Cryopreservation of *Panax ginseng* cells

**Abstract** The impacts of cryoprotectants (CP) and cell status during the growth cycle on *Panax ginseng* cell viability during cryopreservation were investigated. The ginseng cells used had a 5–7 times proliferation rate (compared with inoculum) in 2–3 weeks and were subcultured at 2- and 4-week intervals in liquid and on solid media, respectively. After testing various CP solutions of glycerol, dimethylsulphoxide, ethylene glycol and sucrose, a combination of 10% (v/v) glycerol and 4% (w/v) sucrose was selected for its least cytotoxicity and highest cell viability after thawing. With this CP solution, cells throughout the growth cycle exhibited a ‘U’-shaped fluctuation of post-thaw cell viability. The highest viability (86.5%) occurred during the lag phase from cells already maintained in suspension culture and then in the late exponential phase (61.4%); the lowest level of 15.4% was in the mid-exponential phase. Callus freshly transferred to liquid medium showed a less obvious fluctuation pattern. The recovered cells were brown-to-reddish at first and gradually returned to a light yellow colour after several subcultures.

**Key words** *Panax ginseng* · Suspension · Cryopreservation · Rapid freezing

**Abbreviations** CP: Cryoprotectant · 2,4-D: Dichlorophenoxyacetic acid · DMSO: Dimethylsulphoxide · TTC: 2,3,5-Triphenyl tetrazolium chloride

**Introduction**

Preventing cell line decline due to genetic drift and changes in genotype after long-term passages is one of the key requirements for commercializing cell cultures. Cryopreservation has been demonstrated to have enormous potential in retaining genetic stability and biosynthetic potential of high-yielding somaclones and hybrids. Considerable progress has been made over the years to make this technology a powerful tool for conserving germplasm on a long-term basis. Many plants have been successfully preserved in liquid nitrogen followed by subsequent recovery in growth, metabolism and pronounced regeneration (see references in Bajaj 1995).

*Panax ginseng*, in spite of its popularity in research areas pertaining to organogenesis, embryogenesis, large-scale bioreactor culture and ginsenoside synthesis and analysis, has not been well studied in the field of cryopreservation. Only limited information relating to the cryopreservation of *P. ginseng* is available (Butenko et al. 1984; Seitz and Reinhard 1987; Yoshimitsu et al. 1996; Zhang et al. 1993). When a slow freezing program was applied to exponentially growing cells, 40% viability was obtained (Butenko et al. 1984; Seitz and Reinhard 1987). For pollen, the post-thaw viability did not exceed 40% and was largely dependent on the moisture content in the anther (Zhang et al. 1993). The recovery growth of hairy roots was influenced by pre-culture treatment and type of cryoprotectant (CP) solution (Yoshimitsu et al. 1996). Furthermore, cryopreservation did not affect the yield and pattern of ginsenoside synthesis (Seitz and Reinhard 1987; Yoshimitsu et al. 1996).

To make the commercialization of ginseng cell culture viable, the establishment of a routine and reliable cryopreservation protocol is imperative. A successful cryopreservation protocol depends on a thorough understanding of the interactions between such factors as cell status, pre-culture, cryoprotectant solution,
freezing scheme and post-thaw treatment. Unfortunately, such information for *P. ginseng* is not available. The Hong Kong Institute of Biotechnology Ltd has launched a cryopreservation program to tackle the problem as part of its effort to commercialize ginseng cell culture. Here, we report on the effects of CP solution, cold hardening, cell origin and cell status at time of cryopreservation, on CP solution-caused cytotoxicity and post-thaw cell viability.

### Materials and methods

**Plant material**

*Panax ginseng* cell culture was initiated from 4-year-old roots (Teng and Nicolson 1997) on MS (Murashige and Skoog 1962) basal salts supplemented with 0.5 mg/l thiamine-HCl, 0.5 mg/l pyridoxine-HCl, 0.5 mg/l nicotinic acid, 2 mg/l glycine, 100 mg/l myo-inositol, 4.5 μM dichlorophenoxyacetic acid (2,4-D), plus 3% sucrose, and 0.8% Difco Bacto agar. The pH of the medium was adjusted to 5.7–5.8 before autoclaving at 121°C and 124 kPa for 20 min. Cells were routinely subcultured in the same medium at an interval of 2 and 4 weeks, respectively, for suspension and solid callus cultures. In suspension culture, agar was omitted. Calli were kept in the dark and the suspended cells were shaken at 110 rpm on an orbital shaker (Model S-103, Firstek Scientific, Taiwan) under 24 h illumination provided by cool-white fluorescent light at 20–30 μmol·m⁻²·s⁻¹ in a 25 ± 2°C culture room. For the experiment requiring cold hardening, cells were kept at 5°C for 4 weeks.

**CP solutions and cryopreservation procedure**

CP solutions tested in the study contained various combinations (%) of glycerol, dimethylsulphoxide (DMSO), ethylene glycol and sucrose. They were sorted out into two groups (Table 1).

At the start with the cryopreservation procedure, cells of the same culture age were incubated in 250-ml flasks containing 50 ml fresh liquid culture medium. After thorough mixing and cell settlement, 25 ml of the supernatant was withdrawn and replaced with an equal amount of CP solution. The flasks were then shaken on an orbital shaker at 110 rpm for 1 day. Following the same procedure, the CP solutions were added into the flasks in an orderly manner with a gradual increase in concentration. For example, cells to be treated with 'E' solution of group I were added first with 'A' for 1 day, and then with 'B', 'C', 'D' and 'E'. For group II CP solution Q, cells were first treated with 'I' for 1 day, followed by 'M', 'N', 'O', 'P' and 'Q', the whole process taking a total of 5 days. Since several days were required for the treatment of some CP solutions, the possibility of cytotoxicity during the treatment was monitored by using cell growth and viability tests.

After CP treatment, cells in the CP solution were transferred to 5 ml Nalgene polypropylene ampoules with the ratio of cells to CP solution adjusted to 1:5. The ampoules were then plunged into liquid nitrogen overnight. Thawing was carried out at 40°C in a water bath. Thawed cells were separated from the CP solution by centrifuging at 200 g for 2–3 min and then decanting the supernatant. To dilute the remaining CP solution, 4 ml fresh culture medium was added drop by drop into the ampoules. After another centrifugation using the same conditions, cells were transferred to solid media for recovery growth.

The impact of the culture age

To study the interaction of CP solution and cell status during the growth cycle, cells from both solid and liquid cultures were used. For solid cultures, callus due for a routine subculture was transferred to the liquid medium, and the date of the transfer was marked as day 0. For cells already maintained in liquid medium, the date for a routine subculture was day 0. In both cases, cells were sampled every other day for CP treatment and subsequent cryopreservation. All experiments were repeated at least two to four times, each having three to six replicates. Data analysis was computed using GraphPad Prism (version 2.0, GraphPad Software).

**Cell viability test**

Cell viability was evaluated using the TTC method developed by Towill and Mazur (1975). The method used a TTC solution prepared by dissolving 6 g of 2,3,5-triphenyl tetrazolium chloride and 0.5 ml of Tween 80 in 1000 ml 0.05 M Na₂HPO₄/KH₂PO₄ (17.91 g/6.805 g) buffer at pH 7.4. When placed in the solution, living cells were able to convert tetrazolium to formazan. The absorbance reading of formazan at 490 nm (reading/ml packed cell volume) was used to quantify the percentage of cells that were viable.

Cells were subjected to three TTC tests at different stages of the cryopreservation procedure: (1) prior to any treatment (T1), (2) after being treated with CP solution but before cryopreservation (T2), and (3) after thawing (T3). The analysis of cell viability was formulated as shown below:

- **Final viability referred to control:** \( V_1 = T3/T1 \times 100 \%
- **Viability referred to after treatment of CP solutions:** \( V_2 = T3/T2 \times 100 \%
- **Cytotoxicity due to treatment of CP solutions:** \( D_1 = (T1-T2)/T1 \times 100 \%
- **Death due to cryopreservation only:** \( D_2 = (T2-T3)/T1 \times 100 \%

### Table 1 Groups of cryoprotectants tested

| Group I |    |    |    |    | A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
|---------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Glycerol (v/v) | 0.0 | 0.63 | 1.25 | 2.50 | 5.0 | 10.0 | 15.0 | 20.0 | 25.0 | 30.0 | 40.0 |
| DMSO (v/v)   | 0.0 | 0.31 | 0.63 | 1.25 | 2.5 | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 | 20.0 |
| Ethylene glycol (v/v) | 0.0 | 0.31 | 0.63 | 1.25 | 2.5 | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 | 20.0 |
| Group II: |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Glycerol (v/v) | 0.0 | 0.63 | 1.25 | 2.5 | 5.0 | 10.0 | 15.0 | 20.0 | 25.0 | 30.0 | 40.0 |
| Sucrose (w/v)  | 0.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |