ginseng* adventitious roots.

Keywords Adventitious roots · Bioreactor cultures · Cryopreservation · Ginsenosides · *Panax ginseng* · Vitrification

Panax ginseng C.A. Meyer is an important Asian medicinal plant with numerous pharmacological activities, including anticancer, anti-aging, and antioxidant effects (Choi et al. 2007; Chen et al. 2008). Its major bioactive ingredients are ginseng saponins (ginsenosides). Based on their structural differences, these ginsenosides can be classified into two groups: 20(S)-protopanaxatriols and 20(S)-protopanaxadiols (Chen et al. 2008). Large-scale bioreactor cultures of *P. ginseng* adventitious roots are a safe and effective alternative to field-grown plants or hairy root cultures (Kim et al. 2007) and allow for the commercial production of ginsenosides (Hahn et al. 2003). This technology requires that the reserve stock of adventitious roots be maintained in 20-L bioreactors and subcultured manually. Such collections, however, are cost- and labor-intensive, and they encumber risks of contamination and operator mistakes. Therefore, cryopreservation is the most reliable and economically effective method for long-term maintenance of clonally propagated plant germ plasm (Sakai and Engelmann 2007). This approach, then, necessitates an efficient cryopreservation protocol that enables one to regenerate adventitious root cultures and also retain their growth and biosynthetic characteristics.

To date, cryopreservation has been successful for the hairy root cultures of numerous medicinal plants (Benson and Hamill 1991; Teoh et al. 1996; Hirata et al. 1998, 2002; Xue et al. 2008; Lambert et al. 2009). However, only a few

S. Y. Oh · C. H. Wu · E. Popova · E. J. Hahn · K. Y. Paek (✉)
Research Center for the Development of Advanced Horticultural
Technology, Chungbuk National University,
Cheongju 361-763, South Korea
e-mail: paekky@cbnu.ac.kr

Present Address:

C. H. Wu
Dalian Agricultural Science Research Institute,
Dalian 116036, China

Present Address:

E. Popova
National Agrobiodiversity Center,
National Academy of Agricultural Science, RDA,
Suwon 441-707, South Korea

reports are available for its use with adventitious roots (Jung et al. 2001). A vitrification method based on the consecutive dehydration of plant material with highly concentrated cryoprotector solutions has proven effective for the cryopreservation of *Hyoscyamus niger* and *Atropa belladonna* root cultures (Jung et al. 2001; Touno et al. 2006). Nevertheless, that method has led to poor results from *Maesa lanceolata* hairy roots due to the toxicity of the cryoprotector solution (Lambert and Geelen 2007). In *P. ginseng* hairy roots, a 54% post-freeze recovery rate has been achieved with a vitrification protocol, but lateral root formation has been suppressed after cryopreservation (Yoshimatsu et al. 1996). In contrast to transformed roots, adventitious roots require auxins for their induction, proliferation, and maintenance (Kim et al. 2007). Thus, the possibility of applying a vitrification protocol to *P. ginseng* adventitious roots should be thoroughly investigated. A desiccation method is routinely utilized for embryogenic cultures. Its advantages are its simplicity and the omission of a toxic cryoprotector treatment (Bomal and Tremblay 2000; Danso and Ford-Lloyd 2004). However, this method has not previously been tested with cultured roots. Preliminary culturing of plant material on a medium supplemented with high sucrose concentrations to induce desiccation and chilling tolerance is considered essential to the success of this method (Grenier-de March et al. 2005).

Here, we examined desiccation and vitrification methods to cryopreserve the adventitious roots of *P. ginseng*. The growth and biosynthetic characteristics of regenerated root cultures were assayed and compared with those of untreated roots to monitor the possible effects of cryopreservation.

Materials and Methods

Adventitious Root Culture in Bioreactors

Adventitious roots of *P. ginseng* C.A. Meyer were induced and proliferated as described by Kim et al. (2007). Cultures were maintained in 5-L bioreactors containing 4 L of a modified MS liquid medium supplemented with 5 mg L⁻¹ of IBA and 50 g L⁻¹ of sucrose (basal medium (BM)) at 22±1°C under darkness (Kim et al. 2007). Air was supplied at 0.1 vvm. Subculturing was performed every 40 days by transferring 5 g L⁻¹ fresh roots to bioreactors with fresh BM.

Desiccation Procedure

Two types of explants were used in our desiccation experiments: (1) root tips (3 to 4 mm long) were excised and incubated for 9 days on a Gelrite-solidified basal

medium then transferred to a preculture medium (see below) or (2) 8- to 10-mm roots were excised and incubated for 9 days on a Gelrite-solidified basal medium before 3- to 4-mm root tips were excised and immediately transferred to the preculture medium. Preculturing was done for 7 or 16 days under darkness on a modified MS medium (Kim et al. 2007) that was supplemented with 10% (w/v) sucrose and 2.2 g L⁻¹ Gelrite without growth regulators. Precultured root tips were placed on 5×25-mm strips of sterile filter paper (Whatman No. 4; Whatman Ltd., England) in open 90-mm Petri dishes. Desiccation was performed under the air current from a laminar airflow clean bench for 0, 20, 40, or 60 min.

To estimate survival, root tips desiccated for certain periods were transferred to a basal medium and kept at 21±1°C in the dark. Survival was defined by the number of root tips that showed callus induction or elongation 35 days after treatment. Three replications were performed, with 10 to 15 root tips per replication.

Vitrification Procedure

Root tips (3 to 4 mm long) were excised from our bioreactor-cultured adventitious roots after a routine 40-day subculture period. They were incubated for 12 days in 100-ml flasks containing 20 ml of liquid BM at 22±1°C in the dark (rotated at 90 rpm). Afterward, 12 to 15 root tips were suspended for 15 min in 2 ml of loading solution (2 M glycerol and 0.4 M sucrose; Nishizawa et al. 1993) in 2-ml cryotubes (Nalgene, USA). They were then placed for various periods into 2 ml of modified Plant Vitrification Solution 2 (PVS2, Sakai et al. 1990) containing 30% (w/v) glycerol, 15% PEG₆₀₀₀, 15% dimethyl sulfoxide, and 0.4 M sucrose. One milliliter of the PVS2 was then discarded, and the cryotubes were sealed and plunged directly into liquid nitrogen. After at least 1 h, the samples were rewarmed rapidly in a water bath at 41°C for 60 s. The root tips were retrieved from their cryotubes and unloaded with 2 ml of liquid BM containing 1.2 M sucrose without growth regulators for 15 min. For reculturing, the root tips were transferred to Gelrite-solidified BM and incubated at 21±1°C under darkness. Survival (i.e., elongation with or without root formation), root formation, and the number of lateral roots per explant were recorded after 35 days and expressed as percentages. Three replications were performed, with 12 to 15 root tips per replication.

Reestablishment of Flask and Bioreactor Cultures

New adventitious roots that had developed from samples cryopreserved by vitrification were further proliferated as shown in Fig. 1. Roots were subcultured twice on

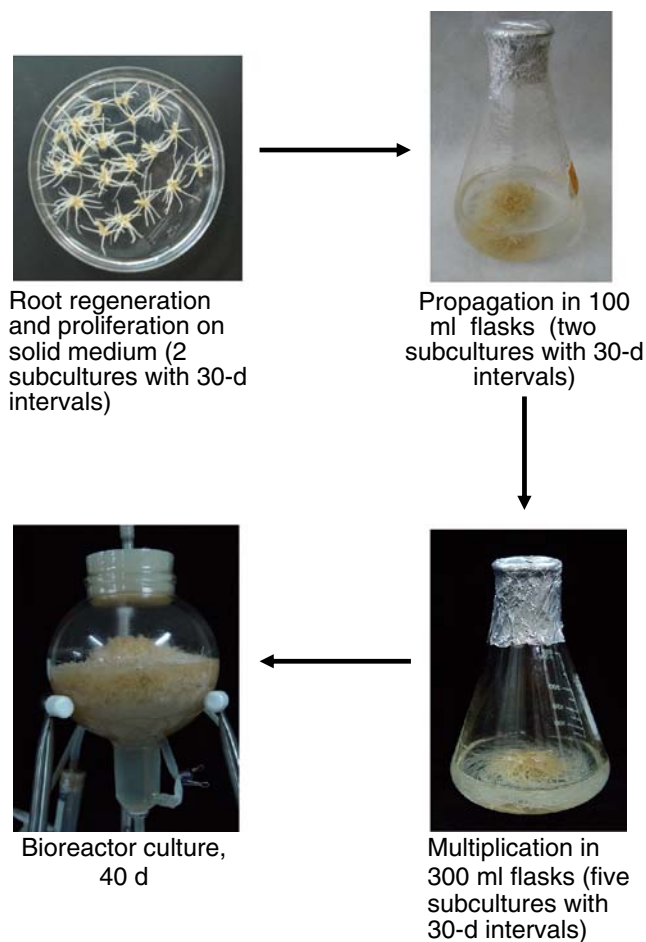


Fig. 1 Reestablishment of *Panax ginseng* adventitious root cultures from cryo-preserved root tips. All cultures were performed in modified MS medium (Kim et al. 2007) supplemented with 5 mg IBA L⁻¹ and 50 g sucrose L⁻¹ at 22±1°C in darkness

Gelrite-solidified BM at 30-day intervals, followed by propagation in 100- and 300-ml flasks. The bioreactors were inoculated from 300-ml flasks at the end of the fifth subculture period. Inoculum sizes were 5 g L⁻¹ fresh weight (fw). Both flask and bioreactor cultures were performed in Basal media at 21±1°C in the dark. Flasks were rotated at 90 rpm, and air in the bioreactors was supplied at 0.1 vvm.

Determination of Growth Curve and Root Weights

In flask cultures, growth profiles were recorded twice at the fourth and sixth subculture intervals. Three to four flasks were harvested every 3 to 5 days for each point on our growth curve. Biomass production in the bioreactors was determined at day 40 after inoculation. Three bioreactors were harvested for each treatment. Roots were washed with running tap water for 2 min followed by distilled water. After filtering under vacuum, root fresh weights were recorded, and their dry weights were determined after drying at 60°C for 3 days.

Extraction and Analysis of Ginsenosides

Extraction and analysis of ginsenosides in bioreactor-cultured adventitious roots were performed as described by Kim et al. (2007), using an HPLC system equipped with an Altec Platinum C18 column (33×7 mm; particle size 1.5 μm). Ginsenosides were eluted with water/acetonitrile (3:1, v/v) for 10 min, then at 63:37 for 25 min, with a flow rate of 1.2 ml min⁻¹. Ginsenosides were detected at 203 nm. The ginsenoside standards were purchased from Chromadex (USA). Triol fractions were calculated as the sum of ginsenosides Rg1, Re, Rf, and Rh1. The diol fraction was equal to the sum of ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Rg3, and Rh2. The total ginsenoside content was calculated as the sum of those

Table 1 Survival of *Panax ginseng* adventitious root tips after desiccation (-LN) and cryopreservation (+LN), as affected by explant type, pre-culture medium, and duration of desiccation (3 replications)

Pre-culture	Explant type I				Explant type II	
	10% sucrose, 7d		10% sucrose, 16d		10% sucrose, 7d	
	-LN	+LN	-LN	+LN	-LN	+LN
Desiccation (min)						
0	75/76 (99%)*	2/59 (3%)	40/40 (100%)	5/36 (14%)	60/60 (100%)	2/39 (5%)
20	49/69 (71%)	7/75 (9%)	32/35 (91%)	1/38 (3%)	55/58 (95%)	5/43 (12%)
40	10/56 (18%)	0/24 (0%)	21/21 (100%)	0/45 (0%)	2/39 (5%)	n/t**
60	0/47 (0%)	0/33 (0%)	8/12 (67%)	0/34 (0%)	0/41 (0%)	n/t

Note: Explant type I – root tips were excised from bioreactor-cultured roots and incubated on a Gelrite-solidified basal medium for 9 d before pre-culture. Explant type II – root tips were excised from 8- to 10-mm adventitious roots incubated on a basal medium for 9 d, then immediately transferred to the pre-culture medium.

* Data are shown as no. of surviving root tips / no. of root tips taken to treatment (%)

** n/t – not tested

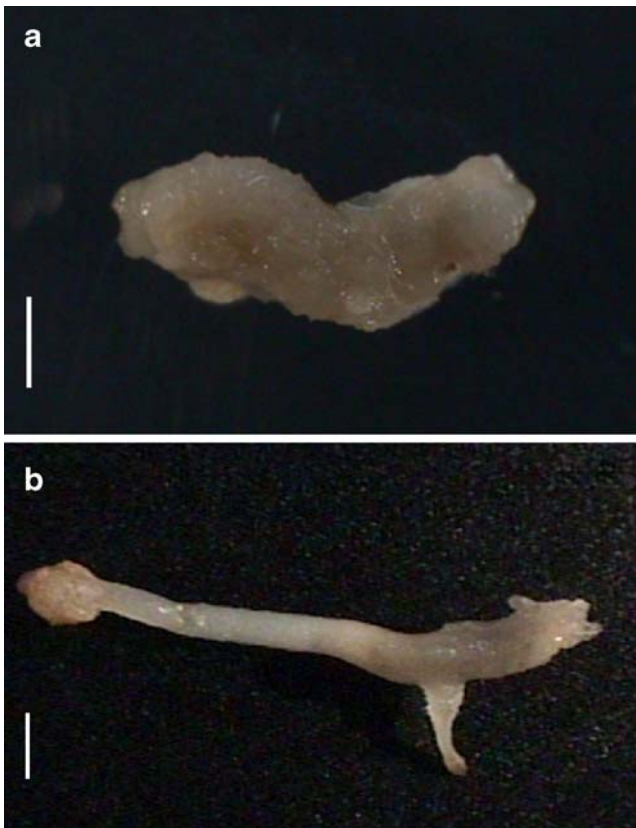


Fig. 2 Callus (A) and elongated root tip with new adventitious root (B) after cryopreservation via desiccation. Tips (3–4 mm long) were pre-cultured on basal medium with 10% (w/v) sucrose for 16 d, then desiccated under flow of sterile air for 60 min. Photos were taken 40 d after treatment. Bar=2 mm

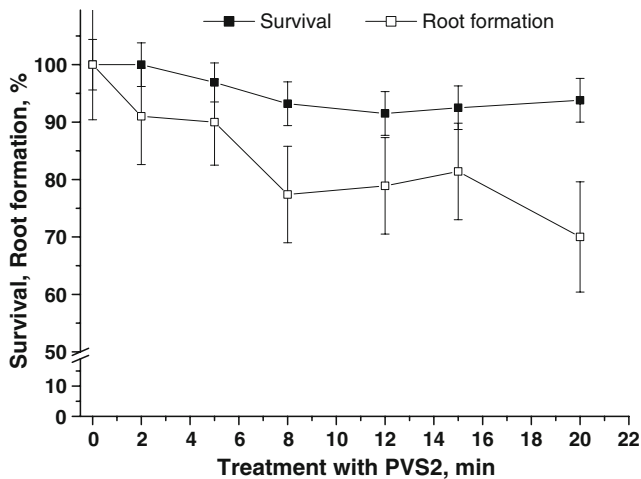


Fig. 3 Toxicity assay of Plant Vitrification Solution 2 (PVS2) for adventitious roots. Tips (3–4 mm long) were incubated in basal medium for 12 d, then treated with loading solution (LS) for 15 min followed by PVS2 for 0–20 min. Tips were unloaded with basal medium containing 1.2 M sucrose for 15 min, and regenerated on Gelrite-solidified BM in darkness for 35 d

triol and diol ginsenoside fractions. Three bioreactors were harvested for each set of experimental conditions, and analyses were performed for triplicate extracts.

Statistical Analysis

All data were analyzed by SAS Version 9.1 (SAS, Raleigh, NC). The significance of differences between treatments was assessed using a Duncan’s multiple range test at $P \leq 0.05$.

Results and Discussion

In all experiments, the first sign of survival was evidenced by swelling of the root tips followed by callus formation or root elongation. New lateral roots developed 10 to 12 days after treatment but only from elongated root tips.

Effect of Cryopreservation Procedure

To avoid lethal intracellular ice formation, freezable water should be removed from samples prior to cryopreservation (Sakai 2000). The simplest desiccation procedure involves sufficient desiccation of plant material in the air current of a laminar airflow cabinet followed by rapid freezing (Engelmann 2004). In our study, root tips of *P. ginseng* could not survive desiccation of >40 min without first being pre-cultured on a sucrose-enriched medium (data not shown). Incubation of root tips in the presence of 10% (w/v) sucrose for 7 or 16 d improved survival to 18 and 100%, respectively, after 40 min of desiccation (Table 1). That desiccation period could be extended to 60 min, with 67% survival, if root tips were

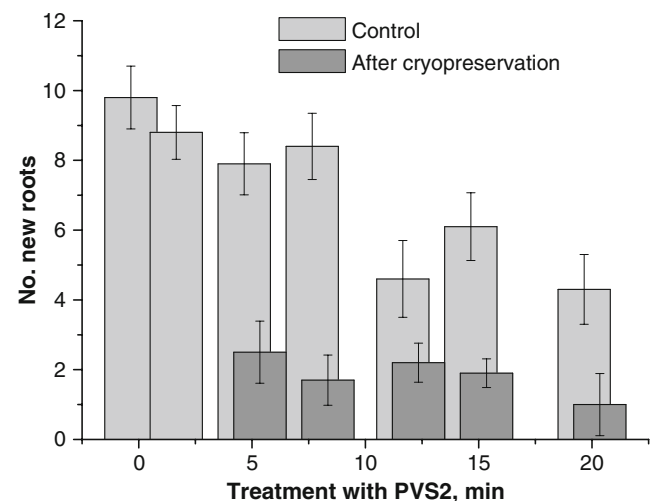


Fig. 4 Effect of cryopreservation on number of new roots developed from *P. ginseng* root tips that had been pre-treated as described for Fig. 3

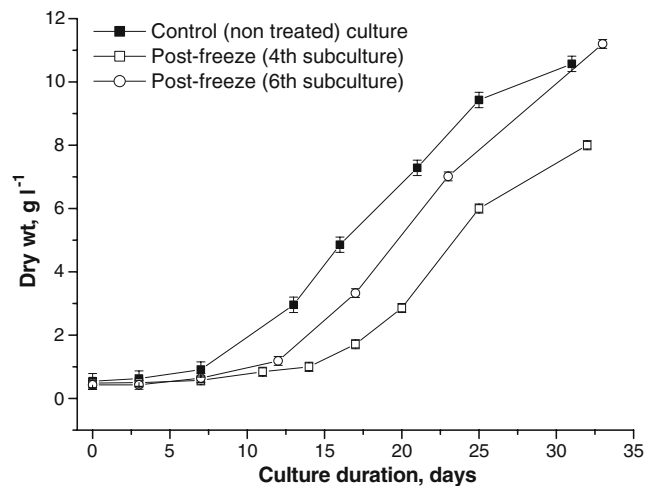


Fig. 5 Growth profiles of control and post-freeze-regenerated adventitious root cultures in 300-ml flasks as determined at 4th and 6th subcultures

first pre-cultured on a sucrose-enriched medium for 16 d. Incubation of excised root tips on the basal medium for 9 d before sucrose preculture (Explant type I) was slightly beneficial when desiccation was performed for 40 min. If desiccation was followed by cryopreservation, survival did not exceed 14% for any of our treatments (Table 1). Moreover, survival of both desiccated and cryo-preserved samples was mostly via callus formation (Fig. 2a). Lateral roots developed occasionally but exhibited slow growth and low capacity to produce new roots (Fig. 2b). Another important disadvantage of this method was that the exact water content of desiccated root tips could not be measured because of their tiny size. Thus it was difficult to determine the proper desiccation time for the explants.

In vitrification method, freezable water is osmotically removed from samples by incubating them in highly concentrated cryoprotector solutions (Sakai and Engelmann 2007). Plant Vitrification Solution 2 (PVS2), designed by Sakai et al. (1990), has been successfully applied to diverse plant materials, including shoot tips, cell suspensions, and embryogenic tissues (Sakai and Engelmann 2007). This solution has been found promising when used in the

cryopreservation of *H. niger* and *A. belladonna* root cultures (Jung et al. 2001; Touno et al. 2006). However, explants usually can be exposed to PVS2 for only a very short time because of the toxicity and severe osmotic stress provoked by its high cryoprotectant concentration. Therefore, we investigated its negative effect on *P. ginseng* root tips before performing cryopreservation. Based on results presented in Fig. 3, we assumed that root formation was more susceptible to PVS2 than was survival. Treatment for more than 15 min was detrimental to lateral root development. However, in contrast to the desiccation protocol, callus formation was sometimes observed after PVS2 exposure and cryopreservation regardless of the duration of treatment. We recorded a maximum survival rate of 90% and a root formation efficiency of 32.5% when root tips were cryopreserved after incubation with PVS2 for 15 min (data not shown). Thus, we demonstrated that *Panax* adventitious roots are more sensitive to cryopreservation than are hairy roots, which have 60% recovery after being cryopreserved by vitrification method (Yoshimatsu et al. 1996). The number of new roots that developed after cryopreservation was significantly lower ($P < 0.05$) compared with tissues that had been PVS2-treated but not cryopreserved, and the length of exposure to the cryoprotector solution had no apparent effect (Fig. 4). Yoshimatsu et al. (1996) also have found that cryopreservation of *P. ginseng* hairy root tips significantly suppresses the formation of lateral roots. However, we observed that the amount of newly regenerated roots was sufficient for their further proliferation and the reestablishment of flask and bioreactor cultures (see Fig. 1).

Post-Freeze Characteristics of Adventitious Root Cultures

Growth profiles of post-freeze-regenerated root cultures were recorded twice, at the fourth and sixth subcultures in 300-ml flasks. These were compared with a standard growth curve for the untreated roots (Fig. 5). The long lag phase in those two subcultures could have been a direct reflection of freezing injury (Kantha and Engelmann 1994). Lower biomass accumulations in the regenerated

Table 2 Biomass and ginsenoside production in untreated (Control) and post-freeze-regenerated *Panax ginseng* adventitious roots, after 40 d of culture in 3-L bioreactors

Treatment	Biomass accumulation			Ginsenoside production (mg g ⁻¹ dry weight)		
	Dry weight, g l ⁻¹	% dry weight	Growth ratio*	Protopanaxa-diols	Protopanaxa-triols	Total ginsenosides
Control	9.1±0.1**	7.8±0.2	2.3±0.1	0.98±0.12	1.00±0.02	3.27±0.11
After cryopreservation	12.5±0.1	8.2±0.2	3.4±0.1	1.86±0.12	1.41±0.02	1.98±0.11

* Growth ratio was calculated as [harvested dry weight (g) – inoculated dry weight (g)] / inoculated dry weight (g)

** Values are means and SE for 3 replications

culture (compared with the control) were recorded at the fourth flask subculture. However, at the sixth subculture, the final amount of biomass produced by the regenerated culture was equal to that by the untreated control. This culture, therefore, retained its growth capability after cryopreservation as confirmed by root dry weight measurements in the bioreactors. At day 40 of bioreactor-culturing, the dw mean root was 12.5 g L⁻¹ for tissues regenerated after cryopreservation compared with 9.1 g L⁻¹ for the control roots (Table 2). A similar enhancement of growth and biomass productivity has been detected in *P. ginseng* cell cultures after cryopreservation (Joshi and Teng 2000), and an increase in fresh weight following cryopreservation has also been reported for *H. niger* adventitious roots (Jung et al. 2001). Finally, no differences in growth rates have been found for either *Astragalus membranaceus* or *Gentiana macrophylla* hairy roots after cryopreservation when compared with their noncryopreserved counterparts (Xue et al. 2008).

Contents of both diol and triol ginsenoside groups increased after cryopreservation (Table 2), with the total ginsenoside content (i.e., the sum of triol and diol fractions) rising to 3.27 versus 1.98 mg g⁻¹ dw for the control culture. To our knowledge, this is the first report on post-freeze enhancement of metabolite production in root cultures. *P. ginseng* hairy roots regenerated after cryopreservation produce five major ginsenosides at a basal level (Yoshimatsu et al. 1996). Production of betacyanin, betaxanthin, and alkaloids in *Beta vulgaris* and *Nicotiana rustica* hairy roots also is retained after cryopreservation (Benson and Hamill 1991). Furthermore, the content of tropane alkaloids in post-freeze-recovered adventitious roots of *H. niger* is comparable to those in the control roots (Jung et al. 2001), and vincamine content remains stable after *Vinca minor* hairy roots are cryopreserved (Hirata et al. 2002). It is possible that cryopreservation induces a stress response in *P. ginseng* adventitious roots which, in turn, stimulates the production of secondary metabolites (Kim et al. 2007; Wu et al. 2007).

Our results suggest that the desiccation method is inappropriate for the cryopreservation of *P. ginseng* adventitious roots because of their extreme sensitivity to such treatment. In contrast, root tips can be successfully cryopreserved through vitrification. Here, adventitious root cultures after cryopreservation resumed their usual growth and biosynthetic characteristics at the same or greater level than the untreated control. Further studies will be conducted to improve post-freeze root formation and to achieve rapid post-freeze regeneration of cultures.

Acknowledgements This work was supported by a Korea Science and Engineering Foundation grant funded by the Korean Government (MOST; R01-2007-000-10543-0).

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