

The Usage of Cryopreservation and Synthetic Seeds on Preservation for Plant Genetic Resources



Tolga İzgü¹ and Yeşim Yalçın Mendî^{2*}

¹Department of Horticulture, University of Ege, Turkey

²Department of Horticulture, University of Çukurova, Turkey

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***Corresponding author:** Yeşim yalçın mendî, Department of Horticulture, Faculty of Agriculture, University of Çukurova, 01330, Sarıçam, Adana, Turkey, Email: ymendi@gmail.com

Introduction

The endangered and endemic species of plant biodiversity in both nature and agricultural fields have led the flora to look for alternatives to in situ conservation. Therefore, cryopreservation is a useful tool for long-term storage of plant germplasm for next generations, requiring only a minimum of space and maintenance. With increasing interest in the genetic engineering of plants, the preservation of cell lines (callus or protoplast) and somatic embryos with incomparable attributes is assuming high significance. Lately, cryopreservation was reported to offer real hope for enhancing the preservation of endangered and endemic plants.

Cryopreservation techniques are based upon different tissues of plant storage such as apical or axillary shoot tips, meristems, scions, seeds, spores, gametophytes, rhizomes, zygotic and somatic embryos, pollens, embryogenic callus lines at ultra-low temperature of liquid nitrogen (LN, -196 °C). The major advantage of this procedure is to diminish in vitro culture costs, required space, contamination and somaclonal variation or genetic altering risk. The long-term conservation of embryogenic cell lines may be a valuable tool in genetic engineering.

Generally two types of cryoprotectants are used for cryopreservation, those that penetrate the plasma membrane (e.g., Dimethyl sulfoxide (DMSO) and glycerol), and the non-penetrating chemicals (e.g. polyethylene glycol (PEG), polyethylene glycol (EG) and sucrose). Synthetic/encapsulated seed technology involving calcium alginate encapsulation of in vitro or in vivo generated explants proved to be a reliable system to deal with propagation and cryostorage. Cryopreservation techniques are separated into two main groups: traditional (two step freezing) and modern (one step freezing) techniques. There are several combinations of the cryopreservation techniques. The combinations of these techniques are now directly applicable for many plant species. These techniques:

- A. Controlled rate cooling (two-step freezing, controlled-rate freezing or slow cooling)
- B. Vitrification (with plant vitrification solution-2 "PVS2") [1].
- C. *PVS2 including 30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% dimethylsulfoxide (DMSO; w/v) in MS (Murashige and Skoog, 1962) basal medium (plant growth regulators free) containing 0.4 M sucrose (pH 5.8)
- D. Encapsulation/vitrification
- E. Encapsulation/dehydration
- F. Droplet method
- G. Desiccation (Laminar flow cabinet or silica gel)
- H. V-Cryo-plate and D-Cryo-plate procedures

Slow cooling is provided by a device named «programmable freezer». In this system, the parameters can be adjusted easily such as starting temperature, freezing rate, final temperature and waiting time. Because this method is expensive, lower technology (Nalgene freezing container, Mr. Frosty®) can be used instead of it. Vitrification is a very effective method for the cryopreservation of plant materials because it would avoid the potentially impairing effects of intracellular and extracellular freezing. This method can be defined as the transition of water directly from the liquid phase into an amorphous phase, while avoiding the formation of crystalline ice. Vitrification is commonly used for embryogenic callus lines, meristems, somatic embryos and etc. in LN. In vitrification methods, embryogenic callus/somatic embryos can be sufficiently dehydrated with PVS2 at 25 °C or 0 °C without causing injury to enable vitrification upon rapid cooling in LN.

Encapsulated or synthetic seeds have advantages such as easy handling, storage, transferring, germ plasm conservation of elite or endemic plant species for cryopreservation, exchange of non-contaminated plant materials among laboratories, gene banks and industries. For preparation of alginate beads, the explants are suspended in calcium-free liquid MS basal medium with 3-4% (w/v) sodium alginate (generally using low viscosity) [2]. This solution generally contains low level sucrose in the MS basal medium. In some cases, explants can also be encapsulated in a MS medium with a slightly higher sucrose concentration. The mixture is dropped with a pipette into liquid culture medium containing calcium chloride (100 mM CaCl₂). This causes the polymerization of alginate in presence of an raised concentration of calcium, hence producing beads around the explants. These synthetic beads are usually 4 or 5mm in diameter and include one shoot tip, somatic embryos (such as globular, heart, torpedo and cotyledon stages or microcorm) or embryogenic callus clusters. They are waited in the CaCl₂ solution for 20-30min after the beads are formed to polymerization. First application of encapsulation dehydration technique was developed for in-vitro pear and potato axillary shoot tips. This method is based on the preservation of synthetic seeds. The standard vitrification protocol takes place in cryotubes while the droplet method is made on aluminum foil strips [3]. The droplet method with PVS2 or DMSO is also used for cryopreservation of several plant types. Drops of different concentration of DMSO or PVS2 solution are placed on aluminum foil strips and immersed into liquid nitrogen (LN). Although the cooling rate is extremely fast, no vitrification is determined within the plant tissues or cell.

The desiccation technique, either in a laminar airflow cabinet or over silica gel, is used for cryopreservation of somatic embryos, synthetic seeds or embryonic axes. Because this method is simple and easy handling, it should be tried first before testing the other techniques. The optimum moisture for cryopreservation ranges from 8 to 20% (time of desiccation, usually 1-2h) depending on explant type and the species. There are two desiccation techniques: dehydration under the air current of a laminar flow cabinet or dehydration in sealed containers with silica gel. Desiccation under the laminar flow can produce variable desiccation rates depending on the airflow rate, air temperature, and relative humidity [2].

A new cryopreservation methods based on vitrification and air dehydration of explants placed on aluminum cryo-plates, named the V-cryo-plate and D-cryo-plate technique. Recently, a vitrification protocol using the aluminum v-cryo-plate method has been reported [5-8]. The cryo-plate method has two main advantages:

- A. A user-friendly procedure and,
- B. Higher cooling and warming rates of treated explants are possible by directly immersion in LN [4] Consequently, efficient regrowth was obtained after cryopreservation of the various plant explants [9]. In the D cryo-plate method, shoot

tips or buds attached to the cryo-plates are dehydrated in the laminar air flow cabinet or over silica gel after loading treatment with glycerol and sucrose solution for inducing tolerance to dehydration. Capacious samples consisting of buds and basal stems can be used for the materials in this technique. So it is a practical and efficient method for cryopreservation [4,10-13] Figure 1.



Figure 1: Basic steps of the different cryopreservation techniques, A) Polymerization of synthetic seeds in CaCl₂ solution (100 mM), B-C) Preculture of synthetic seeds and somatic embryos on MS medium with 0.3M sucrose before immersion in LN, D) Treatment with PVS2 solution of synthetic seeds and somatic embryos in cryovials, E) Somatic embryos placed on aluminium plates (Droplet method), F) Dessication with silica gel of synthetic seeds, G) Isopropanol included in a Mr Frosty® (Nalge Nunc, Rochester, New York, USA) unit provides a 1°C•per min. cooling rate (Controlled-rate freezer), H-I) Samples are directly immersion into liquid nitrogen (LN, -196°C at least 1 h) and the standard thawing cryogenic vial from the storage container to a water bath held at 35–40°C.

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