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Original Paper

Simple cryopreservation protocol with an encapsulation technique for tobacco BY-2 suspension cell cultures

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Abstract Tobacco BY-2 cells were successfully cryopreserved by a simple slow prefreezing (equilibrium freezing) method using an encapsulation technique. After the cells were immobilized in alginate gel beads, the beads were treated with cryoprotectant solution (2 M glycerol, 0.4 M sucrose) for 45 min. The beads were then transferred to a laboratory freezer at −30°C, stored for 2 h, and then immersed in liquid nitrogen. To initiate the regrowth of cells, the beads were warmed in a water bath. Following dilution of cryoprotectant solution, the beads were suspended and cultured in normal medium. With this method, suspension cell cultures were regrown within 7 days. There were no differences in the morphology or growth profiles between cryopreserved cell cultures and the original cell cultures.

Key words: Cryopreservation, encapsulation, slow prefreezing method, tobacco BY-2.

The tobacco BY-2 suspension cell line is a model system used extensively in cytological and molecular studies, especially in the visualization of cellular processes, in combination with cell synchronization and transformation techniques (Kumagai et al. 2001; Kutsuna et al. 2003). However, the maintenance of cell cultures by successive subculture is cumbersome and entails the risk of losing the cell line due to disease, contamination, or technical errors. The cryopreservation of cells and organ cultures in liquid nitrogen (LN) can overcome these problems, and provides a reliable and cost-effective method for the long-term storage of plant genetic resources (Engelmann 2000; Sakai 2002).

Cultured plant cells are successfully cryopreserved with the slow prefreezing (equilibrium freezing) method (Schrijnemakers and Van Iren 1995; Kim et al. 2001). The two freezing steps are key stages in this procedure: one involves cooling the cells to −30°C at a slow and controlled rate in a programmable freezer, and the other involves holding them for an adequate time at −30°C in the presence of cryoprotectant. These freezing steps work for dehydration of cells at optimal level. After the freezing steps, when the cells are sufficiently dehydrated, they are immersed in LN. By rapid immersion into LN, the cells were completely vitrified without crystallization and stored without cryoinjury, which is caused by the formation of intracellular ice crystals. However, this freezing method requires complicated procedures and the use of a programmable freezer.

Cryopreservation with encapsulation, in which cells and organs are embedded in vitro in alginate gel, has been successfully achieved with many plant materials in combination with vitrification methods (Niino and Sakai 1992; Hirai and Sakai 2003; Charoensub et al. 2004) or air dehydration methods (Bachiri et al. 2000; Wang et al. 2002; Fang et al. 2004).

The characteristics of the materials to be cryopreserved are relevant to successful cryopreservation. Undifferentiated cell suspensions, which consist of large vacuolated cells, are prone to severe cryoinjury compared with embryogenic cultures and apical organs, which contain small cytoplasmic-rich meristematic cells (Häggman et al. 1998; Wang et al. 2002). In addition, suspension cells are sensitive to environmental stresses, such as dehydration, high osmotic pressure, and low temperatures. Moreover, the viability rate of cryopreserved suspension cells must be high to aviod the selection of particular types of cells (Ishikawa et al. 1995; Menges and Murray 2004). The retention of high cell viability also ensures the rapid regrowth of cells after thawing, because the proliferation of suspension cells depends on the initial cell density (Matsubayashi et al. 1996).

In this study, we successfully established a simple cryopreservation protocol that retains a high level of cell viability and allows the rapid regrowth of suspension

Abbreviations: LN, liquid nitrogen; LS medium, Linsmaier and Skoog medium.

cultures. This method is a combination of the encapsulation technique and a simple slow prefreezing (equilibrium freezing) procedure. We report here the optimum conditions for the cryopreservation of BY-2 cells.

Materials and methods

Cell culture

Suspension cultures of tobacco BY-2 cells derived from *Nicotiana tabacum* L. cv. 'Bright Yellow-2' were maintained as described by Nagata et al. (1992). Suspension cells were grown in Linsmaier and Skoog (LS) medium containing sucrose (30 g l^{-1}, 0.09 M) and 2,4-dichlorophenoxyacetic acid (0.2 mg l^{-1}) with shaking (130 rpm) at 27°C in the dark and subcultured in fresh medium at 1-week intervals. Cells used for cryopreservation were taken from the exponential growth phase on day 3 after subculture.

Encapsulation of suspension cells in alginate gel

BY-2 cells were collected by centrifugation at 100 *g* for 5 min and resuspended in LS medium containing 2% (w/v) sodium alginate. Cell density was adjusted to 0.1–0.2 ml of packed cells in 1 ml of solution (approximately 10^6 cells ml^{-1}). The mixture of cells and alginate was dripped into culture medium containing 0.1 M calcium chloride, which caused gelation of the alginate forming beads approximately 4 mm in diameter. The alginate beads were kept in the calcium-enriched medium for 10 min. The medium was removed with a pipette and the beads were then incubated in normal medium with gentle shaking for 20 min.

Cryopreservation and regrowth procedures

The beads containing BY-2 cells were incubated in LS medium containing 2 M glycerol and 0.4 M sucrose (cryoprotectant solution) with gentle shaking at 25°C. Three beads were transferred to a 2.0 ml cryovial with 0.3 ml of the cryoprotectant solution. The cryovials were placed in a laboratory freezer at −30°C and stored for a definite length of time (prefreezing step). Finally, the vials were plunged into LN. After more than 30 min storage, the vials were warmed in a water bath at 40°C. To dilute the cryoprotectant solution, the beads were incubated in LS medium containing 1.2 M sucrose with gentle shaking for 15 min at 25°C. The medium was replaced with LS medium containing 0.5 M sucrose and then with normal LS medium at 15 min intervals. The beads were suspended in LS medium and cultured with shaking (130 rpm) at 27°C. Figure 1 summerizes the cryopreservation and regrowth procedures.

Determination of the optimal cryostorage conditions

In order to determine optimal prefreezing duration, beads containing BY-2 cells were treated with the cryoprotectant solution for 45 min at 25°C, and then prefrozen in a freezer at −30°C for 0–3.0 h, before they were plunged into LN. We also examined the effect of cryoprotectant treatment on cryopreservation. The beads were treated with cryoprotectant solution for 0–60 min at 25°C, prefrozen in a freezer for 2 h, and then plunged into LN. In addition, various concentrations of glycerol and sucrose were used as cryoprotectants. In the control experiments, the prefrozen beads were re-warmed without immersion in LN (frozen control) or the cryoprotectant-treated beads were transferred to dilution medium (treated control). The viability of the cells was determined 1 day after re-warming.

Determination of cell viability and mitotic index

The beads containing BY-2 cells were incubated in Evans blue solution (1 mg ml^{-1} in LS medium) for 15 min and then washed with fresh medium to remove excess dye. The beads were crushed with a cover glass and the cells were counted under a differential interference contrast microscope (BX62, Olympus, Tokyo, Japan). Stained cells were considered dead. The viability of cells was expressed as the percentage of unstained living cells in the total cells. Mitotic index was expressed as the percentage of cells with condensed chromatin after staining the cells with an acetocarmine solution (Qian et al. 1996). We used 3 beads in each treatment and scored at least 400 cells in each bead (1200 cells per treatment).

Regrowth of suspension cell cultures

Suspension cell cultures were established by culturing 3 beads in 3 ml of liquid LS medium in a 12-well microplate after dilution of cryoprotectant solution (Figure 1). The cultures were maintained with shaking (130 rpm) at 27°C in the dark for 3 days. The embedded cells were released into the culture medium by crushing the beads. The cell suspensions were cultured for a further 4 days and then transferred into 95 ml of fresh LS medium in a 300 ml flask. The cell lines were subcultured 3 times at 1-week intervals.

Results

Identification of optimal cryostorage conditions

Figure 2A shows an alginate gel bead encapsulating BY-2 cells. The viability of BY-2 cells embedded in alginate beads was determined by the Evans blue exclusion method. The living cells had normal intracellular structures after cryopreservation, as did the encapsulated control cells (Figures 2B, C). In contrast, the dead cells had coagulated cytoplasm stained with Evans blue

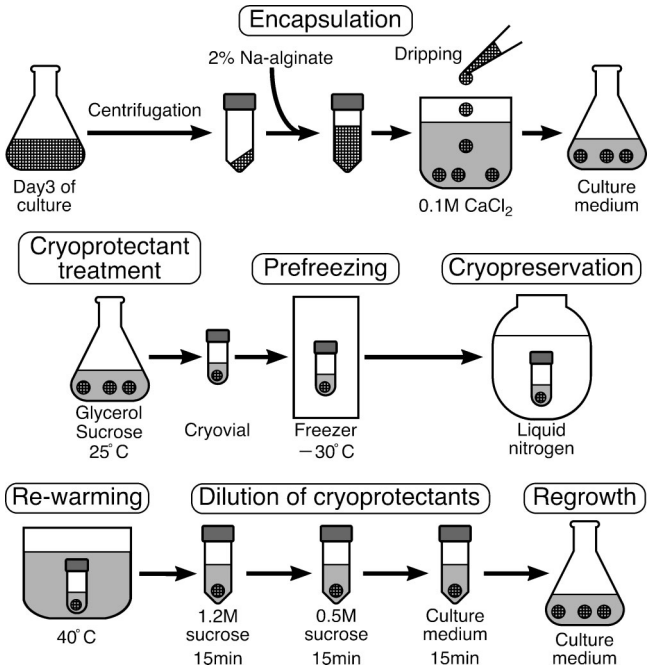


Figure 1. Scheme of cryopreservation procedure for tobacco BY-2 suspension cells.

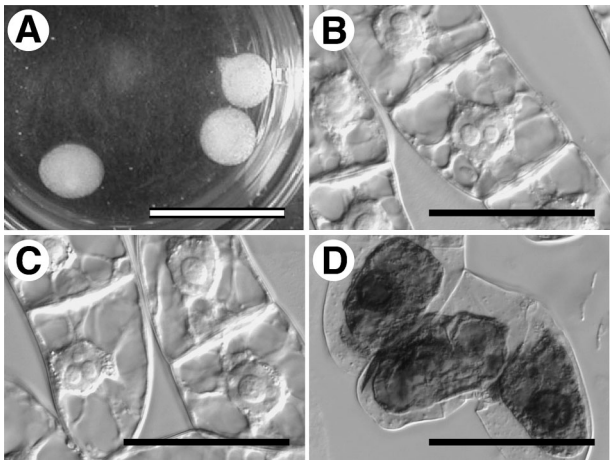


Figure 2. Encapsulation of BY-2 cells in an alginate gel bead. (A) The alginate bead. Bar, 1 cm. (B) Control cells without treatment. (C) Cells remain alive after cryopreservation. (D) Dead cells after cryopreservation. The cells were stained with Evans blue on day 1 of culture and observed under a differential interference contrast microscope. Bars in (B)–(D), 50 μm.

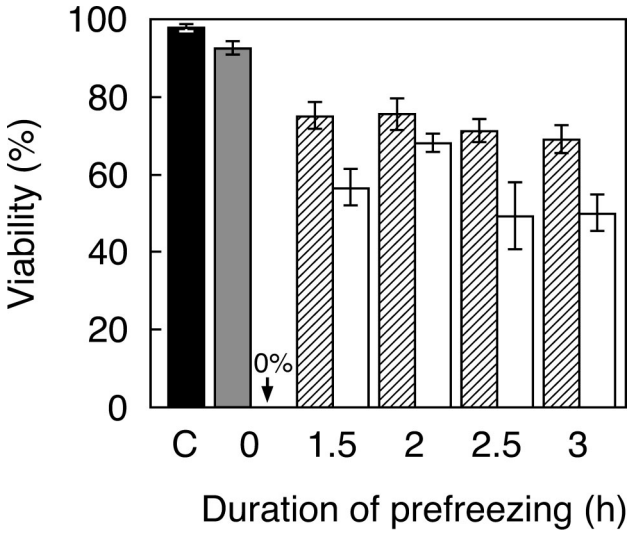


Figure 3. Effect of prefreezing duration on the viability of BY-2 cells after cryopreservation. The encapsulated cells were treated with cryoprotectant solution containing 2 M glycerol and 0.4 M sucrose for 45 min at 25°C and stored in a freezer at −30°C for the indicated times, then plunged into LN. Closed columns, cells without treatment (encapsulated control); gray columns, cells treated with cryoprotectant (treated control); hatched columns, cells cooled to −30°C (frozen control); open columns, cells cryopreserved in LN. Results represent the means and standard deviations of 3 independent experiments.

(Figure 2D).

Encapsulation in alginate gel itself had little effect on the viability of BY-2 cells (Figure 3). The viability of prefrozen cells stored in a freezer at −30°C for 1.5–3.0 h was around 70–75%. However, the duration of prefreezing changed the viability of cells after storage in LN from 50% to 69%, whereas cells without prefreezing were not viable. Prefreezing in a freezer at −30°C for 2 h produced the highest level of cell viability after storage in LN at 69%.

The duration of treatment with cryoprotectant solution was crucial to successful cryopreservation (Figure 4). The treatment (0–60 min) alone, without prefreezing or storage in LN, had no effect on the viability of the cells. Almost all cells without cryoprotectant treatment were unviable after either prefreezing or storage in LN. In contrast, cells treated with cryoprotectant survived after prefreezing and even after storage in LN. The viability of

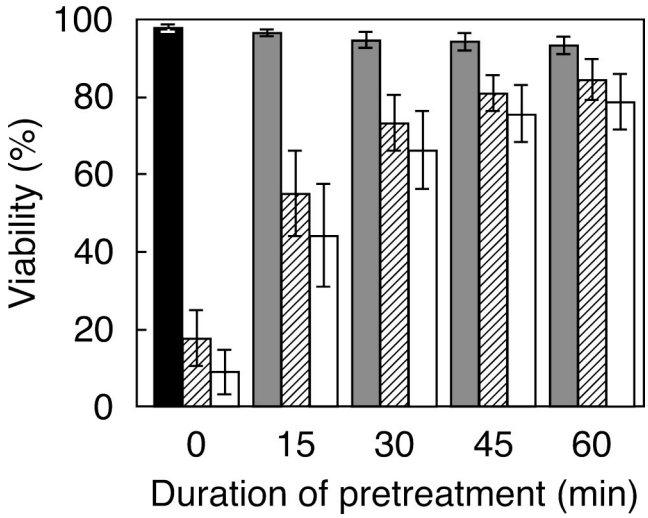


Figure 4. Effects of duration of pretreatment in cryoprotectant solution on the viability of BY-2 cells after cryopreservation. The encapsulated cells were treated with cryoprotectant solution containing 2 M glycerol and 0.4 M sucrose for the indicated times, prefrozen in a freezer for 2 h at −30°C, and then plunged into LN. Closed columns, cells without treatment (encapsulated control); gray columns, cells treated with cryoprotectant (treated control); hatched columns, cells cooled to −30°C (frozen control); open columns, cells cryopreserved in LN. Results represent the means and standard deviations of 3 independent experiments.

cells after storage in LN increased with increasing duration of the cryoprotectant treatment, and reached to around 79% after 45 min treatment.

Concentrations of sucrose from 0.09 M to 0.6 M in the cryoprotectant solution containing 2 M glycerol had a slight effect on the viability of cells (Figure 5A). The highest cell viability after storage in LN was 66%, achieved with 0.4 M sucrose. On the other hand, concentrations of glycerol from 0 M to 2 M in the cryoprotectant solution containing 0.09 M sucrose had a significant effect on the viability of cells. The highest cell viability after storage in LN was 51%, achieved with 2 M glycerol (Figure 5B). No cells were viable after treatment with cryoprotectant solution containing 0.09 M sucrose alone. These results suggest that glycerol is a key factor in cryopreservation.

In conclusion, we determined the optimal conditions for the cryopreservation of BY-2 cells to be as follows: cryoprotectant solution, 2 M glycerol and 0.4 M sucrose in LS medium; cryoprotectant solution treatment, 45 min at 25°C; duration of prefreezing at −30°C in a freezer, 2 h. Cryopreservation experiments using this protocol revealed that the mean cell viability from 3 independent experiment with 3 beads each was 67% and the regrowth efficiency of the cell suspension cultures from 7 independent experiments with total 33 vials was 100%.

Regrowth of suspension cell cultures

Using the optimal conditions established in the present study, the proliferation potential and mitotic activity of

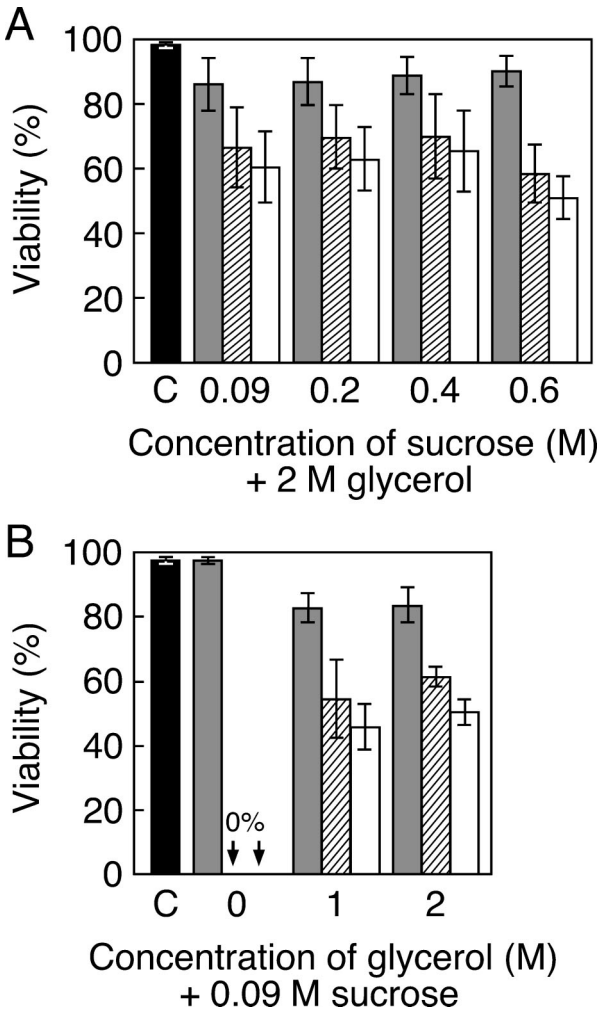


Figure 5. Effects of the concentrations of sucrose (A) and glycerol (B) in the cryoprotectant solution on the viability of BY-2 cells after cryopreservation. Encapsulated cells were treated with cryoprotectant solution supplemented with the indicated concentrations of sucrose and glycerol for 45 min at 25°C, prefrozen in a freezer for 2 h at −30°C, and then plunged into LN. Closed columns, cells without treatment (encapsulated control); gray columns, cells treated with cryoprotectant (treated control); hatched columns, cells prefrozen at −30°C (frozen control); open columns, cells preserved in LN. Results represent the means and standard deviations of 3 independent experiments.

cryopreserved BY-2 cells were examined relative to those of encapsulated control cells without immersion in LN. The number of dead cells increased markedly after cryopreservation, which caused a reduction in viability on day 1 of culture (Figure 6A). Following a 1-day lag phase, the number of living cells increased as the duration of culture increased. The growth rate of the cryopreserved cells was similar to that of encapsulated control cells. The mitotic index of cryopreserved cells on day 1 of culture was equal to that of the encapsulated control cells (Figure 6B), indicating that the cryopreserved cells recovered their capacity for growth. The mitotic indices of both the cryopreserved and control cells were constant during culture.

The cryopreserved cells rapidly proliferated in the

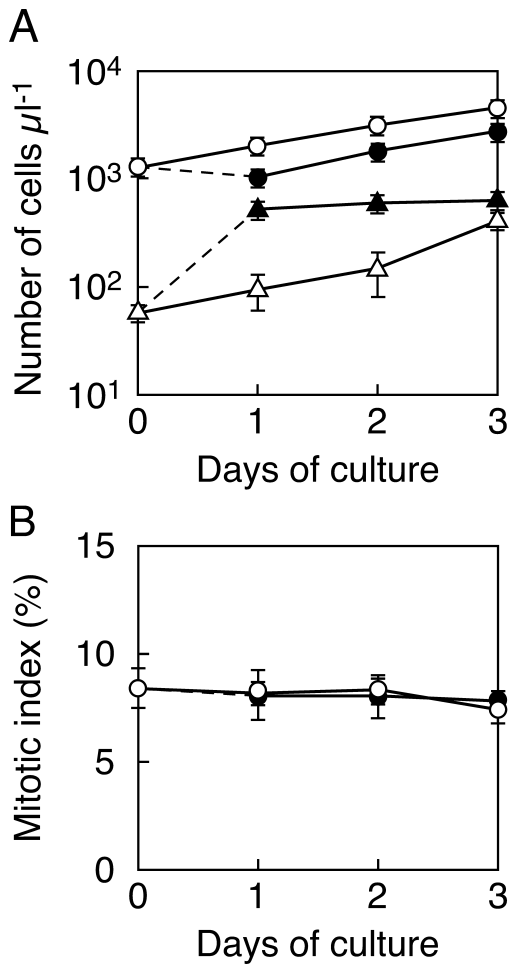


Figure 6. Recovery of proliferation potential and mitotic activity by BY-2 cells after cryopreservation. (A) Proliferation potential. Open circles, living cells among encapsulated control cells; open triangles, dead cells among encapsulated control cells; closed circles, living cells among cryopreserved cells; closed triangles, dead cells among cryopreserved cells. (B) Mitotic activity. Open circles, encapsulated control cells; closed circles, cryopreserved cells. Results represent the means and standard deviations of 3 independent experiments.

alginate beads (Figures 7A, B, C). The beads were then crushed to release the cells into the culture medium on day 3 of culture (Figures 7D, E), and suspension cell cultures were grown within 7 days (Figure 7F). The cell suspension was transferred to fresh medium and subcultured at 1-week intervals (Figure 7G). The cells of this cell suspension that recovered from cryopreservation were examined for morphological differences and changes in growth profiles compared with cells of the normal cell line. There were no obvious morphological differences between the cryopreserved cells and the untreated control cells (Figures 8A, B). The growth profiles of the cryopreserved cell lines were identical to that of the control line (Figure 8C).

Discussion

Cell viability tests using 2,3,5-triphenyltetrazolium chloride or fluorescein diacetate frequently produce inaccurate results, due to technical difficulties (Ishikawa et al. 1995, 1996; Verleysen et al. 2004). In this study, the Evans blue exclusion method was effective in determining the viability of BY-2 cells (Figure 2). Living cells extrude Evans blue dye via the intact plasma membrane, whereas dead cells accumulate the dye through the damaged membrane (Kadota et al. 2004). The viability of cryopreserved cells was determined on day 1 of culture when the cells started to propagate, because we could not evaluate the viability of cells that were still recovering from plasmolysis immediately after re-warming.

BY-2 cells treated with cryoprotectant solution for 45 min acquired tolerance to cooling to −30°C, subsequent storage at −30°C, and exposure to LN (Figure. 4). Previous reports have shown that prolonged preculture in a medium enriched with sugars or sugar alcohols for several days is indispensable for the cryopreservation of suspension cells (Gazeau et al. 1998; Moran et al. 1999; Swan et al. 1999; Zhang et al. 2001). However, preculture of BY-2 cells in medium containing 0.3 M sucrose, mannitol, or sorbitol for 1day resulted in a marked reduction in cell viability (data not shown). Therefore, short-term treatment with cryoprotectant solution may be appropriate for the cryoprotection of cultured cells that are sensitive to long-term preculture.

Cryoprotection with a combination of 2 M glycerol and 0.4 M sucrose resulted in the highest level of cell viability after cryopreservation (Figure 5). Glycerol appears to be essential for the cryopreservation of BY-2 cells. This result is consistent with a recent study demonstrating that glycerol is a more effective cryoprotectant than other polyalcohols and sugars (Turner et al. 2001). That report proposed that the superior cryoprotective qualities of glycerol are due to its stereochemical arrangement of hydroxyl groups and small molecular size. Moreover, glycerol and sucrose are less cytotoxic than other common cryoprotectants such as dimethyl sulfoxide, propylene glycol, and ethylene glycol (Sakai et al. 1991; Joshi and Teng 2000).

Freezing at a slow cooling rate in the presence of cryoprotectants causes the freeze-induced dehydration of cells (Schrijnemakers and Van Iren 1995). Sufficiently dehydrated cells are vitrified by rapid immersion into LN and able to survive without cryoinjury caused by the formation of intracellular ice crystals. Slow prefreezing (equilibrium freezing) procedure with a slow cooling rate can be achieved with simple cooling in a laboratory freezer rather than a programmable freezer (Sakai et al. 1991; Menges and Murray 2004; Winkelmann et al. 2004). With our convenient procedure, BY-2 cells are cryopreserved by simple cooling in a laboratory freezer at −30°C without specialized equipment (Figure 3).

Suspension cells immobilized in alginate gel beads

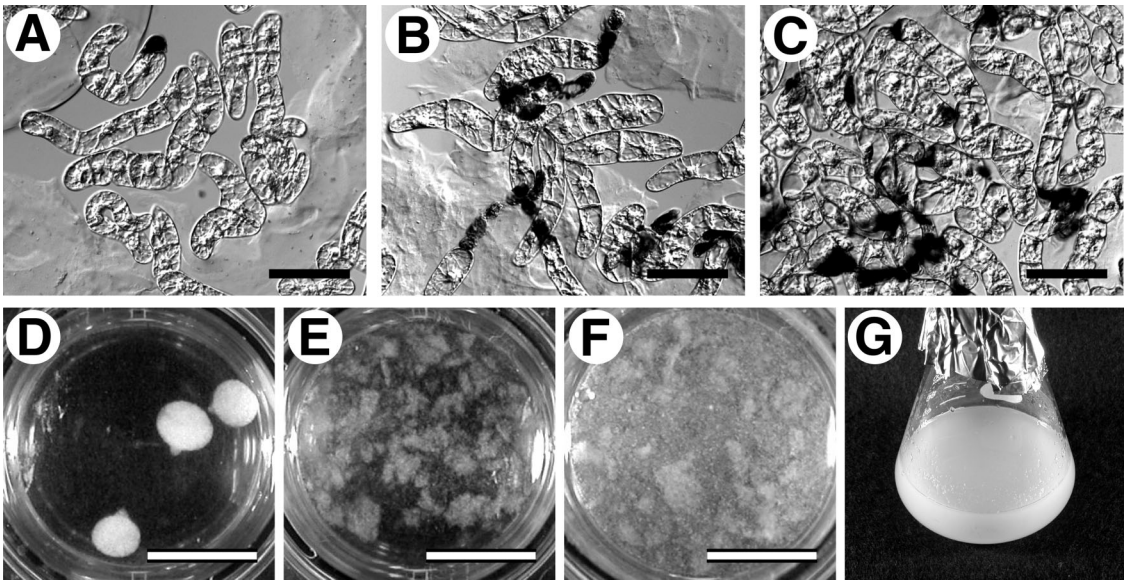
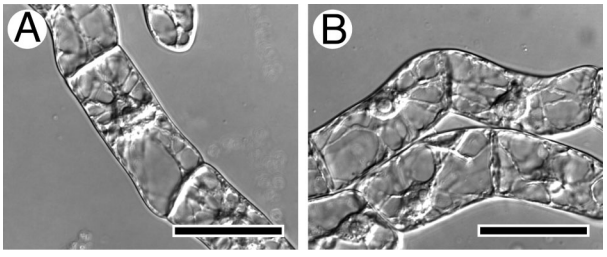


Figure 7. Regrowth of BY-2 cells after cryopreservation and re-establishment of cell suspension cultures. (A) Encapsulated cells before cryopreservation. (B) Cryopreserved cells on day 1 of culture. (C) The cryopreserved cells on day 3 of culture. The dead cells were stained black with Evans blue. Bars in (A)–(C), 100 μm. (D)–(F) Regrowth of cell suspension culture. (D) Alginate beads on day 3 of culture. (E) Beads crushed to release the embedded cells. (F) Suspension cultures after a further 4 days. Bars in (D)–(F), 1 cm. (G) Re-established suspension culture.



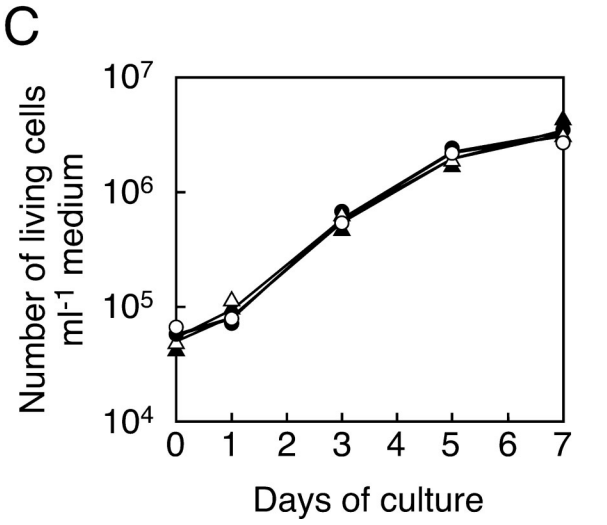


Figure 8. Growth characteristics of BY-2 cell lines recovered from cryopreservation. Morphology of untreated control cells (A) and cryopreserved cells (B) on day 3 of subculture. Bars, 50 μm. (C) Growth profiles of untreated control cell line (open circles) and three cryopreserved cell lines (closed circles, open and closed triangles). The three cryopreserved cell lines were subcultured 3 times at 1-week intervals after cryopreservation.

were efficiently cryopreserved in LN. The alginate gel protects the embedded cells from mechanical stress, direct exposure to concentrated cryoprotectants, and severe changes in osmotic pressure by modulating the diffusion of the cryoprotectant (Draget et al. 1988). Furthermore, the immobilization of suspension cells in alginate beads allows the replacement of culture medium without centrifugation and easy handling (Hirai and Sakai 2003).

The retention of high cell viability during cryopreservation ensures the regrowth of cell cultures that is equivalent to the original cell lines, while minimizing genetic changes and the selection of particular types of cells (Ishikawa et al. 1995; Menges and Murray 2004). Many reports have shown that cell and organ cultures regenerated after cryopreservation retain characteristics identical to those of the original cell lines with respect to DNA polymorphisms (Ahuja et al. 2002; Hao et al. 2002; Liu et al. 2004), cytogenetic properties, (Urbanová et al. 2002), transgene expression (Ryynänen et al. 2002), and biochemical properties (Yoshimatsu et al. 1996; Jung et al. 2001). However, our knowledge of the influence of cryopreservation on the genetic stability of plant cells is still limited. We are planning genome-wide expression studies using microarray techniques and cryopreserved lines of *Arabidopsis* T87 suspension cells to assess whether the cultured cells regenerated from cryopreservation retained their genetic identity.

This simple cryopreservation protocol is potentially a good cryogenic technique for suspension cell lines. Further study is required to examine the applicability of

this procedure to a wide range of cell lines of plant species.

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