Cryopreservation of Plant Cells, Tissues, and Organs

Concepts

- Cold tolerance and dehydration tolerance can be induced in many types of plants, and this improves their recovery from cryopreservation.
- Meristematic cells and cells with few or small vacuoles are most suitable for cryopreservation.
- Osmotic dehydration, air drying, and cryoprotection decrease the water content of cells and allow vitrification of the cytoplasm.
- Cryoprotectants may remain outside the cell and cause osmotic dehydration.
- Cryoprotectants may enter the cell and add to the cell solution, thus reducing the chance of crystallization.
- Culture techniques are very important in successful cryopreservation protocols.
- Seeds can be desiccation and/or cold tolerant, or desiccation and/or cold sensitive.
- Embryos/embryonic axes of desiccation and cold sensitive seeds can be cryopreserved using the desiccation technique.

Cryopreservation, storing biological materials at liquid nitrogen temperatures (–196°C), is now a viable option for most types of plant materials. The earliest studies of plant cryopreservation were in the 1960s. Sakai (1960) showed that cold-acclimated dormant buds could be slowly cooled to low temperatures, immersed in liquid nitrogen, rewarmed, and retain viability.

Several methods were developed in the early 1990s that allowed for rapid cooling of shoot tips, cells, or callus cultures. These techniques expanded the number of species and the types of plants that could be cryopreserved. Cryopreservation is now used for storing laboratory specimens, germplasm collections, stock culture collections, and rare or endangered species.

Tissue Culture Aspects

Many of the plant materials stored by cryopreservation come from tissue culture systems. It is vitally important that the culture system produce healthy plants for the process. Cultures in less than optimum condition have lower regrowth than those in good condition. Small plant cells with few vacuoles are the easiest to cryopreserve.

Most cryopreservation protocols involve preconditioning stages in culture. These may include growth on highly osmotic media, cold acclimation, or extended culture without transfer. Sequential transfer to medium with increasing sucrose concentrations, cold acclimation, growth on abscisic acid, or glycerol are used to increase osmotic tolerance when plants are desiccation sensitive. Most of these techniques improve cryopreservation tolerance by adding solutes to the cytosol or developing desiccation tolerance in the cells. Recovery following cryopreservation is also dependent on in vitro culture systems in most cases. The optimal growth room temperature and lighting as well as the proper recovery medium can greatly affect regrowth.

General Steps in Cryopreservation

All cryopreservation techniques are based on a similar premise: condition the plant cells and dehydrate them to the point that they will remain alive. The physiological condition of the plant is the first concern. Ideally, the plant is naturally adapted to cold or dry conditions. In reality, that is not the case for most in vitro grown plants or cell cultures.

The first step is pretreatment. This involves growing the plant under conditions that improve resistance to cold or dry conditions. Cultures can be cold-acclimated with long cold nights and short warm days, grown for a long time without transfer, or cultured on osmotic media. The pretreatment method chosen should be suitable for the type of plant in question. A temperate plant will usually respond to cold acclimation, while a tropical plant might die from such treatment. A plant conditioned to grow in moist environments would require a more gradual increase in osmotic medium than a naturally desiccation tolerant plant. Once the plant is pretreated, the shoot tips can be further conditioned by preculture for a few days on specialized media. Standard preculture for many controlled-rate cooling protocols includes medium with 5% dimethyl sulfoxide (DMSO) for 2 days. Vitrification protocols may include high molarity sucrose preculture.

Exposure to cryoprotectant solutions varies with the technique. Controlled rate cooling cryoprotectants are relatively mild, and exposure is often 30 min to as long as 2 h. Vitrification solutions are highly osmotic, and exposure is usually carefully timed to avoid overexposure that results in damage or death of explants. Once the optimum cryoprotectant exposure is achieved, slow controlled cooling is initiated, or vials are plunged in liquid nitrogen in the case of vitrification protocols.

Rewarming is a critical procedure in all of the techniques. Rapid rewarming of the vials without overheating the plant materials requires exposure to hot water (~40°C) for 1–2 min followed by cool water (~25°C) to protect from overheating. Movement of vials from the liquid nitrogen to the hot water should be immediate, or the vitrified solutions may crystallize rather than liquefying and death will result. Removal of the cryoprotectant and reculture of the plant material is the next important step. For dedifferentiated cultures, decanting and not rinsing may produce better regrowth than rinsing. For shoot cultures complete rinsing is important. Culture explants under low or no light for the first week, followed by standard growth conditions favors regrowth. The recovery medium is often the standard multiplication medium; however, in some cases, auxin in the medium can cause callus formation rather than shoot tip growth and should be avoided.

Cryopreservation Techniques

There are three main types of cryopreservation techniques, controlled cooling, vitrification, and desiccation, and some involve combinations of these (Table 38.1). The first type of cryopreservation developed for organized tissues involved controlled rate cooling. This technique is applied mostly to cell cultures and to organized tissues of temperate plants, both dormant buds and shoot tips of in vitro grown plantlets.

TABLE 38.1
Commonly Used Cryopreservation Techniques

Technique	Procedure
Controlled rate cooling (slow cooling, two-step cooling)	Dormant wood collected from trees during the coldest winter months are given additional chilling, dehydrated gradually by slow cooling, and stored in liquid nitrogen vapor. Thawed buds are grafted or budded onto rootstocks for recovery. Shoot tips, callus, or cell cultures are pretreated in cryoprotectants and frozen at <1°C/ min to –40°C, and then plunged into liquid nitrogen.
Solution-based vitrification (rapid cooling)	Several protocols and cryoprotectants are available. After a brief loading phase and a brief cryoprotectant pretreatment, the vials or foils are directly plunged into liquid nitrogen.
Encapsulation-dehydration (vitrification)	Explants are encapsulated in alginate and dehydrated osmotically for about 20 h and then dried in airflow or over silica gel before direct immersion in liquid nitrogen.
Encapsulation vitrification	Encapsulated explants are immersed in vitrification solutions and directly plunged in liquid nitrogen.
Desiccation	Orthodox seeds are dried over silica gel until they retain 3%–7% seed water content. Recalcitrant seed embryonic axes are dried to 7%–20% water (fresh weight basis) before direct immersion in liquid nitrogen.

In controlled rate cooling, the samples are preconditioned, cryoprotected, and then slowly cooled to the freezing point of the cryoprotectant where ice is initiated (about –9°C). Then they are slowly cooled to –35°C or –40°C before plunging in liquid nitrogen (Figure 38.1). The speed of cooling determines the amount of time that the dehydration can continue. If the rate is too fast, then freezable water will remain in the cell and crystallize at low temperatures. If the rate is too slow, then the cell might be lethally dehydrated. At the terminal transfer temperature (–35°C to –40°C), the plunge into liquid nitrogen causes the cytoplasm to vitrify (turn to a glass) and avoid ice crystal damage. Rewarming must be fast so that the cytoplasm liquefies rather than crystallizing.

FIGURE 38.1 Cryogenic freezers used for controlled rate of cooling of cell cultures and shoot tips.

Vitrification techniques involve the transition of cellular liquids to amorphous glass without crystal formation. Water can transition from liquid to solid (ice) or liquid to an amorphous glass (vitrified). A vitrified solution is in an amorphous state that has the physical properties of a liquid. This amorphous state can easily revert to a liquid or to ice crystals if the conditions change (during slow warming, for example). Glass is an excellent freeze avoidance system since a glass does not increase in volume like ice. Vitrified samples must be quickly rewarmed, or ice formation will occur. There are two main techniques that use this principle, solution-based and desiccation-based vitrification (Figure 38.2).

Solution-based vitrification protocols use loading solutions of sucrose and glycerol to add solutes to the cell and to osmotically dehydrate the cell, followed by highly viscous cryoprotective solutions that will vitrify as the samples are plunged into liquid nitrogen. The main solution used is Plant Vitrification Solution #2 (PVS2). In any vitrification technique, the preculture of plants, loading solution, time and temperature of the cryoprotectant solution, and rewarming procedure are all critical to success.

Encapsulation-dehydration is a vitrification technique that employs sucrose loading and dehydration to remove water from the cells, and the cytoplasm forms a glassy state (vitrifies) on exposure to liquid nitrogen. In this technique, in vitro plantlets are pretreated by cold acclimation or sucrose preculture, and then shoot tips are removed and encapsulated in alginate beads. The beads are cultured overnight in a 0.75M sucrose solution to add sucrose to the cells, and then they are blotted dry and allowed to desiccate under laminar flow or with silica gel until they have about 20% moisture content. At this point the cells will vitrify on contact with liquid nitrogen.

Cryopreservation of seeds is accomplished using desiccation methods. This normally involves direct drying of orthodox seeds over silica gel, or in the case of recalcitrant seeds, the embryonic axis is removed and dried under laminar flow, over silica gel, or flash dried.

Cryoprotection

All forms of cryopreservation require some type of cryoprotection. A wide range of cryoprotectant solutions are available for use (Table 38.2). Cryoprotectants can be penetrating and add to the osmolality of the cell or nonpenetrating and aid in osmotic dehydration of the cell. Penetrating cryoprotectants such as glycerol, sugars, and DMSO are useful for increasing solutes in the cell and in the case of DMSO for conditioning membranes.

TABLE 38.2
Cryoprotectants Commonly Used in Plant Cryopreservation in Combination with the Normal Growth Medium

Cryoprotectant	Composition	Sample Type	Technique
DGS	1 M DMSO 1 M Glycerol 2 M Sucrose	Dedifferentiated cultures	Controlled rate cooling
DGP	1 M DMSO 1 M Glycerol 2 M Proline	Dedifferentiated cultures	Controlled rate cooling
DGSP	1 M DMSO 1 M Glycerol 1 M Sucrose 1 M Proline	Dedifferentiated cultures	Controlled rate cooling
DGlu	2.5 M DMSO 1.1 M D-Glucose	Dedifferentiated cultures	Controlled rate cooling
DMSO	5 or 10 % DMSO	Embryogenic cultures of conifers, algal cultures, mosses	Controlled rate cooling
PGD	10% PEG 10% Glucose 10% DMSO	Cell cultures, somatic embryos, shoot tips	Controlled rate cooling
DMSO-sucrose	10% DMSO 0.6 M sucrose	Embryogenic cultures	Controlled rate cooling
Sucrose	0.75M sucrose	Cell cultures, somatic embryos, shoot tips, algal cultures, ferns, and mosses	Encapsulation-dehydration
PVS2	30% glycerol (w/v) 15% ethylene glycol (w/v) 15% DMSO (w/v) in medium containing 0.4 M sucrose (pH 5.8)	Cell cultures, somatic embryos, shoot tips	Vitrification and encapsulation-vitrification
PVS3	40% glycerol (w/v) 40% sucrose (w/v)	Shoot tips	Vitrification

Nonpenetrating cryoprotectants serve to osmotically dehydrate the cells. Vitrification solutions are highly viscous and cause rapid dehydration of cells, so their use can be toxic if timing and temperature of application are not carefully controlled.