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Cryopreservation of Plant Genetic Resources

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1. Introduction

The plant genetic resources is preserved by pollen, seed, branch, bulb, or tissue culture at the gene bank. Since these need to be updated periodically (several months ~ several years), I point out the problems involving the great labor and space spent for the maintenance of plant genetic resources.

Cryopreservation is a storage method of plant genetic resources at ultra-low temperature, for example, that of liquid nitrogen (LN; -196 °C). It is a preservation method that enables plant genetic resources to be conserved safely, and cost-effectively.

For successful cryopreservation, it is essential to avoid intracellular freezing and induce the vitrification state of plant cells during cooling in LN. In addition, the cryopreservation method should be a simple protocol for everyone to use easily. Since the 1970's, cryopreservation techniques have been researched using different plant organs, tissues and cells. As a result, different cryopreservation procedures have been developed (for example, slow-prefreezing method, vitrification method, dehydration method). With the development of these cryopreservation methods, tissues of tropical plants, which have been conventionally thought to be not cryopreserved, also were successfully preserved in LN (Bajaj, 1995; Towill & Bajaj, 2002). In this Chapter, I describe different types of cryopreservation methods.

In addition, I often ask my colleagues why cryopreservation of plant tissue did not succeed irrespective of the method. As the cause, it is possible that there is a problem in the character of plant species (stress resistance and polyphenol production), or the ways used in the cryopreservation technique. Then, I also would like to present some knowledge about some improvements for making cryopreservation of plant genetic resources more successful in this chapter.

2. Methods of cryopreservation of plant genetic resources

In the section (2.1), I would like to introduce cryopreservation methods of plant genetic resources that have been developed. In the section (2.2), I would like to describe the approach when cryopreserving plant samples from the past literatures or my own experience in order to enhance the regrowth percentage after cryopreservation,

2.1 Cryopreservation method of plant genetic resources

In this section, I introduce cryopreservation procedures by using figures.

2.1.1 Slow programmed freezing (also known as “prefreezing”)

Slow programmed freezing was a major cryopreservation method for plant genetic resources until the 1980's. The procedure in this method is shown in Fig. 1.

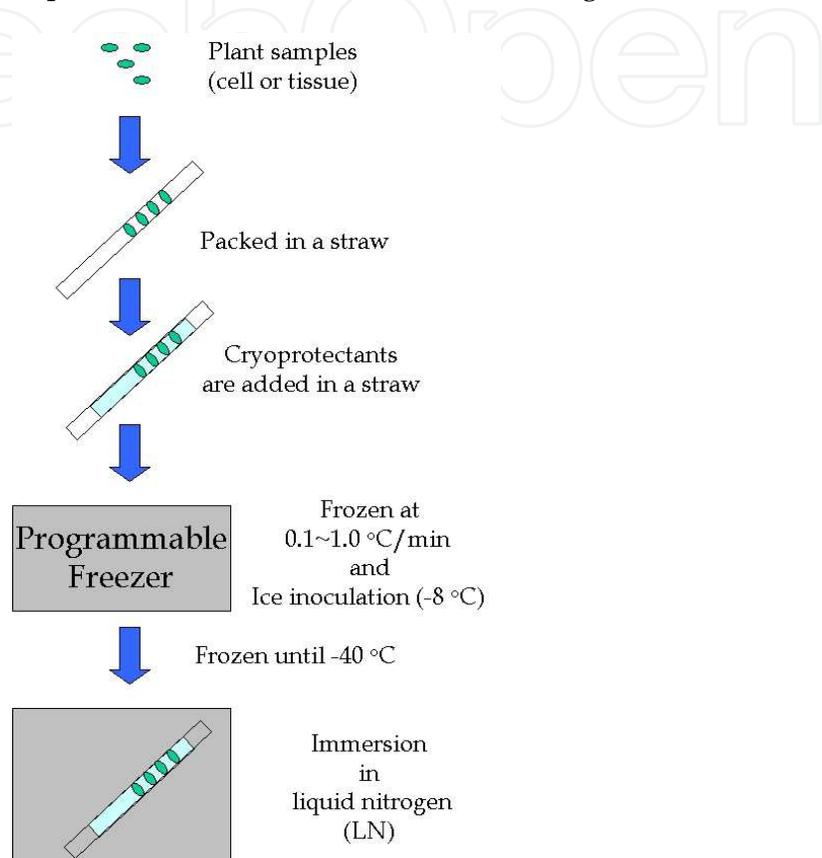


Fig. 1. The protocol of slow programmed freezing (from Kumu et al., 1983).

Plant genetic resources (cells and tissues) were packed in cryotube or straw, and cryoprotectants were added. In this method, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and glucose were utilized as cryoprotectants. In many cases, these were used independently, but Finkle & Ulrich (1979) reported that the regrowth percentage of germplasm after cryopreservation was higher when mixing cryoprotectants in sugar cane cells. Packed specimens were gradually cooled from -20 °C to -100 °C using a programmable freezer or ethanol baths. Processing which freezes cryoprotectant in a tube artificially is performed near -7~-8 °C in the middle of the freezing. In this treatment, ice is made to form out of a cell under gradual cooling. Intracellular moisture penetrates a plasma membrane, and arrives at the surface of the ice besides a cell, and freezes. This is called 'extracellular freezing'. Intracellular moisture is removed and a cytoplasm is contracted by 'extracellular freezing'. Kindly refer to the book of Kartha (1985) to understand the principle of this phenomenon. After making specimens freeze to a predetermined freezing-temperature, they are immersed in LN. The freezing-temperature is arranged by -40 °C in many species. Cryopreserved tubes are warmed using hot water (40 °C) for 1~2 min, and cryoprotectants

are removed from a tube. After rewarming, samples are moved from the cryotube, and recultured. The cooling rate in this method is important. It differs from 0.5 °C/min to 50 °C/min with plant species and the size of the plant germplasm. However, in the case of the freezing speed of 2 °C/min or more, the regrowth after preservation tends to fall (Sugawara & Sakai, 1974; Uemura & Sakai, 1980). The disadvantage of this method is that there are many species for which the prefreezing method is not utilized at all. In addition, there are plant tissues which freeze to death partially, and cases in which the decrease in subsequent viability induced also exists (Grout & Henshaw, 1980; Haskins & Kartha, 1980).

2.1.2 Slow unprogrammed freezing (also known as “simple freezing”)

This cryopreservation method was reported using samples of several species in the early 1990's. The advantage of this method is that researchers can cryopreserve without a special programmable freezer, compared with slow programmed freezing.

The slow unprogrammed freezing is shown in Fig. 2. Plant tissues are added to the tube containing cryoprotectants. Tubes are treated for about 10 min at room temperature (25 °C), and are kept at -30 °C for 30~120 min. They are then immersed in LN thereafter. Cryopreserved tubes are warmed using hot water (40 °C) for 1~2 min, and cryoprotectants are removed from a tube. After rewarming, samples are moved from the cryotube, and recultured. In this cryopreservation method, mixtures of glycerol and sucrose or DMSO and sorbitol are used as cryoprotectants (Sakai et al., 1991; Niino et al., 1992; Maruyama et al., 2000). In this cryopreservation method, although ‘naked’ samples are used, Kobayashi et al. (2005) utilized cells encapsulated with alginate beads in the suspension cells of tobacco.

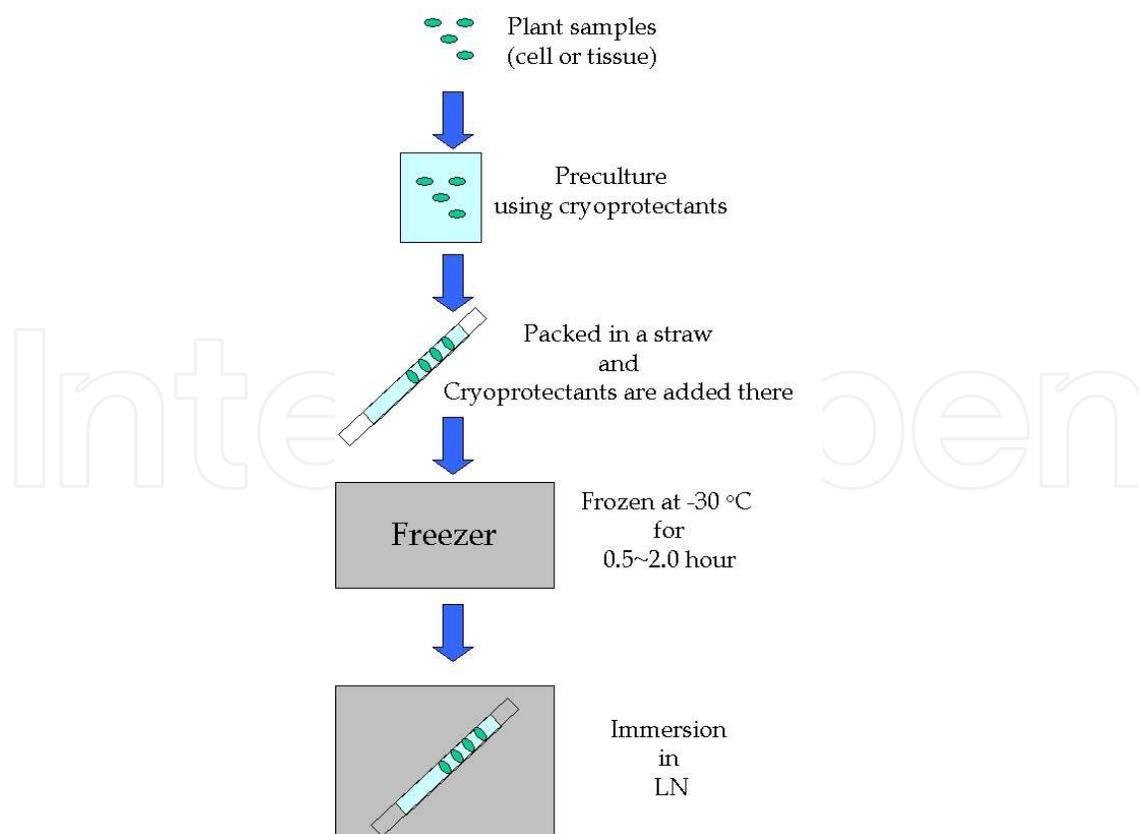


Fig. 2. The protocol of slow unprogrammed freezing (from Sakai et al., 1991).

2.1.3 Vitrification

The vitrification method has been the major cryopreservation method since Uragami et al. (1989) developed it using asparagus culture cells. This cryopreservation method is shown in Fig. 3. Plant tissues are added to the tube containing the loading solution (LS) for the osmoprotection. Beads in tubes are osmoprotected for about 30 min at room temperature (about 25 °C). LS is the liquid culture medium in which sucrose (0.4 mol/L) and the glycerol (2.0 mol/L) were contained. After loading, LS is removed from a tube, and new vitrification solution is added for the dehydration of plant tissues.

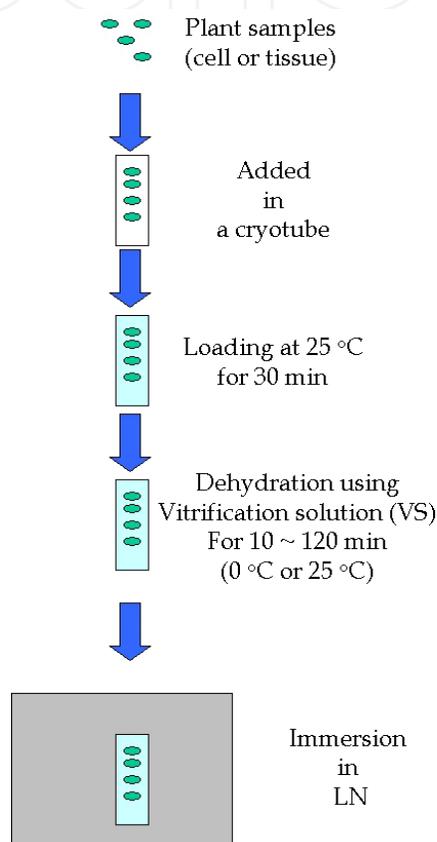


Fig. 3. The protocol of vitirication method (from Sakai et al., 1990).

Many cryoprotectants are dissolved in the vitrification solution, and the optimal dehydration time using the solution changes greatly with treatment temperature.

In many cases, the dehydration using the vitrification solution is performed at 0 °C by the reason of the toxicity to plant cells. Plant Vitrification Solution 2 (PVS2; Sakai et al., 1990) is utilized most as the vitrification solution. Besides PVS2, there are many vitrification solutions. Please refer to Table 1 for the composition. They are immersed in LN after that. Cryopreserved tubes are warmed using hot water (40 °C) for 1~2 min, and the vitrification solution is removed from a tube. After the removal of vitrification solution, unloading solution (the liquid medium supplemented with 1.2 mol/L sucrose) is added to a tube, and cryoprotectants are removed from plant tissues for 30 min at 25 °C. In many cases, the above-mentioned liquid mediums (LS, PVS and unloading solution) were adjusted by pH

5.7~5.8, but without plant growth regulators. After unloading, samples are removed from the cryotube, and recultured.

Component (g/L)	PVS1	PVS2	PVS3
Glycerol	220.0	300.0	500.0
Ethylene Glycol	150.0	150.0	
Propylene Glycol	150.0		
Dimethyl Sulfoxide (DMSO)	70.0	150.0	
Sucrose		136.9	500.0
Sorbitol	91.1		

Table 1. Components of major plant vitrification solutions. Components of three plant vitrification solutions are referred from previous reports (Uragami et al., 1989; Sakai et al., 1990; Nishizawa et al., 1993).

2.1.4 Encapsulation-vitrification

The encapsulation-vitrification method was reported first by Matsumoto et al. (1995) using shoot apices of *Wasabia japonica*, and then spread all over the world. The advantage of this method is that regrowth of plant germplasm after cryopreservation is markedly increased by encapsulating plant samples with alginate beads. The encapsulation of plant germplasms makes for less damage to samples during vitrification procedures (loading treatment and dehydration treatment). On the other hand, due to encapsulation-dehydration, treatment time becomes long compared with that of vitrification and the cryopreservation operation becomes complicated (for example, encapsulation).

The procedure for encapsulation-vitrification is shown in Fig. 4. Plant tissues are immersed in the calcium-free liquid medium supplemented with 0.4 mol/L sucrose, 30.0 g/L sodium alginate and glycerol (1.0~2.0 mol/L). The mixture (including a plant cell or tissue) was added drop by drop to the liquid medium containing 0.1 mol/L calcium chloride, forming beads about 5 mm in diameter. The above-mentioned liquid mediums (30.0 g/L sodium alginate and 0.1 mol/L calcium chloride) were adjusted by pH 5.7, but without plant growth regulators. Encapsulated specimens are added to the culture bottle containing LS for osmoprotection. Beads in the bottles are osmoprotected for 16 hours at room temperature (25 °C). LS is the liquid culture medium in which sucrose (0.75~0.8 mol/L) and the glycerol (2.0 mol/L) were contained. After loading, LS is removed from a bottle, and PVS is added newly for the dehydration of plant tissues. The same as with vitrification, the dehydration using PVS is performed at 0 °C in light of the toxicity to plant cells.

After dehydration of PVS, encapsulated samples are moved to a cryotube containing fresh PVS, and immersed in LN. Cryopreserved tubes are warmed using hot water (40 °C) for 1~2 min, and the vitrification solution is removed from the tube. After removal of the solution, unloading solution (supplemented with 1.2 mol/L sucrose; pH 5.7) is added to a tube, and cryoprotectants are removed from plant tissues for 30 min at 25 °C. After unloading, samples are moved from the cryotube, and recultured.

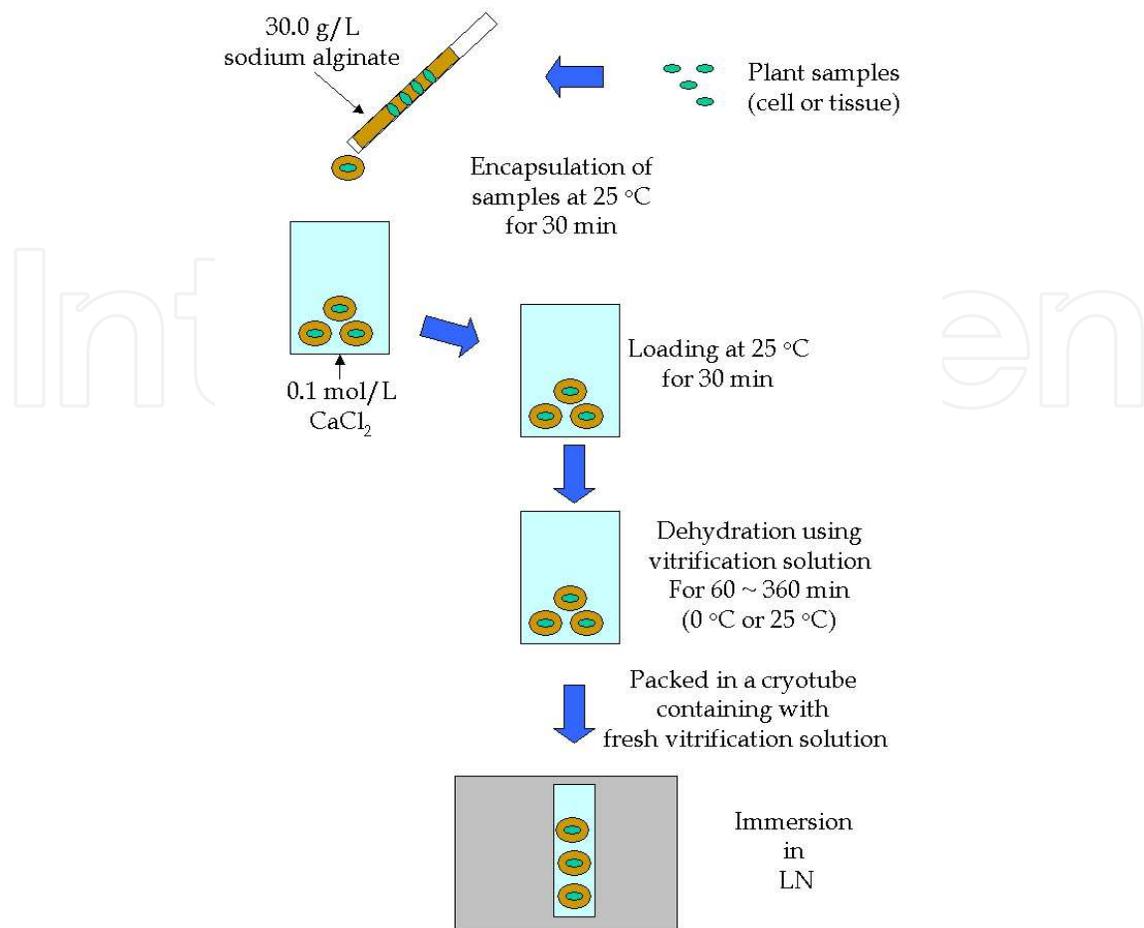


Fig. 4. The protocol of encapsulation-vitrification method (from Matsumoto et al., 1995)

2.1.5 Simplified encapsulation-vitrification

The simplified encapsulation-vitrification method was first reported by Hirai & Sakai (2002) using shoot apices of sweet potato. The operating procedure in this method is the same as encapsulation-vitrification (see Fig. 4), however, the composition of LS differs. LS of simplified encapsulation-vitrification includes high-concentration glycerol (2.0 mol/L) and sucrose (1.6 mol/L), and the viscosity of LS is high. Although this method succeeded with sweet potato, there are some plant species which cannot be cryopreserved using high concentration glycerol (Hirai & Sakai, 1999).

2.1.6 Droplet method

The droplet method was first reported by Schäfer-Menuhr et al. (1994, 1997) using potato apices. The operating procedure is the same for vitrification. However, the LS immersion protocol differs compared with that in the vitrification method. This cryopreservation method is shown in Fig. 5. After treatments by LS and PVS, plant samples are put on aluminum foil which is sterilized and cut small. One drop of PVS is dripped onto plant samples, and the whole aluminum foil is immersed in LN. The aluminum foil after cryopreservation is taken out from LN, and one drop of unloading solution supplemented with 1.0 mol/L sucrose is dripped onto to freezing samples. After rewarming, samples are

moved from the cryotube, and recultured. In the droplet method, in order to make a plant sample cool quickly, Wesley-Smith et al. (2001) used not liquid nitrogen but a slush nitrogen (-210 °C) and an isopentane (-160 °C). In addition, the droplet method can reportedly obtain a high regrowth percentage after cryopreservation in tropical plants difficult to cryopreserve (Pennycooke & Towill, 2000, 2001; Leunufna & Keller, 2003; Panis et al., 2005).

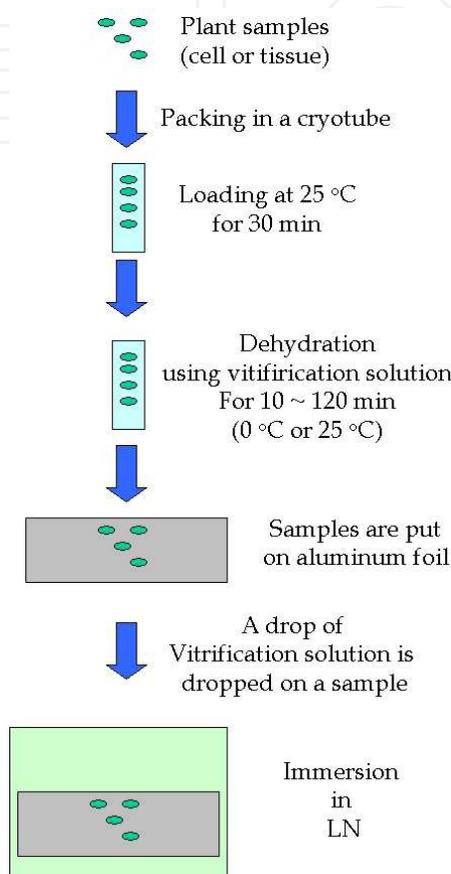


Fig. 5. The protocol of Droplet method (from Schäfer-menuhr et al., 1997).

2.1.7 Dehydration

Dehydration was first reported by Uragami et al. (1990) using asparagus lateral buds. A dry technique is superior to vitrification in that it does not need to produce PVS. Therefore, there is no influence of medical toxicity at low cost. Problems of dehydration include ready influence of humidity on drying by air flow and dried samples are easily crushed with tweezers.

The cryopreservation procedure is shown in Fig. 6. Plant tissues are put on the filter paper or nylon mesh sterilized and cut small. Samples are dehydrated by silica gel (Uragami et al., 1990) or air flow (Shimonishi et al., 1992; Kuranuki & Yoshida, 1996) before immersion in LN. It is reported that the optimal moisture of the sample is 10%~30% for survival after cryopreservation in the dehydration method (Uragami et al., 1990; Shimonishi et al., 1992; Kuranuki and Yoshida, 1996). After the dehydration, germplasms are moved to a cryotube and immersed in LN. Cryopreserved tubes are warmed at room temperature or using hot

water (40 °C) for 1 ~ 2 min. After rewarming, samples are removed from the cryotube, and recultured.

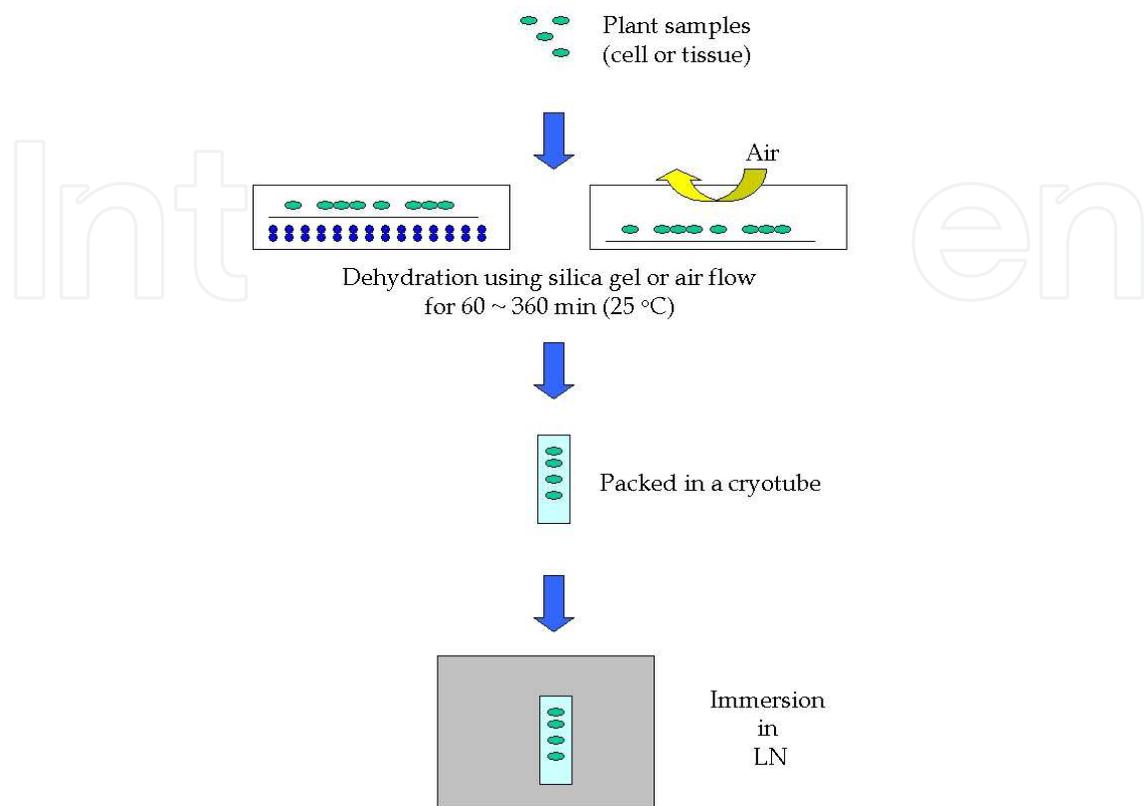


Fig. 6. The protocol of Dehydration method (from Uragami et al., 1990).

2.1.8 Encapsulation-dehydration

The encapsulation-dehydration method was first reported by Fabre & Dereuddre (1990) using shoot apices of potato, and spread worldwide the same way as vitrification and encapsulation-vitrification. This method excels that of dehydration in that regrowth of plant germplasm after cryopreservation is markedly increased by encapsulating plant samples with alginate beads. In addition, encapsulated samples are difficult to be crushed with tweezers compared with the dehydration method.

The encapsulation-dehydration procedure is shown in Fig. 7. Plant tissues are immersed in a calcium-free liquid medium supplemented with 0.4 mol/L sucrose and 30.0 g/L sodium alginate. The mixture (including a plant cell or tissue) was added drop by drop to the liquid medium containing 0.1 mol/L calcium chloride, forming beads about 5 mm in diameter. The above-mentioned liquid mediums (30.0 g/L sodium alginate and 0.1 mol/L calcium chloride) were adjusted by pH 5.7~5.8, but without plant growth regulators. Encapsulated germplasms are added to the culture bottle containing LS for the osmoprotection. Beads in the bottles are osmoprotected for 16 hrs at room temperature (25 °C). LS is the liquid culture medium in which sucrose (0.75~0.8 mol/L) is contained. After loading, LS is removed from the bottle. Loaded samples are put on sterilized filter papers, and samples are dehydrated by silica gel for 3~7 hours before immersion in LN. After dehydration by silica gel, encapsulated samples are moved to a cryotube, and immersed in LN. Cryopreserved tubes are warmed using hot water (40 °C) for 1 ~ 2 min. After rewarming, samples are moved from the cryotube, and recultured.

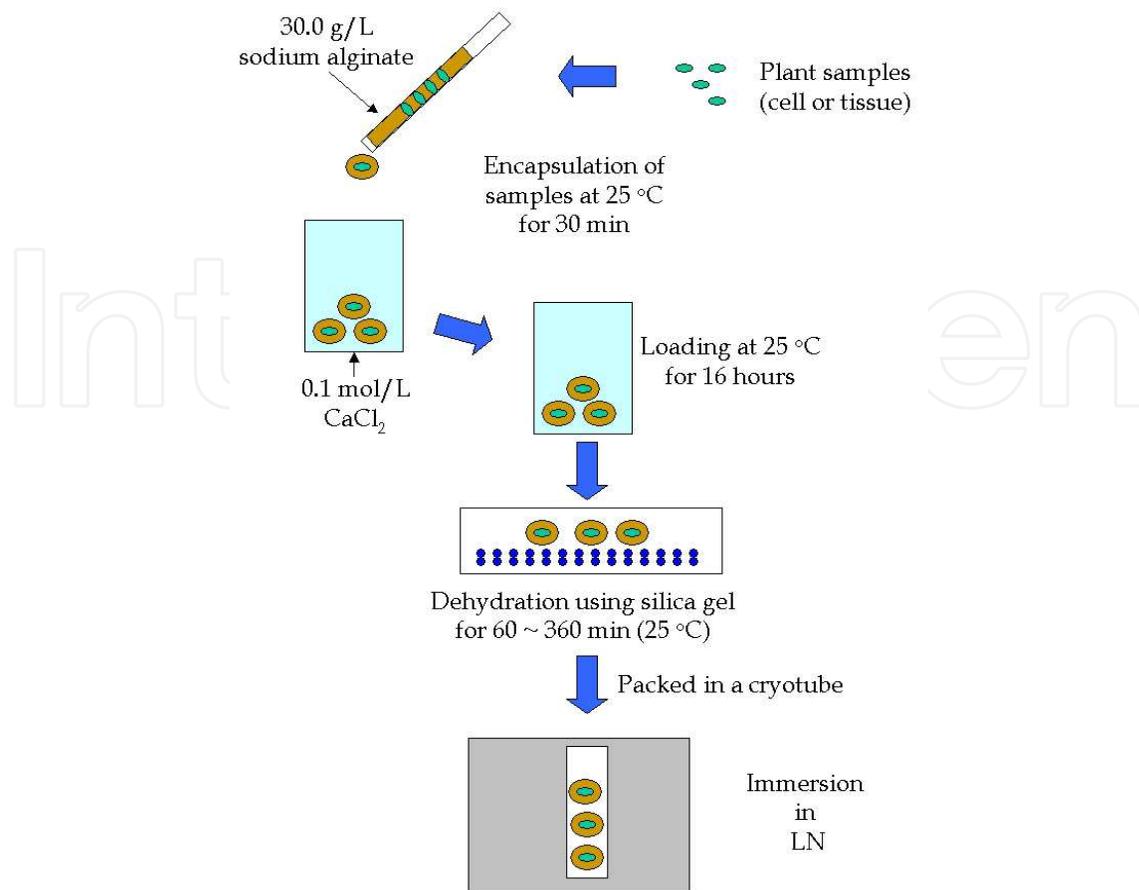


Fig. 7. The protocol of Encapsulation-dehydration method (from Fabre & Dereuddre, 1990).

In encapsulation-dehydration, the addition of glycerol besides sucrose in LS reportedly enhances the regrowth percentage of cryopreserved samples. The optimal concentration of glycerol in LS is 0.5~2.0 mol/L for regrowth of cryopreserved specimens (Matsumoto & Sakai, 1995; Kami et al., 2005, 2007, 2008).

2.1.9 Newly-developed encapsulation-dehydration

A newly developed encapsulation-dehydration method was first reported by Sakai et al. (2000). The operating procedure is the same as for encapsulation-dehydration (see Fig. 7), however, the LS composition differs. LS of the newly developed encapsulation-dehydration includes a high concentration (2.0 mol/L) of glycerol besides sucrose. Therefore, the loading time of this method (1 hour) is shorter than that of encapsulation-dehydration (16 hours).

2.2 Methods of improvement of cryopreservation efficiency

In this section, I introduce some approaches to increase regrowth of samples after rewarming with past reports and actual experimental data I obtained.

2.2.1 Plant material

Before performing cryopreservation of plant samples, it is necessary to grasp the characteristics of the given plant species. For example, it is better to utilize encapsulation-

dehydration rather than vitrification for plant species which are subject to toxicity from cryoprotectants. Moreover, if you want to cryopreserve the plant germplasms readily susceptible to toxicity in DMSO with the vitrification method, it is better to use PVS3 rather than PVS2 as the vitrification solution.

Next, I would like to explain this paragraph with actual experimental data I obtained. In cryopreservation, the extracted size of plant material also becomes important. When plant tissues are greatly (3 mm x 3 mm) trimmed, the extraction labor will decline with small tissue size (1 mm x 1 mm). However, the regrowth percentage of large tissues after cryopreservation seems to decrease more than that of small tissues (Fig. 8; Kami et al., 2010). From previous reports, the reason is that the smaller the size of the extracted plant, the more the osmosis cryoprotectant decreases (Kim et al., 2004, 2005).

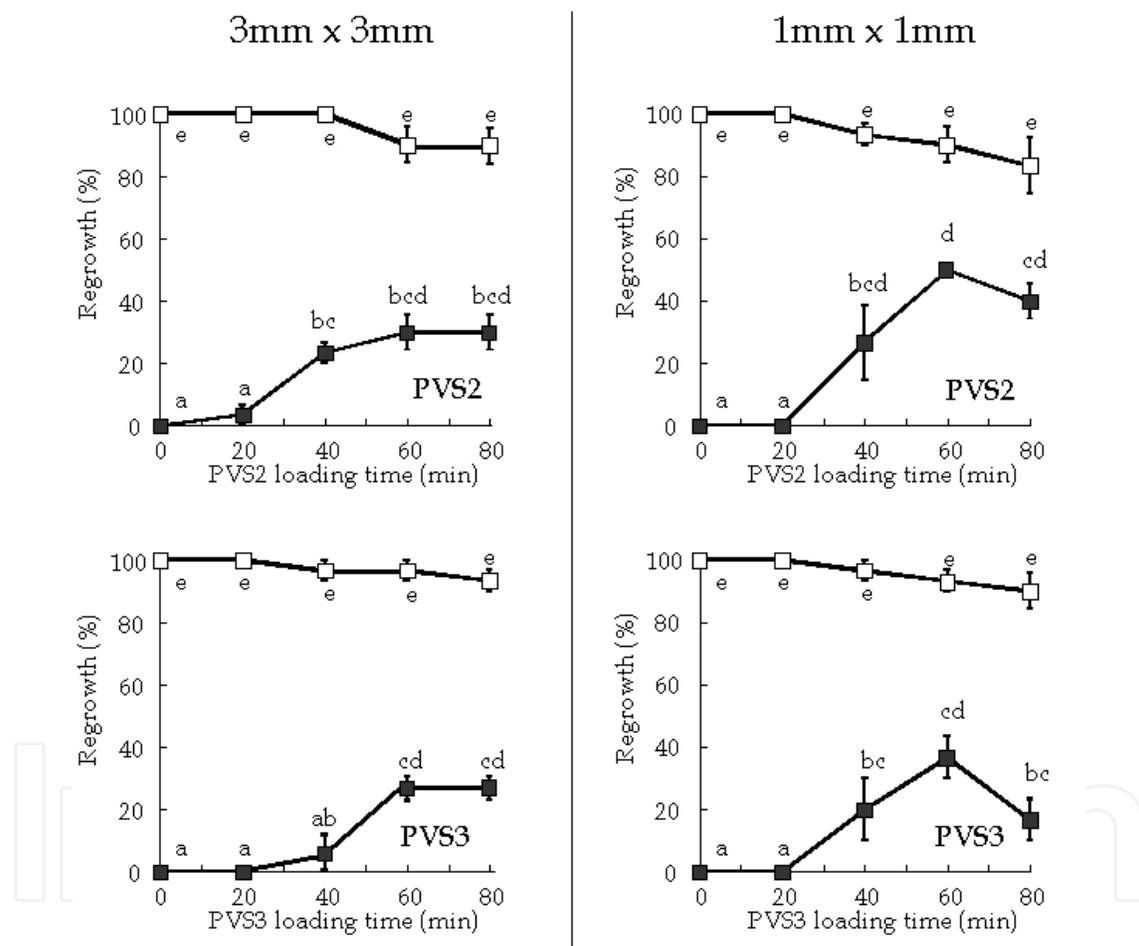


Fig. 8. Effects of excised apex size and exposure time to plant vitrification solution (PVS) on the regrowth of shoot apices immersed in liquid nitrogen (LN) using vitrification. Apices were dehydrated with two types of PVS at 0 °C for various lengths of time prior to cooling (Cryopreserved) or without cooling to -196 °C (Treated Control). The PVS in a cryovial was exchanged just after PVS loading treatment to prevent deterioration of PVS by a loading solution in this study. After cooling for 1hour in LN, rewarming apices were transplanted into regrowth medium. Values represent mean \pm SE of three determinations. Differences in mean values of regrowth of treated control and cryopreserved apices with different letters are statistically significant (Tukey's HSD at $p < 0.05$) in all data. (from Kami et al., 2010)

2.2.2 Treatment before cryopreservation

Before cryopreservation, cold-acclimation and preculture are done, so survival percentages will increase after cryopreservation.

Cold-acclimation is a treatment by which plantlets are cultured at about 5 °C for one week to two months. However, Chang et al. (2000) reported that cold-acclimation was performed at -1 °C in grass species (*Zoysia* and *Lolium* sp.). The freezing resistance of plant specimens reportedly increases by cold-acclimation (Chang et al., 2000). However, since cold-acclimation cannot be adapted for a tropical plant, you should not perform this operation. Moreover, optimal acclimation periods differ by plant germplasms. In addition, prolonged cold-acclimation may curve and lower the survival percentage of plant specimens after cryopreservation. Therefore, I recommend that you closely consider the optimal cold acclimation period before trying cryopreservation.

Preculture is the treatment which gives plant cells or tissues dehydration tolerance. In many cases, plant samples are cultivated for 24~48 hours by culture medium supplemented with high-concentration the sucrose (0.3~0.7 mol/L). And some plant species are moved gradually from low to high concentration of sucrose medium (Niino et al., 1992; Niino & Sakai, 1992a,b; Suzuki et al., 1994; Niino et al., 1997). In addition, there are also cases in which glycerol (Matsumoto et al., 1998; Niino et al., 2003), DMSO (Fukai, 1990), or abscisic acid (ABA; Kendal et al., 1993; Tsukazaki et al., 2000) is mixed with a sucrose culture medium, and culture medium containing sorbitol without sucrose are used (Yamada et al., 1991; Maruyama et al., 2000). In many cases, room temperature is used for treatment (20~25 °C). However, some plant species can be processed by -1 °C (Chang et al., 2000) or 5 °C (Niino & Sakai, 1992a,b; Kuranuki & Sakai, 1995; Tanaka et al., 2004).

2.2.3 Treatment under cryopreservation

I would like to explain this paragraph with actual experimental data I obtained. In vitrification, I examined the effect of exchange times of fresh PVS2 during a 60-min PVS2 loading treatment on shoot apices (*Cardamine yezoensis* Maxim.) immersed in LN using a vitrification protocol (Fig. 9). The shoot regeneration percentages after cryopreservation was enhanced up to 96.7% when two PVS2 exchanges were used. Moreover, above 80% of shoot regrowth was maintained also by three or more PVS2 exchanges. From this experiment, it became clear that the injury by too much dehydration and medical toxicity are not induced by the exchange of fresh vitrification solution. However, the increase in the exchange time of vitrification solution carries a complex risk of losing the shoot apex and operating. Therefore, I considered that even 2 exchanges during 60-min PVS2 loading treatment on shoot apices of *Cardamine yezoensis* was appropriate (Kami et al., 2010).

Since PVS2 at 0 °C has high viscosity and the circulation in the cryobial is poor, it is thought PVS2 around a shoot apex was diluted by the moisture flowing out of the plant tissue. Therefore, by exchanging for fresh PVS2, the dilution of PVS2 around a shoot apex was prevented and the dehydration maintained.

Furthermore, adding an ice blocking agent to PVS reportedly enhances regeneration of cryopreserved sample in recent years (Zhao et al., 2005).

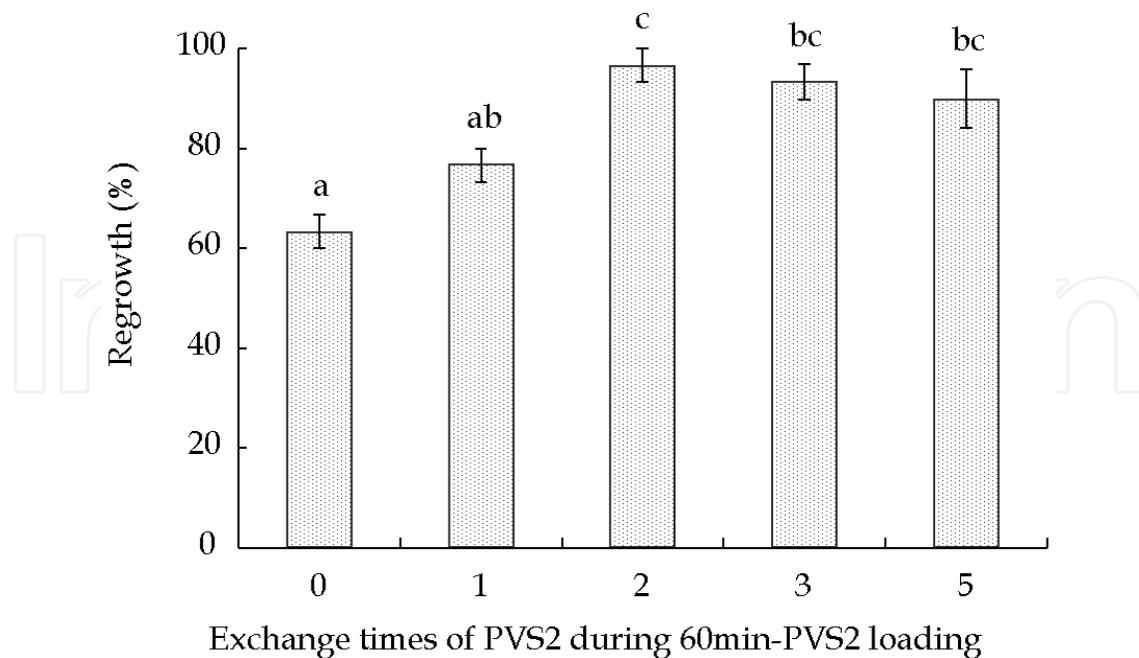


Fig. 9. Effects of exchange times of PVS2 during 60-min PVS2 loading treatment on shoot apices immersed in LN using vitrification. 2 ml of fresh PVS2 were exchanged at 0°C for 60-mins prior to cooling. A PVS2 exchange just after PVS2 loading treatment was not counted as the exchanging time of PVS2 in this study. After cooling for 1hour in LN, rewarming apices were transplanted into 1/4MS. Values represent mean \pm SE of three determinations. Differences in mean values of regrowth with different letters are statistically significant (Tukey's HSD at $p < 0.05$) in each treatments. (from Kami et al., 2010)

2.2.4 Treatment after cryopreservation

In cryopreservation of plant genetic resources, regeneration after rewarming is the key. Surviving cells or tissues after cryopreservation readily succumb due to different environmental agents because they have been injured by the dehydration or temperature change during the cryopreservation procedure. Moreover, when plant specimens were injured by the cryopreservation process, polyphenol can be produced. Thus, this may threaten the survival of plant specimens after cryopreservation. In that case, regeneration of tissues after preservation reportedly increased when activated charcoal (Bagniol & Engelmann, 1992) and polyvinyl pyrrolidone (Niino et al., 2003), an adsorbent of polyphenol, was mixed with a culture medium.

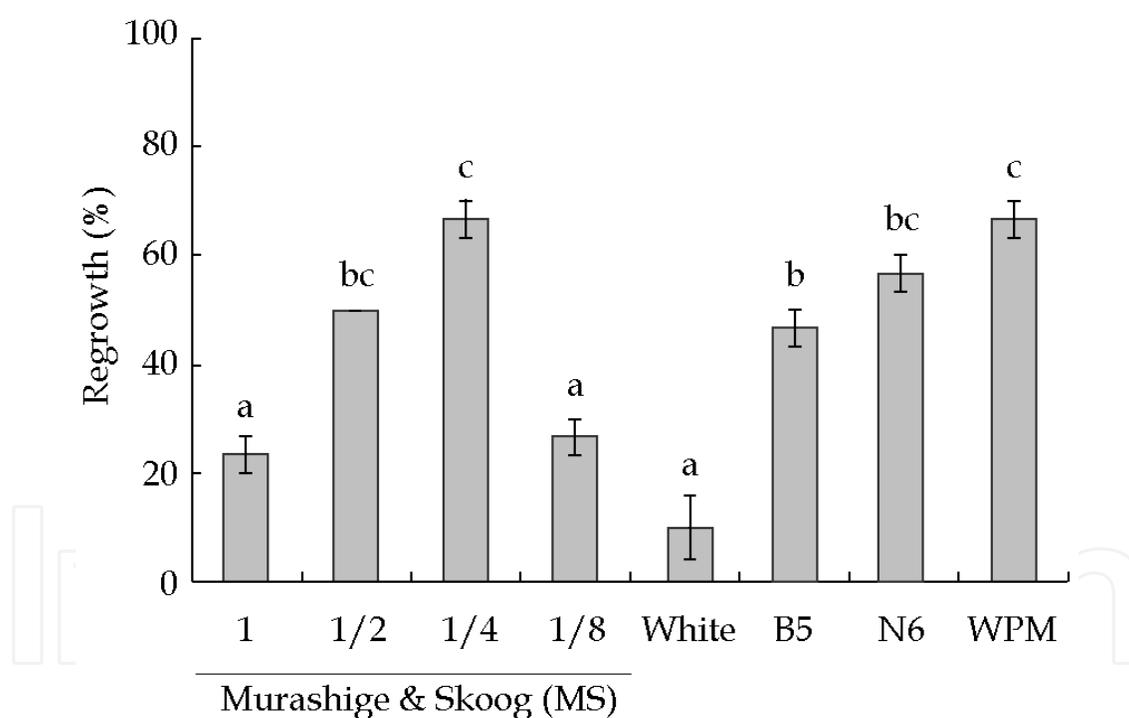
In recent years, it is also reported that regrowth percentages of rewarming tissues increased by mixing surfactant with regrowth medium (Anthony et al., 1996; Niu et al., 2010). Therefore, special consideration must be given to certain plant species. From previous reports, the regrowth after preservation increases sharply also by decreasing NH_4^+ concentration in a culture medium (Niino et al., 1992a, 1992b; Suzuki et al., 1994; Pennycooke & Towill, 2001).

Next, I would like to explain this paragraph with actual experimental data I obtained. I examined the effects of various nutrient media (Table 2) on regrowth of cryopreserved

apices (*Cardamine yezoensis* Maxim.). It was demonstrated that 4-fold dilution of inorganic salts of Murashige and Skoog's medium (1/4MS) or Woody Plant medium (WPM) as basal medium resulted in higher regrowth percentages (both 66.7%) than six other media (Fig. 10; Kami et al., 2010).

Component (mmol/L)	Murashige & Skoog (MS)				White	B5	N6	WPM
	1	1/2	1/4	1/8				
NO ₃ ⁻	45.39	22.70	11.35	5.67	0.79	26.77	30.25	6.30
NH ₄ ⁺	20.61	10.31	5.15	2.58	0.00	2.33	7.01	5.00
PO ₄ ³⁻	1.25	0.62	0.31	0.16	0.12	1.09	2.94	1.25
K ⁺	20.05	10.02	5.01	2.51	1.63	24.73	30.94	12.61
Ca ²⁺	2.99	1.50	0.75	0.37	1.27	1.02	1.13	3.01
Mg ²⁺	3.07	3.07	0.77	0.28	5.98	2.08	1.54	3.07

Table 2. Compositions of eight types of nutrient medium for the regrowth of cryopreserved shoot apices



Nutrient media used for regrowth of cryopreserved apices

Fig. 10. Effects of nutrient media on the regrowth of shoot apices immersed in LN using vitrification. Apices were dehydrated with PVS2 at 0°C for 60-mins prior to immersion in LN. The PVS2 in a cryovial was exchanged once just after PVS2 loading treatment. After cooling for 1hour in LN, rewarming apices were transplanted into 8 types of basal medium. Values represent mean \pm SE of three determinations. Differences in mean values of regrowth with different letters are statistically significant (Tukey's HSD at $p < 0.05$) in each treatments. (from Kami et al., 2010)

3. Conclusion

The cryopreservation technique for plant genetic resources has developed since the 1990s. However, since there are plant species which cannot yet be cryopreserved, improvement of the technology is a pressing need. I have limited my remarks to the introduction of the cryopreservation technique in this section. This seems like a personal comment, not a part of your conclusion. Kartha (1985), in his detailed book on these principles, provides a valuable addition to this chapter, not but provides an explanation of cryopreservation technology.

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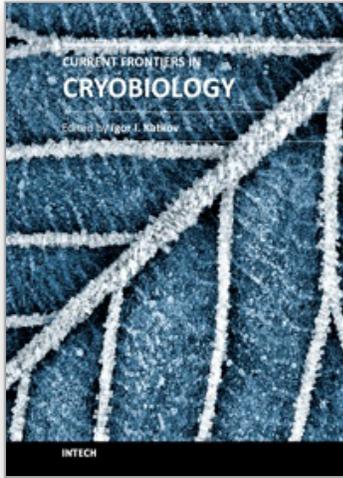
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Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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