IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

CONSERVATION OF PLANT GENETIC RESOURCES USING CRYOPRESERVATION

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ABSTRACT

Plant Cryopreservationis the preservation of plant species in the form of tiny plant materials outside natural habitats (ex situ conservation), including DNA, pollen, buds, dormant buds, embryonic axes, zygoticand somatic embryos, and seeds at ultra-low temperature (-130°C) but generally prefer to store plant materials in liquid nitrogen at -196°C. The physical and metabolic cellular processes are effectively stopped, and then cryopreserved plant materials can be recovered and grown to regenerate a whole plant after cryopreservation. Plant cryopreservation has been developed since 1960 with the principles of slow cooling and vitrification. There are 7 major steps (1. preculture, 2. osmoprotection, 3. dehydration, 4. store in liquid nitrogen, 5. rapid thawing, 6. unloading, and 7. recovery) generally used for successful plant cryopreservation. The developed methods from the beginning are: 1. Dormant buds, 2. Slow freezing, 3. Vitrification, 4. Encapsulation-dehydration, 5. Encapsulation-vitrification, 6. Droplet-vitrification, 7. V cryo-plate, and 8. D cryo-plate. It is an alternative method for a long-termed plant conservation using less space, less cost, and no environmental effects. Although in some cases, such as storing seeds at -20°C, is more appropriate and less expensive than storage in liquid nitrogen and buds can be stored at -4 to -80°C, but the storage cannot last long. At present, the recent cryopreservation methods use a shorter time, unskillful labors, simple equipment with high survival. In addition, many countries adopt these methods to use for their germplasm storage.

Keywords: Plant cryopreservation, Developed methods

INTRODUCTION

Plant Cryopreservation is the preservation of plant species in the form of tiny plant materials outside natural habitats(ex situ conservation), including DNA, pollen, buds, dormant buds, embryonic axes, zygoticand somatic embryos, and seedsat ultra-low temperature (-130°C) but generally prefer to store plant materialsin liquid nitrogen at -196°C, which is the temperature level that stops biological activities, including all biochemical reactions of cells which will lead to cell aging (and cell death) and no genetic changes during storage (Engelmann, 2004). Therefore, it is a reliable method for long-term storage of plant

materials, although in some cases, such as storing seeds at -20°C, is more appropriate and less expensive than storage in liquid nitrogen and buds can be stored at -4 to -80°C, but storage cannot last long. Therefore, plant cryopreservation is another option for long-term storage, but cryopreservedplant materials should be checked periodically.

Steps in Plant Cryopreservation

There are 7 major steps in plant cryopreservation. Some plants may not require every step (skipping steps 1, 2, 5, or 6) in preservationdepending on plant species and cryopreservation methods. The whole protocol is as follows:

1. **Preculture** The cells will be adjusted to low temperature using 0.3 M sucrose (1-3 days) **for** 1. Adding sucrose and 2. Adding ABA (abscisic acid) to stabilize cell membrane under dry condition.
2. **Loading solution** Using chemicals to protect osmotic pressure, such as 2 M glycerol + 0.4 M sucrose (20 minutes) **for** dehydrating the cells to reduce osmotic pressure and help to withstand highly concentrated solution, such as PVS2 solution and adjust to dry and vitrification conditions.
3. **Dehydration** Nowadays, scientists combine dehydration chemicals at a suitable concentration for each plant material. These chemicals are called plant vitrification solutions (PVS) which have many formulas. The formula used in the present 4 formula is PVS1 (Usagami et al., 1989), PVS2 (Sakai et al., 1990), PVS3 (Nishigawa et al., 1993), and PVS4 (Sakai et al., 2000). Highly concentrated solution, such as PVS2 solution at 25^{o}C or 0^{o}C (for suitable time) is used **for** dehydrating the cells sufficiently. These chemicals have different functions during the cold and warm process. Sugar helps stabilize cell membrane and proteins. DMSO will capture free radicals, increase the absorption of membrane cells and protect the cell structure during the cold condition. Glycerol helps stabilize cell membrane. But the work of these chemicals is not yet fully understood.
4. **Storage in liquid nitrogen** All the cryopreserved cells will be vitrified from -115^{o}C to -196^{o}C (in liquid nitrogen).
5. **Rapid warming** Warm the cryopreserved cells rapidly at 35-40 ^{o} C in water bath to change from glass state to liquid state.
6. **Unloading solution** Move the cells to concentrated 1.2 M sucrose for 20 minutes to reduce osmotic pressure and not let water move into the cells too fast. This step is for acclimation before culture.
7. **Culture on suitable medium** Culture the cells on suitable medium for plantlet development. Then, transfer the plantlets to the greenhouse, applying acclimatization for 2 weeks.

Methods in Plant Cryopreservation

Preservation of plant materials in cold conditions need to understand the basic mechanism of this principle which can be done in two ways: 1. Slow coolingor slow freezing (cell dehydration due to cold temperature) and 2. Vitrification (glass formation). In slow cooling, dormant bud and slow freezing methods are concerned. In vitrification, cells are dehydrated by exposure to highly concentrated chemicals or air drying before freezing, followed by rapid warming to avoid intracellular ice formation. Six different vitrification-based methods are: 1. vitrification, 2. encapsulation-dehydration, 3. encapsulation-vitrification, 4. droplet-

vitrification, 5. vitrification cryo-plate method (V cryo-plate), and 6. dehydration cryo-plate (D cryo-plate). Up till now, there are eight methods chronologically as follows:

1. Dormant bud method

This is the first plant cryopreservation method that dormant buds were collected during winter when buds were dehydrated at -10 to -30^{o}C before storage in liquid nitrogen (-196^{o}C). This method was successful in willows (*Salix koriyanagi*) and poplar (*Populus sieboldii*) (Sakai, 1960). Later, dormant bud method was studied in apple (Sakai and Nishiyama, 1978; Forsline et al., 1998; Towill and Bonnart, 2003), cherry (Towill and Forsline, 1999), blueberry (Jenderek and Reed, 2017), etc.

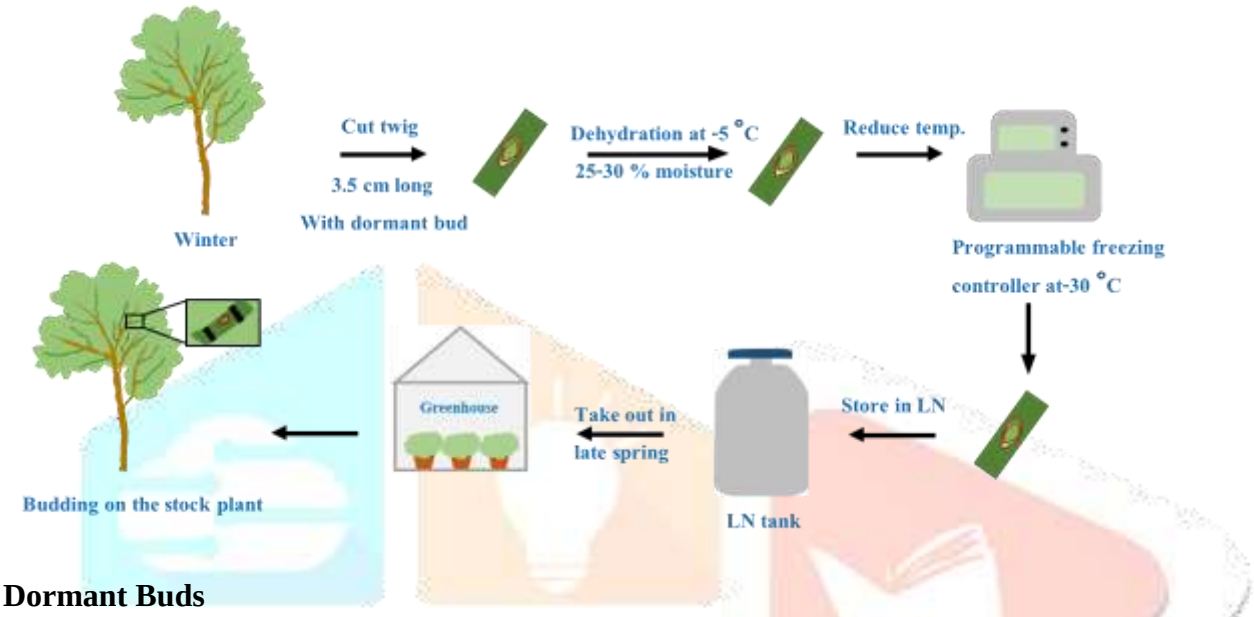

Figure 1. Diagram of Dormant bud method.

The protocol for preserving dormant buds is shown in Figure 1. This method works well with cold perennials, such as apples, willows, mulberry, conifers, etc., and is another option for plant cryopreservation.

2. Slow freezing method

Slow freezing was the standard method in the early time(Panis and Lambardi, 2005), but need a programmable freezing controller to reduce the temperature at the rate of 0.5-2°C/min depending on the plant species and the growth stage until about - 40°C, then store in liquid nitrogen. The protocol takes many hours to complete and requires expensive tools. This method was popular during 1980-1990 to store undeveloped plant tissues, such as suspended cells and calli of various plants.

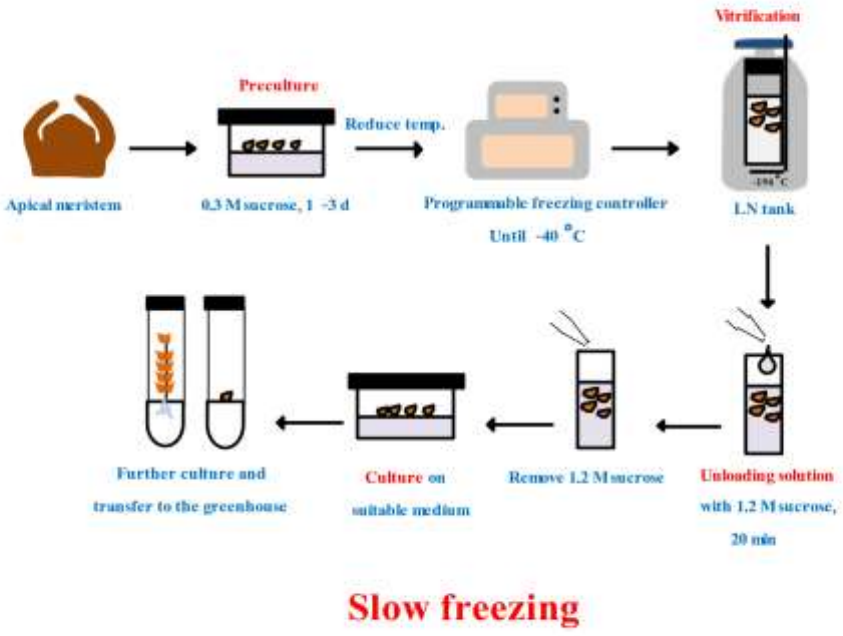

Figure 2. Diagram of Slow freezing method.

The protocol for Slow freezing method is shown in Figure 2.Slow freezing method is used to preserve plant species in extremely cold conditions compared to other methods. Due to complications,it takes a long time and must use a programmable freezing controller but may work better in some plants in China at the National Gene bank in Beijing of the 82 plants collected, only 3 mulberry plants were stored by slow freezing method and had a survival rate of 90% (Zhang et al., 2014).

3. Vitrification method

Vitrification is a method developed to reduce the temperature and to warm rapidly, all parts of cells and tissues are in a glass state. Vitrification method involves treatment of explants with plant vitrification solution (PVS), such as PVS1,PVS2, PVS3, and PVS4to induce dehydration of explants during cooling and warming to avoid intracellular ice-crystal formation (Uragami et al., 1989; Sakai et al., 1990). The key to successful cryopreservation by vitrification method is to prevent injury by optimizing exposure time to PVS for dehydration (Niino et al., 2007) because over exposure time to PVS may result in cell injury and intracellular ice formation during cooling. The optimum exposure time to PVS depends on explant size and species specific. The suitable dehydration duration was related to the sample size, the composition, and loading solution (Chen and Wang, 2002). Sakai et al. (1990) succeeded to cryopreserve nucellar cells of

naval orange using PVS2 solution, after that many plants were experimented with success.

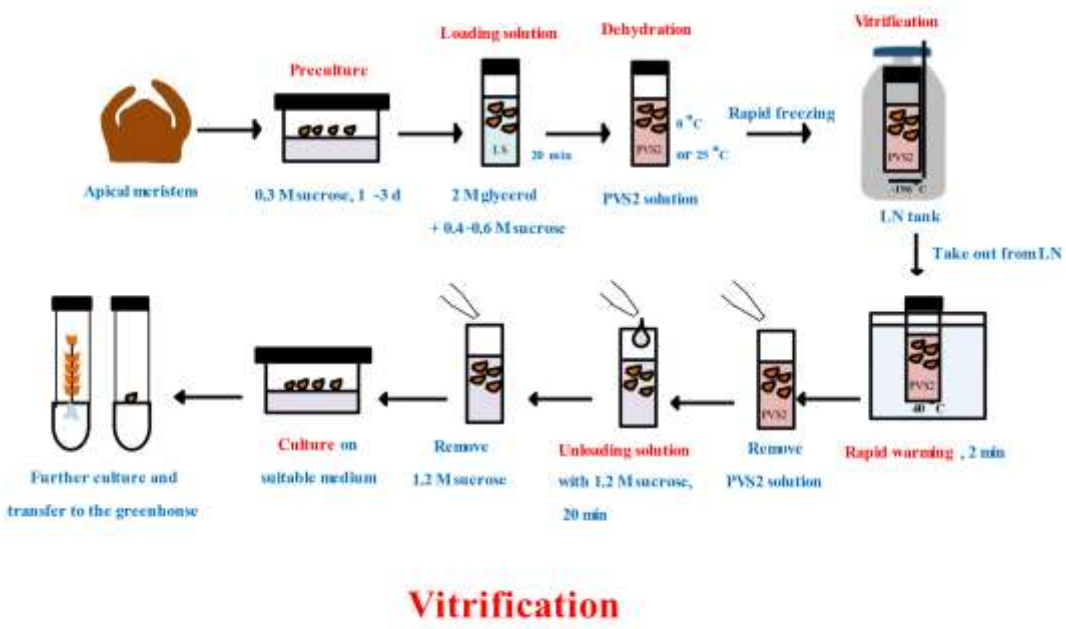

Figure 3. Diagram of Vitrification method.

The protocol for Vitrification method is shown in Figure 3.Vitrification is a method developed with short processing time, low cost, and less skilled work. It gives high survival, but it needs accurate time because of a short protocol time. In addition, the use of chemicals to dehydrate cells may be toxic to cryopreserved plant materials.

4. Encapsulation-dehydration method

Encapsulation-dehydration method is developed from artificial seed production that explants are encapsulated in alginate beads, precultured with high sucrose, desiccated by air-drying in a laminar air-flow cabinet or with silica gel, and then plunged into liquid nitrogen (Sakai et al., 2000; Matsumoto and Sakai, 1995). The advantages of this method are easy for manipulation of encapsulated explants (Hirai et al., 1998), preventing direct contact of toxic chemicals with plant materials, and non-toxic cryoprotectants are applied to protect during dehydration (Niino and Sakai, 1992). However, this method is the longer dehydration procedure than vitrification method (Thammasiri, 2000). Fabre and Dereuddre(1990) succeeded to cryopreserve tomato apical meristems by using this method. There are many plant species to be cryopreserved by encapsulation-dehydration.

Jitsopakul et al. (2008a) successfully cryopreserved protocorms of *Vanda coerulea*by encapsulation-dehydration in combination with a loading solution (Figure 4). Protocorms were selected 70 days after sowing seeds, harvested from 7-month-old fruits. After encapsulation in an alginate matrix composed of 2% Na-alginate, 2 M glycerol plus 0.4 M sucrose (loading solution),

the protocorms were precultured in modified VW liquid medium (Vacin and Went, 1949) supplemented with 0.7 M sucrose on a shaker (100 rpm) at 25±3ºC for 20 h. Encapsulated protocorms were

then dehydrated in a sterile air-flow in a laminar air-flow cabinet at 25±3^{o}C for 1-10 h, and then directly plunged into liquid nitrogen for 1 d. After thawing at 49^{o}C for 2 min, cryopreserved beads were cultured on modified VW agar medium for regrowth. The highest regrowth of 40% was observed with cryopreserved bead with 35% water content for 8 h dehydration. No morphological variation was detected between non-cryopreserved and cryopreserved plantlets, and ploidy level was unchanged because of cryopreservation.

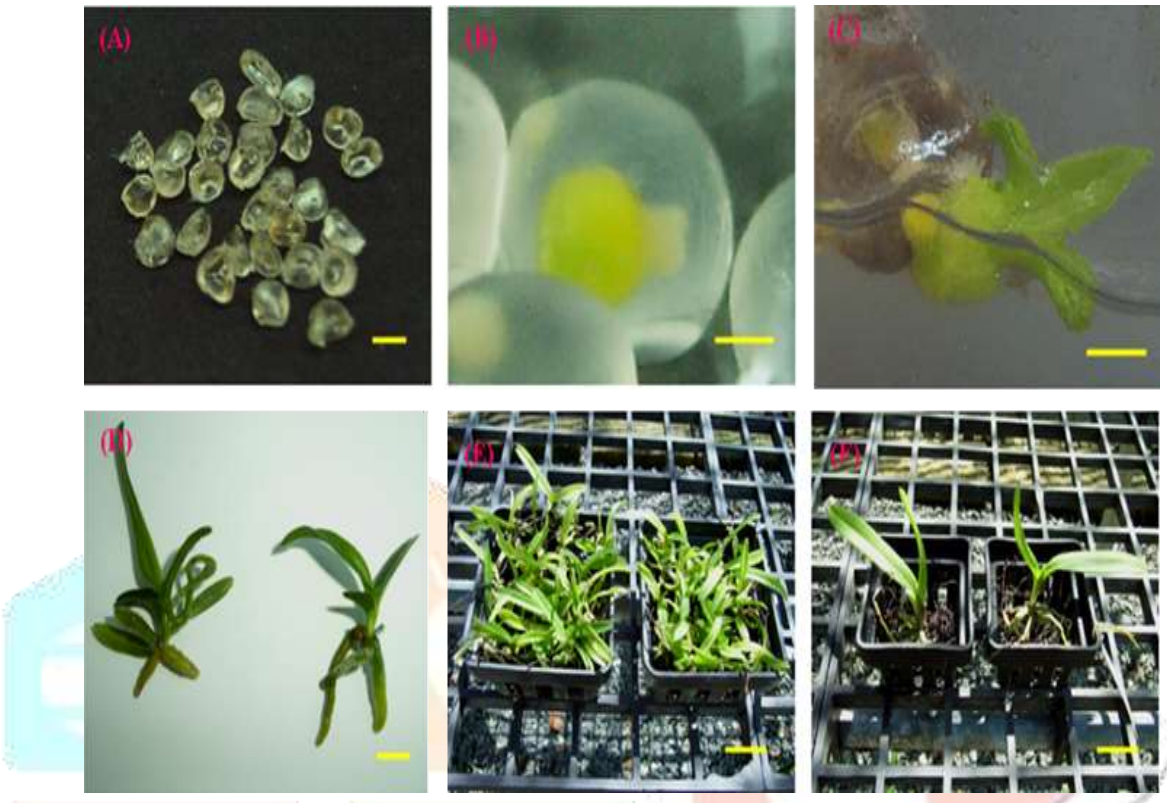

Figure 4. Regrowth of *Vanda coerulea*protocorms after cryopreservation by encapsulation-dehydration in combination with loading solution.
(Source: Jitsopakul et al., 2008a (with permission from CryoLetters Journal)

A: Precultured beads after sterile air-flow dehydration for 10 h. B: Cryopreserved protocorms after 20 days of regrowth. C: 3 months of culture on modified VW agar medium showing shoot growth. D: Plantlets after 8 months of culture on modified VW agar medium (left: non-cryopreserved, right: cryopreserved plantlet). E: Plantlets derived from cryopreserved protocorms after 5 months and F: 15 months of culture in the greenhouse. Bar for A-C = 1 mm, for D = 0.5 cm, and for E and F = 1 cm.

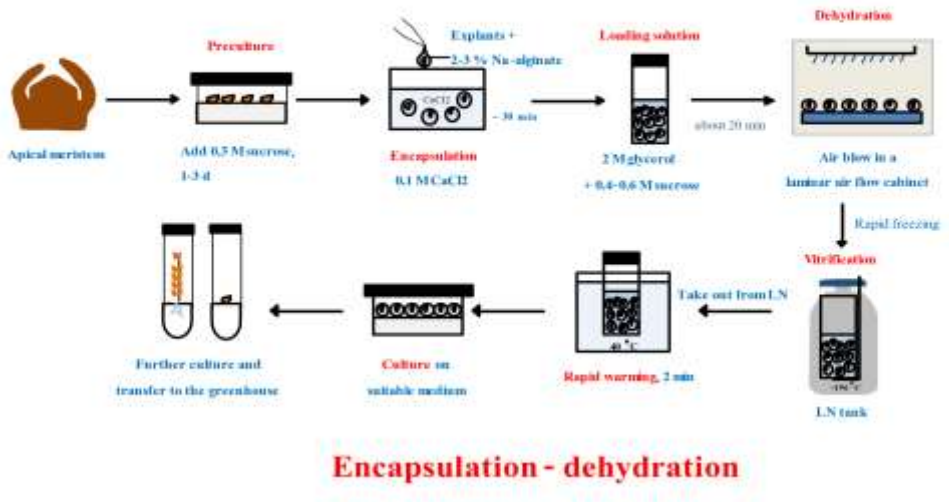

Figure 5. Diagram of Encapsulation-dehydration method.

The protocol for Encapsulation-dehydration method is shown in Figure 5. No highly concentrated chemicals used for dehydration, but air blow is used instead; therefore, it is not toxic to plant materials and environment. Encapsulation helps to reduce the damage from moving plant materials and air blow, as well as taking longer time (20-30 minutes more) for operation which help flexible operation. In addition, there is no unloading solution after rapid warming since the water from outside the cells will not move in fast due to the presence of calcium alginate encapsulation and in a dry condition including without highly concentrated chemical solution. The disadvantages of this method are long operation of 2-3 days, medium or low survival depending on adjusted protocols, and high sucrose concentration after dehydration which some plant species cannot tolerate.

5. Encapsulation-vitrification method

Encapsulation-vitrification method is a combination of encapsulation-dehydration method and vitrification method. The explants are encapsulated in alginate bead, and then subjected to dehydration by highly concentrated plant vitrification solutions, such as PVS1, PVS2, PVS3, and PVS4. This method gives higher survival than encapsulation-dehydration (Hirai and Sakai, 1998; Sakai et al., 2008; Matsumoto, 2017).

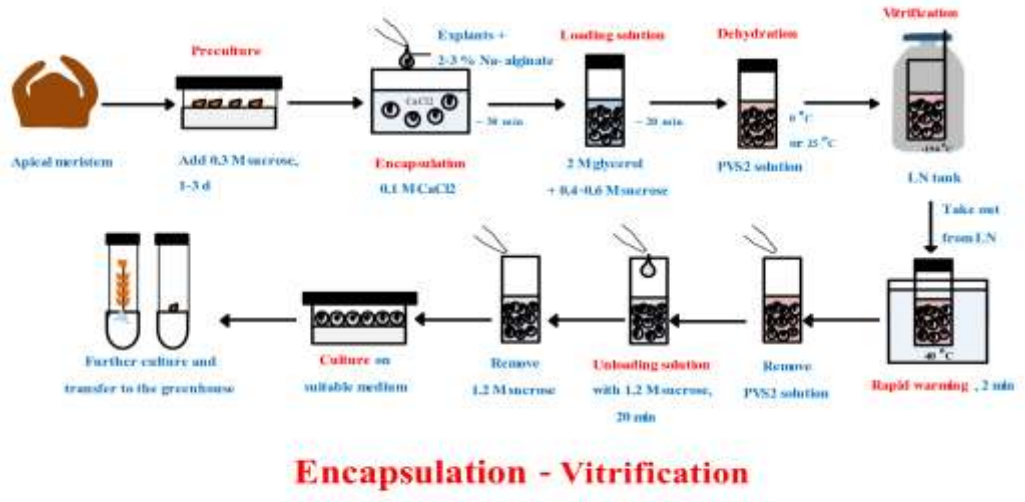

Figure 6. Diagram of Encapsulation-vitrification method.

The protocol for Encapsulation-vitrification method is shown in Figure 6. Encapsulation-vitrification method is the method that is applied from Encapsulation-dehydration. Survival is higher than the Encapsulation-dehydration methodand close to or slightly lower compared with the vitrificationmethod.

6. Droplet-vitrification method

This recent method is the fast freezing from small drops of plant vitrification solution (PVS) on aluminium foil. Droplet-vitrification method is a combination of droplet-freezing and solution-based vitrification, then placed on aluminium foil strip in droplet of vitrification solution and then frozen by rapidly immersion in liquid nitrogen (Sakai and Englemann, 2007). Rapid warming was done by dipping the aluminium foil strips in unloading solution without using a water bath (Kim et al., 2006). During the cooling and warming procedures, rapid heat transfer is needed to avoid freezing injury (Agrawal et al., 2004; Kim et al., 2006; Yoon et al., 2006). Aluminium foil has an efficient thermal conductivity, resulting in quick and uniform heat distribution among tissue (Halmagyi et al., 2005; Kim et al., 2006; Yoon et al., 2006). A high warming rate was employed to avoid recrystallization of intracellular ice or addition cell dehydration by extracellular ice (Kim et al., 2006). The first success of droplet-vitrification method was studied in potatoes (Pennycooke and Towill, 2000). Later, successes in papaya (Ashmore et al., 2001), prunes (De Boucaud et al., 2002), jam (Leunufna and Keller, 2003), chrysanthemum (Halmagyi et al., 2004), banana (Panis et al., 2005), rose (Halmagyi and Pinker, 2006), *Bletilla striata*(Jitsopakul et al., 2008b), *Grammatophyllumspeciosum*(Sopalun et al., 2010), *Vanda coerulea* (Jitsopakul et al., 2008a), Sugar cane (Barraco et al., 2011), *Vanilla* orchid (Gonzalez-Arnao et al., 2009; Hernandez-Ramirez et al., 2014), Oil Palm (Gantait et al., 2015), Grapes (Pathirana et al., 2015), Orange (Volk et al., 2012), etc.

Jitsopakul et al. (2008b) studied droplet-vitrification method for cryopreservation of*Bletilla striata* mature seeds (0 day after sowing), zygotic embryos (3 days after sowing)and protocorms (6, 9, and 12 days after sowing) (Figure 7). Mature seeds were surface-sterilized and sown on solidified New Dogashima(ND) medium supplemented with 3% sucrose and cultured under illumination provided at an intensity of 62.0 μmol.

$m^{-2}.s^{-1}$ for 16 h/dat 25°C for preparation of zygotic embryos and protocorms. Mature seeds, zygotic embryos, and 6-day-old protocorms were precultured in liquid ND medium supplemented with 0.3 M sucrose for 3 h on a shaker (110 rpm) and then dehydrated with 2 M glycerol and 0.4 M sucrose in ND liquid medium (loading solution) for 15 min, followed by exposure to PVS2 solution for 60 min at 25°C. Then, plant materials were soaked in liquid nitrogen by droplet-vitrification method, and then were cultured on solidified ND medium supplemented with 3% sucrose, germination rates of cryopreserved mature seeds, cryopreserved zygotic embryo, and survival rate of cryopreserved 6-day-old protocorms were 93%, 91%, and 84%, respectively. Cryopreserved 9-day-old protocorms gave the highest survival rate of 66% when precultured with 0.5 M sucrose for 3 h on a shaker, dehydrated with loading solution for 15 min, followed by exposure to PVS2 solution for 40 min at 25°C and cultured on solidifiedND medium supplemented with 480 mg/l ammonium nitrate and 3% sucrose. No survival was observed in cryopreserved 12-old-day protocorms.Figure7 (7A-7E) showed development of mature seeds. Mature seeds developed into zygotic embryos at 3 days after sowing (Figure7B). Protocorms formed green spots on zygotic embryos at 6 days of sowing (Figure7C). Protocorms formed apical meristems at 9 days of sowing (Figure7D) and formed primary leaves at 12 days of sowing (Figure7E).

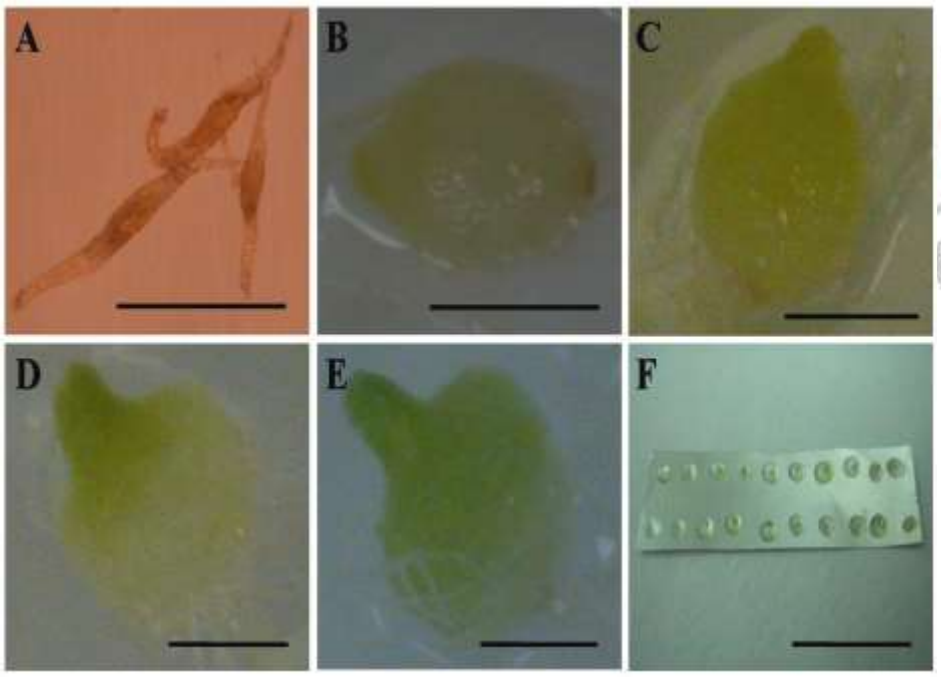

Figure 7. Mature seeds of *Bletilla striata*.
(Source: Jitsopakul et al., 2008a(with permission from CryoLetters Journal)

A: Development of mature seeds sown on solidified ND medium supplemented with 3% sucrose under illumination provided at an intensity of 62 μM $m^{-2}.s^{-1}$ for 16 h/d at 25°C for 3 days, B: 3 days, C: 6 days, D: 9 days, E: 12 days, and F: Twenty droplets of PVS2 solution (2 μl) with protocorms, placed on sterilized aluminium foil strip (7 x 20 mm^2). Bar: A-E = 0.5 mm, F = 1 cm.

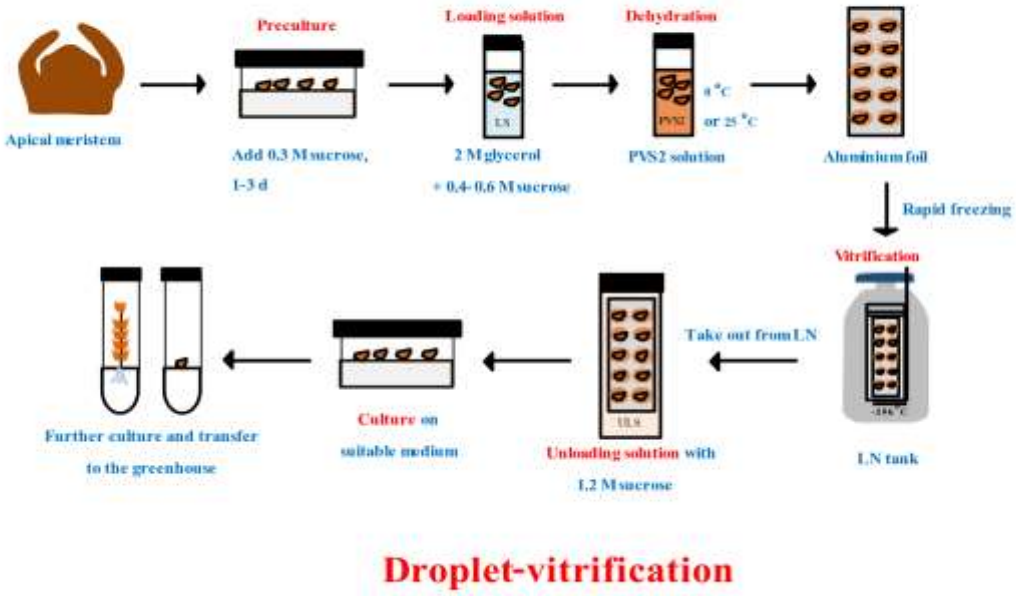

Figure 8. Diagram of Droplet-vitrification method.

The protocol for Droplet-vitrification method is shown in Figure 8. Droplet-vitrification method is a relatively new method which is improved from Vitrification. The explants are dehydrated by using just 1 drop of highly concentrated plant vitrification solution and place on an aluminum foil which contributes to heat. It is convenient to move 10 -12 explants on the aluminum foil at the same time. High survival is obtained from rapid cooling and warming from aluminium foil.Rapid warming is skipped from this protocol.

7. V cryo-plate method

A new method for preserving plant species in cold conditions was developed 13 years ago by Yamamoto et al. (2011) is the V cryo-plate method. It is the combination of encapsulation-vitrification and droplet-vitrification methods to make artificial seeds to attach well (high quality) on aluminium sheets (cryo-plate) size 7 mm × 37 mm × 0.5 mm, which has 10-12 holes (hole size 1.5 mm × 0.75 mm) in which the aluminium sheet is thicker than the aluminum foil (aluminum foil) used with the droplet-vitrification method. The thermal conductivity of the aluminium sheet is better than the aluminium foil which has a cooling rate of approximately 4,000 °C/min and an increase of temperature of around 3,000 °C/min; while, aluminium sheets have a temperature reduction of around 5,000 °C/min and an increase of temperature around 4,500 °C/min. Therefore, V cryo-plate method gives higher survival than droplet-vitrification method because the cooling and warming rates are faster. The advantages of the V cryo-plate method are: 1. The cooling and warming rates are very fast. Therefore, providing high survival. 2. The protocol is easy and convenient because the plant materials are attached to the aluminium sheet throughout the operation, not in a suspended state in a super cool solution, such as PVS2 and PVS3. Using this method does not need special skills.

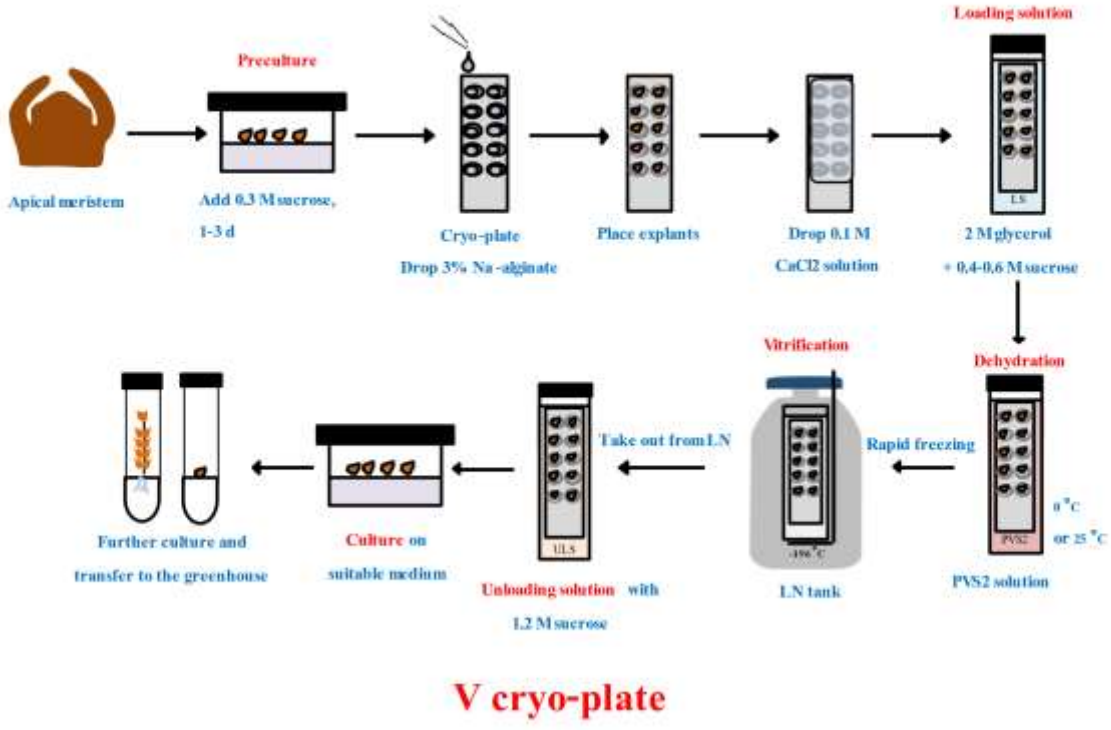

Figure 9. Diagram of V cryo-plate method.

The protocol for V cryo-plate method is shown in Figure 9. The V cryo-plate method is developed from a combination of Encapsulation-vitrification and Droplet-vitrification method. The advantages of the V cryo-plate method are: 1. The cooling and warming rates are very fast. Therefore, providing high survival. 2. The protocol is easy and convenient because the plant materials are attached to the aluminium sheet throughout the operation, not in a suspended state in a super cool solution, such as PVS2 and PVS3. Using this method does not need special skills.

D. cryo-plate method

Niino et al. (2013) developed the D cryo-plate method, which is a combination of encapsulation-dehydration with the V cryo-plate method (Niino et al., 2014). As some plants may be affected using PVS2 solutions, there is low survival. Therefore, to avoid this damage, cells were dried by blowing air from a laminar air-flow cabinet. In addition, the cryopreserved plant materials can be larger than those using the V cryo-plate method because there is no problem of dehydration and the toxicity of the PVS2 solution after prolonged immersion. Both V cryo-plate and D cryo-plate methods have the same protocol except for the cell dehydration by air-drying, silica gel, or drying beads, instead of using concentrated chemical solutions, such as PVS2 solution.

Later, Cordova II and Thammasiri (2016) developed D cryo-plate method using silica gel and drying beads for dehydrating cells (Figure 10). Regrowth was observed at the 2nd week of transfer to ½MS media. Regrowth was observed to be 73.8% and 76.5%using silica gel and drying beads for dehydration, respectively. Dehydration using silica gel or drying beads did not significantly affect regrowth rate. Protocorms dehydrated using silica gel or drying beads developed into normal plantlets.

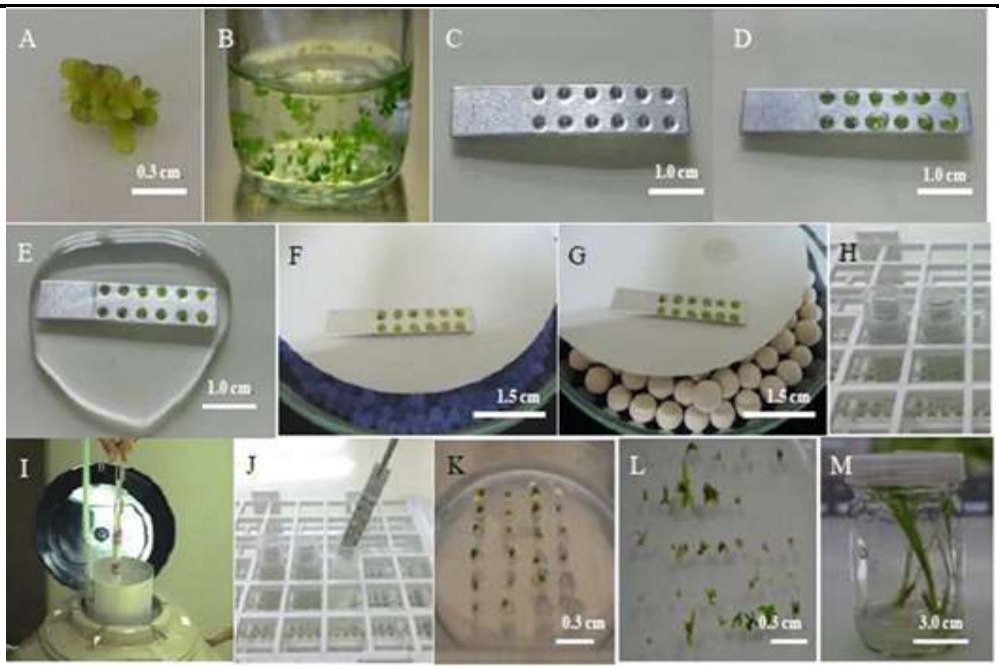

Figure 10.Cryo-plate method dehydrated with silica gel or drying beads.

A: Protocorm development, B: Preculture of protocorms in ½MS liquid medium with 0.7 M sucrose for 1 d, C: Pour the alginate solution containing 2% (w/v) sodium alginate in calcium-free ½MS basal medium with 0.4 M sucrose in the wells, D: Place the precultured protocorms in the wells one by one, E: Pour the calcium chloride solution containing 0.1 M calcium chloride in ½MS basal medium with 0.4 M sucrose, F: Dehydration with 50 g silica gel, G: Dehydration with 30 g drying beads, H: Put each cryo-plate in a 2 ml cryotube, I: Plunge 2 ml cryotubes into liquid nitrogen for 1 d, J: Warming in 1.2 M sucrose solution for 20 minutes, K: Plate on ½MS agar medium, L: Regrowth, and M: Regrowth after 60 days.

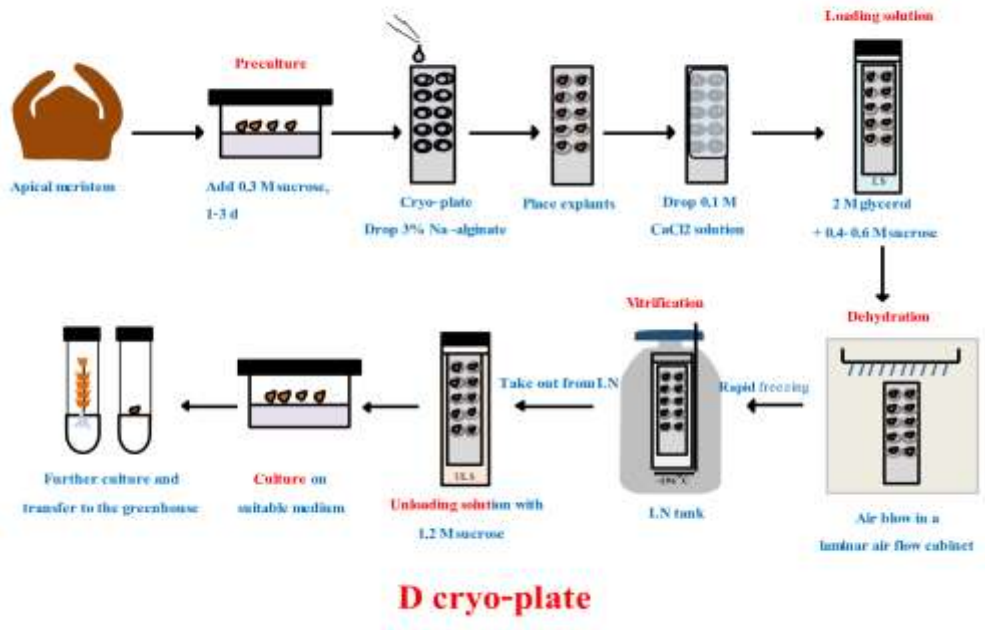

Figure 11. Diagram of D cryo-plate method.

The protocol for D cryo-plate method is shown in Figure 11. The development of the V cryo-plate method, which is a combination of Encapsulation-vitrification and Droplet-vitrification method and followed by the D cryo-plate method which is a combination of Encapsulation-dehydration with V cryo-plate method by using aluminum sheet that conducts heat,as well as a vehicle to carry 10-12 plants at the same time throughout every step. Therefore, making the operation convenient, fast, efficient, and high survival. Since it has a cooling rate of 5,000°C/min and a warming rate of 4,500°C/min.

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