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Cryopreservation of Plant Cell Suspensions

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Summary

The cryopreservation of dedifferentiated cells, grown in suspension culture, is one of the portfolio of techniques employed for the long-term conservation of higher plant germplasm. Suspension cultures are also important in biotechnology, particularly in transformation studies and for the production of specific metabolites, and, here, there is also a pressing need for genetically stable, long-term storage of cell lines.

Cryopreservation of suspension cell cultures can be exploited by either slow, or rapid, cooling techniques. During slow cooling the extracellular solutions are nucleated and the cells cryodehydrate during controlled cooling as a consequence of extracellular ice, to the point where their intracellular fluids will vitrify on subsequent transfer to liquid nitrogen. In the rapid cooling protocols, the cells are prepared by extreme osmotic dehydration, with cryoprotection, before plunging the samples directly into liquid nitrogen to achieve vitrification. Extensive success has been achieved with both techniques but rapid cooling is, currently, widely favored because of its simplicity.

Key Words: Higher plant; cell suspension; cryopreservation; slow cooling; vitrification; genetic conservation; long-term storage.

1. Introduction

Suspension cultures may be considered being dedifferentiated and at the simplest level of organization of higher plant material held in vitro. Typically, they are diverse in morphology and development and contain varying proportions of single cells and aggregates of differing sizes and morphologies. These include cells that range from densely cytoplasmic to massively vacuolate, with varying vacuolar volumes and cell wall properties.

Plant suspension cultures are valued for a diversity of reasons, including:

1. Their role in genetic transformation studies and the development of transformation technology (e.g., **refs. *1–5***).

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2. The production of specific metabolites, often following transformation (e.g., **refs. *6–10***).
3. Their utility as starting material in the process of embryogenesis and whole-plant recovery (e.g., **refs. *11–15***).

In many instances more than one of these attributes may be combined in a single program of research and development, particularly in biotechnology.

It is widely assumed, from a genetic conservation standpoint, that the cell population in suspension is clonal but, if not, then any genetic diversity that might exist is uniformly distributed throughout the culture, albeit that it might be restricted to particular phenotypes. Further diversity, evident in most cultures, is the differing placement of individual cells in the suspension within the mitotic cycle. Few suspension cultures can be brought to high levels of mitotic synchrony without significant effort ***(15,16)***, but the high levels of survival (90%) reported following cryopreservation of *Arabidopsis* suspension cultures ***(17)*** may indicate that such efforts are worthwhile.

It follows from the morphological and developmental diversity within most plant cell suspension cultures that it is unlikely that any particular cryopreservation protocol, however devised, will be successful for more than a limited portion of the cell population. Clearly, this understanding should influence estimates of successful post-thaw viability.

The consequences and advantages of cryoconservation and its application to plant suspension cultures are well documented ***(18–23)***. The first generation of techniques, still current and widely employed, were based on a protocol reliant on relatively slow-cooling rates, at or near 1°C/min ***(18–24)*** and a cryoprotectant mixture containing dimethyl sulfoxide (DMSO), glycerol, and sucrose. For many culture types, acceptable levels of success with this basic method depended on empirical optimization of:

1. The duration and nature of any pretreatments used to take advantage of natural cell adaptation.
2. The type, mixture, and concentration of cryoprotectants.
3. The incubation time in the protectant solution.
4. Precise cooling rate.
5. The duration of any necessary subzero-holding temperatures during the cooling protocol that increased cryodehydration.
6. The recovery conditions.

Typically, suspensions are concentrated from the growth medium at a point in the culture cycle where the proportion of small, more densely cytoplasmic cells is at a maximum, with these being viewed as the likely survivors of the freezing protocol. Dependant on the particular genotype, the suspensions may benefit from pregrowth with enhanced concentrations of an osmoticum such as

mannitol or sorbitol and proline and DMSO have also been used in pretreatment ***(17,19,25,26)***. This pregrowth takes advantage of any natural, adaptive responses that result from osmotic stress and/or activation of the synthesis of natural protectants ***(27)***. Pregrowth at low temperature may also be beneficial ***(28,29)***. Thereafter, the cells are exposed to a cryoprotectant solution containing, in the original protocol, DMSO, glycerol, and sucrose at final concentrations of 0.5, 0.5, and 1 *M*, respectively ***(19,30)***. The optimum concentrations will vary with circumstance, and empirical optimization is likely to be needed. Other protectant compounds used include proline and polyethylene glycol (*see* **Note 1**). Once pretreated, volumes of cell suspension of less than 2 mL are cooled at a rate typically between 0.5 and 2.0°C/min to –40°C, before plunging into liquid nitrogen. At these slow-cooling rates, the unfrozen suspension cells become embedded in ice following nucleation of the extracellular solution, resulting in ongoing cryodehydration ***(22,23)***. As temperature decreases, the dehydration continues and intracellular solutions become increasingly concentrated and viscous. A point is reached where the residual intracellular solutions will vitrify when the cell sample is plunged directly into liquid nitrogen. A holding period at an appropriate subzero temperature may be required to achieve a sufficient level of dehydration. This pattern of cooling, followed by the plunge to ultra-low temperature, provides the classic "two-step" freezing protocol. The expectation is that, with rapid rewarming to avoid intracellular ice crystallization, a proportion of the cells in suspension will retain organization and viability following thawing ***(19,30,31)***.

A second major step in the cryopreservation of plant cell suspensions is seen in the more recent proliferation of vitrification techniques, whereby cells can be cooled rapidly to ultra-low temperatures avoiding the phase transition from liquid water to crystalline ice and, instead, producing an amorphous glass ***(31)***. Two mechanisms are at work in the successful vitrification protocol, the first being extensive cytoplamic dehydration resulting from high concentrations of essentially nonpenetrating protectants, e.g., glycerol at 30% (w/v) in the extracellular medium ***(31)***. The second is the increase in viscosity of the cytoplasmic solution that follows from the penetration of high concentrations of protectant, such as DMSO, included in the added vitrification medium into the cells.

Following treatment with vitrification medium, both the extra- and intracellular solutions are radically modified as a consequence of osmotic dehydration and loading with high concentrations of cryoprotectants. The cells that survive are those that become sufficiently dehydrated so that their intracellular solutions form a stable glass when, with rapid cooling by direct immersion of the sample in liquid nitrogen, they pass the appropriate glass transition point. Typically, the cooling rates employed are in excess of 200°C/min. The extracellular solutions

must undergo a similar transition. For the widely used plant vitrification solution PVS2 containing high levels of glycerol, ethylene glycol, and DMSO, this occurs in the region of –115°C ***(31)***. The presence of a glass provides stability in the system to be preserved, but successful recovery depends on a thawing procedure that transforms the aqueous glass phase directly to a liquid without the intervention of crystalline material, which can only be achieved by rapid rewarming. Precise rates are not usually quoted for this part of published protocols, perhaps because of the difficulties in making appropriately cited, accurate measurements. However, immersion of 2-mL cryovials, foil packets, or straws in water at 40°C for 1–2 min is widely reported ***(29,31,32)***. On occasion, the vitrification protocol can be modified to include an encapsulation stage, whereby the cells are embedded in alginate beads prior to the vitrification procedures ***(33)***.

2. Materials

2.1. Slow-Cooling Protocols

1. A cell suspension selected at a point in the culture cycle where the proportion of smaller, more densely cytoplasmic cells are at a maximum. The culture may have been subjected to pretreatments, such as growth in osmoticum-supplemented medium (e.g., sorbitol or mannitol at 0.3–0.5 *M*) and/or at low temperatures (6–0°C). Compounds such as desferrioxamine may also be used (*see* **Note 2**).
2. An apparatus capable of providing uniform, slow cooling to temperatures between –35 and –40°C. Reliable, programmable freezers that achieve this are commercially available, but simpler constructions relying on cooled liquid baths may be used (*see* **Note 3**).
3. An appropriate storage Dewar that allows for storage in the liquid or vapor phase. For secure cryoconservation, the storage Dewar should have an effective inventory system (drawers, canes) so that the precise location of samples can be achieved without unnecessary movement of samples from the storage environment. Duplicate samples should be stored in separate Dewars, ideally in a separate facility. Level alarms for liquid nitrogen should be fitted to prevent compromise of the storage temperature.
4. Autoclaved/sterile polypropylene cryopreservation vials, typically of 2-mL volume. Alternatives will include the straws used for semen cryopreservation, with volumes up to 1 mL, and self-made foil packets made from double layers of aluminium foil.
5. Suitable filtration or low-speed centrifugation to allow cell suspensions to be concentrated before freezing.
6. Cryoprotectant solutions: a double-strength solution of 1 *M* DMSO, 1 *M* glycerol, and 2 *M* sucrose to be added to an equal volume of cells, and pregrowth medium is widely employed. Other protectants and concentrations may be more appropriate (*see* **Note 1**).
7. A thawing bath set a 40°C, containing smaller vessels holding sterile water. Thawing vials in this water reduces the incidence of post-thaw contamination.

8. Suitable post-thaw recovery media, either liquid or solidified.
9. A solution of 0.1% (w/v) fluoroscein diacetate (FDA) in acetone, to carry out a post-thaw assessment of potentially viable cells. A microscope with an ultraviolet (UV) light source, and excitation and barrier filters at 490 and 530 nm, respectively, is also needed.

2.2. Vitrification Protocols

1. The materials and requirements for pregrowth and the selection of samples at an appropriate place in the culture cycle is as for slow cooling (*see* **Subheading 2.1.**). The requirements for concentrating the cell suspension, the sample vials for cryopreservation, frozen storage, a thawing bath, and the fluorescence viability assay are also similar.
2. A "loading" or osmoprotection solution that produces a final concentration of 2 *M* glycerol and 0.4 *M* sucrose.
3. The PVS2 solution ***(34)*** comprising 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) DMSO in the appropriate growth medium supplemented with 0.4 *M* sucrose.
4. An "unloading" or dilution medium of 1.2 *M* sucrose in the appropriate growth medium.
5. A suitable recovery medium.

3. Methods

3.1. Slow Cooling

1. Aliquots of the suspension culture are transferred to pregrowth medium for a period of 2–7 d, the optimum period being determined by investigation.
2. Pregrown cultures are concentrated by settling, filtering, or centrifugation and the more concentrated suspension cooled on ice for 30 min.
3. A volume of prechilled cryoprotectant equal to the volume of cell suspension, and at twice the required final concentration, is added to the suspension culture in three aliquots over a 20-min period, with mixing by gentle agitation at each addition.
4. After an incubation of longer than 1 h in the final concentration of protectant, excess solution is pipetted off to leave a 30% loosely packed cell volume.
5. An appropriate aliquot is transferred to the containers for freezing (1 mL into a 2-mL cryovial) and then transferred to the cooling apparatus. A programmable freezer should be precooled to 4°C.
6. The samples are cooled at 1°C/min to –40°C, with a holding period of up to 40 min at this temperature, or higher, to enhance cryodehydration.
7. Frozen samples are transferred into a small Dewar of liquid nitrogen as rapidly as possible, and once equilibrated (when bubbling ceases) can be transferred to the storage Dewar.
8. To thaw, samples are removed from the storage Dewar and immediately plunged into sterile water at 40°C, with continuous agitation. The samples are thawed when no more ice is visible in the sample.

9. To recover the cells, the thawed container is wiped with 70% (v/v) ethanol to minimize surface contaminants, is then opened, and the cells are returned to a recovery medium, when any dilution procedures can take place.
10. An estimate of initial, post-thaw viability can be made at this stage using 0.5 mL of cell suspension to which several drops of FDA stain is added. The sample is viewed microscopically for UV fluorescence.

3.2. Vitrification

1. Aliquots of the suspension culture are transferred to pregrowth medium for a period of 2–7 d, the optimum period being determined by investigation.
2. Pregrown cultures are concentrated by settling, filtering, or centrifugation.
3. Cells are transferred to a loading solution adjusted to give a final concentration of 2 *M* glycerol and 0.4 *M* sucrose for 20–30 min at room temperature.
4. Cells concentrated and transferred to PVS2 at 0°C for 60–90 min.
5. During the PVS2 incubation the cells are transferred to the vials/straws for freezing.
6. Direct immersion of the sample containers (vials/straws) into liquid nitrogen, with subsequent transfer to the storage dewar.
7. To thaw, samples are removed from the storage Dewar and immediately plunged into sterile water at 40°C, with continuous agitation. The samples are thawed when no more ice is visible in the sample.
8. To recover the cells, the thawed container is wiped with 70% (v/v) ethanol to minimize surface contaminants and then opened, and the cells are returned to an unloading solution of 1.2 *M* sucrose for 20–30 min at room temperature before transferring the cells returned to a recovery medium, when any further dilution procedures can take place.
9. An estimate of initial, post-thaw viability can be made at this stage using 0.5 mL of cell suspension to which several drops of FDA stain is added. The sample is viewed microscopically for UV fluorescence.

4. Notes

1. The selection of an optimally effective protectant solution is likely to require empirical investigation. Variants previously reported ***(19,35,36)*** have included:
   a. DMSO at 5–10% (v/v).
   b. DMSO and glycerol both at 5% (v/v).
   c. 4% (v/v) DMSO, 4.6% (w/v) glycerol, and 11.5% (w/v) proline.
   d. 10% (v/v) DMSO, 8% (w/v) glucose, and 10% (w/v) polyethylene glycol (PEG2000).
   e. 10% (v/v) DMSO and 18% (w/v) sucrose.
   f. 18.4% (w/v) glycerol and 14.4% (w/v) sucrose.
2. Desferrioxamine is intended to reduce oxidative stress by reducing cation availability, thereby reducing the Fenton reaction and the production of hydroxyl radicals.
3. Alternatives to commercial programmable freezers will include baths of suitably low freezing point solutions (e.g., methanol, glycol) with a dip cooler to lower the temperature and a stirring device to ensure uniform cooling ***(19,24)***. Where

laboratory freezers with temperatures below –30°C are available, suitably insulated containers (polystyrene) may allow sufficient control of the cooling rate to provide successful cryopreservation.

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