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# Cryopreservation of bromegrass (*Bromus inermis* Leyss) suspension cultured cells using slow prefreezing and vitrification procedures[1]

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**Abstract**

Cryopreservation of a non-embryogenic bromegrass suspension culture was attempted using a slow prefreezing method (two-step method) and a vitrification method. We examined 17 solutions as cryoprotectants for the slow prefreezing method. The cryoprotectant (CSP1) comprising sucrose, dimethylsulfoxide (DMSO) and glycerol (10, 10, 5%, w/v) gave the best survival (> 80%) of bromegrass cells exposed to liquid nitrogen (LN) as determined by regrowth and fluorescent diacetate (FDA) staining. The optimum conditions were: exposure time to CSP1 (30 min), cooling rates (0.3–0.5°C/min) following ice-inoculation at −8°C, prefreezing temperature (−30 to −40°C). This protocol worked well when glass vials were used as containers and not plastic cryotubes. With CSP1, the slow dropwise addition of the protectant to the cells on ice that has frequently been used could be circumvented; direct addition of CSP1 to the cells at 25°C did not affect the survival. However, dilution of CSP1 by 5-fold with 3% sucrose following a freeze-thaw had to be done slowly (taking 15 min). The same cryoprotectant (CSP1) was also effective as a pretreatment for cryopreservation of bromegrass cells by vitrification. Following direct treatment with CSP1 at 25°C for 30 min, cells were incubated in a vitrification solution, PVS2 (Sakai et al., 1990) for about 2–4 min at 25°C prior to submersion in LN. This procedure gave 30–40% survival of bromegrass cells based on regrowth and FDA staining. Without the CSP1 pretreatment, bromegrass cells did not survive LN exposure by vitrification with PVS2. Preculture with abscisic acid or with high osmoticum (0.3–0.7 M sucrose) could not be used as a substitute for pretreatment with CSP1.

*Keywords:* Bromegrass (*Bromus inermis*); Carrot (*Daucus carrota*); Cryopreservation; Vitrification; Slow prefreezing; Suspension cultures

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## 1. Introduction

During the last two decades a number of plant culture systems in vitro have been developed for clonal propagation, production of virus-free plants, transformation and regeneration, or as an experimental system for basic research. These include protoplast cultures, cell suspension cultures, cultures of meristems, somatic embryos or shoot primordia, embryogenic calli and transgenic cultures. Problems encountered in the maintenance of these cultures are that the cultures or their specific characters can be easily lost due to prolonged subculturing, contamination, limitation in labor and personnel changes. Cryopreservation has attracted attention as a method to safely preserve these cultures and their characters.

Cryopreservation is also an ideal tool for stable long-term storage of variable plant genetic resources, especially vegetatively propagated plants and recalcitrant seeds. For cryopreservation of either plant genetic resources or in vitro culture systems, development of a protocol with a high survival rate is necessary to avoid genetic changes and selection of a particular type of cell. There is also a need for a simple and widely applicable method of cryopreservation. Suspension cultures are a reliable and convenient system to use for the development of such methods since they are fast growing and homogeneous.

The objective of this study was to develop an improved cryoprotectant for cryopreservation of cell cultures that may realize high survival rates, simple and easy handling and wide applicability. Thereby we present an efficient protocol for cryopreservation of a non-embryogenic bromegrass suspension culture using a slow prefreezing method (2-step). We also demonstrate successful cryopreservation of fine suspension-cultured cells by vitrification, which has been only marginally successful in earlier studies [1].

## 2. Materials and methods

### 2.1. Cultured cells

A non-embryogenic suspension culture of *Bromus inermis* Leyss cv. Manchar was mainly used. It was maintained as previously described [2] with a slight modification. Briefly, cultures were initiated biweekly by transferring 3 ml of cells to fresh Erickson's (ER) medium (pH 5.8, 0.5 ppm 2,4-D) and incubated on a rotary shaker (80 rpm) at 25°C. Ten-day old cells were harvested and processed for cryopreservation. In an experiment to determine the effect of abscisic acid (ABA) pretreatment, cells were incubated for 7 days at 25°C in ER medium containing 20 ppm ABA as previously detailed [2,3]. Experiments were replicated three times.

### 2.2. Cryopreservation methods

#### 2.2.1. Slow prefreezing (2-step) method

Equal amounts of bromegrass cells (usually 0.2–0.3 g) were dispensed into conical centrifuge tubes (10 ml, graduated glass) with screw caps. This was done by placing equal aliquots (0.4 ml) of a stirred cell suspension into the tubes and subsequently discarding the medium using a pasteur pipette. Cryoprotectant solutions with varying concentrations of sucrose (0–20%, w/v), dimethylsulfoxide (DMSO)(0–10%, w/v), and glycerol (0–20%, w/v) were prepared (Fig. 1) by dissolving them in Mili Q water (not in the medium) and autoclaving at 121°C for 15 min. One ml aliquots of the cryoprotectant solutions were directly added to the cells and incubated at 25°C for 30 min unless otherwise noted. Then, 0.5 ml aliquots of the solutions containing only cryoprotectants were removed from the tubes and discarded, while the remainder (cells plus cryoprotectants) was processed for cryopreservation.

The tubes were directly placed at between −8 and −12°C where freezing of the cryoprotectants was initiated by touching the tubes with a liquid nitrogen (LN)-cooled steel rod for about 10 s. To complete ice inoculation, the tubes were exposed to between −8 and −12°C for another 15 min. The tubes were then cooled at 0.3°C/min to the prefreezing temperature (−30°C), unless otherwise specified, either with a program freezer or in an ethanol bath cooled manually by the addition of aliquots of LN. They were held at the prefreezing temperature for 5 min prior to immersion into LN. Following storage in LN for 1–48 h, the tubes were rapidly rewarmed in 40°C water. The cryoprotectant was slowly diluted 5-fold by dropwise addition of 3% (w/v) sucrose over 15 min at 25°C, unless otherwise noted, and processed for viability assays. Control cells treated with the cryoprotectant only were diluted and processed in the same manner.

In one experiment, the cryoprotectant was added to cells slowly in a dropwise manner (over 15 min) at 25°C and the cryopreserved cells, following a freeze-thaw cycle, were slowly diluted as described above (slow addition/slow dilution). In another experiment, the entire cryoprotectant was rapidly added to cells at 25°C and diluted rapidly by direct addition of 3 ml sucrose (3%) at 25°C (rapid addition/rapid dilution).

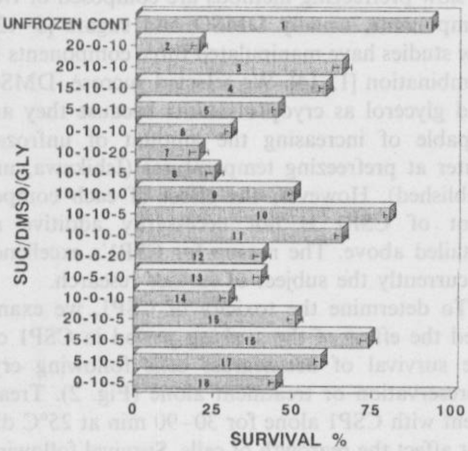


Fig. 1. Effect of different cryoprotectant mixtures (combinations of sucrose, DMSO and glycerol) on the survival of bromegrass cells exposed to LN with a slow freezing method. Cells were treated with cryoprotectants for 30 min, ice-inoculated between −8 and −12°C (1–2°C below the freezing point of each cryoprotectant), and cooled at −0.3°C/min to −30°C prior to immersion in LN. Survival was determined by FDA staining and expressed as the percentage of the control untreated and unfrozen. Data are presented as mean ± S.E. ($n = 3$). Regrowth analyses showed similar survival rates.

### 2.2.2. Vitrification method

Cells (0.2–0.3 g) dispensed in conical centrifuge tubes as described earlier were treated with 0.3 ml of a vitrification solution (PVS2: 15% (w/v) ethylene glycol, 30% (w/v) glycerol, 15% (w/v) DMSO, 0.4 M sucrose) [4] for 1–7 min at 25°C. The tubes with cells were immediately submerged in LN and stored there for more than 1 h. Following rapid thawing in 40°C water, the cells were diluted by direct addition of 2 ml of 1.2 M sucrose. After 10 min, they were further diluted with 2 ml of liquid ER medium and centrifuged at 1000 rpm for 3 min, if necessary, and processed for viability assays. Cells treated with PVS2 only were diluted in the same manner.

In some experiments the cells were preincubated in 1 ml CSP1 for 30 min (followed by complete removal of CSP1) prior to treatment with PVS2.

### 2.2.3. Viability assays

Viability of the cells was determined by regrowth or fluorescein diacetate (FDA) staining. For regrowth, the cells were transferred onto a piece of filter paper, which was blotted on paper towels to remove excess dilutant. The filter paper with cells was incubated on fresh semi-solid medium (2 pieces on 25 ml medium) at 25°C. Untreated control cells and cells killed by boiling were cultured in the same manner. The cells were harvested after 9 days of culture (in the exponential growth stage) and washed well with distilled water. The dry weight of the cells was determined by oven-drying at 70°C for 24 h. Survival rates were calculated from the dry weights of the untreated control, totally killed control, and the samples as described before [3].

FDA staining was conducted according to Ishikawa et al. [3]. Cells cryopreserved by slow prefreezing were stained with FDA 30 min after the completion of dilution. Cells cryopreserved by vitrification were stained with FDA following 2 days of incubation on the semi-solid medium because staining immediately after dilution gave erroneously higher survival scores for unknown reasons [3].

## 3. Results and discussion

### 3.1. Slow prefreezing (2-step) method

#### 3.1.1. Cryoprotectants

To find a cryoprotectant suitable for bromegrass cells, we examined a series of cryoprotectants (Fig. 1). The best survival following cryopreservation was obtained with a combination of 10% (w/v) sucrose, 10% (w/v) DMSO and 5% (w/v) glycerol (Fig. 1, lane 10). Hereafter we call this mixture CSP1 which is short for cryoprotectant for slow prefreezing # 1 and primarily used it in the rest of the experiments reported.

The effect of each component (sucrose, DMSO and glycerol) when used in various combinations on cell survival is also shown (Fig. 1). When 10% DMSO and 5% glycerol were present (lanes 10, 15–18), sucrose added at a concentration of 10% (CSP1) gave a higher survival rate than lower or higher sucrose concentrations. In the presence of 10% DMSO and 10% glycerol, increasing concentrations (0–20%) of added sucrose resulted in higher survival rates of cryopreserved bromegrass cells (lanes 3–6 and 9). In the absence of DMSO, however, increasing concentrations (10–20%) of sucrose caused a decrease in the survival (lanes 2 and 14) whereas increasing concentrations (10–20%) of glycerol resulted in higher survival rates (lanes 12 and 14). Surprisingly, there was some survival even without DMSO. DMSO has usually been thought to be an indispensable component of cryoprotectants in slow prefreezing methods [5].

In cocktails containing 10% sucrose and 10% DMSO in combination, 5% glycerol gave the best survival (CSP1) and higher concentrations of glycerol (10–20%) decreased the survival of cells (lanes 7–11). Increasing concentrations (0–10%) of added DMSO gave increased survival in the presence of 10% glycerol (lanes 2–3, 9, 13 and 14) but decreased survival (lanes 7 and 12) in the presence of 20% glycerol. It seems that glycerol and DMSO are not compatible with cell survival when both were used in high concentrations. 10% DMSO-5% glycerol cocktails (lanes 15–18 and 10) gave higher survival rates than 10% DMSO-10% glycerol cocktails (lanes 3–6 and 9) at any concentration of sucrose used.

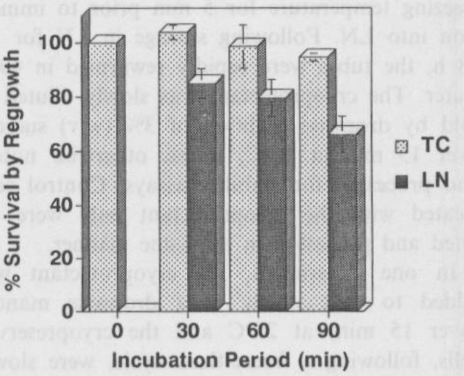


Fig. 2. Survival of bromegrass cells cryopreserved following exposure to CSP1 for different periods of time. Cooling rate: −0.3°C/min to −30°C (prefreezing) from −8°C (ice inoculation). Survival was determined by regrowth. TC, CSP1-treated controls; LN, CSP1-treated and LN-exposed. Data are presented as mean ± S.E. ($n = 3$).

To our knowledge, most cryoprotectants used in slow prefreezing methods are composed of two components, usually DMSO and sugars [6–12], few studies have manipulated three components in combination [13,14]. We selected sucrose, DMSO and glycerol as cryoprotectants because they are capable of increasing the amount of unfrozen water at prefreezing temperatures (Ishikawa, unpublished). However, the effect of each component of CSP1 is not necessarily additive as detailed above. The reason for CSP1's excellence is currently the subject of further research.

To determine the toxicity of CSP1, we examined the effect of the soaking period in CSP1 on the survival of bromegrass cells following cryopreservation or treatment alone (Fig. 2). Treatment with CSP1 alone for 30–90 min at 25°C did not affect the regrowth of cells. Survival following cryopreservation was best (85%) at 30 min soaking period as determined by regrowth. Thus we adopted 30 min soaking regime in subsequent experiments.

#### 3.1.2. Cooling rates and prefreezing temperatures

Regrowth results show that the best survival was obtained when cells were cooled at −0.3°C/min and prefrozen to −30 or −40°C (Figs. 3

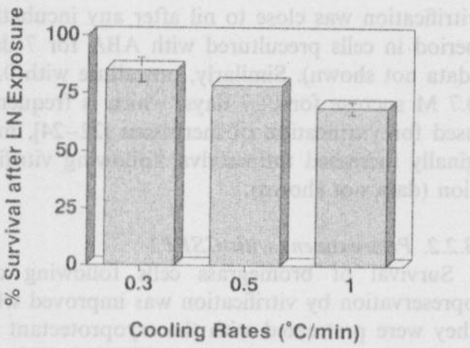


Fig. 3. Survival of bromegrass cells immersed in LN following prefreezing to −30°C at different cooling rates. Cells were treated with CSP1 for 30 min and ice-inoculated at −8°C. Survival was estimated by regrowth and expressed as the percentage of untreated control cells. Data are presented as mean ± S.E. ($n = 3$).

and 4). To make the protocol time shorter, the cells were prefrozen to −30°C at −0.3°C/min. The optimum range for cooling rates was broad (Fig. 3).

### 3.1.3. Addition and dilution of CSP1

In most cases of cryopreservation by slow prefreezing that have been reported, cryoprotectants were added slowly in a dropwise or gradual manner and/or on ice [6–19]. Surprisingly, dropwise addition of CSP1 gave a lower survival rate than rapid (direct) addition when the dilution step was done slowly (Fig. 5). The mechanism of this phenomenon is not known, but the cumbersome slow addition step can be circumvented by the use of CSP1. However, a comparison of the dilution step done slowly and rapidly shows that this step is crucial and has to be conducted slowly at 25°C (Fig. 5). Finkle and Ulrich [14] found that dilution of their cryoprotectant at 22°C gave better survival than dilution at 0°C while a slow gradual addition of the cryoprotectant at 0°C was essential.

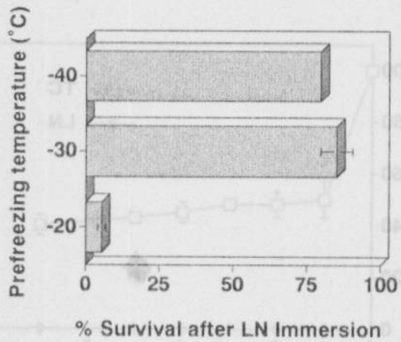


Fig. 4. Effect of prefreezing temperatures on the survival of bromegrass cells submerged in LN. Cells were treated with CSP1 for 30 min and cooled at −0.3°C/min to the prefreezing temperatures following ice inoculation. Survival was determined by regrowth and expressed as the percentage of untreated control cells. Data are presented as mean ± S.E. ($n = 3$).

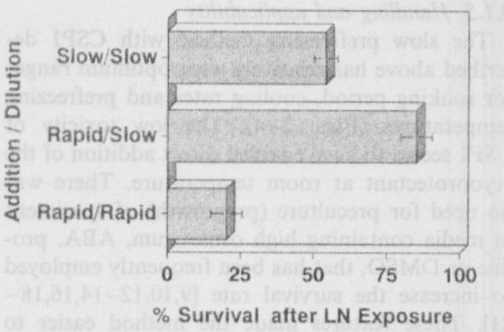


Fig. 5. Effect of differentially combined addition (rapid or slow) and dilution (rapid or slow) of the cryoprotectant (CSP1) on the survival of cryopreserved bromegrass cells. Cells were processed as described in Fig. 1. Survival was estimated by regrowth and expressed as the percentage of untreated control cells. Data are presented as mean ± S.E. ($n = 3$).

### 3.1.4. Containers

In all the experiments shown above, glass centrifuge tubes were used. We attempted to see if they could be replaced by plastic cryotubes. When the identical protocol (30 min CSP1, ice-inoculation at −8°C followed by prefreezing to −30°C at −0.3°C/min, rapid addition/slow dilution) was used, cryotubes gave lower survival (20%) than glass tubes (> 80%), as determined by regrowth. This is probably due to the lower heat conductivity of the plastic tubes. Thawing of cells in glass tubes were completed in about 30 s whereas it took 3 or 4 times longer in plastic cryotubes. This protocol is therefore to be used with glass tubes.

### 3.1.5. Handling and applicability

The slow prefreezing method with CSP1 described above has relatively wide optimum ranges for soaking period, cooling rates and prefreezing temperatures (Figs. 2–4). The low toxicity of CSP1 seems to have enabled direct addition of the cryoprotectant at room temperature. There was no need for preculture (pregrowth) of specimens in media containing high osmoticum, ABA, proline or DMSO, that has been frequently employed to increase the survival rate [9,10,12–14,16,18–21]. These features made the method easier to handle and less time-consuming. The whole process from the harvest of cells to submersion into LN took no more than 3 h. This method has been successfully applied to cryopreservation of carrot suspension-cultured cells (Ishikawa, unpublished data), melon somatic embryos (Niwata et al., unpublished), cultured shoot primordia of melon (Ogawa et al., unpublished) and embryogenic calli of taro [21]. Each had more than 70% survival rates based on regrowth and the method seems to have a reasonably wide applicability, especially for liquid cultured materials and/or fine cultured cells. This method seems to be applicable to cultures derived from both chilling sensitive plants (melon, taro) and cold-hardy plants (bromegrass, carrot). Bromegrass and carrot cultures recovered from cells cryopreserved with this method were very similar to the non-cryopreserved cultures in the morphology and growth characteristics. Survival rates following cryopreservation of 70–85% may be sufficiently high to avoid selection of a particular cell type and/or genetic changes.

## 3.2. Vitrification method

### 3.2.1. With or without preculture

When bromegrass cells were directly treated with PVS2 without pretreatment, there was a marked loss of cell viability in the control treated and unfrozen (TC, Fig. 6). This indicates that PVS2 is toxic to bromegrass cells. There was no survival following a LN plunge after any period of incubation with PVS2 (LN, Fig. 6). Application of exogenous ABA is known to induce increased freezing and salinity tolerance in bromegrass cells [3]. However, survival following vitrification was close to nil after any incubation period in cells precultured with ABA for 7 days (data not shown). Similarly, preculture with 0.3–0.7 M sucrose for 2–7 days, which is frequently used for vitrification of meristems [22–24], marginally increased the survival following vitrification (data not shown).

### 3.2.2. Pretreatment with CSP1

Survival of bromegrass cells following cryopreservation by vitrification was improved when they were pretreated with the cryoprotectant developed for slow prefreezing (CSP1) for 30 min prior to incubation with PVS2. With CSP1 pretreatment, the best survival (about 40%) was obtained after 3 min incubation in PVS2 as determined by FDA staining and regrowth (Fig. 7).

This method (CSP1-PVS2) was applied to carrot suspension-cultured cells which are more resistant to high osmotic stress than bromegrass cells but marginally tolerant to a LN plunge with PVS2 alone (data not shown). Carrot cells pretreated with CSP1 for 30 min then cryopreserved by vitrification with PVS2 for 1–3 min gave over 85% survival (Ishikawa, unpublished). The CSP1-PVS2 vitrification method seems to be suitable for culture materials (cells, meristems) that are moderately resistant to osmotic stress but sensitive to

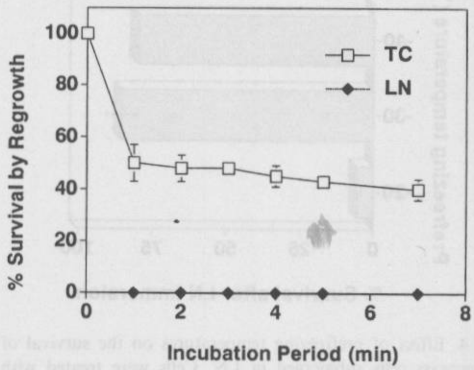


Fig. 6. Survival of bromegrass cells treated with PVS2 for various periods of time (TC) and then exposed to LN (LN). Survival was determined by regrowth and expressed as the percentage of untreated control cells. Data are presented as mean ± S.E. ($n = 3$).

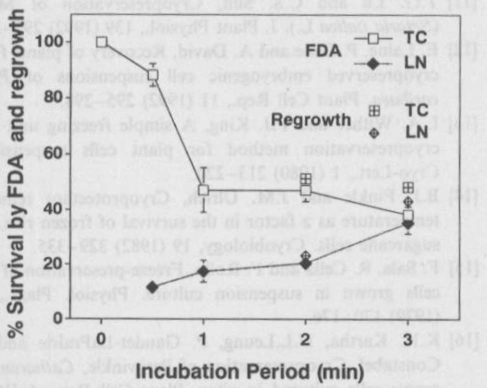


Fig. 7. Improved survival by pretreatment with CSP1 for 30 min of bromegrass cells cryopreserved by vitrification following incubation with PVS2. TC, PVS2-treated cells; LN, cells treated with PVS2 and plunged into LN. Survival was estimated by FDA staining and regrowth (2–3 min incubation period only) and expressed as the percentage of untreated control cells. Data are presented as mean ± S.E. ($n = 3$).

direct exposure to PVS2. The slow prefreezing method using CSP1 described earlier in this paper, was suitable for fine and/or liquid cultured materials, that are more sensitive to high osmotic stress.

PVS2 was first developed with a suspension culture of navel orange [4]. However, to our knowledge, this nucellar cell culture is an exceptional case as it is extremely tolerant to stresses. Cryopreservation of many other suspension cultured cells by vitrification had poor results [1]. Cell suspension cultures of bromegrass and carrot are no exceptions (Fig. 6). This is in contrast to meristems, many of which have been successfully cryopreserved by vitrification [22–25]. The reason for poor survival in suspension cultured cells could be that the penetration of the vitrification solution to fine cells is very rapid and makes the cells leaky simultaneously. The cells may lose vital components which easily defuse to the solution due to the large relative surface area and can not be readily reabsorbed. In the case of more complex tissues, the penetration of the vitrification solution is more gradual and substances leaked into the intercellular space, if any, are easily reabsorbed. Pretreatment with CSP1 resulted in increased survival following vitrification from 0 to 40% in the case of bromegrass cells and from 0 to 85% in the case of carrot cells. The mechanism of improvement of survival by CSP1 was yet to be studied.

### 3.3. Conclusions

We examined various combinations of sucrose, DMSO and glycerol and developed a cryoprotectant for the slow prefreezing method (CSP1): The effect of sucrose was equivocal whereas DMSO and glycerol were not compatible with cell survival when both of them were used at high concentrations. DMSO is not necessarily an indispensable component of an efficient cryoprotectant. CSP1 (sucrose, DMSO, glycerol: 10, 10, 5% w/v) has relatively wide optimal ranges and low toxicity, and is easy to handle. Over 80% of bromegrass cells survived cryopreservation when the cells were soaked in CSP1 for 30 min at 25°C, ice-inoculated at −8°C, then cooled at 0.3–0.5°C/min to −30 or −40°C prior to submersion in LN. CSP1 could be directly added to the cells at room temperature prior to cryopreservation, but slow dilution of CSP1 with 3% sucrose following cryopreservation and the use of glass vials are essential for a high rate of cell survival. This protocol does not require preculture of cells in high osmoticum or DMSO containing medium and is less time-consuming than previously published slow prefreezing methods. It is applicable to cryopreservation of cultured cells of carrot, melon and taro and survival rates of 70% or more is possible. This method is suitable for liquid cultured and/or fine cultured cells, especially ones that are sensitive to high osmotic stress.

CSP1 is also effective as a pretreatment for vitrification of bromegrass cells. Soaking in CSP1 for 30 min at 25°C followed by 3 min exposure to PVS2 at 25°C gave about 40% survival of vitrified bromegrass cells and >85% of carrot cells (regrowth estimates). The vitrification procedure using CSP1-PVS2 is suitable for cultured materials that are sensitive to direct exposure to PVS2 but moderately resistant to high osmotic stress.

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