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Cryopreservation of Suspension Cultures of *Camptotheca acuminata*

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Abstract Suspended cells of *Camptotheca acuminata* were observed to lose their ability to synthesize camptothecin and its derivatives as a result of repeated cultures. Accordingly, the maintenance of high-yield cells by cryopreservation was studied to overcome this stability problem, and various factors involved were optimized. Pregrowing the cells in 8% myo-inositol for 4 days was found to be the most effective in improving survival. The highest survival was obtained when the pregrown cells were cryoprotected with a mixture of 10% DMSO, 0.6 M mannitol, and 10% glycerol. When the cryopreserved cells were maintained in a freezer at -70°C, 94% survival was obtained after 4 months. The survivals after 5 and 8 months of storage decreased to 52% and 45%, respectively. No loss of biosynthetic capability of camptothecin was observed after short to medium term cryopreservation of *C. acuminata*.

Key words: Cryopreservation, *Camptotheca acuminata*, plant cell suspensions

Plant cell cultures are potential sources of useful chemicals. However, they have to be routinely maintained for extended periods of time by periodic subculturing, which is a time consuming and labor intensive process, but that can result in the loss of the cultures due to microbial contamination, equipment failure, or human error. Long-term maintenance by subculturing can also result in mutations, including changes in the structure and number of chromosomes [3, 6], and subsequent loss of the morphogenetic potential [12, 15]. The production of useful chemicals by *in vitro* cultures varies between cell lines and in certain individual lines can be fairly stable. However, the product content and spectra in other cell lines require repeated selection to maintain a high production level; accordingly, cryopreservation would appear to be a possible alternative to overcome these stability problems [8, 24].

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Camptothecin is an antitumor alkaloid first isolated from *Camptotheca acuminata*, a native tree of China. In China, camptothecin has been widely used in the treatment of cancer. In 1966, Dr. Wall and Mansukh Wani successfully isolated an active ingredient, camptothecin, from bark extracts [21]. Clinical trials began in the early 1970s, however, the drug was proved to be too toxic for clinical use. Efforts have been made to reduce the toxicity of camptothecin for clinical use. 10-Hydroxy camptothecin is less toxic and so far is one of the promising camptothecin derivatives. Topotecan is an analogue of 10-hydroxy camptothecin [26]. SmithKline Beecham Pharmaceuticals discovered topotecan and markets it as Hycamtin(R), which has been approved by the FDA for refractory ovarian cancer. Daiichi Pharmaceutical Co. Ltd. discovered CPT-11(irinotecan) [18], another analogue of 10-hydroxy camptothecin, which is approved in Japan for refractory ovarian cancer. In a series of clinical trials, topotecan showed activity against a variety of solid tumors including ovarian cancer, small-cell lung cancer, and others. In one study, comparing topotecan and taxol for the treatment of advanced recurrent ovarian cancer, topotecan showed a 20% response rate, whereas taxol had only a 12% response rate [11]. Topotecan is as significant as taxol in terms of its unique mechanism of action and novel approach [20].

Camptothecin and its derivatives can be produced by plant cell cultures of *C. acuminata*. Sakato *et al.* [13, 14] induced callus from the stem of *C. acuminata* on a MS medium containing 0.2 mg/l 2,4-D and 1 mg/l kinetin. Giberellin, L-tryptophan, and a conditioned medium stimulated the cell growth. After 15 days of suspension culture, the concentration of camptothecin in the cells was 0.0025% dry weight, which was about 1/20 of the level in the intact plant. Hengel *et al.* [7] also established a suspension culture system of *C. acuminata*. The highest level of camptothecin accumulated in cells harvested in a MS medium containing 4 mg/l NAA. Song *et al.* [16, 17] reported an enhanced production of camptothecin by various elicitors, however, in suspension cultures of *C. acuminata*, the production of camptothecin and its derivatives

was hampered due to a stability problem. Suspended cells lose their ability to synthesize camptothecin and its derivatives during the repeated cultures. To overcome this instability problem, the cryopreservation of cells is indispensable for *C. acuminata* cultures. In this paper, various factors involved in the cryopreservation of cell suspension cultures of *C. acuminata* were studied, and the cryopreservation procedure was optimized at each stage.

MATERIALS AND METHODS

Cell Cultures

Seeds of *C. acuminata* were obtained from the Cheolipo Botanical Garden in Taean, Korea and propagated. Young stems of *C. acuminata* were surface sterilized by immersing them for 5 min in a sodium hyperchlorite solution (20% v/v) containing 0.1% Tween 20. The plant material in the solution was sonicated for 30 sec and then sterilized for another 10 min. Thereafter, the plant material was rinsed five times with sterilized distilled water. For callus induction, young stems were cut and placed on agar plates with different media. The basic media were hybrids of MS (Murashige and Skoog) and B5 (Gamborg) salt medium (1:1) supplemented with 2,4-D (5 μM), kinetin (0.5 μM), and 25 g/l of sucrose as the carbon source. 0.5% (w/v) of the agar was added to prepare the solid medium for callus maintenance. The pH was adjusted to 5.8 with 1 N KOH. For the maintenance of the suspension culture, 16 g of cells (fresh cell weight) was transferred into 200 ml of the medium in a 500-ml Erlenmeyer flask every 8 days. Callus subculturing was carried out every 40 days by transferring a spoonful of healthy callus onto 40 ml of the solid medium. One-hundred-twenty-five-ml Erlenmeyer flasks containing 50 ml of the growth medium were used for the experimental batch cultures on a gyrotory shaker at 180 rpm. The temperature of the culture room was 26°C.

Pregrowth and Cryoprotectant Treatment

Various pregrowth conditions were tested. The cells were pregrown in the hybrid medium described above, or supplemented with 5% (w/v) of different sugars, such as glucose, mannitol, myoinositol, sorbitol, or sucrose. Five grams of seven-day-old fresh cells were transferred directly to 50 ml of the pregrowth medium, and then used for cryopreservation after four days of culture.

The optimum combination of cryoprotectants consisting of DMSO, glycerol, and sugar or sugar alcohol (sucrose, mannitol, sorbitol, glucose, myo-inositol) was studied in combination with each of the pregrowth treatments. Two-tenth gram (fresh weight) of the suspension cells were filtered and transferred to 2 ml cryotubes (screw-cap polypropylene ampoules) containing 1.7 ml of the liquid hybrid medium supplemented with cryoprotectants. The cryoprotectants were prepared in double strength, and filter sterlized and chilled on ice. The cells in the cryotubes were suspended on ice for 1 h.

Freezing and Thawing

The cell suspensions (1.7 ml) in the cryotubes cooled in the freezing container (NALGENE™ Cryo 1°C) at a rate of -1°C/min and 250 ml of iso-propanol, an antifreezing agent, were added into a 500-ml freezing container. To achieve a cooling rate of 1°C/min, the container was cooled in a nonprogrammable freezer at -70°C. After 1 day of cooling and storage, the cryotubes were rapidly thawed in a 40°C water bath and the cell viability was tested.

Viability and Regrowth

The thawed cell suspensions were immediately used for assessing the viability. The rate of viability of the frozen-thawed cells was compared with the controls by means of TTC reduction assay [19]. For the TTC reduction assay, the contents of the ampoules were filtered, 90 mg of fresh cells were transferred to 15-ml centrifuge tubes, and 3 ml of the TTC solution [19] was added. The mixture was then left in total darkness for 16 h at 26°C. The tubes were centrifuged at 100 ×g for 5 min and the supernatant was removed after centrifugation. After 1.5 ml of 95% EtOH was added, the mixture was sonicated for 30 min. Then, 1.5 ml of 95% EtOH was added again and the mixture was left at room temperature for 4 h. After centrifugation at 100 ×g for 5 min, the absorption of the supernatant was measured at 485 nm using a Shimatzu UV-1201 Spectrophotometer, and the viabilities were determined by comparing the values obtained from the cryopreserved cells with those of the control cells. The viability tests were performed in triplicates. The viability of the cells after various treatments was expressed as a survival percentage compared with the nontreated, nonfrozen control cells.

For regrowth of the cryopreserved cells, filter papers (Whatman No. 1, 5 cm in diameter) sterilized by autoclaving were placed on Petri dishes containing 20 ml of the hybrid medium solidified with 0.5% agar. To this, 1.7 ml of the thawed cell suspensions containing about 0.2 g cells (fresh weight) was dispensed onto a double layer sterilized filter paper placed over the solid medium. After 5 days, the filter papers with cells were transferred to other Petri dishes containing the same medium. After 2 weeks, the cells were transferred either onto the solid medium for callus maintenance or into the liquid medium for suspension cultures.

Analysis of Camptothecin

The cells were harvested by vacuum filtration and the filtrate was collected for an analysis of the extracellular alkaloids in the medium. For the measurement of the intracellular camptothecin and 10-hydroxy camptothecin concentrations,

5.0 g of cells (FCW) were extracted with 30 ml of methanol and the suspension was sonicated at 125 W for 10 min. Forty milliliters of water and 70 ml of chloroform were then added and the solution was mixed vigorously. After centrifugation for 10 min at 3,000 rpm, the chloroform phase was recovered and evaporated to dryness. The remaining residue was dissolved in methanol and filtered through 0.45 μm membrane filters for HPLC analysis. For extracellular alkaloid analysis, 10 ml of the medium filtrate was added to 10 ml of chloroform and mixed vigorously. After centrifugation for 10 min at 3,000 rpm, the chloroform phase was recovered and evaporated to dryness. The remaining residue was dissolved in methanol and filtered for analysis. HPLC analysis was performed using an HPLC system (Spectra System, Thermo Separation Products, Co.) with a reversed phase column (Vydac C-18, 250×4.6 mm, 10 μm, 90 Å) and fluorescence detector (FL2000, Thermo Separation Products, Co.). The flow rate was 1.2 ml/min. A gradient variable mobile phase composition was used for a better resolution. For the first 15 min, the ratio of the buffer solution (0.075 M ammonium acetate with 1 mM tetrabutylammonium phosphate, pH 6.4) to acetonitrile was maintained at constant 78:22 (v/v). Then the ratio was increased from 78:22 to 50:50 in 5 min as the linear gradient mode. For the last 10 min, the ratio was maintained at constant 50:50. The excitation wavelength was 350 nm, and the emission wavelength was optimized at 450 nm for camptothecin and at 550 nm for 10-hydroxy camptothecin.

Results and Discussion

It was observed that the suspended cells lost their abilities to synthesize camptothecin and its derivatives as subcultures proceeded. Figure 1 shows fluctuation of the camptothecin production in the suspension cultures of *C. acuminata*. Unfortunately, the concentration of camptothecin produced in the suspension cultures decreased over a period of 20 months. This loss of synthetic ability can be an obstacle in the production of camptothecin and its derivatives using the plant cell cultures of *C. acuminata*. It is still not clear how the suspended cells lose their biosynthetic abilities. However, long-term repeated subculturing may possibly result in mutations of cells including changes in the structure and number of chromosomes. This problem of stability in cultures of undifferentiated plant cells can be solved by repeated screening of high-yield cell lines. Alternatively, maintenance of high-yield cell lines by cryopreservation would appear to be another way to overcome stability problems.

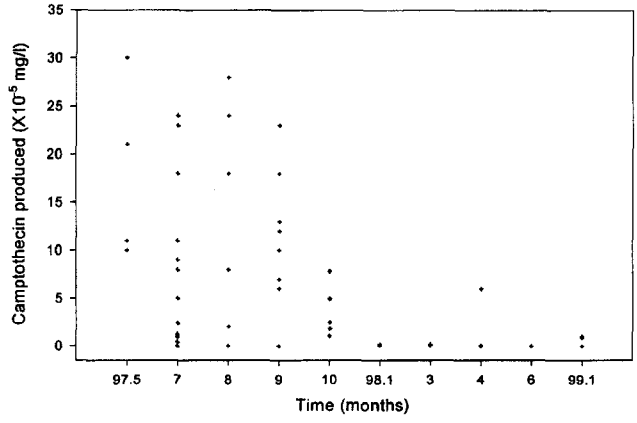

Fig. 1. Fluctuation of camptothecin production in suspension cultures of *Camptotheca acuminata* over 20 months.

During the cryopreservation process, the survival of cell suspensions depends on various factors. The nature and physiological state of the cells determine their ability to withstand the freezing process. Freeze tolerance can be increased by pregrowing the cells in a high osmotic medium supplemented with osmotic additives such as mannitol, sorbitol, various sugars, or proline prior to freezing. During freezing, plasmolysis is the major cause of injury in highly vacuolized cells like plant cells. Various osmotic compounds can minimize this damage by exerting forces that inhibit cell expansion, thereby reducing the volume of the cell and its vacuole. When the suspended cells of *C. acuminata* were cryopreserved using different pregrowth additives, differences were observed in their viability (Table 1). Large increases in viability were observed when the cells were pregrown with mannitol, myo-inositol, and sorbitol. The highest viability was obtained in the hybrid medium supplemented with 5% myo-inositol. This was an unexpected result, because mannitol is the most commonly used pregrowth additive [25], and pregrowing cells in sorbitol has also been established to be effective in improving survival where mannitol is not successful [9]. The concentration of the pregrowth additive is also important to maintain an adequate osmolality. Table 2 shows that 8% was the optimum concentration of myo-inositol for maintaining the highest viability. When the cells were pregrown in the hybrid medium supplemented with 8% myo-inositol, a proper reduction in

Table 1. Effect of various pregrowth additives on survival of *Camptotheca acuminata* during the process of pregrowing. The cells were pregrown for 4 d in the hybrid medium with various pregrowth additives. The pregrown cells were then frozen without a cryoprotectant and their viabilities analyzed after thawing.

Pregrowth additive	Survival (%)
Control	18
5% glucose	19
5% mannitol	27
5% myo-inositol	32
5% sorbitol	30
5% sucrose	20

Table 2. Effect of myoinositol concentration on survival of *Camptotheca acuminata* during the process of pregrowing. The cells were pregrown for 4 days in the hybrid medium with various myo-inositol concentrations. The pregrown cells were then frozen without a cryoprotectant and their viabilities analyzed after thawing.

Myo-Inositol (%)	Survival (%)
Control	19
1%	25
2%	20
3%	36
4%	33
5%	32
6%	30
7%	37
8%	55
9%	32

the cell volume, the cytoplasm to vacuole ratio, and the cellular water content were all believed to change the osmotic behavior of the cells, making them more tolerant to any freezing damage. The pregrowing time was another important factor for improving the viability. Pregrowing the cells for 4 days with myo-inositol considerably improved their viability. When the cells were pregrown for more than 4 days, the cells darkened and reduced viability was observed.

Cell damage occurs during the process of freezing due to either the formation of intracellular ice crystals or the solution effects resulting from excessive dehydration [10]. Cryoprotectants applied prior to freezing reduce the freezing injury in a number of ways. Cryoprotectants are divided into two classes; penetrating and nonpenetrating compounds. Penetrating compounds cause repression of the freezing point in the cytoplasm, and also provide increased volume to reduce the damage caused by the increased concentration of toxic compounds and electrolytes in the cytoplasm. Nonpenetrating compounds act by osmotic action reducing the water content of the cell, thereby exerting protective dehydration and preventing ice damage. Therefore, the effects of various cryoprotectants on the cell survival during the process of freezing were studied. When different concentrations of DMSO were supplemented as a cryoprotectant, the survival ranged from 40 to 62% (Table 3). Therefore, DMSO alone was not an effective cryoprotectant. Ten percent of DMSO produced the least toxic effect with a 62% survival rate. When sugars or sugar alcohols were used as cryoprotectants, mannitol improved the survival considerably (Table 3), and 0.4 M of mannitol produced a 76% survival rate, which was higher than the highest survival obtained with DMSO.

Using a mixture of cryoprotectants was superior to using a single compound (Table 4). Two combinations resulted in better survival rates than those of single cryoprotectants. A 97% survival was obtained when a mixture of 10% DMSO and 0.4 M mannitol was supplemented as the cryoprotectant. This mixture of DMSO and mannitol is believed to induce an additional cryoprotective effect along with a reduction in the individual toxicity. The mannitol concentration in the mixture with 10% DMSO was optimized. Table 5 shows the optimum concentration of mannitol to be 0.4 M to 0.6 M. Glycerol is one of the most commonly used cryoprotectants in the cryopreservation of microbial and animal cells. However, glycerol is not effective in preventing freeze damage when used alone. A reduced uptake at the temperature of application can even cause injury due to excessive plasmolyzation [23], because glycerol acts either as a penetrating compound or a

Table 3. Effect of various cryoprotectants on survival of *Camptotheca acuminata* during the process of freezing. Cells were pregrown in 8% myo-inositol for 4 days. The pregrown cells were frozen at a rate of - 1°C/min with various cryoprotectants and thawed for a viability analysis.

Cryoprotectant	Survival (%)
0	54
5% DMSO	56
10% DMSO	61
15% DMSO	46
20% DMSO	54
0.4 M sucrose	60
0.4 M glucose	54
0.4 M sorbitol	64
0.4 M mannitol	76
0.4 M myo-inositol	69

Table 4. Effect of a mixture of cryoprotectants on survival of *Camptotheca acuminata* during the process of freezing. Cells were pregrown in 8% myo-inositol for 4 days. The pregrown cells were frozen at a rate of - 1°C/min with various cryoprotectants and thawed for a viability analysis.

Cryoprotectant	Survival (%)
10% DMSO+0.4 M glucose	57
10% DMSO+0.4 M mannitol	97
10% DMSO+0.4 M sorbitol	73
10% DMSO+0.4 M sucrose	68
10% DMSO+0.4 M myo-inositol	82

Table 5. Effect of mannitol in a mixture of cryoprotectants on survival of *Camptothca acuminata* during the process of freezing. Cells were pregrown in 8% myo-inositol for 4 days. The pregrown cells were frozen at a rate of 1/min with various cryoprotectants and thawed for a viability analysis.

Cryoprotectant	Survival (%)
10% DMSO+0.2 M mannitol	44
10% DMSO+0.4 M mannitol	97
10% DMSO+0.6 M mannitol	98
10% DMSO+0.8 M mannitol	71
10% DMSO+1.0 M mannitol	66

Table 6. Effect of glycerol in combination of 10% DMSO and 0.6 M mannitol on survival of *Camptothca acuminata* during the process of freezing. Cells were pregrown in 8% myo-inositol for 4 days. The pregrown cells were frozen at a rate of -1°C/min with various cryoprotectants and thawed for a viability analysis.

Cryoprotectant	Survival (%)
10% DMSO+0.6 M mannitol+5% glycerol	81
10% DMSO+0.6 M mannitol+10% glycerol	101
10% DMSO+0.6 M mannitol+15% glycerol	88
10% DMSO+0.6 M mannitol+20% glycerol	87

nonpenetrating compound depending on the temperature of application [8]. Accordingly, an optimal concentration of glycerol in combination with 10% DMSO and 0.6 M mannitol was studied. A 101% survival rate was obtained when the cells were cryoprotected with a mixture of 10% glycerol, 10% DMSO, and 0.6 M mannitol (Table 6). This result indicates that the suspended cells of *C. acuminata* were completely protected from freeze damage, when pregrown with 8% myo-inositol for 4 days and then cryoprotected with a mixture of 10% DMSO, 0.6 M mannitol, and 10% glycerol.

Cells can be frozen by slow freezing, stepwise freezing, or rapid freezing. Freezing can cause injury due to the solution effects caused by extracellular freezing, or mechanical damage caused by intracellular freezing. In this study, however, the freezing method was not optimized. Slow freezing with a cooling rate of -1°C/min was applied because it is the most widely used procedure, especially for the cryopreservation of cell suspensions [5, 24]. Thawing can be achieved either rapidly at rates of 500-750°C/min by plunging the frozen sample into water at 37-40°C, or slowly by exposing the specimens containing cryovials to blown warm air, air at room temperature, or to liquid nitrogen vapor followed by air at room temperature. The thawing method applied in this study was rapid thawing, because it reduces the chance of recrystallization. Recrystallization of ice crystals occurs during slow thawing, particularly at temperatures above -45°C, resulting in the formation of large ice masses which can damage cell membranes and cytoplasmic organelles. This zone of recrystallization passes rapidly during rapid thawing, so that the ice crystals melt before they have an opportunity to recrystallize [2].

The optimized procedure for cryopreservation was used successfully for maintenance of *C. acuminata*. The cryopreserved cells were maintained in a freezer at -70°C. They were then regrown and used for callus and suspension cultures. The effect of the term of storage on the survival of the cryopreserved cells was observed. A 94% survival was obtained for the cryopreserved cells stored for 4 months. The survival after 5 and 8 months' storage, however, decreased to 52% and 45%, respectively. The loss of viability in this study is believed to be due to the storage temperature. The storage temperature should be low enough to inhibit all metabolic activity and prevent any biochemical injury. Storage at a higher temperature causes structural damage to the cells as a result of the progressive recrystallization of ice, resulting from the migration of water in a solid state [23]. At -20°C, protein denaturation takes place due to metabolic activity, leading to cell death. In addition, changes in the concentration of the solutes and subsequent pH variations also take place at these temperatures [1]. Temperatures within the range of -4°C to -70°C are not recommended because cell deterioration is observed at such temperatures [22]. The storage temperature in this study was on the border of the temperature range for long-term storage. For the short term (4 months), maintenance of cryopreserved cells of *C. acuminata* at -70°C may be adequate. However, the temperature should be lower for long-term storage. Even ice recrystallization cannot be completely prevented when a storage temperature above -130°C is used [4], hence, liquid nitrogen is recommended for long-term storage. The cryopreserved cells of *C. acuminata* were observed to maintain the ability to biosynthesize secondary metabolites, proved by the measurement of the camptothecin produced in the suspension cultures of the thawed and regrown cells. The camptothecin produced in the control cultures ranged between 4.5×10^{-4} and 6.2×10^{-4} mg/l. On average, 5.4×10^{-4} mg/l of camptothecin was produced in the suspension cultures of the regrown cells that had been cryopreserved for 4 months. Concentrations of 5.9×10^{-4} and 4.8×10^{-4} mg/l of camptothecin were produced from the cells cryopreserved for 5 and 9 months, respectively. Accordingly, no serious loss of biosynthetic capability of camptothecin was observed in the short to medium term cryopreservation of *C. acuminata*. This indicates that the procedure optimized for cryopreservation in this study can be successfully used for the maintenance of *C. acuminata*.

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