ChinaRxiv 中X

AI translation · View original & related papers at
chinarxiv.org/items/chinaxiv-201806.00249

Effects of Different Cryopreservation Conditions on the Viability and Regeneration Capacity of Sugarcane Protoplasts: Postprint

Authors: Li Suli, Song Yani, Li Zhigang, Liu Fangjun, Lai Peiheng, Qin Xiaoyi, Li Jiajun

Date: 2018-06-25T00:00:00+00:00

Abstract

To obtain sugarcane protoplasts with high viability and strong regenerative capacity, this study investigated the cryopreservation solution composition, cryopreservation temperature, and tissue source of sugarcane protoplasts. The results revealed: (1) Different cryopreservation solutions, cryopreservation temperatures, and tissue sources for protoplast cryopreservation and recovery exerted significantly differential effects on the viability of sugarcane protoplasts. Among three cryopreservation solution combinations, combination 2 (70% medium + 20% serum + 10% DMSO) yielded the highest viability after 30 days of cryopreservation and recovery, reaching 72%. For recovery within 90 days of cryopreservation, no significant difference in viability was observed between protoplasts cryopreserved in liquid nitrogen at -196°C and those in a -80°C freezer, with both achieving rates above 75%; however, after 90 days of cryopreservation and recovery, protoplasts cryopreserved in liquid nitrogen at -196°C exhibited stronger viability than those from -80°C freezer storage. Comparing different tissue sources, protoplasts recovered from young leaves after 30 days of cryopreservation showed relatively high viability at 79.2%, whereas those from shoot tips showed only 42.7% viability. (2) For different cryopreservation solutions and temperatures, the timing of initial cell division and cell cluster formation showed no significant differences; generally, the cell wall became essentially complete after 5-6 days of culture, cell division initiated after 6 days, and cell clusters formed after 15 days. Comparing different tissue sources, protoplasts obtained from enzymatic digestion of shoot tips demonstrated the strongest regenerative capacity, forming cell walls 3 days earlier and initiating first division 2 days earlier than protoplasts from young leaf enzymatic digestion. The research findings will provide a scientific basis for ultra-low temperature preservation of sugarcane protoplasts and supply materials and technical support for somatic cell fusion breeding, genetics, and transgenic research.

ChinaRxiv 中X

Full Text

Preamble

Effects of Different Freezing Conditions on the Vitality and Regeneration Ability of Sugarcane Protoplasts

Li Suli, Song Yani, Li Zhigang*, Liu Fangjun, Lai Peiheng, Qin Xiaoyi, Li Jiajun
(College of Agriculture, Guangxi University, Nanning, 530005, China)

Abstract: To obtain sugarcane protoplasts with high vitality and strong regeneration capacity, this study investigated the effects of cryoprotectant composition, storage temperature, and tissue source on protoplast viability after cryopreservation. The results showed: (1) Significant differences in protoplast vitality were observed among different cryoprotectants, storage temperatures, and tissue sources. Among the three cryoprotectant formulations, Combination 2 (70% medium + 20% serum + 10% DMSO) yielded the highest post-thaw viability after 30 days, reaching 72%. Within 90 days of storage, no significant difference in viability was found between liquid nitrogen (-196°C) and -80°C freezer storage, with both maintaining viability above 75%. However, after 90 days, liquid nitrogen storage resulted in significantly higher viability than -80°C storage. Comparing different tissue sources, protoplasts derived from young leaves showed higher viability (79.2%) than those from stem tips (42.7%) after 30 days of cryopreservation. (2) No significant differences were observed in the timing of first cell division and cell cluster formation among different cryoprotectants and storage temperatures. Generally, after 5-6 days of culture, cell wall formation was essentially complete; cell division initiated after 6 days, and cell clusters formed by 15 days. However, protoplasts from stem tips demonstrated stronger regeneration capacity than those from young leaves, forming cell walls 3 days earlier and initiating division 2 days earlier. These findings provide a scientific basis for the cryopreservation of sugarcane protoplasts and offer material and technical support for somatic cell fusion breeding, genetic research, and transgenic studies.

Keywords: sugarcane, protoplast, cryopreservation, vitality, regeneration

Funding: This work was supported by the National Science and Technology Support Program of China (2008BADB8B00), the National Natural Science Foundation of China (31460373), the Natural Science Foundation of Guangxi (2011GXNSFA01806), and the Guangxi Science and Technology Key Project (1222014-2B).

Corresponding Author: Li Zhigang, Ph.D., Professor, specializing in plant stress physiology. Email: lizhigangnn@163.com

---

Introduction

Plant somatic cell fusion breeding is a technique based on cell totipotency and membrane fluidity principles, wherein cell walls are enzymatically removed and protoplasts from different species, genera, or even families are artificially induced to fuse and regenerate into hybrid plants through in vitro culture. This approach breaks through sexual incompatibility barriers, enabling broad genetic recombination to create novel hybrids unattainable through conventional breeding. Unlike genetic engineering, cell fusion avoids complex gene manipulation procedures such as isolation, purification, cleavage, and ligation, and does not raise biosafety concerns since transferred genes originate from the plants themselves. The technical requirements and equipment are less complex, requiring lower investment and facilitating widespread research and application (Li Zhiyong, 2003). Moreover, somatic hybridization involves complex genetic recombination rather than simple parental gene stacking, making it highly desirable for crop improvement.

Protoplasts are essential materials for somatic cell fusion and are also ideal for mutant screening and genetic transformation research. However, plant material availability is constrained by seasonal and developmental factors, limiting the flexibility of protoplast fusion experiments. Currently, most laboratories obtain protoplasts through immediate sampling and enzymatic digestion, which is time-consuming and inefficient—excess protoplasts must either be cultured or discarded, and experiments may be suspended during off-seasons due to material shortage (Zhuang Chunhui, 2015). Cryopreservation technology effectively addresses these issues, as ultra-low temperatures enable excellent germplasm preservation. With increasing success in protoplast regeneration across species, protoplasts from major cereals such as maize, rice, and wheat have become important materials for germplasm storage (Takeuchi et al., 1982).

The primary damage to protoplasts during freezing comes from ice crystal formation. Dimethyl sulfoxide (DMSO) is a highly effective permeable cryoprotectant that rapidly penetrates cells and lowers the freezing point of cytoplasm, preventing damage from severe dehydration during freeze-thaw cycles (Huang Ling, 2011). Many laboratories widely use DMSO as a cryoprotectant due to its low cost, excellent protective effect, and ease of removal (Lü Weimin et al., 2014). However, different DMSO concentrations can trigger extensive apoptosis upon thawing, exerting toxic effects on cells (Li Zhongwen et al., 2016). Optimizing cryopreservation conditions to minimize cryoprotectant toxicity and maximize preservation of protoplast biological characteristics is crucial for successful somatic cell fusion breeding.

Sugarcane is a vital pillar of China' s sugar industry and a source of renewable energy, fiber, sugar-based chemicals, and feedstock. However, sugarcane production faces challenges from limited cultivar diversity and inadequate new varieties, severely impacting industry competitiveness. Enhancing sugarcane breeding innovation to provide superior varieties has become an urgent eco-

nomic priority. As a subtropical crop with a long growth period, sugarcane production is restricted by season and geography. Without appropriate cryopreservation methods, protoplast fusion research cannot access materials outside the growing season or subtropical regions, while abundant experimental materials during the growing season cannot be utilized or preserved long-term, severely limiting research progress. This study addresses these issues by investigating the effects of different cryoprotectants, storage temperatures, and tissue sources on the viability and regeneration capacity of sugarcane protoplasts after cryopreservation, aiming to provide reserve materials for future somatic cell fusion and genetic transformation research.

Materials and Methods

Protoplast Isolation

Sugarcane protoplasts were isolated from *Saccharum officinarum* L. cv. ROC22 using the method described by Huang Ling (2011) with minor modifications. Plant materials were obtained from the sugarcane experimental base at Guangxi University College of Agriculture. The enzyme solution consisted of 2% cellulase, 0.5% pectinase, 0.1% macerozyme, and 0.3% hemicellulase. The washing solution was CPW medium containing 9% mannitol. Both enzyme solution and washing solution were adjusted to pH 5.8.

Tissue Sources and Enzymatic Digestion

Two tissue sources were compared: young leaves and stem tips at the early elongation stage.

Young Leaves: Healthy sugarcane leaf sheaths were selected as starting material. After removing 2-3 outer leaf sheaths, the tissue was surface-sterilized with 75% ethanol for 30 seconds and rinsed three times with sterile water. The outer layers and both ends were excised to expose the pale yellow heart leaves. The top 1-5 cm of tender leaves above the shoot apex was retained and sliced into ~1 mm thick sections. Approximately 0.5 g of leaf tissue was collected and treated with 5 mL CPW medium containing 13% mannitol for 0.5-1 hour to induce plasmolysis. After removing the plasmolysis solution, 5 mL of enzyme mixture was added for enzymatic digestion at room temperature for 4 hours. The digested material was sequentially filtered through 100-mesh and 200-mesh cell strainers, and protoplasts were purified by gradient centrifugation.

Stem Tips: Sugarcane stem tips at the early elongation stage were collected. After removing young leaves, the tissue was surface-sterilized with 75% ethanol for 30 seconds and rinsed three times with sterile water. Outer layers and both ends were excised, and several large young leaves enclosing the shoot apex were removed, retaining only the 3rd to 6th young leaves or leaf primordia near the apical meristem. The stem tip was trimmed into 1 cm $\times$ 1 cm $\times$ 2 cm blocks

containing leaf primordia, shoot apex, primary thickening meristem, and partial mature tissue, then sliced into ~1 mm sections. The subsequent plasmolysis and enzymatic digestion procedures were identical to those for young leaves.

Cryoprotectant Formulations and Freezing Protocol

Three cryoprotectant formulations were prepared using KM8P medium, bovine serum albumin (BSA), and varying DMSO concentrations: - Combination 1: 75% medium + 20% BSA + 5% DMSO - Combination 2: 70% medium + 20% BSA + 10% DMSO - Combination 3: 65% medium + 20% BSA + 15% DMSO

Isolated protoplasts were adjusted to a density of 1×10 cells/mL using a hemocytometer. Three storage temperatures were tested: 4°C, -80°C, and -196°C, with three replicates per treatment. Each cryovial contained 200 L of protoplast suspension. Samples were stored for 7, 30, 60, and 90 days before thawing to assess viability and regeneration capacity.

A slow-freezing protocol was employed: cryovials were first placed at 4°C for approximately 40 minutes, then transferred to -20°C for 30-60 minutes, followed by overnight storage at -80°C, and finally transferred to liquid nitrogen at -196°C for long-term storage.

Thawing and Recovery

Rapid thawing was performed by immersing cryovials from liquid nitrogen or -80°C freezer directly into a 37°C water bath with continuous agitation. After thawing, the cryoprotectant solution was removed by centrifugation, and protoplasts were washed three times with CPW medium containing 9% mannitol before being resuspended in KM8P culture medium.

Viability Assessment

Protoplast viability was determined using trypan blue exclusion according to Li Yuzhu (2012). A 4% trypan blue stock solution was prepared by dissolving 4 g trypan blue in distilled water, adjusting to 100 mL, filtering, and storing at 4°C. For viability assays, the stock solution was diluted to 0.4% with PBS (Widholm, 1972). Dead cells stained blue, while viable cells remained unstained. Viability was calculated as: (number of viable protoplasts / total number of protoplasts) × 100%.

Cell Wall Regeneration Observation

Cell wall formation was monitored using fluorescent brightener VBL according to Liu Lin et al. (2014). VBL was dissolved in 9% CPW solution (pH 5.8) at a concentration of 0.1%, centrifuged at 85×g for 10 minutes, and the supernatant was used for staining (Huang Xianghui and Yan Jiqiong, 1980). An equal volume of VBL staining solution was added to protoplast suspensions and incubated at room temperature for 5 minutes. After washing 3-4 times with 9% CPW

solution, stained protoplasts were observed under a fluorescence microscope at 345 nm excitation and 100× magnification to assess cell wall development.

Results

Effects of Cryoprotectant Formulation on Protoplast Viability

As shown in Table 1 , all three cryoprotectant formulations resulted in reduced protoplast viability compared to fresh controls after various storage durations. The extent of viability loss followed the pattern: Combination 1 (75% medium + 20% BSA + 5% DMSO) > Combination 3 (65% medium + 20% BSA + 15% DMSO) > Combination 2 (70% medium + 20% BSA + 10% DMSO), with significant differences among formulations.

Combination 2 proved optimal for sugarcane protoplast cryopreservation. After 7 days of storage, Combination 2 yielded 27.69% higher viability than Combination 1 and 18.43% higher than Combination 3. After 30 days, these differences were 27.13% and 16.17%, respectively. Even after 90 days, Combination 2 maintained 28.45% and 18.36% higher viability than Combinations 1 and 3, respectively.

Effects of Tissue Source on Protoplast Viability

Table 2 demonstrates that protoplasts derived from young leaves consistently exhibited significantly higher viability than those from stem tips after 7, 15, and 30 days of cryopreservation, with viability differences of 35.91%, 37.56%, and 36.5%, respectively. While both tissue sources showed approximately 10% viability loss after 7 days of storage, no significant differences in viability decline were observed among the different storage durations.

Effects of Storage Temperature on Protoplast Viability

Figure 1 [Figure 1: see original paper] reveals that after 7 days of storage, no significant differences in viability were detected among the three temperature treatments (4°C, -80°C, and -196°C). However, after 30 and 60 days, protoplasts stored at -80°C and -196°C showed significantly higher viability (by 40% and 60%, respectively) than those stored at 4°C, though no difference was observed between the two subzero temperatures. After 90 days, -196°C storage yielded the highest viability, while 4°C storage produced the lowest. Specifically, -196°C storage resulted in 22.81% higher viability than -80°C and 71.37% higher than 4°C, with these differences being statistically significant.

Effects of Cryoprotectant Formulation on Cell Wall Regeneration

Cell wall formation was a gradual process, with fluorescence intensity increasing over the culture period. After 30 days of cryopreservation and subsequent cul-

ture, no significant differences in cell wall formation were observed among the three cryoprotectant formulations. On day 1 of culture, no blue fluorescence was detected around cells, indicating absence of cell wall formation (Plate III: A). By days 2-3, faint blue fluorescence began to appear around cells due to VBL binding with cellulose (Plate III: B). Around days 5-6, uniform, intense blue fluorescence was observed along the entire cell periphery, indicating complete cell wall formation (Plate III: C).

Effects of Tissue Source on Cell Wall Regeneration

After 30 days of cryopreservation and culture, stem tip-derived protoplasts formed complete cell walls by days 3-4, whereas young leaf-derived protoplasts required until days 5-6. Despite this temporal difference, the process of cell wall formation was similar between the two tissue sources (Plate IV).

Effects of Storage Temperature on Cell Wall Formation

No significant differences in cell wall formation timing were observed among protoplasts stored at 4°C, -80°C, and -196°C. All treatments showed no cell wall formation on day 1 (Plate V: A), faint blue fluorescence appearing on days 2-3 (Plate V: B), and complete cell wall formation by days 5-6 (Plate V: C).

Effects of Cryoprotectant Formulation on Cell Division

After 90 days of cryopreservation, no differences in the timing of division initiation were observed among the three cryoprotectant formulations. On day 0 of culture, no dividing cells were observed, with protoplasts appearing spherical with clear membrane boundaries (Plate VI: A). By day 6, numerous dividing cells were visible, including many first divisions (Plate VI: B). Around day 15, small cell clusters with tightly packed cells began to appear (Plate VI: C).

Effects of Tissue Source on Cell Division

Table 3 shows that stem tip-derived protoplasts initiated division significantly earlier than those from young leaves. The first division occurred 2 days earlier in stem tip protoplasts, the second division 3 days earlier, small cell cluster formation 5 days earlier, and small callus formation 10 days earlier. However, the division process itself showed no qualitative differences between tissue sources.

Effects of Storage Temperature on Cell Division

All storage temperatures resulted in similar timing for the first cell division, occurring around day 7 of culture with no significant differences observed (Plate VIII: A, B, C).

Discussion

DMSO is a highly effective permeable cryoprotectant widely used in ultra-low temperature preservation. At room temperature, DMSO exerts mild phytotoxicity, but this effect diminishes as temperature decreases (Lü Weimin et al., 2014). Previous studies have identified optimal DMSO concentrations varying by species: Lin Xiaoyuan (2011) found 15% DMSO yielded highest viability in *Chlorella*, while Xue Jianping et al. reported 5% DMSO produced maximum viability (65%) in *Rehmannia glutinosa* shoot tips. Our study demonstrates that cryoprotectant formulation significantly affects sugarcane protoplast viability, with Combination 2 (70% medium + 20% BSA + 10% DMSO) being optimal. The superiority of Combination 2 over Combinations 1 and 3 likely reflects inadequate protection at 5% DMSO (resulting in ice crystal damage) versus toxicity at 15% DMSO (reducing viability). Cheng Fei et al. (2009) reported similar findings for *Robinia* callus cryopreservation using 10% DMSO + 0.5 $mol \cdot L^{1}$ sucrose + 10% (w/v) polyethylene glycol.

Different tissue sources exhibit varying post-thaw viability, division capacity, and regeneration potential. Recent cryopreservation research has focused primarily on shoot tips (Li Xiang et al., 2018) and callus tissues (Hong Senrong et al., 2018). Chen Yong et al. (2000) investigated cryopreservation of *Dendrobium candidum* protoplasts from leaves, stems, and callus, finding young leaves and callus most suitable. Jian Lingcheng (1987) demonstrated that cryopreserved sugarcane callus could recover and regenerate new plants. Our results show that young leaf-derived protoplasts exhibited higher post-thaw viability than stem tip-derived protoplasts, yet stem tip protoplasts initiated division earlier. This may be attributed to the inherent characteristics of stem tip cells, which are small, have thin cell walls, large nuclei, and dense cytoplasm, conferring strong and sustained division capacity that facilitates earlier division initiation upon culture.

Storage temperature significantly affects post-thaw viability. Liu Hongquan (2004) reported that *Porphyra* protoplasts stored in liquid nitrogen at -196°C showed highest viability (66.5%) and could regenerate into thalli. Dong Jinjiang and Xia Zhenao (1996) found that foxtail millet embryogenic cell lines stored at -40°C showed poorer recovery than those at -196°C, possibly because ice crystals still formed at -40°C, whereas ice crystal formation was prevented at -196°C. Our results indicate that for short-term storage (within 90 days), no significant difference exists between -80°C and -196°C storage. However, for long-term preservation, viability declined in -80°C stored samples, while 4°C storage caused significant viability loss after 30 days, making it unsuitable for long-term preservation. These findings align with previous reports. Additionally, we found that storage temperature did not significantly affect regeneration capacity, with protoplasts forming complete cell walls by day 5 and initiating first division by day 7. Ma Fengwang and Li Jiarui (1998) reported similar results for apricot protoplasts stored at -196°C, which began dividing 5-6 days after thawing with a plating efficiency of 2.2%, indicating that cryopreservation did not impair

regeneration capacity.

This study successfully optimized cryopreservation conditions for sugarcane protoplasts. Key findings include: (1) Young leaf-derived protoplasts showed significantly higher post-thaw viability than stem tip-derived protoplasts; (2) The optimal cryoprotectant formulation (70% medium + 20% serum + 10% DMSO) maintained viability up to 72.3% after 90 days; (3) For young leaf protoplasts stored up to 3 months, no significant difference was observed between -80°C and -196°C storage, but beyond 3 months, -196°C was significantly superior; (4) Ultra-low temperature preservation did not substantially alter protoplast regeneration capacity. These results provide a scientific foundation for sugarcane protoplast cryopreservation and offer material and technical support for somatic cell fusion breeding, genetic research, and transgenic studies.

References

Cheng F. 2009. Cryopreservation of callus of *Robinia idahoensis* and in vitro plant regeneration [D]. Henan Agricultural University.

Chen Y. 2000. Protoplasm of *Dendrobium candidum* cryopreserved by vitrification [J]. Journal of Wenzhou Teachers College (Natural Sciences), (03): 40-41.

Dong JJ, Xia ZA. 1996. Ultra-low temperature cryopreservation of embryogenic cell lines of foxtail millet (*Setaria italica* L. Beauv) [J]. Chinese Journal of Biotechnology, 12(04): 400-403.

Hong C. 2000. Study on primary factors influencing protoplast culture and isolation in sugarcane [D]. Fujian Agriculture and Forestry University.

Hong SR, Ning BS, Ye SY, et al. 2018. Dark culture and semi-thin section observation of regenerated plants after callus cryopreservation [J]. Acta Agriculturae Zhejiangensis, 30(01): 65-70.

Huang L. 2011. Protoplast culture and plant regeneration of head cabbage [D]. Zhejiang Normal University.

Huang XH, Yan JQ. 1980. Application of calcofluor white VBL in studying protoplast cell wall regeneration [J]. Acta Agriculturae Zhejiangensis, (2): 207-211.

Jian LC. 1988. Low temperature biology and long-term preservation of plant germplasm [J]. Chinese Bulletin of Botany, (2): 65-68.

Jian LC, Sun DL, Sun HL. 1987. Some factors in sugarcane callus tissue cryopreservation [J]. Journal of Integrative Plant Biology, (2): 123-131+232.

Li X, Su Q, Song H. 2018. Preservation and regeneration of stem tip of peach leaf [J]. Jiangsu Agricultural Sciences, 46(5): 140-143.

Li ZY. 2003. Cell Engineering [M]. Beijing: Science Press: 3-21.

Liu HQ. 2004. Transgenic study of *Porphyra yezoensis* [D]. Ocean University of China.

Li ZW, Wu T, Fu YX, et al. 2016. Low concentration of DMSO protects SSMC7721 cell viability after cryopreservation [J]. Chinese Journal of Histochemistry and Cytochemistry, (1): 70-74.

Liu L, Che Y, Chen L, et al. 2014. Detection of rice protoplast cell wall stained with fluorescent whitening agent VBL [J]. Guizhou Agricultural Sciences, 42(11): 70-72.

Lin XY. 2014. Cryopreservation and mutagenesis breeding of high-yield EPA marine microalgae [D]. Guangxi University for Nationalities.

Li YZ. 2012. Study on protoplast culture and somatic cell hybridization between alfalfa and birdsfoot trefoil [D]. Gansu Agricultural University.

Liu HQ, Yu S, Lin XY, et al. 2017. Study on vitrification cryopreservation of two green algae [J]. Jiangsu Agricultural Sciences, 45(21): 183-186.

Lü WM, Huang GM, Zeng Y, et al. 2014. The operation mechanism of cryoprotective agents and experimental study on cryopreservation of biological materials [J]. Cryogenics and Superconductivity, 42(5): 12-16.

Ma FW, Li JR. 1998. Cryopreservation of apricot protoplast [J]. Acta Horticulturae Sinica, (4): 18-21.

Takeuchi M, Matsushima H, Sugawara Y. 1982. Totipotency and viability of protoplasts after long-term freeze preservation [M]. *Plant Tissue Culture*: 797-798. Japanese Association for Plant Tissue Culture.

Widholm JM. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells [J]. Stain Technology, 47(4): 189-194.

Xue JP. 2004. Induction of tuberous roots and vitrification cryopreservation of shoot tips in *Rehmannia glutinosa* in vitro [D]. Huazhong Agricultural University.

Zhuang CH. 2015. Ultra-low temperature preservation of germplasm resources and establishment of seed embryo regeneration system [D]. Northeast Forestry University.

Note: Figure translations are in progress. See original paper for figures.

Source: ChinaXiv —Machine translation. Verify with original.