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Cryopreservation Procedure Technique in Medicinal Plants

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ABSTRACT

Preservation is a suitable method to preserve the plant for consecutive years. The motivation of the initial studies for the protection of plants is to identify freezing methods for the preservation of plant organs during consecutive years. Preservation to preserve plant organs includes almost all plant parts in liquid nitrogen at very low temperatures (usually -196 degrees Celsius), which is a practical and economical method compared to field preservation methods. In order to preserve the genetic treasury of threatened plants, it is necessary to use freezing protection technology. It is necessary to use appropriate pretreatments for freezing protection. The most important and widely used pretreatment is coating and dewatering as a practical technique. Gene banks based on cryopreservation and biotechnology-based techniques are expanding in the world, and the protection and optimal use of germplasm reserves are of great importance in all countries. Preservation of plant genetic resources guarantees the sustainability of genetic progress in other cultivars, especially commercial cultivars, which have high economic value. In other words, every plant is an important gene store for a breed. Plants are exposed to various risks caused by adverse environmental conditions, both biotic and abiotic stresses. These unfavorable and inappropriate conditions can remove plants from the planet and subsequently, the valuable gene treasury is removed. Protection and maintenance of plant biodiversity are very necessary for plant breeding programs, genetic engineering, etc. Of course, it should be noted that the preservation for successive years of plant organs using the cryopreservation method is only successful if the formation of ice crystals (with appropriate and standard plant dehydration) is prevented inside the cell.

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1. Introduction

The need to protect biodiversity due to its direct relationship with human life has been accepted by world scientists. Nowadays, creating a DNA bank is the most important way to preserve threatened species in the world, which is provided with the help of biotechnology. Preparation of the DNA bank of these species can be done with the cryopreservation technique and this guarantees the preservation and continuation of human life. Cryopreservation is considered an ideal strategy for long-term plant preservation. Early studies in plant conservation were motivated by the identification of cryopreservation methods for the long-term conservation of plant genetic resources. Cryopreservation is known as an applied aspect of cryobiology or the study of life at low temperatures. Ultra-low temperatures stop metabolic and biochemical reactions in the cell after plant tissues are sufficiently dehydrated to prevent the formation of intracellular ice crystals, which can cause cell death and destruction of cell organelles during the cryopreservation process. Cryopreservation is currently the most innovative and cost-effective biotechnological approach that allows long-term safe protection of plant biodiversity without the risk of genetic changes (Benelli, 2021; Kaviani and Kulus, 2022; Whelehan *et al*., 2022). Plant genetic resources constitute a repository of genomic information and are important for world food security, crop improvement and maintaining genetic diversity (Tirado-Pérez and Sandoval-Cancino, 2022; Salgotra and Chauhan, 2023). In breeding programs, due to changing weather

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patterns, it is important to obtain new or more productive plants that are resistant to biotic and abiotic stresses (Benelli, 2021; Gonzalez Guzman *et al*., 2022). Despite the availability of genetic reserves, the farm method cannot be a suitable method for plant protection due to the high cost and exposure to pest and disease attacks and adverse weather conditions (Ahmadian and Kakaei, 2019). Therefore, the freezing preservation method can be a suitable method for preserving and maintaining the genetic reserves of plants. Storage in low-temperature conditions slows down or stops metabolic processes and prevents biological spoilage (Ahmadian and Kakaei, 2019; El-Shaieny *et al*., 2022).

2. History of general advances in plant freezing

Cryopreservation at -196 degrees Celsius is considered an ideal means for the long-term preservation of plant germplasm. Through freezing, endangered hybrids, apart from being easy for international exchange, can be maintained with little effort. Freezing through cryopreservation methods reduces the damage caused by intracellular crystallization and provides a high regeneration rate before immersion in liquid nitrogen. The most suitable part of the plant used for meristem culture is because it produces virus-free cultures (Reed, 2008; Rajasekharan, 2015). However, each plant species has a different genetic structure and reacts differently to different freeze protection treatments (Margesin *et al*., 2007). Therefore, improving the freezing method at each stage is very important for post-healing growth. Freezing-based methods use cryoprotectants such as Sucrose, DMSO, PVS2, Glycerol, and PVS3 (Benelli *et al*., 2013), which help remove free water, thereby preventing lethal cryoinjury during immersion in liquid nitrogen. The main reason for losing water is survival. In other words, germplasm preservation by freezing in liquid nitrogen (Cryopreservation) (Chicaiza-Cabezas *et al*., 2023) is another method of plant preservation in ex-situ conditions, which helps in the long-term preservation of hereditary reserves, maintaining the genetic stability of the maternal base, and reducing costs. Maintenance in the field is considered as a backup for plants that are propagated as colonies or as a protection system for important crops. In this technology, using different techniques, the germplasm material is dehydrated as much as possible and stored at a very low temperature (-196 ºC) (Comizzoli *et al*., 2022) in liquid nitrogen for a long time (dozens of years). By using the liquid nitrogen preservation technique, it is possible to provide a method of preserving seeds and other plant tissues, in which plant materials can be stored indefinitely without losing their potency. For cryopreservation of plant tissues, PVS2 solution has been widely used, which was presented by (Sakai *et al*., 1990). This solution has a high potential in preserving plant tissues. Cryopreservation can be done in the liquid nitrogen or gas phase of this material, in order to prevent the formation of ice crystals, the samples should be placed at a temperature lower than -130 degrees Celsius. Rapid thawing in 35-40°C water is usually used to recover samples to minimize the possibility of crystal re-formation and tissue damage (Towill, 1991). Seed cryopreservation has been successfully performed in many plant species, but some researches have pointed out problems such as abnormal germination and seed death due to internal damage (Chmielarz, 2010; Vollmer *et al*., 2021). The subject of cryopreservation of plants started in 1965 when Hirai was studying the biological activities that took place during the freezing of biological samples. Therefore, after three years, it was done as the first successful follow-up to freeze callus cells. In the following years, new freezing methods such as direct immersion, slow freezing, and glass freezing were developed as well as more and more applied to different plants and diverse plant tissues (Reed, 2017).

Ice has been around since 2000 BC, as archeological findings show that ice was used throughout Mesopotamia to store food. In simple words, the subject of freezing is related to biological research at relatively low temperatures. In other words, the freezing of plants refers to the preservation method for keeping various samples in very low temperatures from -135 to -196 degrees Celsius for many years. The damage of making changes is usually extremely small. Freezing methods are very necessary and necessary to preserve *in vitro* cultures, secondary metabolite cell cultures, embryonic organs, endangered species and transgenic plants for many years. The usefulness of freezing plant genetic material is very high with many advantages of freezing compared to other methods. Cryotherapy to destroy and reduce the virus has a great potential for spreading the germplasm of plants. There are many methods of freezing. However, the number of plants that have been introduced and placed in cryo-

banks and research on them has been applied is very small. All countries should feel the need to carry out this technique regarding the plants that are useful for them so that this useful method can be spread all over the world and can benefit from its relative advantage. It seems that other researchers and scientists in universities and research centers and related public and private organizations as well as interested people should come together and manage this very important issue (Acheampong Amankwaah *et al*., 2023). As mentioned earlier, freezing refers to keeping biological samples such as cells, tissues and organs at very low temperatures such as liquid nitrogen (usually 196 degrees Celsius). The main factor of the early studies was the creation of freezing methods for the long-term preservation of plant genetic resources (Sakai, 1960). Since the first success of plant freezing using two-stage freezing of mulberry (*Morus alba*), many and significant successes have been made in this field (Wang *et al*., 2021a). Extensive and successful freezing research led to several technical advances, including the development of various freezing methods. It has been stated in various experiments that cryopreservation preserves or even enhances the regeneration capacity of embryogenic tissues, which was widely used in genetic transformation in various plant species (Wang *et al*., 2014; Wang *et al*., 2021a). In research, the use of frozen embryogenic tissues for genetic transformation improved the transformation efficiency and regeneration frequency of transformed plant tissues (Wang *et al*., 2012). Freezing to preserve transgenes in transformed material was shown to provide a safe and reliable strategy for the long-term preservation of genes (Wang *et al*., 2012; Wang *et al*., 2021a). These studies showed the usefulness of cryo-biotechnology in the genetic engineering of plants (Wang *et al*., 2021a). The general process of cryopreservation by (Wang *et al*., 2021a) is shown in Fig. 1.

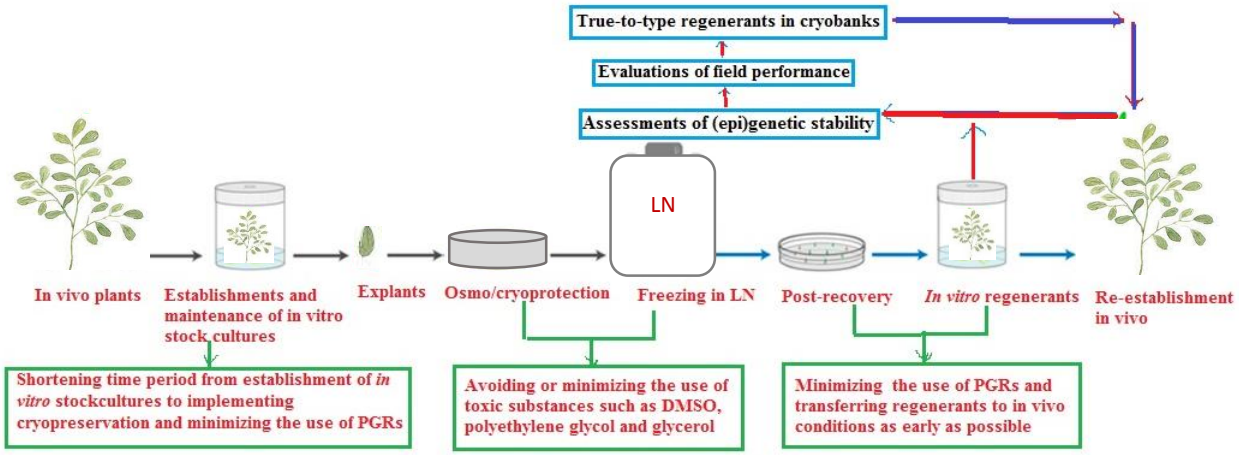

Figure 1. Different stages of freezing (black arrows), after cultivation for recovery and re-establishment of plants (blue arrows), genetic stability study (epi) and field performance study in cold-derived recoveries/plants (red arrows), and Measures taken to ensure genetic stability (epi) and actual regenerator/plants recovered after freezing (green arrows). DMSO: dimethyl sulfoxide; LN: liquid nitrogen; PGRs: plant growth hormones. (This figure is taken from the Wang *et al*., 2021a).

3. Methods and application of materials in a cryogenic protection technique

In the cryopreservation method, the freezing molecules protect the optimal environment of cell cold protection of plant cells. In this sense, dimethyl sulfoxide (DMSO) is the most common chemical pretreatment. It is used both for pre-growth and during cold protection (Bekheet *et al*., 2020; Kartha *et al*., 1979). DMSO reduces the electrolyte concentration in the cold contents remaining in and around a biological cell during freezing. In practice, PVS2 (plant freezing solution #2) containing 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide (DMSO) (all v/v) and 0.4 M sucrose is most commonly used (Sakai *et al*., 1990; Bekheet *et al*., 2020). This solution is more effective for dehydration and has less toxicity for plant materials (Sakai *et al*., 1991). For successful cryopreservation procedures, careful control of cryopreservation is necessary to achieve adequate dehydration while avoiding injury from chemotoxicity and sudden osmotic stress (Panis *et al*., 2001; Bekheet *et al*., 2020). Another solution used in cryoprotection is

PVS3, which contains 40% glycerol and 40% sucrose (WV). Keeping germplasm at liquid nitrogen temperature will always lead to concerns about damage to germplasm, so it is necessary to use relevant pre-treatment materials to prevent damage. The role of these pre-treatments is actually to reduce the stress caused by liquid nitrogen on cells and other plant organs. There are various pretreatments including vitrification (Matsumoto, 2017), encapsulation-dehydration (Ozden-Tokatli *et al*., 2008), cryogenic plates (Wang *et al*., 2021b), use of chemicals (Gonzalez-Arnao *et al*., 2014), rapid drying and slow freezing (Panis, 2019) and air drying (Engelmann, 2011), etc. In the freezing preservation technique, the role of the amount of water in plant cells is effective in the success or failure of this technique. Each type of plant has a standard amount of water content, therefore the standard level of water in the cells of each plant should be determined for preservation. Because overwatering of plant cells due to dehydration and under-watering of plant cells due to the formation of ice crystals inside the cells destroys them and removes them from the purpose of protection (Seydi *et al*., 2021). Dewatering can be done by two methods: physical dewatering and chemical dewatering.

4. The necessity of protecting plant species in comparison with animal species

The issue of the protection of plants and vegetation is being followed seriously in related research centers and various measures have been taken to prevent them from disintegrating. Along with other methods that have been carried out in connection with this issue, the freezing preservation method can be used as a safe and complementary method in addition to their protection in the gene bank and seed bank, or their protection in the botanical garden, or Even their protection can be used in natural flooded areas. Freezing protection method to protect animal species and especially endangered species has been a common method for many years, but this cold protection method has been limited for plant species, so the use of this method in the protection of banks of a plant gene seems essential (Kakaei and Mansouri, 2019a). Plant genetic materials include indigenous populations, traditional and old varieties, wild forms and weeds, genetic foundations, inbred lines and modern cultivars. Today, the genes in natural germplasms are used for gene transfer, as a result, the protection of plant germplasms becomes very important (Tay, 2005; Salgotra and Chauhan, 2023). Preservation of plant germplasm is a healthy storage of the genetic diversity of plants and related species in the form of seeds or live plants for future use. There are various methods for the same purpose, and the cryopreservation method using liquid nitrogen is a more promising and cost-effective method to protect germplasm in the long term (Bagheri and Saki, 2017). Other requirements or relative advantages of the cryopreservation technique can be mentioned in the laboratory preservation of plant cells or cultured calluses. which is a valuable tool not only for preserving genetic resources Rather, it pointed to the preservation of plant materials in a form ready for further manipulations such as the production of secondary metabolites in laboratory conditions (Shibli *et al*., 2006).

5. A review of research related to cryopreservation

Kakaei and Mansouri (2019a) in the study of freezing protection of sesame (*Sesamum indicum* L.) seeds, stated that sesame is among the most ancient and valuable plants and is considered including most important oil crops in all countries. For this purpose, they use two anti-freezing substances PVS2 and PVS3 were used and determined the relationship between the measured traits and the treatment time in antifreeze agents and stated that regarding the germination trait, increasing the duration of treatment with antifreeze agents increased the percentage of germination. In another research, Kakaei (2020) in the study of the effect of cryopreservation by vitrification method on the germination of cotton seeds, stated that it is possible to store cotton germplasm in liquid nitrogen and also suggested the use of embryos as well as different genotypes of cotton to evaluate the protection capability, Cryopreservation in future studies. In research on black cumin seeds (*Nigella sativa*), Kakaei and Mansouri (2019b) used two anti-freezing treatments called PVS2 and PVS3 and expressed that PVS3 has more effectiveness in black cumin seeds in cryopreservation. Mansori et al. (2014) in the study of rapeseed cryopreservation by vitrification method stated that rapeseed treatment for 100 minutes with PVS2 solution and 20 and 60 minutes with PVS3 solution compared to other treatment combinations showed the highest percentage of germination. Fig. 2

shows the cryopreservation activity of rapeseed carried out by the author.

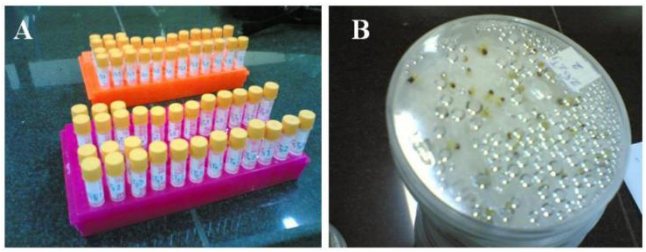

Figure 2. Treatment of rapeseed seeds with different protective solutions before cryopreservation (A), rapeseed seed samples cultured in Petri dishes containing the filter paper after cryopreservation (B) (Mansori *et al*., 2014).

Bekheet et al. (2020) in a study to develop a suitable way to freeze in a liquid nitrogen tank Shoot tips and artichoke callus culture using dimethyl sulfoxide (DMSO) and plant freezing solutions 2 (PVS2) as freezing solutions, shoot tips for twenty, forty, sixty and eighty minutes before immersion in liquid nitrogen (LN) exposed to DMSO or PVS2, and their results showed that the use of PVS2 treatment for 60 minutes as a cold protectant in freezing the tip of the branch and Korean artichoke callus culture was more effective compared to DMSO. Seydi et al. (2021) in the study of micropropagation and encapsulation-watering method for ultra-cold protection of inverted tulip plant (*Fritillaria imperialis*) stated that the lowest explant survival rate was related to control explants (without pretreatment). Htwe et al. (2023) in the study of cryopreservation in bananas stated that cryopreservation methods have been implemented in germplasm storage tanks as a model for the preservation of asexually propagated plant genetic resource sets when they are needed again. Various models have been used for the effective freezing of plant tissue. Examining the above sources shows the importance of gene reservoirs in the plant sector, because the preparation of future human food is directly or indirectly dependent on plant preservation techniques, especially freezing preservation techniques, so research in this direction is very necessary and practical (Rajasekharan and Rohini, 2023; Nguyen *et al*., 2023; Zhang *et al*., 2023; Kim and Popova, 2023; Höfer and Flachowsky, 2023; Gowthami *et al*., 2023; Aparna *et al*., 2023). It seems that in the future, the benefits and functions of freezing protection for humans will be more visible.

Conflict of Interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present research.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

The original draft of this version was written by the first researcher and revised and revised by the second researcher.

Informed Consent

The authors declare not to use any patients in this research.

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