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CRYOPRESERVATION OF PLANT GENETIC RESOURCES

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ABSTRACT

PGRs (Plant Genetic Resources) have a significant impact on agriculture and are mostly stored in field and seed genebanks. As an in vitro preservation approach, cryopreservation is essential for long-term storage of PGRs. Cryopreservation was not perfected until the 1960s. Two common processes associated with this technique are vitrification and encapsulation/dehydration. V cryopanels and D cryopanels are just two examples of recent cryogenic processes using cryopanels. Cryo-plate technologies have many advantages, such as being easy to work with during the therapy and resulting in rapid cell regeneration after cryopreservation. For the purpose of successfully preserving plant germplasm, it is essential to conduct research on the genetic stability of plants that have been tissue-cultured for extended periods of time as well as plants that have been cryopreserved. This study provides an overview of cryopreservation methods that are currently in use. According to a variety of morphological, biochemical, and molecular assessments, investigations conducted up to this point have found either no changes or modest variations between cryopreserved and non-cryopreserved samples that were kept under optimum circumstances.

Keywords: *cryo-plate, cryopreservation, genetic stability, shoot tips, vitrification*

INTRODUCTION

Plants that have been domesticated and some of their wild relatives are both included in the category of plant genetic resources, often known as PGR. This is due to the fact that domesticated plants are typically more useful than their wild counterparts. When it comes to food security and agricultural biodiversity, plant genetic resources (PGR) are of paramount importance as they can

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be used to breed new or more productive crops that are resistant to biological and environmental challenges. is. Therefore, plant germplasm (PGR) is very valuable. This article discusses the topic of cryopreservation, an essential technique for long-term preservation of plant germplasm, as most storage and maintenance can preserve the genetic integrity of regenerated plants. Cryopreservation has rapidly become an essential technique for long-term preservation of plant germplasm due to its small footprint and low maintenance requirements. Cryopreservation is becoming increasingly important for long-term preservation of plant germplasm. Cryopreservation is a method of long-term preservation of plant material by exposing it to extremely cold temperatures such as liquid nitrogen. This method can be applied to various plants (LN). At these temperatures, cellular metabolic processes are completely halted (Engelmann, 2004) and the genome remains unchanged. For this reason, cryopreservation has become an effective method to ensure the long-term viability of genetic material. Additionally, new experimental materials have been developed with unique properties for cryopreservation. These materials require little storage space or maintenance and do not introduce genetic modifications into the stored DNA. Through the cryopreservation process, new experimental materials with unique properties have been identified, making this technique a powerful tool for long-term preservation of germplasm. Cryopreservation methods are used by institutions worldwide to preserve plant germplasm (Niino, 2006). Cryogenic therapy often involves one of two processes he follows.

Vitrification (Sakai et al., 1990) or encapsulation/dehydration (Fabre and Dereuddre, 1990). Encapsulation/vitrification (Matsumoto et al., 1995a) and droplet vitrification (Matsumoto et al., 1995b) are two examples of innovative cryopreservation methods that combine vitrification with other components. Scientists have been working hard to find ways to reduce the potential for genetic alterations in cryopreserved and regenerated plant tissues. These analyzes showed that under ideal conditions, cryopreserved material is virtually indistinguishable from its non-cryopreserved counterpart. Optimal conditions include:

The principle of cryopreservation

Cryopreservation failure is due to intracellular freezing and can only be prevented by slow cooling in liquid nitrogen. The cryogenic process requires sufficient dehydration of cells and shoot tips to prevent freezing and allow vitrification after rapid cooling in liquid nitrogen. This is very important for the success of the surgery. Cryopreservation of cultured cells, shoot tips, and somatic embryos could have far-reaching implications if simple and reliable cryopreservation techniques can be developed.

The conventional cryogenic methods

More than 40 years have gone into perfecting the science of plant cryopreservation (Reed, 2008).

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Slow freezing and simple freezing are two examples of the more conventional cryopreservation techniques that emerged in the 1970s and 1980s. Treat samples (shoot tips, embryos, cells) with cryoprotectants (DMSO, glycerol, ethylene glycol, sucrose) before placing them in straws or cryotubes. Samples are contaminated on ice at -7°C and then flash frozen to -40°C at a rate of 0.3-0.5°C/min in a programmed freezer. Finally, the sample is placed in a container with liquid nitrogen. Kumu et al. (1983) developed the progressive freezing method. For easy freezing, samples are packed in straws or cryotubes, treated with cryoprotectant at 25 degrees Celsius, flash frozen at -30 degrees Celsius to remove excess water, and finally immersed in liquid nitrogen. is needed.

Currently, vitrification and encapsulation/dehydration are the most commonly used cryogenic processes. Treatment of dehydration is the main difference between the two approaches. Remove water from the cells using a highly concentrated solution such as B. PVS2 solution to generate vitrified cells. After the cells are encapsulated and dehydrated in ambient air, they are air-dried to remove residual moisture before vitrification with liquid nitrogen. This allows cells to be stored for long periods without risk of damage. Since its introduction, scientists have discovered the most effective means of protecting various plant species, including tropical plants (Sakai, 2008). Advantages of the vitrification strategy, such as faster post-recovery recovery and shorter treatment times, are not shared with the encapsulation/dehydration approach.

Vitrification

The invention of vitrification technology has allowed for the preservation of many different plant species, including those that thrive in tropical and subtropical climates. To successfully dry tissues without causing damage, a highly concentrated fluid is required for this method. This allows the tissues and the extremely concentrated fluid surrounding them to condense into a stable glass upon exposure to liquid nitrogen. Before transferring cells or shoot tips to liquid nitrogen for vitrification, they should be dried at 0 or 25 degrees Celsius using a highly concentrated vitrification solution. This should be done at the same time as the vitrification process. First, a safer vitrification solution for navel orange nuclei was developed. The medium of PVS2 solution contained 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO and contained 0.4 M sucrose (pH 5, 8th). This solution can be supercooled to temperatures below -70 °C as it turns into a metastable glass at a temperature of about -115 °C on rapid cooling. Gradual heating reveals that the vitrified PVS2 undergoes a glass transition at -115 °C, followed by exothermic devitrification (crystallization) and finally endothermic melting.

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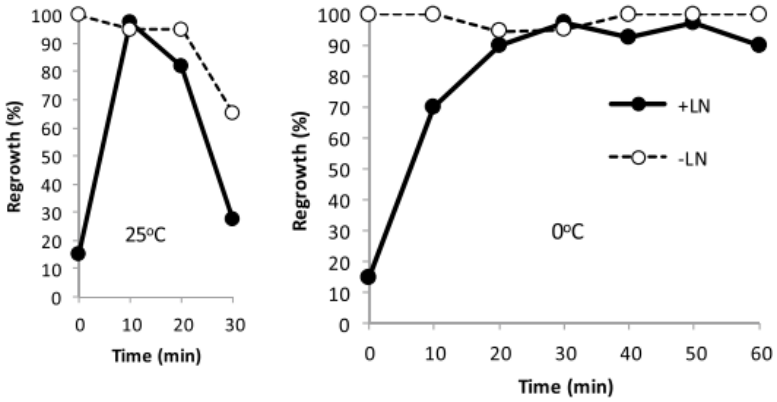

Fig.1. Recovery growth from vitrified wasabi shoot tips cooled to -196 °C: The influence of PVS2 exposure period at 25 or 0 °C. One day of precultivation in 0.3 M sucrose was followed by 20 minutes of treatment in a 2 M glycerol/0.4 M sucrose (LS solution) solution at 25 °C for 1 mm shoot tips. After being exposed to PVS2 for varying amounts of time, these shoot tips were then submerged in LN (Matsumoto et al., 1994).

The shoot tips of many plants are too delicate to withstand direct desiccation by PVS2 during cryopreservation. This is because cryopreservation damages cells and tissues through osmotic stress and chemical toxicity, resulting in poor or no regeneration after thawing. Induction of osmotic tolerance and achieving high regeneration rates after cryopreservation can be achieved by either sucrose pre-incubation or LS treatment (2 M glycerol + 0.4 M sucrose solution). Both treatments are very effective. After vitrification-based cryopreservation, application of two osmoprotective strategies has shown remarkable recovery growth rates in several plant species. The rate of shoot development depends on how long plants are exposed to PVS2. Wasabi sprout tips treated with PVS2 for 10 minutes at 25 degrees Celsius or 30-50 minutes at 0 degrees Celsius showed the greatest recovery (Matsumotora, 1994). Wasabi sprout tips maintained a high level of recovery after being treated with PVS2 for up to 20 minutes at 25 degrees Celsius or up to about 60 minutes at 0 degrees Celsius (without liquid nitrogen cooling).

Encapsulation/dehydration

It was first detailed by Fabre and Dereuddre (1990) that exemplification/drying out happened. A medium containing 0.3-0.6 M sucrose is utilized to preculture the shoot tips or undeveloped organism for 1-3 days. They are put in alginate dots and treated with an exceptionally focused sucrose arrangement (around 0.8 M) for the following 16 hours. The examples are presently more powerful against drying out on account of these cycles. Tests are dried to the proper degrees of mugginess by putting them on silica gels or in a laminar stream bureau after they have been cleaned. As the sucrose arrangement dries, its molarity rises significantly and could become

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supersaturated. The glass progress happens at temperatures around - 196 degrees Celsius. Cryoprotectants like as DMSO and ethylene glycol, which have been connected to the improvement of hereditary adjustments post-cryopreservation, are superfluous with this methodology. The epitome and parchedness handling approach has been valuable for a large number of plant species and cultivars. Rather than vitrified shoot tips, non-vitrified shoot tips showed fundamentally more slow paces of both starting and recuperation development (Matsumoto et al., 1995a).

Exemplification and drying out, a type of cryopreservation, is basic and exceptionally successful. This procedure permits us to evade the issues that emerge while working with plant materials that are delicate to PVS2 vitrification arrangement (Engelmann et al., 2008). Significant components in accomplishing high paces of plant recovery following cryopreservation with this technique are the length of the drying up process and the osmo-security treatment of the shoot tips. Matsumoto et al. (1995a), Figure 2, portrays the rehydration of wasabi shoot tips of shifting water contents when cooling in fluid nitrogen. Diminished water accessibility prompted expanded shoot creation, which crested at around 65% at water accessibility levels of 18-20%.

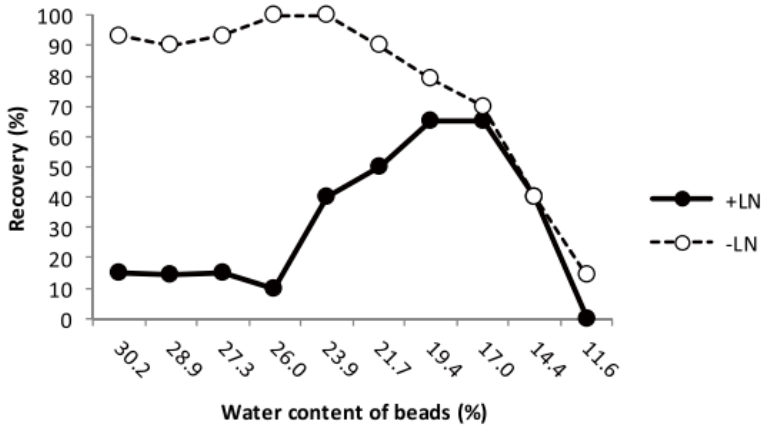

Fig.2. Growth recovery of wasabi shoot tips after being encapsulated and frozen at -196 °C. In order to dehydrate the 1 mm shoot tips, they were first precultured in 0.3 M sucrose for 1 day, then treated with 0.8 M sucrose solution for 16 hours at 20 °C.

Vitrification based methods

The encapsulation/dehydration process is more time-consuming and labor-intensive than the vitrification approach, despite being user-friendly and simplifying dehydration. In contrast, vitrification is time-consuming and inefficient when dealing with a large number of shoot tips at once due to its complexity, delicate nature, and need for special attention. New techniques, such

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as encapsulation/ vitrification and droplet vitrification, have been developed using cryogenic technologies based on vitrification to overcome these issues.

The encapsulation/vitrification method begins with enclosing the shoot tips in alginate beads before submitting them to the vitrification procedure. This makes the materials easy to work with, making it practical to treat a large number of shoot tips simultaneously. In addition, smaller specimens like callus and suspension cultures, as well as hairy roots, may be more suited to the encapsulation and vitrification method. Horseradish shoot primordia, as well as those of lilies, statice, and strawberries, were given this treatment. The regrowth rate of wasabi was greatly increased when the shoot tips were encapsulated, air-dried, and then kept. Encapsulation/vitrification's growth rate in terms of recovery was equivalent to that of vitrification. Comparisons were made between three distinct cryogenic processes and the production of shoots on wasabi shoot tips that were frozen to -196 degrees Celsius. Encapsulation followed by dehydration led to much lower amounts of shoot development than encapsulation followed by vitrification or encapsulation followed by vitrification (Table 2). In addition, both the creation of new shoots and the development of existing shoots upon reculture occurred more quickly in vitrified shoot tips, with or without encapsulation, in comparison to dried shoot tips that had been encapsulated. When compared to the time required for dehydration in the encapsulation/vitrification process, the time required in the encapsulation/vitrification process was significantly reduced.

Kartha et al. (1982) invented the droplet-vitrification process, and Leunufna and Keller made certain modifications to it (2003). In this technique, the tips of the shoots are given a vitrification treatment with PVS2, after which they are individually encapsulated in 5–10 l droplets of PVS2 that are put on a sheet of aluminium foil. Finally, the treated shoot tips are submerged in liquid nitrogen. The very little amount of cryoprotective media that the explants are kept in is the primary reason why this method is so beneficial. As a result, it is possible to achieve very high rates of freezing and warming with this method. This technique has been used on the growing tips of potatoes, yams, and other root vegetables.

Table 1: Effects of preculture and LS treatment on recovery of wasabi shoot tips cryopreserved by vitrification.

Preculture	LS treatment	Recovery (%)		
-	-	10.0	±	1.4
-	+	73.3	±	2.4

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+	-	61.2	±	2.7
+	+	100.0	±	0

The precultured shoot tips were cooled in fluid nitrogen subsequent to being dried out with PVS2 in the wake of being treated with 2 M glycerol and 0.4 M sucrose for 20 minutes at 25°C. Regrowth following recultivation is estimated by the number of replanted shoot tips formed into sound new development 21 days after the fact.

Table 2: Wasabi shoot tips were chilled to -196 degrees Celsius using three distinct cryogenic techniques. Recovery, shoot length, and the amount of time necessary for dehydration were all measured.

Cryogenic protocol	Recovery (%±S.E.)			Shoot length (mm)			Time used for dehydration (min)
Vitrification	97.5	±	1.0	11	±	4.0	10 at 25 ℃
Encapslation/dehydration	67.1	±	8.9	6	±	3.6	420 at 25 ℃
Encapsulation/vitrification	96.7	±	2.9	12	±	3.6	100 at 0 ℃

Following 20 minutes in an answer of 2 M glycerol and 0.4 M sucrose at 25 degrees Celsius, the precultured shoot tips were chilled in fluid nitrogen to stop the course of photosynthesis. The precultured shoot tips were treated with 0.8 M sucrose for 16 hours at 20 degrees Celsius in the wake of being typified in alginate gel globules. From that point forward, they were chilled in fluid nitrogen to eliminate all dampness. Level of recultured shoot tips that delivered solid new shoots 21 days following replanting (otherwise called "recuperation").

Cryo-plate methods

Ongoing years have seen the improvement of various novel cryogenic procedures, including the V cryo-plate and the D cryo-plate. The D cryo-plate procedure depends on air lack of hydration (Niino et al., 2013), while the V cryo-plate method depends on PVS2-vitrification parchedness (Yamamoto et al., 2011) (Fig. 3). Prior to being dried out with PVS2 arrangement (V cryo-pate strategy) or in a laminar stream cupboard (D cryo-pate technique), shoot tips precultured in 0.3 M sucrose are connected to the little wells of a cryo-plate utilizing alginate globules. Cryoprotected and dried out shoot tips are then lowered in a tank of fluid nitrogen. The connected shoot tips were segregated from the cryoplate, set in an answer containing 1 M sucrose, quickly warmed to 25

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degrees Celsius for 15 minutes, and afterward plated on a culture medium, coming about in regrowth.

Fast freezing and defrosting times related with cryo-plate advancements would lead one to anticipate comparably quick recovery. Strawberries, as indicated by the V cryo-plate method, can be frozen. The latest journalists to introduce discoveries utilizing the D cryo-plate approach are Niino et al. (2013). Cryopreserving the shoot tips in that examination was fruitful in light of the fact that they stayed connected to the cryoplates all through the whole cycle. These techniques have a high recovery rate because of the quick cooling and warming cycles, and they are easy to use because of the utilization of lightweight aluminum plates to house the examples. Safeguarding immense examples, for example, buds canvassed in base sheaths and basal stems, is attainable and successful with the D cryo-plate approach (Niino et al., 2014). The D cryo-plate strategy for cryopreservation requires precultivation with sucrose and stacking arrangement treatment to actuate osmoprotection of shoot tips, and the estimation of the ideal parchedness time frame. Parchedness times are abbreviated for this particular objective. A few harvests for which this technique has been reported incorporate mat rush, date palm, persimmon, blueberry, and potato. The D cryo-plate strategy can be utilized for bigger examples and needs less work than elective cryopreservation draws near, as found by Niino et al. (2013). D cryo-plate approach lessens the dangers of inappropriate or unreasonable lack of hydration, as well as material weakening or misfortune during extraction and control.

Assessment of genetic stability after cryopreservation

Cryopreservation is a vital technique for the drawn out stockpiling of germplasm in light of the fact that it considers the utilization of trial materials with specific characteristics to save space and support costs without causing hereditary changes. To this end, it is feasible to utilize materials created through exploring different avenues regarding materials that have unmistakable properties. Notwithstanding, in the event that you don't have any idea what you're doing with regards to cryopreservation, you can wind up with hereditary adjustments and calluses on your recently recovered shoot tips. Calluses, as suggested by the work of Charoensub et al. (2004), may promote the development of genetic mutations. However, cryopreservation conditions that are tuned for regrowth after thawing can speed up plant regeneration with minimum to no genetic alterations (Sakai and Engelmann, 2007). However, there is serious concern among specialists that cryopreserved plant specimens would undergo genetic changes. Many studies have focused on finding ways to reduce or eliminate the possibility of genetic mutation in cryopreserved and regenerated plant tissues. However, the discussed studies showed that under ideal conditions, cryopreserved and non-cryopreserved samples showed either no changes or very slight alterations. Morphological analyses have yielded similarly inconclusive results. In this study, scientists took a

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biological tack.

Size: 7 mm × 37 mm × 0.5 mm with ten wells (diameter 1.5 mm, depth 0.75 mm).

Fig.3. Aluminum cryo–plate.

Analysis of medicinal plants' secondary metabolite content after cryopreservation found it to be comparable to that of controls. Even though the plants had been cryopreserved, this was nevertheless discovered.

Wasabi shoot tips were vitrified and cryopreserved for a considerable length of time at - 150 °C (profound cooler), for 2 hours at - 196 °C (LN), for a treated control with no cooling, and for a non-treated control with no cooling; the genetic stability of recovered plants was evaluated during a 26-month period. These specimens were re-heated to provide them with a new beginning. The average sample recovery rate was more than 90%. To check if any of the regenerated wasabi plants had kept their original genetic makeup, morphological, biochemical, and molecular analyses were performed. Minor differences in glucose, fructose, and glutamic acid concentrations were found at the outset, but had disappeared eight months later. Using RAPD-PCR (five primers), we found no statistically significant differences in the samples' appearance, biochemistry, or molecular structure. Therefore, it's possible that after 10 years of storage under cryopreservation, there wouldn't be any discernible genetic changes in regenerated wasabi plants.

Recently, the well-established method of methylation-sensitive amplified polymorphism (MSAP) has been applied to the investigation of cytosine methylation levels and patterns in plants. Thawed cryopreserved or cold-stored hops plants showed no genetic changes according to RAPD or AFLP, however MSAP revealed methylation modifications. Cryopreservation and cold storage did not hinder the efficacy of this method for analyzing epigenetic variation. Changes in methylation status are possible in long-cultivated shoots.

CONCLUSION

The results provided here provide more support for the hypothesis that epigenetic modification may account for the great phenotypic variety observed in cryogenically preserved cocoa SE. Our

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results further suggest that the observed heterogeneity may not be completely random, but rather a response to the environmental challenges that plant cells experience during in vitro cultivation and cryopreservation. This discovery is significant because it raises the possibility that the observed variability is not completely attributable to chance. It stands to reason that the low temperature has a significant role in the observed unpredictability. In fact, temperature reduction promotes RNA-directed DNA methylation. While low temperatures play a role in the cryopreservation process, previous studies have demonstrated that they cannot fully explain the observed variability. For example, the use of the cryoprotectant DMSO, which chelates to nucleic acids, may result in several changes to the DNA's methylation pattern in addition to introducing genetic modifications, as was previously seen. In addition, Harding et al. discovered that the usage of high amounts of sucrose during vitrification causes conditions of high osmotic stress, which may cause alterations in the methylation of DNA regions as an adaptive reaction. This discovery is especially intriguing since it implies that organisms respond to high osmotic stress by methylating DNA sequences. Once we learn how these factors affect the fidelity of the regenerant plants, we can develop new cryopreservation techniques with reduced phenotypic, genetic, and epigenetic variability.

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