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# Cryopreservation of *Scutellaria baicalensis* Cells by Two-step Cooling Method

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A two-step cooling technique has been developed for cryopreservation of suspension cultured *Scutellaria baicalensis* cells. Efficient regrowth of cryopreserved cells was obtained in cryoprotected cells with a mixture of 1.5 M glycerol and 0.4 M sucrose in Schenk and Hildebrandt medium without pretreatment in high osmotic medium. Optimum freezing conditions were found to be a cooling rate of 0.5°C/min from 4°C to -40°C, and then retaining samples at -40°C for 30 min prior to plunging into liquid nitrogen. A regrowth rate of approximately 95% was obtained after three month storage in liquid nitrogen. Callus cultures established from the cryopreserved cells were found to produce the same patterns of flavonoid accumulation and retain their baicalin producing activity.

Plant cell culture technique provides an alternative means for the production of useful metabolites of plant origin. However, the loss of biosynthetic capability during the relatively long period of subculture is often raised as a major problem for its practical application. To minimize such an activity loss, new cultures have to be frequently initiated. The most reliable method for long-term preservation of plant cells is known to be cryopreservation in liquid nitrogen (10).

There have been reports on successful preservation of cultured plant cells in liquid nitrogen without loss of the biosynthetic capabilities of useful metabolites such as biotin (8) and alkaloids (1). However, such a conventional cryopreservation method did not result in a satisfactory regrowth for the cells of *Scutellaria baicalensis* which produced useful flavone glycosides including baicalin (6). Preservation of the flavonoid synthesizing capabilities of this cell line is not only important for an ongoing study of flavonoid biosynthesis, but also for industrial exploitation of scutellaria cell cultures.

In the present study, we have developed an efficient cryopreservation method of *S. baicalensis* cells, by which both regrowth of the cells and maintenance of the biosynthetic capability of flavonoids were satisfactorily achieved.

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Key words: *Scutellaria baicalensis* Georgi, cell suspension culture, cryopreservation, two-step cooling, baicalin

## MATERIALS AND METHODS

### Cell Material

A cell line of *S. baicalensis*, H3, was used in this study (6). The cell line was derived from hypocotyl and was maintained on Schenk and Hildebrandt (SH) (7) agar medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin (SHDK). To initiate cell suspension cultures, one gram of calli were placed in a 250 ml Erlenmeyer flask containing 40 ml of SHDK liquid medium on a gyratory shaking incubator (80 rpm) at 25°C under dark conditions. They were subcultured in 10% (v/v) inoculum at 10 days interval.

### Pretreatment

Unless specified otherwise, cells were cultured in SHDK medium. For preculture, 4-day-old cells were harvested by filtration and resuspended in SHDK liquid medium supplemented with 3% mannitol. Resuspended cells were incubated for another 24 h. The cryoprotectant solution was prepared in a concentration of 2 M glycerol plus 0.4 M sucrose in SH basal salt medium and sterilized by filtration. A 5-day-old culture containing 2 ml of packed cell volume was placed in a 15 ml conical tube with a screw cap. After draining the culture medium, 10 ml of prechilled cryoprotectant was added to the tube and mixed well. The suspension was then placed in an ice bath for 30 min. Five ml of the cell suspension in the cryoprotection solution was finally prepared by draining off the supernatant.

**Freezing**

One ml of the cryoprotected cell suspension was dispensed into cryotubes with screw cap (2 ml in volume, Nalgene). The tubes were capped and sealed with parafilm (Whatmann). The sealed tubes were placed in a freezing chamber (CRYOMED 1010 freezer) precooled to 4°C. The specimens were cooled at a cooling rate of 1°C/min to -40°C, then retained at that temperature for 40 min before plunging into liquid nitrogen.

**Regrowth**

After being stored longer than 24 h in liquid nitrogen, the tubes were rapidly thawed in a 40°C water bath. The thawed cell suspensions without washing were plated over pieces of sterilized filter paper (Whatman No. 1, diameter: 7 cm), which were then placed on a petri dish (87×15 mm) containing 20 ml of SHDK agar medium. After 3 to 4 h, small cell clumps (approximately 2 mm in diameter) were transferred to other plates containing the same medium. One hundred cell clumps were plated for every experiment. They were then incubated in a growth chamber at 25°C under dark condition. After 15 days of incubation, the calli grown were counted.

**Analyses**

Calli were extracted with hot water and the baicalin content was determined by HPLC according to the procedure described previously (6). All experiments were duplicated and the results were averaged.

## RESULTS

So far, appropriate cryopreservation methods for *S. baicalensis* cells have not been reported. Cryopreservation of *S. baicalensis* cells by the vitrification method reported by Nishitawa *et al.* (5) did not show any regrowth activity. To establish a suitable preservation method, therefore, two types of cryoprotectants were tested; a mixture of 2 M DMSO and 0.4 M sucrose and a mixture of 2 M glycerol and 0.4 M sucrose. The cells suspended in the cryoprotectant were cooled at a rate of 1°C/min from 4°C to -40°C and then held at -40°C for 40 min before plunging into liquid nitrogen. The results are shown in Table 1 and Fig. 1. The cryoprotected cells with a mixture of glycerol and sucrose showed a higher regrowth rate than those with a mixture of DMSO and sucrose. Pretreatment in a high osmotic medium was not necessary. To determine their optimum concentrations, cells were treated with different concentrations of glycerol and sucrose before freezing. The results showed that the combination of 1.5 M glycerol and 0.4 M sucrose produced the highest level of regrowth (Table 2).

A two-step cryoprotective cooling was found to be most effective in obtaining a high level of regrowth; an initial cooling to -40°C, holding for 30 min, then plunging cooled cells into liquid nitrogen. The optimum cooling rate was 0.5°C/min (Table. 3). Cooling rates faster than 1°C/min resulted in lower regrowth rates after cryopreservation.

Established calli from cryopreserved cells produced yellowish-green cell clumps which turned into whitish ones after approximately 8 days, and continued to grow actively. After approximately 4 weeks of the culture, these colonies grew to bright-yellow cell clumps which showed the characteristics of normal calli.

The effect of the culture age on callus regrowth after cryopreservation is shown in Fig. 2. Cells which were harvested at the lag phase or the stationary phase pro-

**Table 1.** Effect of cryoprotectant solution and preculture on regrowth of suspension cultured *S. baicalensis* cells after cryopreservation by two-step cooling method[a].

| Cryoprotectant solution | Culture condition | Percentage of regrowth |
|---|---|---|
| no cryoprotection | | 0 |
| 2 M DMSO | no preculture[b] | 32 |
| + 0.4 M sucrose | preculture[c] | 0 |
| 2 M glycerol | no preculture[b] | 89 |
| + 0.4 M sucrose | preculture[c] | 0 |

[a]Data are the means of two independent experiments; standard deviation of the values are <10%. [b]Cells were harvested at 5 days of culture in SH medium. [c]Cells were harvested at 24 h of culture in SH medium containing 3% mannitol + 3% sucrose after 4 days of culture in SH medium.

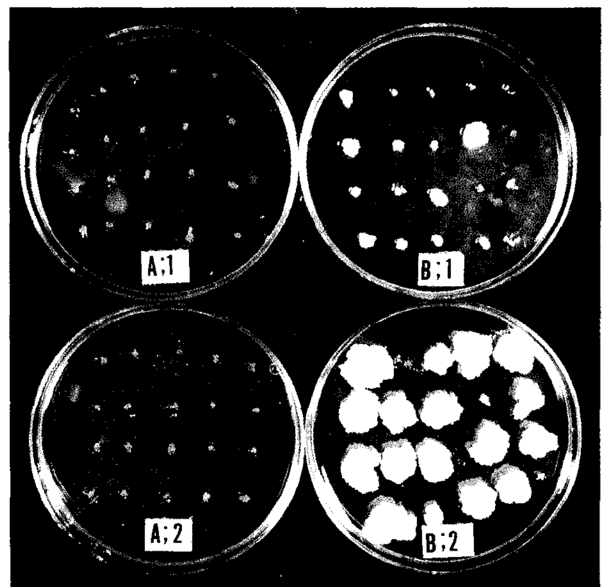


**Fig. 1.** Effects of cryoprotectant and preculture on cell regrowth on cryopreserved cells.

A: Cells were preculturd in SH medium containing 3% mannitol + 3% sucrose for 24 h. B: Cells were cultured in SH medium containing 3% sucrose. 1, Solution of 2 M DMSO + 0.4 M sucrose was used as a cryoprotectant; 2, Solution of 2 M glycerol + 0.4 M sucrose was used as a cryoprotectant.

**Table 2.** Effect of concentration of cryoprotectant solution on regrowth of suspension cultured *S. baicalensis* cells after freezing and storage in liquid nitrogen by two-step cooling method[a].

| Concentration of cryoprotectant | Percentage of regrowth |
|---|---|
| Experiment 1[b] | |
| 3 M glycerol | 30 |
| 2.5 M glycerol | 46 |
| 2 M glycerol | 91 |
| 1.5 M glycerol | 92 |
| 1 M glycerol | 20 |
| 0.5 M glycerol | 0 |
| Experiment 2[c] | |
| 0.6 M sucrose | 90 |
| 0.5 M sucrose | 93 |
| 0.4 M sucrose | 95 |
| 0.3 M sucrose | 50 |
| 0.2 M sucrose | 31 |
| 0.1 M sucrose | 5 |

[a]Data are the means of two independent experiments; standard deviation of the values are < 10%. [b]0.4 M sucrose was used as a non-permeable element of cryoprotectants. [c]1.5 M glycerol was used as a permeable element of cryoprotectants.

**Table 3.** Effect of cooling condition of cryoprotected cells on regrowth of suspension cultured *S. baicalensis* cells after cryopreservation[a].

| Freezing condition | Percentage of regrowth |
|---|---|
| Direct cooling[b] | 0 |
| Two step cooling[c] | |
| 0 min | 8 |
| 10 min | 25 |
| 20 min | 65 |
| 30 min | 94 |
| 40 min | 90 |
| Two step cooling[d] | |
| 0.5°C/min | 96 |
| 1°C/min | 92 |
| 2°C/min | 85 |

[a]Data are the means of two independent experiments; standard deviations of the values are < 10%. [b]Cryoprotected cells were directly plunged into liquid nitrogen. [c]Cryoprotected cells were cooled from 4°C to -40°C at the speed of 1°C/min and then plunged into liquid nitrogen after holding for the specified time at -40°C. [d]Cryoprotected cells were cooled from 4°C to -40°C at the specified speed and then plunged into liquid nitrogen after holding for 30 min at -40°C.

duced a low regrowth rate. A high level of regrowth was observed in the cells harvested during the logarithmic growth phase on the fourth or sixth day of culture.

Regardless of the storage period, approximately 95% regrowth was observed (data not shown). The capabilities for growth and baicalin biosynthesis of cryopreserved cells were maintained stably after three months in liquid nitrogen. The average values of regrowth rate and baicalin content in calli before and after the cryopreservation are shown in Table 4. There were no significant differences in either cell growth or baicalin production. The HPLC patterns of cultured cell extracts before and after the cryopreservation were the same as shown in Fig. 3.

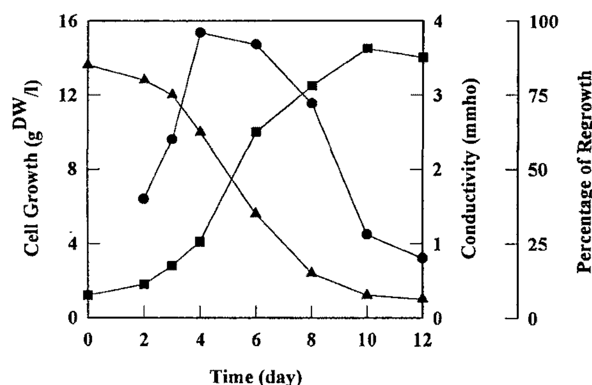


**Fig. 2.** Effect of harvest time on regrowth of cryopreserved *S. baicalensis* cells.
■, cell growth; ▲, conductivity; ●, percentage of regrowth.

**Table 4.** Cell growth and baicalin production from callus cultures of *S. baicalensis* cells before and after cryopreservation.

| Callus No. | Cell growth ($g^{FW}$)[a] | | Baicalin production ($mg/g^{FW}$) | |
|---|---|---|---|---|
| | before | after | before | after |
| 1 | 2.56 | 2.19 | 3.19 | 2.28 |
| 2 | 2.76 | 2.79 | 2.22 | 3.01 |
| 3 | 2.15 | 2.05 | 2.84 | 2.83 |
| 4 | 2.64 | 2.77 | 3.05 | 2.86 |
| 5 | 2.47 | 2.56 | 3.21 | 3.16 |
| 6 | 2.75 | 2.58 | 2.12 | 2.01 |
| 7 | 2.62 | 2.66 | 2.34 | 2.69 |
| 8 | 2.18 | 2.25 | 2.65 | 2.83 |
| 9 | 2.65 | 2.50 | 2.99 | 3.21 |
| 10 | 2.47 | 2.43 | 3.06 | 3.19 |
| 11 | 2.61 | 2.09 | 2.95 | 3.06 |
| 12 | 2.39 | 2.63 | 2.89 | 3.04 |
| average value | 2.52 | 2.45 | 2.79 | 2.87 |

[a]0.05 $g^{FW}$ calli were transfered on SH agar medium and harvested at 30 day of culture.

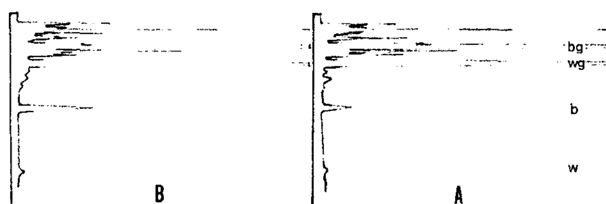


**Fig. 3.** HPLC profiles of flavonoids from callus extracts of *S. baicalensis* before (A) and after (B) cryopreservation.
bg, baicalin; wg, wogonin glucuronide; b, baicalein; w, wogonin.

## DISCUSSION

A variety of cryoprotectants including DMSO, glycerol, proline and their mixtures with other compounds such as sucrose and sorbitol have been widely used for cryopreservation of plant cells (9). However, it was also required to apply an improved cooling process for practical applications. In this study, the use of a mixture of DMSO and sucrose as a cryoprotectant produced a low regrowth rate (32%) after cryopreservation. DMSO, as a permeable element of the cryoprotectant, was considered either toxic to cells or not effective in inducing cell shrinkage and in reducing intracellular ice crystallization. The combination of 1.5 M glycerol and 0.4 M sucrose dramatically improved the regrowth rate (to 95%) of the cryopreserved cells.

Although preculture in a high osmotic medium has been used to obtain a high survival rate of the cryopreserved cells (1, 2), it was found not to be necessary in the two-step cooling process used in this study.

Regrowth rate of cryopreserved cells in liquid nitrogen depends on the cooling condition of cryoprotected cells. The two-step cooling process involved a slow prefreezing step at a rate of 0.5°C/min to -40°C, followed by holding at -40°C for 30 min before plunging into liquid nitrogen, was essential to obtain a high level of regrowth. It is thought that the first, slow prefreezing step induces sufficient cell shrinkage to protect the cells against damage during the second, rapid cooling (3).

Age of the suspension cultured cells was critical in obtaining a high regrowth rate. It was found that 4- to 6-day-old cultures (logarithmic growth phase) were most suitable for cryopreservation because the cells at this stage were considered to have undergone active division and to have relatively dense cytoplasm with small vacuoles (4).

It was also noted that the activities for growth and baicalin production of the cells after cryopreservation were successfully maintained by the method described in this study. Therefore, in conclusion, the cryopreservation method reported here is believed to be suitable for the prolonged preservation of *S. baicalensis* cells.

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