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Cryopreservation strategies for trees: Leveraging organogenesis and somatic embryogenesis for effective conservation

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Abstract: Trees are fundamental to ecosystems, supporting biodiversity, regulating climate, storing carbon and providing vital resources. Several species are under severe threat from deforestation, habitat destruction, urban expansion and climate change, endangering both the species and the ecosystem services they provide. Traditional conservation methods, such as seed genebanks and field genebanks, face challenges like genetic degradation and high maintenance costs. Cryopreservation offers a promising alternative, preserving genetic material at ultra-low temperatures, halting biological processes and minimizing storage space needs. This method is especially effective for species with recalcitrant seeds or difficulty in propagation. Integrating techniques like somatic embryogenesis and organogenesis further enhances cryopreservation. Somatic embryogenesis enables the development of embryos from somatic cells, facilitating the regeneration of trees from cryopreserved tissues. Similarly, organogenesis promotes the development of *in vitro* cultures for species that are otherwise challenging to conserve, allowing for cryopreservation of *in vitro* explants. The combination of these techniques not only preserves genetic material but also ensures the regeneration of viable plants, providing a comprehensive approach to tree conservation. This review highlights the critical role of cryopreservation in preserving tree diversity, with a focus on somatic embryogenesis and organogenesis, exploring current practices, challenges and future directions.

Keywords: Biodiversity, cryopreservation, conservation, extinction, genebanks, genetic diversity, organogenesis

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Introduction

Trees are essential components of life on Earth, playing crucial roles in ecological, cultural and economic spheres. Among the estimated 350,000 vascular plant species on Earth, approximately 59,000 are described as tree species (BGCI, 2021). The latest Global Tree Assessment (GTA) has classified 92.7% of the 57,922 tree species evaluated with conservation status for inclusion on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species. The GTA has identified nine major threats to tree species globally, including agricultural expansion affecting 29% of species, overexploitation affecting 27%, livestock farming affecting 14%, urban development affecting 13%, changes in fire regimes affecting 13%, energy production and mining affecting 9%, establishment of wood and pulp plantations affecting 6%, invasion by non-native species affecting 5%, and climate change affecting 4% (BGCI, 2021).

These combined pressures have already caused the decline and, in some cases extinction of numerous tree species, with profound implications for biodiversity across all trophic levels. In response to these challenges, there is an urgent

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need for targeted and effective conservation efforts aimed at preventing further species extinctions. Therefore, this review discusses targeted conservation strategies for trees, with emphasis on *ex situ* conservation methods – particularly cryopreservation approaches using diverse explants such as seeds, zygotic embryos or embryonic axes, shoot tips and somatic embryos to safeguard genetic diversity and support restoration and sustainable use.

Conservation of trees

In conservation biology, *in situ* and *ex situ* strategies are essential for preserving tree genetic diversity. *In situ* conservation involves protecting tree populations within their natural habitats, such as national parks and reserves, maintaining evolutionary processes and helping species adapt to environmental changes. According to the Protected Planet Report of 2024, protected and conserved areas presently encompass 17.6% of the global terrestrial and inland water area, underscoring the scale of *in situ* conservation worldwide (UNEP-WCMC and IUCN, 2024).

In situ conservation is a dynamic practice that maintains ongoing evolutionary processes, thereby fostering both genetic diversity and adaptation. *In situ* conservation is typically implemented at local or experimental scales, focusing on farmers, traders, processors, or community groups. However, it is not readily structured to ensure large-scale germplasm availability or to guarantee long-term conservation security (Lusty *et al*, 2014). *Ex situ* conservation complements *in situ* methods by preserving genetic material outside natural habitats. Techniques include botanical gardens and arboreta for managing diverse species collections, seedbanks for storing seeds including endangered species, and nurseries for cultivating seedlings for reforestation. Although *ex situ* conservation does not replicate natural ecosystems, it offers cost-effective storage of genetic diversity and supports breeding programmes. For species where traditional methods are inadequate, such as with recalcitrant seeds or species difficult to propagate, *in vitro* (*in vitro* genebank) and cryopreservation strategies provide controlled environments for short- to long-term storage (Gowthami *et al*, 2024a; 2024b).

Cryopreservation

Cryopreservation of tree species represents a cutting-edge approach in conservation biology, enabling the long-term storage of genetic material at ultra-low temperatures (-196°C in liquid nitrogen) to preserve genetic diversity and safeguard endangered or economically valuable species (Agrawal *et al*, 2022; 2024). This technique cryopreserves plant tissues – seeds, embryos, pollen, or meristems – achieving a vitrified state that suspends biological activity and maintains genetic integrity, surpassing conventional seedbanking. It is particularly valuable for species with recalcitrant seeds or those difficult to propagate traditionally, reducing genetic drift and serving as a backup to living collections (Panis *et al*, 2020). The field was significantly advanced by Sakai's (1960) experiments with precooling techniques, which minimized ice crystal formation and cellular damage, laying the groundwork for modern cryopreservation practices and expanding its applications in plant conservation and biotechnology.

Cryopreservation principle and cryoprotection

Water is vital for plant cells, making up about 80% of their tissue mass and performing crucial roles in growth and metabolism, and in maintaining turgor pressure. Exposure to temperatures below 0°C without cryoprotectants can lead to cell death due to ice formation, which disrupts cellular membranes and causes detrimental biochemical changes and intracellular ice crystallization leading to increased solute concentration, both compromising cell integrity. To mitigate these effects, employing cryoprotectants and optimizing cooling and thawing rates are essential (Pegg, 2007). Methods to enhance solute concentration inside cells include air drying, metabolic adjustment, freeze dehydration, osmotic dehydration, and using permeating molecules (Nagel *et al*, 2024).

Cryoprotectant agents (CPAs) protect cells during freezing by increasing solute concentration, which prevents ice crystal formation and minimizes damage. CPAs are categorized into penetrating (e.g. glycerol, dimethyl sulfoxide (DMSO) and non-penetrating types (e.g. sucrose, mannitol). Penetrating CPAs enter cells, lower the freezing point, and interact with biological molecules, while non-penetrating CPAs reduce ionic forces outside cells and inhibit ice formation. Typically, a combination of both types improves cryoprotection, but balancing their effectiveness and toxicity is crucial (Pegg, 2007).

History of cryopreservation techniques

The modern era of cryopreservation began with the discovery that glycerol could protect sperm cells during freezing, laying the foundation for the development of cryoconservation methods now widely applied to plant germplasm (Polge *et al*, 1949; Pegg, 2002). In plant systems, A. Sakai initiated cryopreservation with the survival of mulberry twigs after exposure to liquid nitrogen (LN: -196°C) and pretreatment at -30°C (Sakai, 1960). In 1968, classical slow-cooling techniques were introduced by Quatrano to preserve linseed cells (Quatrano, 1968), where samples were freeze-dehydrated at a controlled rate (1K/h) of cooling to about -40°C, then plunged into LN. Since this method causes substantial extracellular ice formation and plasmolysis, it has been most effective for unorganized tissues (cell suspensions and callus) and for apices of cold-tolerant species (Panis, 2019). In the late 1980s, Uragami and colleagues successfully applied a vitrification-based, one-step freezing technique to *Asparagus* embryos, which became a widely applicable cryopreservation method (Engelmann, 2004).

Classical slow-cooling (or slow-freezing) or two-step freezing protocol

The classical slow-cooling (slow-freezing) and two-step freezing protocols are the original standard methods for cryopreserving hardy and hydrated plant tissues. These techniques differ critically in equipment needs, cooling mechanisms, and overall success rates (Van Iren *et al*, 1995). Both techniques rely on controlled extracellular ice formation to dehydrate cells slowly, preventing lethal intracellular ice crystals. The cells cryodehydrate until intracellular fluids vitrify upon LN transfer. This avoids the 'solution effect'

injury and ice crystal damage that kill cells. However, both techniques are significantly less effective than modern vitrification protocols (Nagel *et al*, 2024).

Desiccation

This technique is one of the simplest methods. It involves dehydrating plant samples to critical moisture content in sterile air or silica gel before direct immersion in LN, bypassing the need for programmable freezers or complex vitrification solutions. Flash drying, or ultra-rapid drying in a compressed dry-air stream, allows for the freezing of explants with relatively high water content with minimal damage (Berjak *et al*, 1992). Air desiccation relies on freeze-induced dehydration: as temperature drops, water is drawn out of cells, forming extracellular ice while intracellular fluids vitrify. Explants generally have the highest survival rates when frozen with a water content of 10 to 20% (fresh weight basis) (Engelmann, 2003).

Encapsulation-dehydration

Based on the principle of synthetic seeds, this technique involves encapsulating precultured explants in sodium alginate (2–4%) and polymerizing with 0.1M $CaCl_2$ for 20–45 minutes to provide physical protection from mechanical injury and oxidative stress (Engelmann *et al*, 2008). The encapsulated beads are then desiccated in a laminar airflow cabinet, silica gel, or through osmotic dehydration to a critical level (~20–30% moisture content), preventing lethal intracellular ice crystals during subsequent rapid freezing in LN (Kaviani and Kulus, 2022). Despite its advantages, the encapsulation–dehydration method is labour-intensive, and the use of an alginate matrix can elevate the risk of tissue hyperhydricity and promote callus formation following LN storage (Kaviani *et al*, 2008; Kulus and Zalewska, 2014).

Vitrification

This widely used technique has been successfully applied to over 100 plant species and is particularly common for cryopreserving shoot tips/embryo/embryogenic axes, etc., of various tree species. It involves achieving complete vitrification, where both intra- and extracellular solutions transform into a glassy state. Explants are first treated with a loading solution (LS) of diluted cryoprotectants to enhance resistance to dehydration, followed by exposure to highly concentrated plant vitrification solution (PVS) to remove all freezable water, followed by rapid cooling in LN (Sakai *et al*, 1990). The choice of LS and PVS solutions and their treatment duration is crucial for desiccation tolerance (Vidal *et al*, 2005).

Encapsulation-vitrification

This technique combines encapsulation-desiccation and vitrification procedures. Explants are first encapsulated in calcium alginate beads, then subjected to dehydration with LS and PVS (Sakai and Engelmann, 2007). The extended exposure to PVS in encapsulated explants allows for slower and safer dehydration. PVS2 exposure is less common due to its tendency to make beads brittle and increase the risk of explant damage (Kaviani *et al*, 2022).

Droplet-vitrification

This method, first reported for sweet potato shoot tips (Pennycooke and Towill, 2000), is an innovative cryopreservation technique that incorporates both droplet-freezing and vitrification. In this procedure, PVS-treated explants are placed on a droplet (10-15μL) of PVS on an aluminium foil strip (~20 × 5mm) and then directly immersed in LN for rapid freezing and thawing. This technique achieves rapid freezing (~130°C/s) and thawing due to the high thermal conductivity of aluminium foil and the direct contact of the explant with LN (Panis *et al*, 2005). It requires considerable technical skill but minimizes handling injury, as the explant adheres to the foil strip, avoiding longer exposure to PVS2, which can be toxic (Panis *et al*, 2005). Owing to its simplicity, high cooling and warming rates, reproducibility and broad applicability across diverse plant species, droplet-vitrification has become one of the most extensively utilized cryopreservation methods for plant genetic resources and is widely adopted in cryobanks worldwide (Sakai and Engelmann, 2007).

Cryo-plate

This innovative cryogenic approach combines droplet-vitrification and encapsulation-dehydration using cryoplates, such as V cryoplate (vitrification cryo-plate) and D cryoplate (dehydration cryo-plate) (Niino *et al*, 2013). In the V cryoplates technique, dehydration is performed using LS and PVS, while in D cryoplates, desiccation occurs in a laminar flow cabinet. These techniques offer easy handling and higher post-thaw regrowth. However, V cryoplates require longer exposure to PVS due to encapsulation. D cryoplates are advantageous for larger explants, being less labour-intensive with reduced risk of chemical or handling injury and minimizing the potential loss of genetic integrity due to PVS treatments. Overall, cryo-plate techniques are among the most efficient and user-friendly cryopreservation methods, often providing higher post-thaw survival and regrowth than droplet-vitrification. However, their widespread application is limited by the requirement for specialized cryoplates.

Vacuum infiltration vitrification

This technique is similar to droplet vitrification, and involves exposing explants to a vacuum (5 minutes) during LS and PVS2 solution incubations using a 200mm ø Kartell vacuum chamber (Funnekotter *et al*, 2015). This reduces the PVS exposure period by half to a quarter compared to the droplet-vitrification technique by creating a pressure differential that forces cryoprotective solution to penetrate deep into intercellular spaces and enhances post-thaw regeneration. This technique is advantageous for sensitive tissues prone to cryoprotectant toxicity, difficult-to-preserve species with high water content and large-scale cryobanking.

Explants and propagules for cryopreservation in trees

Various types of plant tissues, known as explants, can be effectively cryopreserved to ensure their viability and genetic integrity over extended periods in tree species. The

present section contains information on different explants used for cryopreservation of trees with the application of organogenesis and somatic embryogenesis. Each type of explant requires specific cryopreservation protocols tailored to its unique characteristics and developmental stage.

Seeds, zygotic embryos and embryonic axes

Cryopreservation techniques designed specifically for seeds of tree species are essential for maintaining their long-term viability and preserving genetic diversity. Seeds are classified into three main categories based on their ability to withstand drying and freezing processes: orthodox (*Alnus glutinosa, Pinus kesiya* etc.*)*, intermediate (*Buxus hyrcana, Emmenopterys henryi,* etc.) and recalcitrant (*Garcinia mangostana, Castanea sativa, Crateva nurvala,* etc.) (Gowthami *et al*, 2024a). Orthodox seeds are capable of tolerating drying (3–10%) and freezing, so they can usually be safely stored or cryopreserved at very low temperatures for long-term conservation. Intermediate seeds have limited desiccation (10–20%) and cold tolerance, while recalcitrant seeds are highly sensitive to drying and cannot be preserved by conventional seedbanking, so cryopreservation of embryos or excised tissues is often needed (Wyse *et al*, 2018; Singh *et al*, 2021). The critical moisture content varies from species to species for cryopreservation, reflecting their unique physiological and biochemical adaptations. This species-specific variation highlights the complexity of conserving recalcitrant seeds, as each type requires tailored conditions to maintain viability during storage and handling.

The first stage of preserving tree seeds through cryopreservation begins with the crucial step of dehydrating the seeds to lower their moisture content and reduce the likelihood of ice crystal formation when the seeds are frozen later on. To further improve their ability to withstand freezing conditions, orthodox seeds may undergo additional treatments such as osmotic dehydration or exposure to cryoprotectants. These treatments are designed to safeguard cellular integrity and uphold seed viability throughout the cryopreservation process. By protecting the seeds in this manner, their potential for successful long-term storage and eventual use in conservation efforts is greatly enhanced. In *Betula lenta,* following cryopreservation, *in vitro* germination above 10% was achieved only for seeds cultured on semi-solid media with 10μmol/L thidiazuron (TDZ) or on liquid media with 10μmol/L 6-benzylaminopurine (BA)(Rathwell *et al*, 2016).

Several studies on recalcitrant and intermediate species have consistently demonstrated that excised embryos and embryonic axes exhibit greater tolerance to desiccation and subsequent cryopreservation compared to whole seeds (Normah and Makeen (2008) and references therein). EA are preferred over ZE because EA can withstand severe dehydration that would otherwise be lethal to the entire seed (Santos and Stushnoff, 2002). Particularly in oily seeds, exposure to LN can lead to cell death in cotyledon tissues, underscoring the necessity for using EA for cryopreservation (Ballesteros and Pence, 2019). Plantlets can be generated from EA and ZE through *in vitro* culture. Despite exhibiting greater desiccation resistance than complete seeds, EAs and ZEs cannot withstand cryogenic storage when separated from seeds and require pre-treatment before plunging into LN. Desiccation is the most widely used technique for effective cryopreservation of ZE and EA of various tree species due to its simplicity and practical applicability. An ideal range of water contents between 7 and 20% on a fresh-weight basis is usually achieved for whole-plant recovery from frozen embryos and axes (corresponding to 0.08–0.25g H_2O per g dry weight (DW)). Other cryopreservation techniques used for ZE and EA are direct freezing, two-step freezing, vitrification, encapsulation-dehydration, and vacuum infiltration vitrification. The choice between these methods depends on the specific species and genotype, balancing the benefits and risks associated with each protocol. After cryopreservation, the isolated embryonic axes require germination on a nutrient-rich medium *in vitro* to replace the nourishment provided by the intact seed before they can be transferred to soil for further growth.

The post-thaw organogenesis of ZE and EA of trees is a pivotal phase in the cryopreservation process, where the ability of these tissues to regenerate and develop into viable plantlets is tested. The success of this regeneration largely depends on the composition of the basal media, plant growth regulators and conditions of the recovery medium used after thawing, affecting germination rates for different species (Figure 1, Table 1). Therefore, achieving high survival rates and effective organogenesis often requires empirical testing and fine-tuning of the recovery medium and environmental conditions to support the best possible growth outcomes.

The long-term viability of cryopreserved plant axes is a critical factor in assessing the success of cryopreservation techniques (Ballesteros and Pence, 2019). In the case of *Aesculus hippocastanum*, the study demonstrated that the axes maintained a remarkably high viability of 100% even after 23.4 years of storage in LN (Ballesteros and Pence, 2019). This indicates that the cryopreservation method used was highly effective in preserving the axes' ability to remain viable over an extended period. Furthermore, the viability of these axes showed no significant changes during subsequent drying or storage periods totalling 9.5 years in LN, suggesting that both the initial cryopreservation and the subsequent storage conditions were optimal for preserving the axes' viability. In contrast, *Aesculus glabra* axes displayed a notable decline in viability over time. After 21.6 years in storage, the viability dropped to 33%, which is a significant reduction compared to the 80% viability observed in freshly harvested axes. This decrease indicates deterioration in the axes' ability to remain viable over the extended storage period. However, when compared to other conditions, the decrease in viability of *A. glabra* was not statistically significant relative to axes that had been dried (with a viability of 70%) or those stored for 9.5 years in LN (with a viability of 75%).

Plumule tissue, part of the plant embryo situated within the seed, consists of the shoot apex (the apical dome) and the developing leaf primordia that give rise to the above-ground part of the plant, including the stem and leaves. Assy Bah and Engelmann (1992) found that, in coconut zygotic embryos cryopreserved by freezing, most embryonic cells were destroyed, except for those in the plumule, which comprises the apical dome and a few leaf primordia. The plumules are about 1mm in size, similar to immature zygotic embryos, which range from 0.1 to 0.3mm. The plumular tissue's small size, high concentration of regenerative meristematic cells, and strong regenerative capabilities contribute to its suitability for successful cryopreservation. This makes it an excellent explant for cryopreservation. Few successful cryopreservations

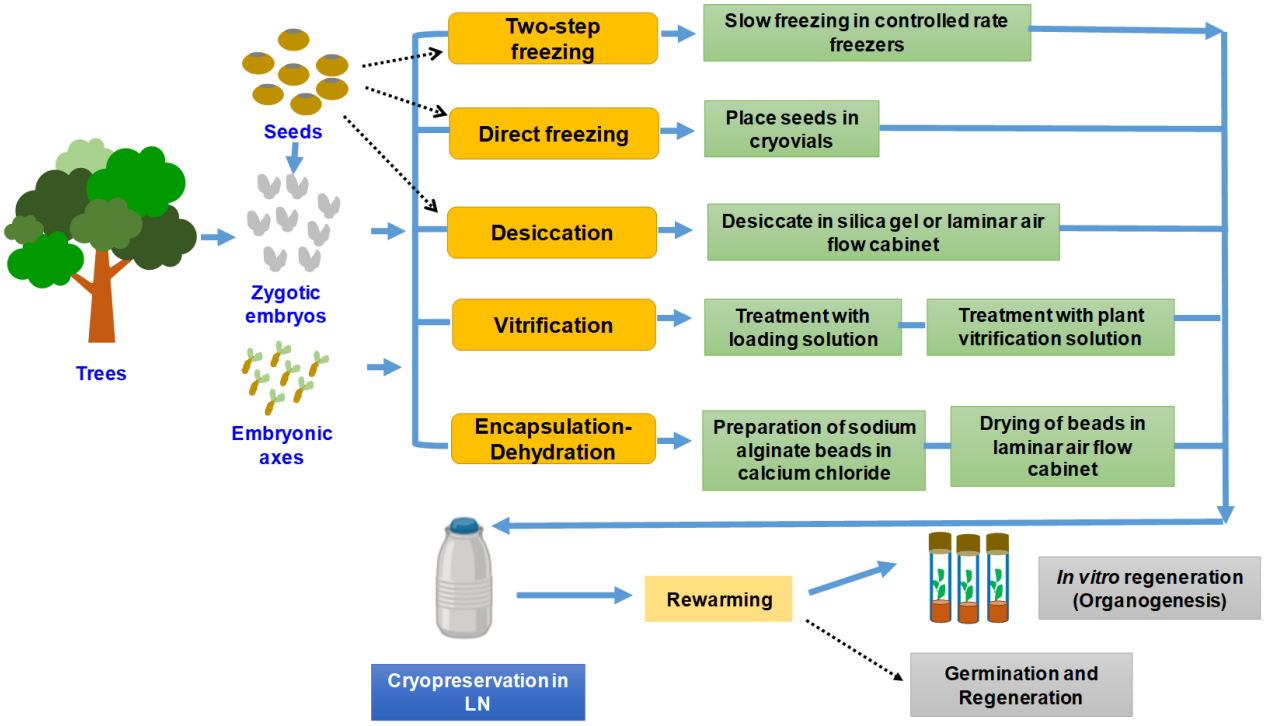

Figure 1. Schematic representation of the integrated cryopreservation workflow for trees using seeds, zygotic embryos or embryonic axes.

of plumular explants were reported in *Quercus robur* (Chmielarz *et al*, 2011; Plitta *et al*, 2014) and *Quercus petraea* (Wasileńczyk *et al*, 2024), using encapsulation-dehydration and droplet-vitrification techniques. Detailed ultrastructural analysis by Alla-N'Nan *et al* (2014) provided insights into the cellular changes occurring during encapsulation-dehydration cryopreservation, enhancing our understanding of tissue integrity under cryopreservation conditions.

Cryopreservation of shoot tips

Shoot tips that include the meristematic dome and several leaf primordia are ideal for long-term conservation, as they contain a uniform population of small, actively dividing cells with high nucleo-cytoplasmic ratios and minimal vacuolation, which improve desiccation tolerance (Varghese *et al*, 2009). It possesses the remarkable ability to regenerate entire plants. Additional distinct advantages include their genetic stability and optimal size for processing. The small, compact nature of shoot tips, coupled with their uniform developmental stage, allows for more precise handling and treatment with cryoprotectants, which are essential for protecting cells from damage during the freezing process. Furthermore, shoot tips typically have lower water content than other plant tissues, reducing the risk of harmful ice crystal formation within cells, which can otherwise lead to cell rupture and decreased survival rates (Agrawal *et al*, 2024; Gowthami *et al*, 2024b).

Organogenesis is important both before and after cryopreservation of shoot tips, and this process is critical as it determines the ability of cryopreserved shoot tips to regenerate into healthy shoots and complete plants, confirming their viability and growth potential after being stored at ultra-low temperatures. Effective organogenesis ensures that the genetic integrity of the plant material is maintained, with the regenerated shoots reflecting the original genetic makeup and not suffering adverse effects from freezing and thawing. This capability directly influences recovery efficiency, allowing a higher percentage of cryopreserved material to develop into viable plants, which is crucial for large-scale propagation. Additionally, organogenesis facilitates the adaptation of regenerated shoots to standard growth conditions post-thaw, ensuring robust development and minimizing stress. Before cryopreservation, optimizing the conditions for multiplication of shoot tip donor plants – including selection of optimum medium with specific plant growth regulators and incubation temperature, pretreatment or preconditioning of shoot tip donor plants, shoot tip size and origin, preculture of shoot tips, cryotechnique, type and concentration of plant vitrification solutions – are essential for organogenesis. Following cryopreservation, where shoot tips are stored at ultra-low temperatures, the post-thaw recovery process becomes essential. The primary challenge is to restore the viability and regenerative potential of the cryopreserved shoot tips. Thawing must be handled with care to prevent thermal shock, and rehydration protocols are implemented to help the tissues recover from the dehydration experienced during freezing. The recovery medium used post-thaw often differs from pre-cryopreservation media, with adjustments made to support the recovery of the tissues and induce organogenesis. The presence of suitable plant growth regulators (PGRs), combined with optimal temperature and light conditions, continues to play a crucial role in this phase. The organogenesis process post-cryopreservation is influenced by the pre-cryopreservation factors and the effectiveness of the thawing and rehydration procedures, and the composition of the recovery medium. Representative examples of cryopreservation of shoot tips of trees are provided in Table 2 and Figure 2.

Table 1. Comparative analysis of different cryotechniques and recovery media for the *ex situ* conservation of seeds, zygotic embryos (ZE) and embryonic axes (EA) in trees. Basal media: MS, Murashige and Skoog (1962); WPM, woody plant medium (Lloyd and McCown, 1981); Phytoregulators & additives: BA/BAP, 6-benzyladenine/6-Benzylaminopurine; KIN, Kinetin; IAA, indole-3-acetic acid; GA3, gibberellic acid; TDZ, thidiazuron; IAPhe, indole-acetyl-phenylalanine; AC, activated charcoal. Cryo solutions: PVS2, plant vitrification solution 2; LN, liquid nitrogen. Symbols: (-) indicates data not specified in the original source or control treatment.

Species	Explant	Cryopreservation technique	Post-cryo germination medium	Germination (%)	Reference
Astronium urundeuva	Seeds	Desiccation	Blotting paper assay in transparent acrylic boxes	69	Paula *et al*, 2022
Betula lenta	Seeds	Desiccation	DKW + 10μmol/L TDZ or BAP	12	Rathwell *et al*, 2016
Cedrela odorata	Seeds	Encapsulation/desiccation	Pots assay	75	Velasco-García *et al*, 2022
Handroanthus impetiginosus	Seeds	Desiccation	Filter paper assay	77.5	Campos *et al*, 2024
Jatropha curcas	Seeds	Desiccation	MS medium	100	Prada *et al*, 2015
Parkia nitida	Seeds	Desiccation	1/4 MS + 20g/L sucrose	94	Maruyama *et al*, 2024
Swietenia mahagoni	Seeds	Desiccation	Pots assay	68	Entensa *et al*, 2022
Aquilaria malaccensis	ZE	Desiccation	MS + 4μM Kinetin	23	Devi *et al*, 2019
Castanospermum australe	ZE	Flash drying	MS	40	Ballesteros *et al*, 2014
Jatropha curcas	ZE	Desiccation	MS medium	100	Prada *et al*, 2015
Pinus elliottii var. *elliottii* x *Pinus caribaea* var. *hondurensis*	ZE	Desiccation	MS + 0.5μM BA + 0.5μM TDZ	86	Ayala *et al*, 2023
Pinus radiata	ZE	Direct freezing	½ modified Quoirin and Lepoivre medium + 5mg/L BA	31.2–100	Hargreaves *et al*, 2004
Strychnos gerrardii	ZE	Flash drying	Full MS	75	Ballesteros *et al*, 2014
Syzygium maire	ZE	Metal-mesh vacuum infiltration vitrification	WPM + 2mg/L zeatin + 1mg/L copper sulphate	19	van der Walt *et al*, 2023
Acer platanoides	EA	Desiccation	WPM	65	Pukacki and Juszczyk, 2015
Acer pseudoplatanus	EA	Desiccation	WPM	70	Pukacki and Juszczyk, 2015
Acer saccharinum	EA	Two-step freezing	WPM	55	Beardmore and Whittle, 2005
Aesculus hippocastanum	EA	Direct freezing	MS+ 2mg/L BAP + 2mg/L IAPhe	100	Ballesteros and Pence, 2019
Aesculus glabra	EA	Direct freezing	MS+ 2mg/L BAP + 2mg/L IAPhe	33	Ballesteros and Pence, 2019
Araucaria angustifolia	EA	Encapsulation-Dehydration followed by flash cooling or two-step freezing	MS+ 3% AC + 0.929μM KIN + 11.40μM IAA	0	Frizzo and Quoirin, 2018
Castanea sativa	EA	Desiccation	MS (half-strength nitrates) + 0.5mg/L BA + 1ml/L Plant Preservative Mixture	93–100	Corredoira *et al*, 2004
Juglans nigra	EA	Direct freezing	MS+ 2mg/L BAP + 2mg/L IAPhe	75–100	Ballesteros and Pence, 2019
Nothapodytes nimmoniana	EA	Desiccation	MS medium	60	Radha *et al*, 2010
Parkia speciosa	EA	Vitrification (PVS2 90 min)	MS +100mg/L myo-inositol	55.5	Sinniah and Gantait, 2013
Syzygium maire	EA	Metal-mesh vacuum infiltration vitrification (PVS2 20 min.)	WPM macronutrients + MS micronutrients + 2mg/L Zeatin + 1mg/L copper sulphate	19	van der Walt *et al*, 2023

Table 2. Cryopreservation protocols for shoot tips of tree species: Preconditioning treatments, cryotechniques, and recovery efficiency. BA, N6-benzyladenine; PVS2, plant vitrification solution 2; GA3, gibberellic acid; IAA, indole-3-acetic acid; NAA, α-naphthaleneacetic acid; WPM, woody plant media; GD medium, Gresshoff and Doy medium (Gresshoff and Doy, 1972); US, unloading solution (basal media + 1.2 M sucrose); BAP, 6-benzylaminopurine; mVSL, modified vitrification solution L (20% glycerol + 5% sucrose + 25% ethylene glycol + 10% dimethyl sulfoxide (DMSO)); DKW, Driver and Kuniyuki basal media (Driver and Kuniyuki, 1984); VS A3, 37.5% glycerol, 22.5% sucrose, 15% ethylene glycol (EG), and 15% DMSO, w/v; KIN, kinetin; V, vitrification; DV, droplet-vitrification; ED, encapsualtion-dehydration; VCP, V cryoplate; DCP, D cryoplate.

Species	Cryo technique	Pregrowth media	Preculture media	Osmoprotection	Dehydration	Thawing	Regrowth media	% Regrowth/ survival	Reference
Alnus glutinosa	V	WPM + 0.5mg/L BA + 0.5mg/L IAA + 0.2mg/L zeatin for 6 week	WPM + 0.2M sucrose for 2 days at 4°C	2M glycerol + 0.4M sucrose for 20 min at 25°C	50% PVS2 for 30 min at 0°C, and then placed in 100% PVS2, for 30 min at 0°C	Rapid thawing in 0°C for 2 min and washed with US twice for 10 min at 25° C	WPM + 0.5mg/L BA + 0.5mg/L IAA + 0.2mg/L Zeatin +20g/L glucose + 0.6% agar	> 50	José *et al*, 2014
Castanea sativa	V	GD + 0.2μM BA	GD + 0.2M sucrose for 2 days at 4°C	LS for 20 min	PVS2 for 60–120 min at 0°C	Rapid thawing in 40°C for 2 min and then washed for 20 with US	GD + 2.2μM BA + 2.9μM IAA+ 0.9μM Zeatin + 0.09M sucrose+ 0.7% agar	63	Vidal *et al*, 2010
Crateva nurvala	V	MS + 0.1mg/L BAP + 3% sucrose for 4 weeks	MS + 0.4M sucrose for 16 h	LS for 20 min at 25°C	PVS2 for 40 min	40°C for 2 min and washed with US for 20 min	MS + 0.1mg/L BAP + 3% sucrose + 0.8% agar	56.6	Sanayaima *et al*, 2006
Garcinia mangostana	V	Not reported	MS + 0.6M sucrose for 2 days	Not reported	PVS2 for 20 min at 0°C	Not reported	Not reported	50	Ibrahim and Normah, 2013
Hancornia speciosa	V	WPM + 2.0mg/L BAP + 0.09M sucrose + 0.7% agar	WPM + 0.3M sucrose for 1 days	LS for 20 min	PVS2 for 60 min at 0°C	39 ± 2°C for 2 min and washed with US for 15 min	WPM + 2.0mg/L BAP +20ppm ascorbic acid + 0.09M sucrose + 0.7% agar	> 70	Santos *et al*, 2015
Nephelium ramboutan-ake	V	MS + 1mg/L BAP	MS + 0.5 M sucrose for 2 days	LS + 0.28mM vitamin C for 20 min at 25°C	PVS2 for 15 min at 0°C	Rapid thawing at 38 ± 2°C for 40–45s followed by rinsed with US for 5 min at 25°C	MS + 1mg/L BAP + 0.28mM vitamin C	3.33	Chua and Normah, 2011
Pinus kesiya	V	MS + 15g/L sucrose + 0.8% agar	½MS + 0.1M sucrose for 11 days followed by 0.4 and 0.7M sucrose treatment for 1 day each	Not reported	mVSL for 10 min at 25 ± 1°C	40°C for 1–2 min and washed with US for 20	MS + 4.0mg/L BA + 3% sucrose	76	Kalita *et al*, 2012
Trichilia emetica	V	MS medium for 2 to 3 months	MS + 0.7M sucrose for 4 days	LS for 15 min	50% PVS2 solution for 5 min followed by 100% PVS2 for 15 min	40°C for 1–2 min and washed with US for 20 min at 25°C	WPM + 0.05mg/L BAP + 0.1mg/L GA3	55	Varghese *et al*, 2009
Ulmus americana	V	Not reported	Preculture medium with 0.1–0.5μM melatonin for 24 hr	Not reported	PVS2 for 10 min	Not reported	Recovery medium supplemented with 0.1–0.5μm melatonin	100	Uchendu *et al*, 2013
Betula lenta	DV	DKW + 5μM BA + 1.0μM GA3 for 2 months	DKW + 0.3M sucrose for 24 h	LS for 20 min at room temp.	VS A3 for 60 min. at 0°C	Rapid thawing in US reduced to 0.8M sucrose	DKW + 5μM BA + 1.0μM GA3	52	Rathwell *et al*, 2016

Table 2 continued

Eucalyptus spp.	DV	MS + 0.04 mg/L BA + 1% sucrose + 0.45% agar for 4–6 week	MS + 0.25M sucrose	Not reported	PVS2 for 30 min	Rapid thawing in washing solution (MS + 1.0M sucrose)	MS + 0.04mg/L BA + 1% sucrose + 0.45% agar	38–85	Kaya *et al*, 2013
Hancornia speciosa	DV	WPM + 2.0mg/L BAP + 0.09M sucrose + 0.7% agar	WPM + 0.3M sucrose for 1 day	LS for 20 min at room temperature	PVS2 for 30 min at 0°C	Rapid thawing in US for 15 min	WPM + 2.0mg/L BAP +20ppm ascorbic acid + 0.09M sucrose + 0.7% agar	>70	Santos *et al*, 2015
Malus domestica	DV	MS + 1.35μM BA + 0.25μM IBA	Not reported	LS for 20 min	PVS2 for 60 min	Rapid thawing in US for 15 min	MS +1.35μM BA + 0.25μM IBA	70	Condello *et al*, 2011
Buxus hyrcana	ED	MS + 0.50mg/L BAP + 1.50mg/L NAA	Shoot tip not precultured	Not reported	Air dehydration in laminar airflow for 60 min	40°C for 2 min	MS + 0.50mg/L BAP + 1.50mg/L NAA	60	Kaviani and Negahdar, 2017
Malus domestica	ED	MS + 0.5mg/L BA + 0.25mg/L IBA	MS + 0.75M sucrose for 24 h	Not reported	Silica gel dehydration for 14 hrs	Beads direct transfer to recovery media	MS + 1.35μM BA + 0.25μM IBA	72	Forni *et al*, 2010
Malus spp.	ED	MS + 0.25mg/L BA + 0.01mg/L IBA	MS + 0.75M sucrose for 5 days	Not reported	Air dehydration in laminar airflow for 6 hrs	Rapid thawing in 38°C for 2 min	MS + 0.25mg/L BA + 0.01mg/L IBA	57	Li *et al*, 2014
Malus domestica var. Gala	ED	MS + 0.25mg/L BA + 0.01mg/L IBA	MS + 0.5M sucrose for 7 days	Not reported	Air dehydration in laminar airflow for 6 hrs	Rapid thawing in 38°C for 2 min	MS + 0.25mg/L BA + 0.01mg/L IBA	75	Feng *et al*, 2013
Prunus armeniaca	ED	WPM + 4mg/L BA + 0.5mg/L 2iP	WPM + 0.75M sucrose for 2 days	Not reported	Air dehydration in laminar airflow for 2 hrs	Rapid thawing at 38°C for 2-3 min	WPM + 1mg/L zeatin + 0.1mg/L IAA	71	Soliman, 2013
Hovenia dulcis	VCP	MS + 0.09M sucrose + 0.8% agar	MS + 0.3M sucrose for 24 h at 26 ± 2°C in darkness	LS for 20 min	PVS2 for 120 min	Rapid thawing in US for 15 min	MS + 0.5mg/l BA + 0.5mg/l KIN + 0.09M sucrose + 0.8% agar	62	Saavedra *et al*, 2021
Prunus cerasifera	VCP	MS + 1mg/L BA + 0.1mg/L IBA + 0.1mg/L GA3	MS + 0.3M sucrose for day at 23ºC in dark	LS for 30 min at room temperature	PVS containing 37.5% (w/v) glycerol, 15% (w/v) DMS, 15% (w/v) EG and 22.5% (w/v) sucrose 30 min at room temperature	Rapid thawing in MS + 0.8M sucrose for 30 min at room temperature	MS + 1mg/L BA + 0. mg/L IBA + 0.1mg/L GA3	44–56	Vujović *et al*, 2015
Diospyros kaki	DCP	Not reported	1/2MS + 0.3M sucrose	LS for 30 min at 25°C	Air dehydration in laminar airflow for 30 min	Rapid thawing in 1/2MS + 1M sucrose solution for 15 min at 25°C	1/2MS + 1mg/L BA	84	Matsumoto *et al*, 2015
Prunus cerasifera	DCP	MS + 1mg/L BA + 0.1mg/L IBA + 0.1mg/L GA3	MS + 0.3M sucrose for day at 23°C in dark	LS 30 min at room temperature	Air dehydration in laminar airflow for 2 hrs	Rapid thawing in MS + 0.8M sucrose for 30 min at RT	MS + 1mg/L BA + 0.1mg/L IBA + 0.1mg/L GA_3	57–77	Vujović *et al*, 2015

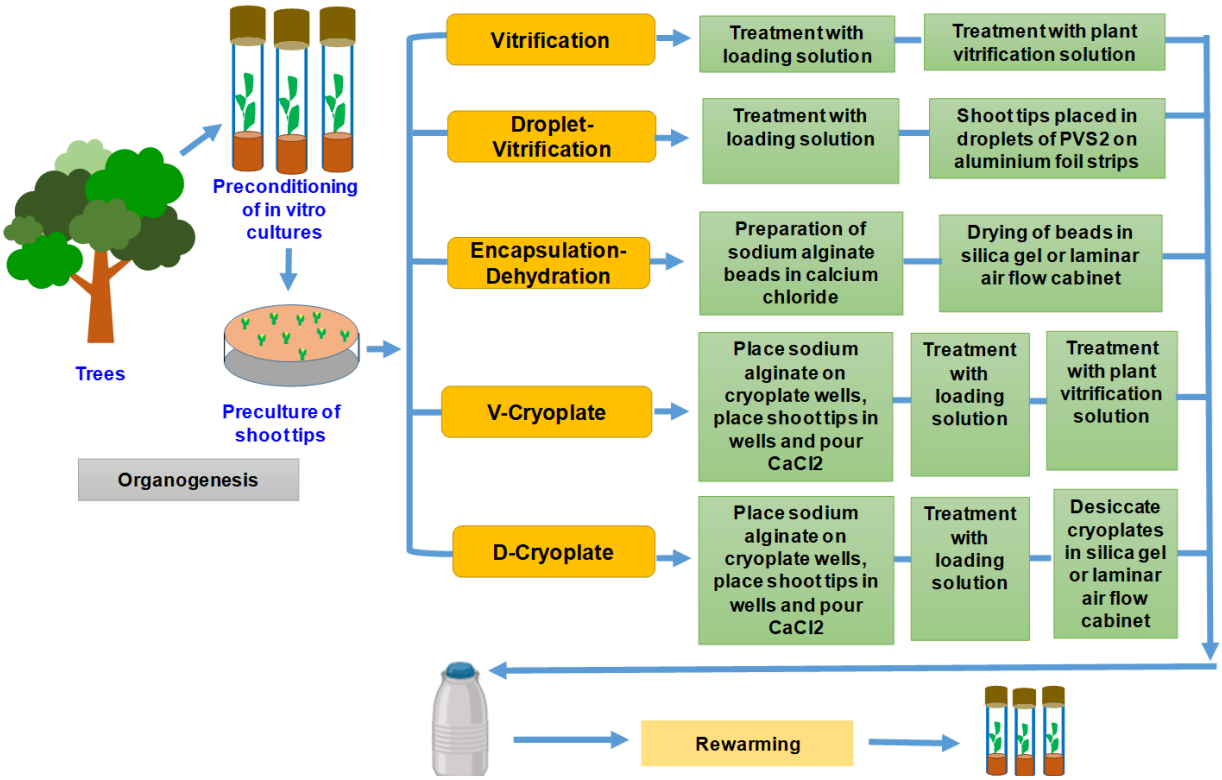

Figure 2. Comprehensive schematic representation of cryopreservation strategies for shoot tips of tree species, illustrating the sequential major steps from pre-conditioning to post-thaw recovery.

Below are some critical factors to consider during the cryopreservation of tree species using shoot tips:

Pretreatment of donor plants

Pretreating donor plants with cold acclimation or exposure to high sugar levels significantly enhances their physiological resilience to the stresses encountered during cryopreservation. Cold acclimation involves gradually lowering the temperature to which plants are exposed, a process that activates stress-responsive genes and strengthens cellular membranes. This pretreatment improves the plant's capacity to endure the harsh conditions of freezing and dehydration by stabilizing cell membranes and enhancing the management of intracellular water content. Cold acclimation has been widely used for temperate crops that naturally possess the capacity to harden to low temperatures (Popova and Kim, 2026). Cold acclimation of shoot cultures in a standard laboratory refrigerator (4°C) for 7 or 14 days resulted in significantly higher regrowth in *Ulmus americana* (Uchendu *et al*, 2013). Another approach to obtain cold hardiness is exposure to high levels of sucrose before cryopreservation, which induces osmotic adjustment, reduces intracellular water content and minimizes the risk of ice crystal formation within cells. Sucrose promotes the production of various substances, including proline, glycine betaine, glycerol, and polyamines, which exert both colligative and non-colligative effects (Hirsh, 1987; Antony *et al*, 2013), strengthen cellular structures and improve membrane stability. The inclusion of 0.1μM melatonin at two key stages in the cryopreservation process proved effective for shoot tips of *U. americana* (Uchendu *et al*, 2013). Melatonin's role at these critical points likely improves the stress tolerance and recovery of the explants by influencing various physiological and biochemical pathways, thus promoting better outcomes in cryopreservation procedures.

Size of explants

The size of excised shoot tips significantly impacts the effectiveness of cryoprotection methods. Typically, shoot tips between 1 and 3mm are used, depending on the species (Bettoni *et al*, 2021). Smaller explants facilitate better uptake of cryoprotectants and efficient water removal, enhancing cryopreservation success. However, very small shoot tips are prone to mechanical damage and loss during handling. Conversely, larger shoot tips can suffer from uneven dehydration and ice crystal formation, leading to cellular damage and poor regrowth. Thus, selecting the appropriate size of shoot tips is crucial for optimizing cryoprotection and recovery. Depending on the species, different sizes of shoot tips are used, such as 0.5–0.8mm shoot tips in *Trichilia emetica* (Varghese *et al*, 2009) and 2.0–4.0mm shoot tips in *U. americana* (Uchendu *et al*, 2013). The survival, shoot regrowth and shoot length of smaller shoot tips (0.5–1.0mm) were significantly better than those of larger tips (2.0mm) in *Castanea sativa* (Vidal *et al*, 2005).

Genotype

The genotype of plant material plays a crucial role for the success of cryopreservation techniques and the efficiency of post-thaw recovery. Variations in genetic backgrounds among cultivars or genetic lines within a species can lead to differing responses to cryopreservation protocols, reflecting diverse characteristics such as age, physiological condition, explant size, and protocol specifics. Genotypic differences in shoot

recovery varied from 37.5% to 54.4% in *C. sativa* (Vidal *et al*, 2005). Later, Vidal *et al* (2010) introduced a vitrification-based cryopreservation technique for 46 genotypes of *in vitro*-cultivated European chestnut. The efficiency of shoot recovery varied greatly, from 0% to 53%, underscoring the notable genotype-specific differences observed in *C. sativa*, emphasizing the need for genotype-specific optimization of cryopreservation protocols.

Preculture

Preculture treatment with sucrose is crucial for optimizing cryopreservation outcomes, significantly enhancing recovery rates of cryopreserved explants. Gradually increasing sucrose concentrations during pre-culture improves the survival and regrowth of shoot tips by optimizing cellular conditions and enhancing cryoprotective effects (Feng *et al*, 2013). Low sucrose concentrations act as a metabolic substrate supporting biochemical changes necessary for cryopreservation, while higher concentrations provide cryoprotection by stabilizing membranes and reducing freezing and thawing damage. High sucrose levels, similar to abscisic acid (ABA), improve cellular tolerance to desiccation through osmotic regulation and membrane stabilization. For instance, in *C. sativa* shoot tips pre-cultured in 0.1 to 0.4M sucrose showed high recovery, whereas 0.7M sucrose reduced recovery to 7.4% (Jorquera *et al*, 2004). Moreover, *T. emetica* shoot tips benefit from sucrose or sucrose-glycerol mixtures through osmotic dehydration and increased protective compounds (Varghese *et al*, 2009).

Plant vitrification solutions

PVS are essential for achieving a glass-like state that prevents ice formation and protects cellular integrity during cryopreservation. Among the most commonly used formulations are PVS2 and PVS3, which have proven effective in a variety of applications. The success of these solutions depends heavily on optimizing exposure duration and temperature to ensure adequate dehydration while avoiding cellular damage. The stepwise PVS2 exposure method was explored because pretreating with a lower PVS2 concentration can mitigate toxicity before using the full concentration. In *Garcinia mangostana*, shoot tips dehydrated with 50% PVS2 for 15 minutes and then exposed to full-concentration PVS2 for 10 minutes had higher survival rates (39.2 ± 10.1%) than other treatments (Ibrahim and Normah, 2013). Also, PVS2 dehydration at a lower temperature (0°C) was shown to be more favourable compared with a higher PVS2 temperature (25°C) (Ibrahim and Normah, 2013). This result may be due to restricted water molecule mobility at low temperatures, which helps prevent ice crystal formation. Conversely, at 25°C, PVS2 penetrates cell membranes more quickly, potentially causing toxic effects or excessive dehydration of the shoot tips (Gowthami *et al*, 2023).

Regrowth medium

Cryopreservation often results in significant cellular damage, necessitating an optimal recovery environment to swiftly address reversible damage, restore normal cell function, and promote tissue proliferation. This process is energy-intensive and requires several key conditions: minimizing stress to avoid additional damage, removing toxins such as CPA residues and byproducts from damaged cells, providing nutrients due to potential disruption in cell connections, supporting repair mechanisms for cellular structures and managing ROS accumulation, supplying appropriate carbohydrate sources for energy, and promoting morphogenic responses like regrowth, embryo development, or new root formation from propagules (Popova *et al*, 2023). Post-culture conditions after cryostorage are crucial for the successful recovery of cryopreserved samples. For instance, in *C. sativa*, a recovery medium supplemented with 0.5mg/L BA and 0.5mg/L IAA was used as regrowth medium (Vidal *et al*, 2010; Jorquera *et al*, 2004). Rathwell *et al* (2016) found that GA3 at 0.35mg/L was superior for plant regeneration from both cryoprotected and cryopreserved *Betula lenta* shoot tips compared to other treatments. *U. americana* shoot tips recovered well with GA3 and BA (Uchendu *et al*, 2013), while BA alone was effective for other *Ulmus* species (Harvengt *et al*, 2004).

Embryogenic explants

Cryopreservation techniques enable the preservation of somatic embryogenic explants, typically in clumps or clusters in tree species. Somatic embryos, whether appearing as clumps or clusters, are stages of development ranging from loosely organized groups of cells to fully developed embryos. These embryos can vary in their degree of differentiation, with some still resembling the initial undifferentiated cells while others have matured into more recognizable embryonic forms. Factors such as age, physiological condition, and size of these explants are critical considerations. Using young embryogenic cultures for isolating initial explants is crucial for successfully recovering materials after storage in LN. Additionally, selecting the appropriate developmental stage of somatic embryos is essential; typically, undifferentiated or early developmental stages are preferred (Engelmann, 2011). These considerations are pivotal in optimizing the cryopreservation process to ensure the viability and genetic integrity of stored plant materials. Cryopreservation of plant material derived from somatic embryogenesis using LN is widely utilized to preserve embryogenic potential and minimize the risk of genetic drift. This technique effectively halts all biological activities, thereby maintaining the viability and regenerative capacity of the tissues over extended periods (Hazubska-Przybył *et al*, 2013). Nevertheless, some studies have indicated that cryopreservation might cause genetic alterations in these types of cultures (Krajňáková *et al*, 2011). Different techniques used for cryopreservation of somatic embryos of tree species are two-step freezing, desiccation, vitrification, droplet-vitrification, and encapsulation-dehydration methods (Figure 3, Table 3).

Here are some critical factors to consider during the cryopreservation of tree species using embryogenic explants:

Selection of explant

The choice of explant is crucial for successful cryopreservation. A range of embryogenic explants, including embryogenic callus, embryogenic masses, embryogenic tissues, embryogenic cell suspensions, proembryogenic masses, nodular embryogenic structures, polyembryoids, and clusters of somatic embryos, are used for cryopreservation (Ballesteros *et al*, 2024). The success is notably affected

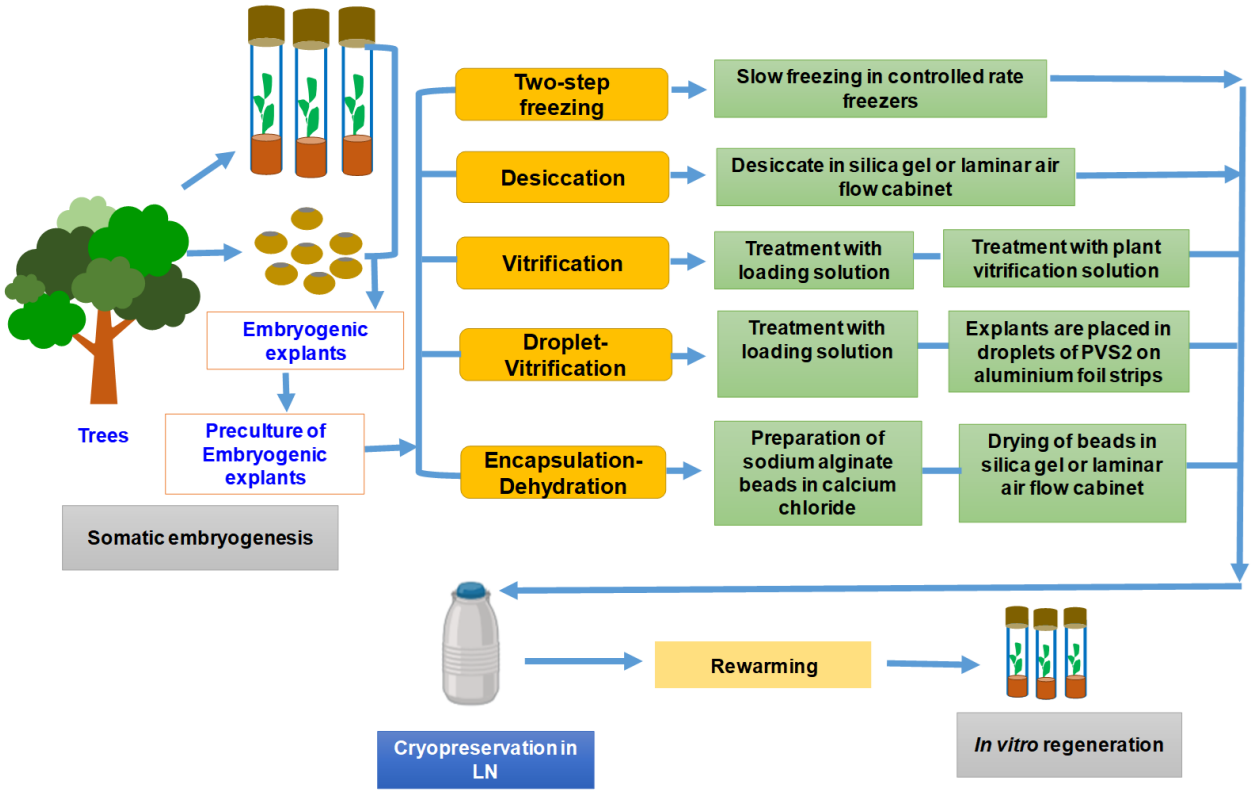

Figure 3. Conceptual diagram of a generalized cryopreservation protocol for embryogenic explants of woody species, illustrating key stages from induction of somatic embryogenesis to post-thaw recovery.

by their developmental stage, with earlier stages generally performing better. In *Quercus* spp., for instance, nodular embryogenic structures demonstrated greater resilience to cryostorage in LN compared to more differentiated cotyledonary embryos (Martínez *et al*, 2022). Similarly, in *Quercus suber*, the recovery rates varied significantly using globular, early torpedo, and cotyledonary embryos from two different embryogenic lines for cryopreservation (Valladares *et al*, 2004). Globular embryos achieved the highest recovery percentage with one embryogenic line, but the lowest recovery with the other line.

Preculture and cryoprotection

Preculture of explants on media containing sugars or sugar alcohols is typically used to subject the tissues to mild osmotic stress, which triggers adaptive responses that enhance desiccation tolerance, such as increased levels of ABA and proteins associated with late embryogenesis (Charoensub *et al*, 1999). In *Q. suber* (Valladares *et al*, 2004) and *Q. ilex* (Barra-Jiménez *et al*, 2015), preculturing on 0.3M sucrose medium was found suitable.

Embryogenic masses of *Picea mariana* achieved the highest survival rates of 50 to 67% by preculturing on semi-solid medium with 0.8M sorbitol for 48 hours, followed by a 30-minute incubation at 0°C in modified cryoprotective agent PVS2 after cryopreservation (Touchell *et al*, 2002). In *Picea abies*, the addition of ABA to sucrose-containing preculture medium improved tolerance to LN storage, resulting in better regrowth and a higher yield of mature somatic embryos in comparison to preculture with sucrose alone (Hazubska-Przybył *et al*, 2013). This combination significantly enhanced both desiccation and LN storage tolerance, leading to more somatic embryos reaching the cotyledonary stage. Combining ABA with sugars in preculture media induces beneficial cellular changes, such as reduced cell size and increased cytosolic density, enhancing cryotolerance.

The reaction to dehydration caused by vitrification solutions can differ significantly across species and even among cultivars of the same species. Assessing each species' or cultivar's sensitivity to this dehydration is a crucial initial step in developing an optimized vitrification protocol. Exposure to PVS2 for up to 120 minutes resulted in no significant decrease in embryogenic levels in *Q. suber*, which typically remained above 95% (Valladares *et al*, 2004). In *C. sativa* cultures, embryogenesis efficiency improved from 33 to 68% post-cryopreservation with a 3-day preculture on high-sucrose medium followed by 60 minutes of PVS2 treatment before storage (Corredoira *et al*, 2004). *Q. ilex* embryogenic lines had 80% growth resumption after cryopreservation following a 0.3M sucrose pretreatment and 30 minutes of PVS2 treatment (Barra-Jiménez *et al*, 2015).

Dormant buds

Dormant buds from field-grown trees are highly valued for conserving genetic resources in vegetatively propagated plants due to their superior genetic stability compared to other explants like individual cells or embryogenic tissues (Zhang *et al*, 2025). Cryopreservation of dormant buds takes advantage of their inherent cold-hardiness, enabling them to endure ultra-low temperatures during storage without the need for cryoprotectants, provided they receive proper pre-treatment (Stushnoff, 1991). Their quiescent state during

Table 3. Cryopreservation protocols for somatic embryogenic explants of tree species. DMSO, dimethyl sulfoxide; PEG, polyethylene glycol; PVS, plant vitrification solution; WPM, woody plant media; RT, room temperature; WC, water content; US, unloading solution; MS, murashige and skoog medium.

Species	Explant type	Cryotechnique	Preconditioning/ Preculture treatment	Dehydration/Cryoprotection	Thawing	% Regrowth/ survival/recovery	Reference
Castanea sativa	6-8mg clumps of globular or heart-shaped secondary embryos	Desiccation	Proliferation medium with 0.3M sucrose for 3 days followed by 0.7M sucrose for 4 days (devoid of glutamine and plant growth regulators)	Desiccation in the air flow of a laminar flow cabinet to water contents of 25%	40°C water bath for 2 min	33%	Corredoira *et al*, 2004
Quercus robur	4–6mg clumps (1.0–1.5mm) of globular or heart-shaped secondary embryos	Desiccation	Proliferation medium with 0.3M sucrose for 3 days followed by 0.7M sucrose for 4 days	Desiccation in the airflow of a laminar flow cabinet to water contents of 24–34%.	40°C water bath for 2 min	56%	Martínez *et al*, 2003
Abies alba	Embryogenic tissue	Two-step freezing	Proliferation medium with 0.5M sorbital for 24 h	DMSO 5% Mr Frosty® with a cooling rate of 1°C/min to -40°C	40°C water bath for 3 min	91.66–100%	Salaj *et al*, 2022
Abies pinsapo	Embryogenic tissue	Slow cooling	Proliferation medium with 0.2M sucrose for 24 h, and with 0.4M sucrose for an additional 24 h	PGD I (10% PEG6000 +10% glucose + 10% DMSO) Mr. Frosty: 1°C/min in an -80°C ultra-freezer until they reached -40°C.	40°C water bath	100%	Cabero-Moreno *et al*, 2025
Larix olgensis	Embryogenic callus	Slow freezing	24 h stepwise preculture on medium containing 0.2 and 0.4mol/L sucrose	0.4mol/L sucrose + 2.5% DMSO + 10% PEG6000 cooling rate of -1°C/min until reaching -80 °C	37°C water bath	100%	Wang *et al*, 2025
Picea abies	Embryogenic tissue	Two-step freezing	Semi-solid medium with increasing sucrose concentration (0.1M for 24 h; 0.2M for another 24 h)	PGD mixture (composed of PEG6000, glucose, and DMSO 10% w/v each) Programmable freezer with a slow cooling rate (0.17°C/min).	37°C water bath for 2 min	87%	Varis *et al*, 2022
Pinus massoniana	Embryogenic callus	Two-step freezing	Proliferation medium with 0.5mol/L sucrose for 48 h	5% DMSO + 80% 0.5mol/L sucrose+15% PEG4000 Programmed cooling: equilibrate at 0°C for 10 min, cool at 1°C/min to -80°C, then hold at -80°C for 30 min.	32°C water bath	20%	Yang *et al*, 2025
Picea pungens	Embryogenic tissue	Two-step freezing	0.4mol/L sorbitol (24 h)	DMSO 5% Nalgene® cooling box of 4 °C gradually reduced to -80 °C, kept for 2 h	37°C water bath for 1 min	High maturation efficiencies (1030E/gFM)	Cao *et al*, 2022
Taxodium hybrid	Embryogenic callus	Slow cooling	0.5M sucrose for 36 h	10% DMSO Programmed cooling at a rate of 1 °C per minute until reaching -80 °C	35°C water bath for 2 min	Proliferation rate of 6.39	Chen *et al*, 2024

Table 3 continued

Castanea sativa	6–8mg clumps of globular or heart-shaped secondary embryos	Vitrification	Proliferation medium with 0.3M sucrose for 3 days (devoid of glutamine and plant growth regulators)	PVS2 at 0 °C for 60 min	40°C water bath for 2 min	68%	Corredoira *et al*, 2004
Euterpe edulis	Somatic embryos	Vitrification	Osmotic conditioning: 30g/L (30 days) → 60g/L (15 days) → 90g/L (15 days),	Modified PVS3 + DMSO 15%	40°C water bath for 2 min	69.50%	Fagundes *et al*, 2026
Fraxinus mandshurica	Embryogenic callus	Vitrification	WPM + 0.5mol/L sucrose for 3 d, followed by loading solution for 60 min	PVS2 at RT for 50 min	40°C water bath for 2 min	> 60%	Liu *et al*, 2023
Kalopanax septemlobus	Embryogenic callus	Vitrification	Precultured in liquid MS with progressively increasing sucrose 0.3 → 0.5 → 0.7M sucrose for 17 → 3 → 3 h	Loading solution for 20 min; Vitrification solution: 33.3% glycerol + 13.3% DMSO + 13.3% ethylene glycol + 20.1% sucrose for 40 min at 0°C	Water bath at 37°C for 30 seconds	> 99%	Shin *et al*, 2012
Mangifera indica	Embryogenic masses	Vitrification	Solid medium containing 0.5M sorbitol for 24 h	PVS3 for 20 min	Water bath at 25°C for 2–3 min	94.3%	Wu *et al*, 2003
Picea mariana	Embryogenic masses	Vitrification	Semi-solid medium containing 0.8M sorbitol for 48 h	PVS2 at 0 °C for 30 min	40°C water bath	0–66.7%	Touchell *et al*, 2002
Quercus ilex	Clusters of globular secondary embryos	Vitrification	Gelled basal medium + 0.3M sucrose for 3 days	PVS2 at RT for 30 min	Water bath at 42°C for 2 min	80%	Barra-Jiménez *et al*, 2015
Quercus robur	4–6 mg clumps (1.0–1.5mm) of globular or heart-shaped secondary embryos	Vitrification	MS + 0.3M sucrose for 3 days	PVS2 at RT for 60 min	40°C water bath for 2 min	70–90%	Martínez *et al*, 2003
Quercus suber	2–4mg (1.5–2.5mm) clumps of globular secondary embryos	Vitrification	MS + 0.3M sucrose for 3 days	PVS2 at 0 °C for 60 min	40°C water bath for 2 min	88–93%	Valladares *et al*, 2004
Euterpe edulis	Somatic embryos	Droplet-vitrification	Osmotic conditioning: 30g/L (30 days) → 60g/L (15 days) → 90g/L (15 days)	Modified PVS3 + DMSO 15%	No thawing direct rewarmimg in US	67.50%	Fagundes *et al*, 2026
Kalopanax septemlobus	Embryogenic callus	Droplet-vitrification	Precultured in liquid MS with progressively increasing sucrose 0.3 → 0.5 → 0.7M sucrose for 17 → 3 → 3 h	Loading solution for 20 min; Vitrification solution: 33.3% glycerol + 13.3% DMSO + 13.3% ethylene glycol + 20.1% sucrose for 40 min at 0°C	No thawing direct rewarming in US	94.2%	Shin *et al*, 2012
Olea europaea	Somatic embryos	Droplet-vitrification	Olive cyclic embryogenesis medium with 0.2M sucrose for 28 days	Loading solution for 20 min PVS2 for 30 min at 0º C	No thawing direct rewarming in US	90%	Bradaï *et al*, 2023
Quercus suber	Somatic embryos	Encapsulation-Dehydration	Encapsulation beads precultured in 0.7M sucrose for 3 days	Desiccation of beads in the airflow of a laminar flow cabinet to 25–35% WC	Water bath at 38°C for 2 min	90%	Fernandes *et al*, 2008

winter reduces metabolic activity and stabilizes genetic material, making them ideal for cryopreservation. Research by Sakai (1960) and Forsline *et al* (1998) demonstrated that pre-cooled, dried twigs can survive storage in LN, enhancing survival rates. Cryopreservation of dormant buds is cost-effective and simpler than traditional methods involving *in vitro*-grown shoots (Tanner *et al*, 2021). This technique requires minimal pre-treatment, offers faster recovery, and is broadly applicable to various plant species. Viability of cryopreserved dormant buds is assessed through methods such as grafting, visual scoring, *in vitro* recovery, direct rooting, and chemical viability testing with 2, 3, 5-triphenyl tetrazolium chloride (TTC). Cryopreservation of dormant winter buds is a well-established technique for preserving tree species such as *Betula* sp. (Endoh *et al*, 2023), *Carya illinoinensis* (Morrissey and Gustafson, 1990), *Castanea ozarkensis* (Jenderek *et al*, 2024), *Diospyros* spp. (Zhang and Luo, 2004; Matsumoto *et al*, 2004; Ai and Luo, 2004; Benelli *et al*, 2008), *Salix* sp. (Towill and Widrlechner, 2004; Jenderek *et al*, 2013; Bonnart *et al*, 2014), *Ulmus* sp. (Harvengt *et al*, 2004; Välimäki *et al*, 2022), and *Populus trichocarpa* (Bonnart *et al*, 2014).

Conclusion and future prospects

The conservation of trees has become increasingly urgent due to escalating threats such as deforestation, habitat destruction and climate change. Cryopreservation stands out as a promising solution by allowing genetic material to be stored at ultra-low temperatures, effectively pausing biological processes. This method is particularly advantageous for trees with recalcitrant seeds or those that are difficult to propagate, thus supporting the preservation of genetic diversity. Nonetheless, the effective application of cryopreservation encounters several challenges, including the need for species-specific protocol development, substantial infrastructure costs, and specialized expertise.

To advance the field, ongoing research is vital for improving cryopreservation techniques and developing more cost-effective, scalable solutions. Future advancements could include the creation of more adaptable protocols for organogenesis, somatic embryogenesis and cryopreservation, which would be applicable to a broader range of species, including those with complex or poorly understood reproductive biology. Progress in cryobiology, the development of better cryoprotectants, and the use of automated systems may address some of the current limitations. Collaboration among researchers, conservationists and policymakers will be crucial to overcoming these challenges and broadening the implementation of cryopreservation.

By tackling these issues and leveraging technological innovations, we can more effectively preserve the genetic diversity of trees. This will ensure their long-term survival and sustain the vital ecosystem services and resources they provide, which are essential for both biodiversity and human well-being.

Author contributions

Gowthami R: Planned, designed the manuscript layout and prepared the draft; Subhash Chander: Compiled and edited the information, prepared tables. GM Puneeth: Prepared tables, formatted references. Anju Mahendru-Singh: Critically edited the manuscript and provided inputs; Marcos Edel Martínez-Montero: Critically edited the manuscript and provided inputs; PE Rajasekharan: Critically edited the manuscript and provided inputs

Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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