Innovations

Cryopreservation of Medicinal and Aromatic Plants: Recent Advances and Challenges

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Abstract: *Medicinal and aromatic plants (MAPs) are regarded as a rich source of secondary metabolites that hold great potential for use in a range of industries, such as herbals, cosmetics, medications, and food flavoring. Medicinal and aromatic plants are currently under a serious threat from unauthorized collection because of their extensive use in the food industry and herbal medicine. Therefore, protecting these natural resources must be a top priority. So, the process of using extremely low temperature (-196℃)is used to maintain living, structurally intact cells and tissues is known as cryopreservation. Explants are used in cryopreservation to preserve biological material for later use are the fundamental component of effective cryopreservation procedures because they provide a consistent source of tissue that may regenerate following freezing, which makes them crucial for biodiversity preservation, agricultural advancements, and genetic conservation. Strategies based on vitrification have been employed, such as droplet-vitrification and cryoplate etc. This review focuses on the core concepts of cryopreservation techniques and how they are applied on different kind of explants, explore the significance of several explant characteristics, including size and source.*

Introduction

Plants with biologically active substances and distinctive aromas that can be used medicinally to cure, prevent, or manage a variety of diseases and conditions are known as MAPs. Secondary metabolites from plants, which are crucial for human health, are found in MAPs. However, new discoveries in plant genomics research have improved our perception of the challenging genetics and biochemistry that go into the manufacture of these secondary metabolites in plants.

In most developing nations, traditional and indigenous health systems continue to rely on generations of knowledge about aromatic and medicinal plants. The usage of aromatic and therapeutic plants has become a major aspect of daily life despite advancements in pharmacology and medicine (Inoue et al. 2019). Many modern drugs, like aspirin and digitalis, are derived indirectly from aromatic and therapeutic herbs. Additionally, several aromatic plants are essential for the

prevention and treatment of human diseases, and some are added to animal feed and cosmetics (Silva and Fernandes 2010). According to numerous in vitro studies, aromatic plants are powerful antioxidants and antimicrobials, and they are also known to contain components that are employed in traditional milk, meat, and baked goods (Dikme et al.2023).

MAPs are protected via a variety of ex-situ methods, including as cryopreservation, gene banks, seed banks, etc. This include cryopreservation, a viable long-term preservation technique that includes storing tissues where their germplasm is kept at the lowest liquid nitrogen temperature (-196 °C).In cryopreservation, tissues or organs are stored in a sub-zero environment with cryoprotective agents (CPAs), also referred to as cryoprotectants. (Jungareet al. 2022).

The initial material used in cryopreservation is an explant. From the given plant, they are carefully chosen and collected. Before cryopreservation, the explant is grown in a controlled setting to preserve metabolic activity and cellular health. Cryopreserved explants include mature seeds, apical or axillary shoot tips, pollen, zygotic and somatic embryos, embryonic axes, shoot primordia, roots, protocorms, protocorm-like bodies (PLBs), cell suspensions, callus, and more (Roque-Borda et al. 2021).

As a result, new cryopreservation methods mediated by vitrification have performed well with all explant types. This paper reviews at the present development and application of Desiccation, Encapsulation-dehydration, (Dereuddre et al. 1990). Vitrification (Sakai et al. 1990). Encapsulation vitrification (D. Hirai et al. 1998), Droplet-vitrification (Kartha et al. 1982), V- cryoplate (Yamamoto et al. 2011), D-cryoplate (Niino et al. 2013) techniquesetc. The physical process of vitrification is important for cryopreservation by vitrification, which involves vitrifying an entire sample, as well as cryopreservation by freezing, which involves cells surviving in glass between ice crystals. (Wowk et al. 2010).These techniques conserve the genetic diversity and potential for future use of medicinal plants with antiallergic properties, such as Prunus domestica, Dendrathema grandiflora, Ponerorchis grandiflora, and Olea europea. (Vujovićet al. 2015), (Martín et al. 2009), (Hiranoet al. 2005), (Lynchet al. 2007), (Gowthami et al.2024). These plants and their genetic material can be preserved indefinitely through cryopreservation, providing a safeguard for their future. This attempts to provide detailed instructions for a variety of plant species, making cryopreservation a more accessible technique for long-term storage.(Reed et al. 2008).

Hurdles and Challenges:

The main cryopreservation problem, according to researchers, is the large plant cell and its internal water, which makes up over 90% of the cell volume. (Bachiri et al. 2000). Freezable water is a significant amount of cellular water that can be frozen (Crowe et al. 1990). There is a small amount of "non-freezable" water,

which is encased in membranes and is essential to maintaining the structure and functionality of cells, even in the presence of extracellular ice (Hoekstra et al. 2001). The formation of ice inside cells is known to disrupt cellular architecture, leading to cell death (Mazur 1984). Later, the main goal was to remove the freezable water from the tissues that were to be cryopreserved.

The success of cryopreservation is significantly influenced by the chemical composition of the recovery medium. Additionally, the PVS must be removed after thawing by treating with a dilution solution, such as reducing the temperature, in order to ensure a high recovery frequency. Maintain the sugar content between 15 and 30 minutes to avoid osmotic shock (Flachsland et al. 2006).

Cryopreservation Techniques

Classical Cryogenic Techniques:

Traditional cryopreservation methods entail rapidly immersing in liquid nitrogen after gradually cooling to a predetermined prefreezing temperature. As the temperature drops during gradual cooling, the exterior medium and cells first supercool, and then ice forms in the medium (Mazur, 1984). In classical freezing processes, samples are first grown, cryoprotected, then slowly cooled to a predetermined prefreezing temperature then submerged in liquid nitrogen, stored, quickly thawed, and recovered. It has been possible to successfully use traditional cryopreservation methods to undifferentiated culture systems such cell suspensions and calluses. (Kartha and Engelmann, 1994; Withers and Engelmann, 1998). These methods can be applied to differentiated structures to freeze the apices of species that are susceptible to cold (Reed and Chang, 1997).

New Cryopreservation techniques:

Procedures based on vitrification have useful advantages over traditional freezing methods. Desiccation, Encapsulation dehydration, Vitrification techniques include encapsulation-vitrification, droplet vitrification and cryo-plate technologies.

Desiccation

Desiccation-based techniques include either exposing the explants to a dry environment using a variety of flows (Ballesteros et al. 2021) or exposing them to a dry environment inside a desiccator with a desiccant (usually activated silica gel, which provides relative humidity (RH) <15%) (Sherlocket al. 2005). It is crucial to modify the desiccation period in order to reach the desired ideal moisture content.

The development of cryopreservation protocols for the embryonic axes of resistant seeds usually involves desiccation (air drying) techniques (Ballesteros et al. 2021).

The usual arrangement for desiccation is an air flow cabinet. A stream of sterile compressed air or silica gel, on the other hand, can create more precise and consistent dehydration conditions. The approach known as "flash drying," which was created by Berjak's group in South Africa, includes a pressurized dry air stream that dries incredibly quickly. This technique enables samples with a relatively high-water content to be frozen, minimizing desiccation damage (Berjak et al. 1989 Wesley-Smith et al. 1992).Samples frozen with a water content of 10% to 20% generally possess the most important survival rates.

Encapsulation dehydration process is based on technologies created for artificial seed manufacturing. After being pregrown in a liquid medium enhanced with sucrose for one to seven days and partially dehydrated in a laminar airflow cabinet or with silica gel to a water content of about 20% (fresh weight basis), the explants are encapsulated in alginate beads and quickly frozen (Dereuddre et al. 1991). The growth recovery and survival rates of cryopreserved samples are often quick and straightforward, with no callus formation. Sakai et al. (2000) proposed a modified procedure that executes pregrowth and encapsulation in a medium containing glycerol and sucrose concurrently. The apices of many species with temperate and tropical origins, as well as cell suspensions and somatic embryos of various species, have all been subjected to this approach (Engelmann, 1997 Engelmann and Takagi, 2000).

Vitrification method was developed by Sakai et al. 1990. Vitrification is the physical process that creates metastable glass without crystallization by supercooling a highly concentrated cryoprotective solution to extremely low temperatures at an easy-to-handle cooling rate. Accordingly, the samples are cryopreserved without the production of harmful intracellular ice (Sakai et al. 2007). Samples are first cultured on medium enriched with cryoprotective substances, then they are treated with a loading solution (such as a mixture of 2M glycerol and 0.4M sucrose) dehydrated with a highly concentrated vitrification solution (such as the glycerol-based PVS2 solution).

Around the world, numerous research teams have created various vitrification solutions. The glycerol-based vitrification solutions known as PVS2 (Sakai et al. 1991) and PVS3 (Nishizawa et al. 1993) however, are the most widely utilized. 0.4 M sucrose (pH 5.8), 30% (w/v), 15% (w/v) DMSO, and 15% (w/v) ethylene glycol glycerol compose up the PVS2 solution. PVS3's basal culture media contains 40% w/v sucrose and 40% w/v glycerol.
A modified vitrification method was recently developed to reduce the vitrification solution's toxicity. This protocol involves treating the apices with a half-strength vitrification solution first, followed by a full-strength one. (Matsumoto et al.2003). Satureja spicigera callus initiated from young leaves was successfully cryopreserved by vitrification (Ghaffarzadeh-Namazi et al. 2017). Applications of

vitrification have proved successful for many different types of plants, like as Actinidia macrosperma, Beta vulgaris, Castanea sativa, Artocarpus heterophyllus with survival rates of 68.3%, 80%, 70%, 37.9%.

Vitrification makes it possible to freeze explants quickly. However, given the length of the sequential steps of a vitrification process is frequently very brief, small-sized explants are challenging to manage. So, **Encapsulation vitrification**is used which combines the processes of encapsulation–dehydration and vitrification, involves surrounding samples via vitrification, followed by freezing them in alginate beads. It is being used on the apices of more and more species. (Sakai, 2000; Sakai et al. 2002). Using vitrification, alginate-coated meristems from potato (Solanum tuberosum L.) axillary buds produced in vitro were successfully cryopreserved. Within three weeks of plating, successfully vitrified meristems produced shoots without the development of an intermediate callus. The average shoot formation rate was close to 70%. (Hirai and Sakai 1999). The first effective cryopreservation method based on encapsulation–vitrification (EnVi) has been created for raspberry (Rubus idaeus L.) shoot tips cultivated in vitro, led to increased cryopreserved shoot tip survival (85%) and regeneration (75%). (Wang et al. 2005).Wasabi shoot tips serve as an illustration of encapsulation-vitrification. Wasabi shoot tips that were cryopreserved using the encapsulation-vitrification method had a 30% higher percentage of shoot formation than those that were cryopreserved using the encapsulation-dehydration method. Dioscorea cayenensis, Mentha spicata L., Malus domestica, Wasabia japonica are some examples which were cryopreserved by using this method have survival rates of 75%, 90%, 70%, 80% respectively.

Droplet vitrification was developed by Kartha et al. (1982) and modified by Leunufna and Keller (2003). Under this technique, cassava shoot tips are first treated with either PVS2 or PVS3 vitrification solution, then put one at a time into 5–10 μl PVS2 droplets and immersed in LN. Due to the small volume of cryoprotective media that the explants are placed in, this technique's primary benefit is its capacity to accomplish extraordinarily high rates of warming and chilling (Matsumoto, 2017). A few important species of aromatic and medicinal plants have been subjected to this procedure using a range of explants, such as seeds, protocorms, and shoot apical meristems/shoot tips. In a droplet-vitrification process, these vitrification solutions were applied to two model species, namely garlic and chrysanthemum, and the results showed that PVS3 and its variants were better than PVS2 and its variants, while the majority of PVS2 variants were on the same level with the original PVS2 (Kim et al. 2009). Colocasia esculanta, Phoenix dactylifera, Pelargonium, were cryopreserved by using droplet vitrification having regeneration rates of 80%, 85.6%, 55.6-96.2%.

Cryo-plate technique there are two established cryopreservation methods based on cryo-plates: V Cryo-plate and D Cryo-plate. These plate-based techniques use calcium-alginate to adhere shoot tips to aluminum cryo-plates (7 × 37 × 0.5 mm) with 10 wells. (Yamamoto et al. 2011).In the V cryo-plate approach, droplet vitrification and encapsulation vitrification are in combination, while calcium-alginate encapsulation in a cryoplate and dehydration are combined in the D cryo-plate method. Because of this, the general cryo procedures for the V and D cryoplate methods are comparable, even though the V method uses PVS to treat the shoot tips. It is possible to employ the D cryo-plate approach for organisms that are PVS2-sensitive. Air dehydration is the foundation of the D cryo-plate approach (Niino et al. 2018)and PVS2-vitrification dehydration of explants on a cryo-plate is the basis of the V cryo-plate method (Yamamoto et al. 2011). By creating the V Cryo-plate technique, Yamamoto showed how to successfully cryopreserve the shoot tips of Dalmatian chrysanthemums (Tanacetum cinerariifolium). According to Yamamoto this V cryo-plate cryopreservation resulted in 65–90% shoot regrowth (average of 77%) for seven Tanacetum cinerariifolium genotypes. The V cryo-plate approach has successfully fully cryopreserved over 25 species to date, and some of these techniques have been used in cryobanks.

The D cryo-plate technique for the effective cryopreservation of mat rush (Juncus decipiens) shoot tips was initially reported by (Niino et al. 2013). The D cryo-plate technique produced shoot regrowth rates ranging from 73 to 97%, with an average of 86.3% for the 20 mat rush genotypes examined (Niino et al. 2013).

The following are the primary benefits of the V and D cryo-plate techniques: Since only the cryo-plates need to be handled during the operation, handling specimens is rapid and simple. PVS2 and loading solution (LS) is capable of providing excellent treatment for specimens put on cryo-plates. The cryo-plates can be quickly and easily cooled or warmed by submerging them in 1.0 M sucrose solution or liquid nitrogen, respectively. This process produces extremely fast cooling and heating rates. Both strategies can lead to high regeneration.

Explant Type in Cryopreservation protocol

Numerous explants have been used in attempts to cryopreserve such medicinal plant species, with consistently positive results. Vitrification-based cryotechniques have been applied to a variety of plant genotypes over the past two to three decades, since there are encouraging results from attempts to cryopreserve these species. (Malik et al. 2019)

The first and foremost convenient and appropriate way for the conservation of medicinal plants are through seed preservation.

Whole seed

Explant seeds already contain food reserves (such as cotyledons or endosperm carbohydrates and oils) that can sustain the plant's early growth and development in culture. As a result, the explant can more easily settle into tissue culture media and start developing without needing outside nutrients right away. The seed bank contained a variety of aged seeds that were either implanted on culture media or used as a source of cotyledon and embryo axis explants.

For the immature seeds of the terrestrial orchid, Bletilla striata a successful cryopreservation technique has been created. The survival rate after preservation was 92% and 81%, respectively, staining with 2,3,5 triphenyltetrazolium chloride demonstrated that the immature seeds, 3 MAP and 4 MAP, that were harvested were cryopreserved by vitrification after being precultured for 3 days on New Dogashima medium supplemented with 0.3 M sucrose.

The immature seeds thus treated did not exhibit a decrease in germination rate in comparison to untreated immature seeds, and they developed into normal plantle ts in vitro (Hirano et al. 2005).Cymbidium finlaysonianum is one of the endangered orchid species in Thailand. In this study, Orchid protocorms were cultivated beforehand for 24 hours in liquid New Dogashima (ND) medium supplemented with 0–1 M sucrose. Following a 24-hour pretreatment in liquid ND medium supplemented with 1.5 M sucrose, the protocorms were encapsulated in 3% w/v Ca-alginate, dehydrated for 0–12 hours, and thereafter submerged for 48 hours in liquid nitrogen.

According to the data, the protocorms with the maximum viability and regrowth (65.56%) (Worrachottiyanon et al. 2018).

A number of dehydration techniques were assessed for their capacity to cryopres erve whole Manilkara zapota seeds.Maximum development rates were 94% for M. zapota (Wenet al. 2013). The majority of agricultural crops are orthodox seeds, this indicates that after they have been dried out to a low moisture content, they may be kept for extended periods of time at low temperatures. However, there are certain significant plant species groups for which seed conservation presents difficulties. The group includes plantains and bananas, which are vegetatively propagated because they do not produce seeds. (Roberts, 1973). There is a continuous struggle to save seeds for a number of intermediate species. (Ellis et al. 1990).

Zygotic Embryo

Tissue culturists typically favor younger tissues, like zygotic embryos, because they have greater potential and competence to create organs and embryos than more mature and differentiated tissues. (Elhiti M., & Stasolla C. (2011).

Podophyllum hexandrum is a highly endangered valuable medicinal plant is related to the Berberidaceae family. Because of its ever-increasing demand, this plant is being unfairly and excessively harvested from the wild, necessitating

conservation efforts. By optimizing several parameters, such as loading time, PVS2 dehydration time, zygotic embryos were cryopreserved utilizing vitrification and V cryo-plate. In order to generate plantlets, zygotic embryos were recovered on various regrowth media. 90% was attained using a V cryo-plate, whereas 73.3% was acquired using vitrification. (Parasher et al. 2023).

Extracted zygotic embryos of Coffea arabica were examined to determine how the precooling temperature affected their survival rate. The highest survival rate (97%) for embryos taken from cryopreserved seeds after they had thawed was seen when the seeds were submerged in liquid nitrogen right after dehydration without being precooled. After two months in culture, all viable embryos transformed into healthy seedlings (Dussert et al. 1997).

This was followed by a three-day preculture of Bletilla straiata zygotic embryos on ND medium supplemented with 0.3 M sucrose at 25°C in complete darkness. After 15 minutes at 25°C, the embryos were covered with a solution of 2 M glycerol and 0.4 M sucrose. After that, they spent three hours dehydrated using a highly concentrated vitrification solution (PVS2) before being submerged in liquid nitrogen for thirty minutes. When embryos were successfully vitrified and heated, they became healthy plantlets. The plant regeneration rate was roughly 60% (Ishikawa et al. 1997).

Although there has long been worry about the inconsistent post-cryo success brought about by the removed zygotic explants that are frequently utilized for cryopreservation. It can be technically difficult to separate zygotes from the surrounding tissues because they are tiny and sensitive. Using zygotic explants from a variety of recalcitrant-seeded species, including sub-tropical monocotyledonous geophytes and sub-tropical dicotyledonous trees, the analysis demonstrated that embryo morphology and anatomy are important factors that determine the drying properties of the various tissues that make up the explant and, consequently, post-cryo survival(Ballesteros et al. 2014).

Somatic embryo

All explants studied exhibited considerable sensitivity to both cryoprotective treatments and freezing. On the other hand, somewhat greater recovery values were accomplished when employing the most varied explants, i.e., somatic embryos 1–6 mm in size. The findings were significantly better when somatic embryos were first cultured on liquid medium for 28 days. At these conditions, 60% of samples resumed embryogenesis, thus ensuring the safe long-term conservation of this type of embryogenic structures. (Bradaï et al. 2017).

Petiveria alliacea L. was used to successfully cryopreserve somatic embryos (SEs) generated from P. alliacea roots using vitrification, encapsulation-dehydration, and D cryo-plate techniques. Using the vitrification procedure, SEs treated with PVS2 solution showed intermediate proliferation recovery and high viability (85%). After 90 minutes of dehydration, cryopreserved SEs using the Dcryo-plate approach showed good viability (85%) and proliferation recovery.

The D cryoplate approach proved to be the most effective cryopreservation method for P. alliacea SEs under the assessed experimental circumstances (Pettinelli et al. 2017).
In vitro roots were used to create somatic embryos (SEs), which were then cryopreserved using the V-cryoplate method. Their feasibility was evaluated using the triphenyl tetrazolium assay, and their recovery was measured by calculating how many secondary SEs were generated for each cryopreserved SE ninety days following exposure to liquid nitrogen (LN). SEs underwent varying treatments with PVS2 and then SEs were submerged in LN and then rewarmed in unloading solution. Following 15 minutes of PVS2 therapy and LN exposure, the TTZ test showed 100% survival. The rate of multiplication recovered after 90 days of culture, with each cryopreserved somatic embryo generating 21 cells.
For the long-term in vitro storage of ex situ plant genetic resources in liquid nitrogen, somatic embryos are the ideal explants. They have made it possible for improvements in survival and recovery after cryopreservation as compared to conventional crystallization-based methods, proving their effectiveness for widespread use with plant embryos and shoot tips. (Gonzalez et al. 2008).

Protocorm-like bodies (PLBs)

Explants of various kinds, particularly protocorms and freshly produced PLBs, have shown effective in regeneration research. Increased synthesis of secondary products and mass plantlet proliferation are made possible by the improved growth of PLB biomass in bioreactors. (Yeung et al. 2024).
The orchid Dendrobium candidum Wall. ex Lindl.'s protocorm-like bodies (PLBs) were successfully cryopreserved with the use of encapsulation vitrification. PLBs that had been dehydrated and encapsulated were submerged straight into liquid nitrogen. After being quickly microwaved in a water bath, the cryopreserved PLBs were rinsed three times at 10-minute intervals using MS media. Plantlets containing the cryopreserved PLBs showed no morphological defects and produced normal shoots and roots. Encapsulated-vitrified PLBs had a survival rate of more than 85%. For the cryopreservation of D. candidum PLBs, this encapsulation-vitrification technique was therefore considered promising (Yin et al. 2009).
Using PLBs of Vanda lilacina Teijsm. & Binn., two novel cryopreservation techniques—the survival rate and outcomes were examined using the V-cryo-plate and D-cryo-plate techniques. Using PVS2 solution for the V-cryo-plate method and silica gel for the D-cryo-plate method, PLBs were dehydrated. The results showed that the V-cryo-plate method gave a survival percentage of 33.33 %. For the D-cryo-plate method, it gave the highest survival percentage of 83.78 %. (Imsomboon et al. 2020).
One of the most significant issues in cryopreservation is the creation of ice crystals. Ice crystals that grow inside or around the PLBs have the potential to penetrate the membranes, resulting in irreversible damage.

Shoot tip explant

In vitro-grown cultures often use shoot tips removed from the apical or axillary buds as explants. Since shoot tips offer a high level of genetic stability for plants that are propagated by cloning and are made up of organized tissues with meristematic cells with dense cytoplasm and small vacuoles, they are the ideal candidates for cryopreservation.

Initially, shoot tip cryopreservation employed techniques developed for plant-cell suspensions, such as controlled-rate cooling (two-step cooling techniques) to dry cells and freeze water in intercellular spaces until they would vitrify (turn to glass) when exposed to liquid nitrogen (LN).Some of the earlier plant species studied using shoot tips were potato and cassava (Normah et al. 2019).

The encapsulation-vitrification method was used to cryopreserve the shoot tips of Artemisia herba-alba that were grown in vitro. The effects of dehydrating the encapsulated Artemisia herba-alba shoot tips with 100% PVS2.After dehydration with a 100% concentrated PVS2 solution, the encapsulated STs exhibited the maximum survival (76%) and regeneration (6%) of Shih STs during cryopreservation. The encapsulated and vitrified shoot tips, on the other hand, showed 68% survival and 12% regeneration rates with 100% PVS2 (Sharaf et al.2012).

Similarly, In vitro shoot tips of Capparis spinosa were successfully cryopreserved using the vitrification, encapsulation-dehydration and encapsulation-vitrification techniques. When encapsulated shoot tips were exposed in PVS2, the method that produced the highest regrowth rate (83%) was encapsulation dehydration, followed by vitrification (70%) and encapsulation-vitrification (resulting in a maximum regeneration rate.) (Shatnawi, 2011).

PVS2 vitrification, encapsulation-vitrification, and droplet-vitrification are the three cryopreservation methods that Ozudogru and Kaya (2012) investigated for the successful cryopreservation of Thymus vulgaris shoot tips in a distinct species. The droplet vitrification procedure, produced the highest survival (85%) and recovery (80%) rates in cryopreserved shoot tips. With encapsulation-vitrification and vitrification (25%), a very low post-thaw recovery (3%) was achieved.

A droplet-vitrification method has been developed for Chrysanthemum morifolium cv cryopreservation. They were subsequently dehydrated using PVS3 vitrification solution. Through direct immersion in liquid nitrogen in tiny drops of PVS3 affixed, the transplants were cryopreserved resulting in regeneration percentages of 81.9% and 84.9% after cryopreservation, respectively (Halmagyi et al.2004).

Although both the apical and axillary shoot tips can be employed as explants, another aspect that could affect recovery following cryopreservation is the homogeneity of the shoot tips (Bettoniet al. 2021).In addition, the ideal pretreatment and cryopreservation conditions are influenced by the size of the removed shoot tips. The ideal explant for cryopreservation techniques is

frequently a shoot tip that is 1–2 mm long, depending on the species(Vollmer et al. 2019).

Root Explants

Using a variety of materials, including roots, cryopreservation in liquid nitrogen (-196 °C) guarantees the safe and economical protection of plant genetic resources. Numerous plant species have so far been successfully cryopreserved root explants that were separated from hairy and adventitious root cultures, as well as plants cultivated in vitro. It has been demonstrated that the cryopreservation procedure has minimal to no effect on the development, biosynthesis, and genetic stability of roots when they have recovered in an ambient growing environment. (Popova et al. 2021).

In vitro plants were used to create an efficient root-based cryopreservation technique for Hypericum perforatum L. a significant medicinal species. After preculturing the root, they created a successful root-based cryopreservation method for Hypericum perforatum L. Furthermore, it was found that the root tips exhibited noticeably slower rates of regeneration than the middle and basal regions, whereas the highest plant recovery following cryopreservation was seen in the root sections extracted from 8-week-old plants and cryopreserved 10 days after excision. Five elite lines of H. perforatum were successfully cryopreserved using this technique, showing regrowth rates ranging from 45 to 87% after cryopreservation. These varying reactions to cryopreservation may have been caused by differences in the lines' growth patterns (Yang et al. 2019).

However, because thin and fragile root tips are extremely sensitive to stressful conditions associated with different steps of a cryopreservation protocol, and because each step of the process necessitates careful optimization, especially the treatment with vitrification solution, structural integrity of roots is crucial for successful cryopreservation (Popova et al. 2021).

Adventitious root cultures

Adventitious roots originate from many cell types and, as a result, from various tissues. Whether ARs are produced intentionally by wounding or hormone treatments, they always come from cells that are close to vascular areas, either as part of the plant's developmental program (as in monocotyledons and naturally vegetatively propagated dicotyledons). This is a conserved characteristic. Interfascicular cambium cells near the phloem cells, phloem or xylem parenchyma cells, juvenile secondary phloem cells, or hypocotyl pericycle cells can all be the starting point for Adventitious roots (Bellini et al. 2014).

Hyoscyamus niger adventitious root culture without auxin was produced, and the roots were successfully cryopreserved by vitrification, exhibiting a high rate of regeneration of 93.3%. After 12 to 14 days of culture in phytohormone-free Murashige and Skoog (MS) liquid medium, the root tips were removed and

recultured at 25 °C in the dark on Woody Plant (WP) solid media supplemented with 0.3 mol/L sucrose (Jung et al. 2001).

In order to cryopreserve the adventitious roots of Panax ginseng, we used desiccation and vitrification methods to determine where ginsenosides that are made commercially come from. The post-freeze survival of 3–4 mm root tips was less than 14% when desiccation was the only treatment applied, regardless of the explant's origin. Conversely, 90% survival and 32.5% root formation efficiency were achieved when a vitrification approach was employed after cryopreservation. From root tips that had been cryopreserved by vitrification, adventitious root cultures were restored in flasks and bioreactors. Post-freeze-regenerated cultures showed a longer lag-phase and poorer biomass output than control roots following four subcultures in flasks. However, the biomass accumulations of the control and regenerated roots were the same by end of the sixth subculturing period. (Oh et al. 2009).

Embryogenic callus

Immature embryos are frequently utilized to induce callus for tissue culture or genetic transformation. Therefore, trustworthy callus induction is necessary for biotechnological study followed by plant regeneration (Özgen et al. 1998). To maintain their embryogenic potential, embryogenic callus that were developed in vitro from the original explant are commonly sub cultured. We term these cultures "embryogenic callus lines" **(**Lambardi et al. 2008).

Through vitrification, Callus from young Satureja spicigera leaves worked out successfully cryopreserved. The suitability of four procedures—desiccation, PVS2 vitrification, PVS3 vitrification, and DMSO freezing—was evaluated. Following PVS3 treatment, the cryopreserved callus's regeneration percentage was noticeably higher (98.7%). This finding suggests that the best method for cryopreserving S. spicigera callus is vitrification using PVS3. These cryopreservation protocols would be useful for the ex-situ conservation of Satureja spicigera callus at very low temperatures (Ghaffarzadeh-Namazi et al. 2017).

Using vitrification, Sen-Rong and Ming-Hua (2012) developed a simple and efficient method for cryopreserving embryogenic Calli of the medicinal plant Anemarrhena asphodeloides. In vitrified embryogenic Calli, the regeneration rate exceeded 60%. The greatest regrowth rate, 62.3–65.7%, was achieved when embryogenic Calli were vitrified after being precultured for two days.

A straightforward and efficient vitrification method for the cryopreservation of S. involucrate callus tissue has been created and refined. A survival rate of almost 56% and a regeneration rate of roughly 40% were obtained using this technique. Including a low-temperature preincubation stage greatly increased survival and regeneration. This boosted the tissue's survival rate to 75%, regrowth rate to 60%, and number of regenerated shoots per explant by more than double (Guo et al.2013).

Cell suspension culture

A sterile (closed) system known as a plant cell suspension culture is typically started by aseptically transferring friable callus fragments into an appropriate sterile liquid media (Dixon et al. 1985).

After that, the callus on the explant is removed, and further subculturing is performed. By continuously shaking the solution, in liquid medium, callus material can be infected. (in flasks) to create cell suspension cultures. Plant to a stable suspension culture of cells, the entire procedure could take six to nine months. The comparatively homogeneous cell population found in cell suspension cultures enables the cells to quickly and consistently access growth hormones, precursors, nutrients, and signaling molecules.

Because of the high rate of cell development, the high reproducibility of conditions, the homogeneity of an in vitro cell population, and the abundance of available material, suspension-cultured cells are a great option for examining complex physiological processes at the molecular and cellular levels. (Moscatiello et al.2013).

For Catharanthus roseus embryogenic cell suspension cultures, a powerful cryopreservation procedure was developed. The greatest frequency of embryogenic culture viability was achieved by this vitrification-based cryopreservation method, wherein embryogenic cells were submerged in LN after being subjected to a preculture medium. When suspension cultures were exposed to sucrose that had been pretreated with DMSO (PT-1), the highest rate of regrowth (88.9%) was noted. The somatic embryos produced on an optimized medium were similar to those from non-frozen embryogenic cultures, and all calluses grew back and resumed their normal growth. Each regenerated plantlet showed typical morphology after being converted from somatic embryos. (Fatima et al. 2009).

With differentiated cultures, tissue-specific biosynthetic pathways that are typically not expressed in cell suspension cultures can be investigated. Because they provide a simplified model system for studying plants, in vitro dedifferentiated plant cell suspension cultures are more practical for the large-scale production of fine compounds in bioreactors as well as for the investigation of cellular and molecular processes (Mustafa et al. 2011).

The complexity of culture preparations varies widely, spanning from isolated single cells to three-dimensional histotypic cell formations. Critical elements of cell culture, including media pH, osmolality, humidity, and cell density, are discussed, along with the fundamentals of culture maintenance and proliferation. (Honegger et al.1999).

Genetic and Biochemical stability assessment

In vitro-cryopreserved plants' genetic stability was investigated through the use of biochemical techniques (bacoside estimate) and molecular techniques (random amplified polymorphic DNA (RAPD) markers) (Sharma et al. 2012).

Maintaining the genetic stability of medicinally significant plant material is the primary goal of cryopreservation techniques, in addition to conservation. Therefore, the validity of this cryopreservation technique is contingent upon the plant material's genetic stability remaining unaltered during exposure to liquid nitrogen. (Zarghami et al. 2008). In order to prevent genetic modification, instead of using undifferentiated plant material for cryogenic preservation, only differentiated tissues—like shoot tips—are selected. In contrast to genetic alterations, epigenetic modifications do not change the original DNA sequence because they typically include changes in DNA methylation (Smulders & Klerk, 2011). Plants that have been cryopreserved can be examined using a variety of methods at the phenotypic, cytological, biochemical, and molecular levels to determine their genetic stability (Harding et al. 2000).Randomly amplified polymorphic DNA (RAPD) fingerprinting is one of the molecular methods that may be used to create DNA profiles, and it is increasingly being used to identify Somaclonal variation (Rani V et al. 1995).The following are some examples of distinct MAP genotypes that were examined to look for any changes following cryopreservation: Ziziphora tenuior (Al-Baba et al. 2015), Chrysanthemum morifolium 'Hangju' (Bi et al. 2016), Bacopa monnieri (Sharma et al. 2017), Allium sativum (Liu et al. 2017), Valeriana jatamansi (Sharma et al. 2021), Gentiana kurroo (Sharma et al. 2021), Dioscorea deltoideia(Sharma et al. 2022).

Discussion and Conclusion

The cryopreservation of medicinal and aromatic plant genetic resources is an important strategy for maintaining biodiversity, ensuring the survival of plant species, and maintaining their genetic integrity. The best option for long-term preservation of biological materials is cryopreservation, which involves storing tissues (usually with a high meristematic zone and low water content, such as shoot tips, seeds, pollen, zygotic or somatic embryos) at extremely low temperatures in liquid nitrogen (LN; -196 °C). (Touchell 2000; Engelmann 2000). Cryoprotectant use, healthy cultures free of microbial infection symptoms, and cultures at the log phase of growth are all necessary for successful cryopreservation. Proline amino acid, sucrose, glycols, sugar alcohols, dextran, and polyethylene glycol are examples of cryoprotectants that reduce cryo-injury. Traditional methods include freeze-induced dehydration, which involves prefreezing to a predetermined temperature and then rapidly immersing in LN. In contrast, contemporary methods use vitrification, which converts water directly from a liquid to an amorphous state without going through the crystalline phase. Although the encapsulation-vitrification method efficiently works with smaller shoot tips and protocorm-like bodies (PLBs), it is shown to be beneficial over traditional cryopreservation techniques and has broader potential for germplasm conservation. "This method is becoming more and more popular because it works with explants of all sizes and is rapid, nonhazardous chemically and physically. A variety of factors, including the survival rate, rate of regeneration,

process duration, and issues with handling the transplants, must be taken into account when choosing a certain cryopreservation technique (Gallard et al. 2006). Usually, the parameters that promote maximum survival do not promote the optimal rate of regeneration. Likewise, Attractylodes macrocephala showed a 76% survival rate for droplet-vitrification, but a lower regrowth frequency of 62% (Zhang et al. 2015).

Around the world, several plant species have been successfully conserved by cryopreservation techniques. (Kundu et al. 2018).

Conflicts of Interest

The authors declare no conflict of interest.

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Data Availability Statement

Data supporting this study are included within the manuscript and no new data was created or analyzed. Any supporting file will be made available upon request from the corresponding author.

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Figure 1.

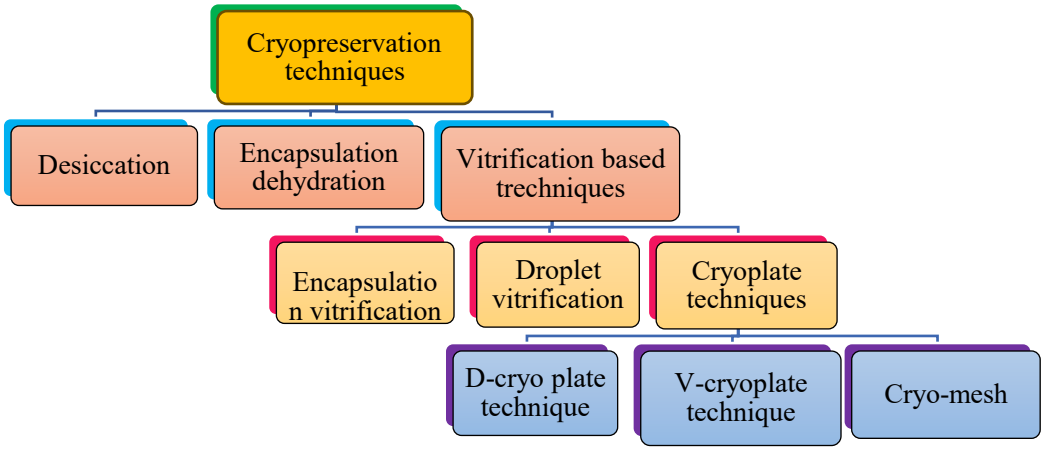

Figure 2.

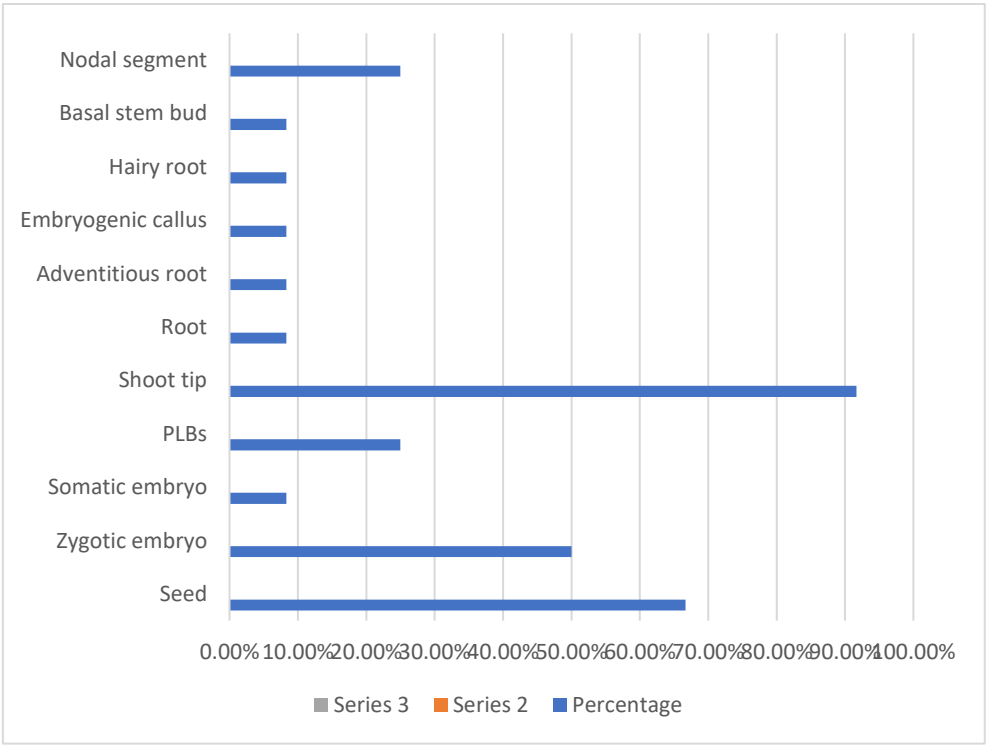

Table 1.
This table represents data on the plant cryopreservation techniques and their effectiveness across various species. It outlines various type of explants (plant part used) like seeds, PLBs, zygotic embryos etc. and the survival of regrowth rates after the preservation process.

Name of plant	Explant used	Regeneration percentage	Cryopreservation Technique	References
Chlorophytum borivilianum	Shoots	33%	Vitrification	Chauhan et al. 2021
Bletillastriata	Seeds	93%	Droplet vitrification	Jitsopakul et al.2008
Satureja bachtiarica	Seeds	76.39%	Desiccation Vitrification	Ghaffarzadeh-Namazi et al. 2015
Gentiana kurroo	Shoot tips	60%	Droplet vitrification	Sharma et al.2021
Decalepis salicifolia	Shoot tip & Nodal explants	92.8%	Encapsulation vitrification	Rodrigues et al.2020
Aconitum heterophyllum	Seeds	75%	Desiccation Vitrification	Kushwaha et al.2010
Podophyllum hexandrum	Zygotic embryos	90%	V cryoplate technique	Parasher et al.2023
Coffea arabica	Zygotic embryos	97%	Encapsulation dehydration	Dussert et al.1997
Petiveriaalliacea	Somatic embryos	85%	D cryoplate technique	Pettinelli et al.2017

Dendrobium candidum	PLBs	85%	Encapsulation vitrification	Yin et al.2009
Artemisia herba-alba	Shoot tips	40%	Encapsulation vitrification	Sharaf et al.2012
Capparis spiunosa	Shoot tips	83%	Encapsulation dehydration	Shatnawi et al.2011
Hypericumperforatum	Roots explants	45-87%	Droplet vitrification	Yang et al.2019
Panax ginseng	Adventitious roots	90%	Vitrification	Oh et al.2009
Satureja spicigera	Callus	98.7%	Vitrification	Ghaffarzadeh-Namazi et al.2017
Curcuma longa	Shoot tips	80%	Vitrification	Babu et al.2012
Piper nigrum	Nodal segments	85%	Encapsulation vitrification	Raju et al.2022
Taxus baccata	Shoots	99.9%	Encapsulation-dehydration	Moghaddam et al.2024
Malaxis acuminata	PLBs	80%	Encapsulation vitrification	Kaur et al.2023
Plumbago rosea	Axillary buds	83.33%	Encapsulation vitrification	Sowmya et al.2017
Eclipta alba	Nodal segment	92.80%	Encapsulation vitrification	Salma et al.2019

Indigofera tinctoria	Axillary shoot meristems	62.2%	Encapsulation dehydration	Nair et al.2009
Juglans regia	Shoot tips	67.8%	Vitrification	Kushnarenko et al.2023
Juncus decipiens	Basal stem buds	86.3%	D cryoplate technique	Niino et al.2013
Ludwigia palustris	Shoot tips	73%	Encapsulation dehydration	Fontanili et al.2015
Maesa lanceolata	Hairy root cultures	90%	Encapsulation dehydration	Lambert et al.2009
Melia azedarach	Zygotic embryo	36%	Encapsulation dehydration	Scocchi et al.2007
Mentha arvensis	Shoot tips	80%	Encapsulation vitrification	Islam et al.2012
Manilkara zapota	Seeds	94%	Desiccation Vitrification	Wen et al.2013
Musa acuminata	Zygotic embryos	86%	Droplet vitrification	Roostika et al.2024
Musa balbisiana	Zygotic embryos	95%	Desiccation Vitrification	Singh et al.2021
Nerium oleander	Shoot tips	60%	Encapsulation vitrification	Hatzilazarou et al.2019
Nymphaea caerulea	Seeds	97%	Vitrification	Lee et al.2022

Plantago algarbiensis	Nodal explants	63.3%	Encapsulation dehydration	Coelho et al. 2014
Epipactis flava	Seeds	33.3%	Vitrification	Linjikao et al.2024
Ranunculus kazusensis	Zygotic embryo	90%	Encapsulation dehydration	Kim et al.2009
Vanda tessellata	Seeds	90%	Encapsulation vitrification	Manokari et al.2021
Dendrobium nobile	PLBs	78.1%	Encapsulation vitrification	Mohanty et al.2012
Colocasia esculenta	Shoot tips	73-100%	Droplet vitrification	Sant et al.2008
Acacia nilotica	Seeds	80%	Vitrification	Jebelli et al.2015