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Plant Cryopreservation: Principles, Applications, and Challenges of Banking Plant Diversity at Ultralow Temperatures

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Abstract

Progressive loss of plant diversity requires the protection of wild and agri-/horticultural species. For species whose seeds are extremely short-lived, or rarely or never produce seeds, or whose genetic makeup must be preserved, cryopreservation offers the only possibility for long-term conservation. At temperatures below freezing, most vegetative plant tissues suffer severe damage from ice crystal formation and require protection. In this review, we describe how increasing the concentration of cellular solutes by air drying or adding cryoprotectants, together with rapid cooling, results in a vitrified, highly viscous state in which cells can remain viable and be stored.

On this basis, a range of dormant bud–freezing, slow-cooling, and (droplet-)vitrification protocols have been developed, but few are used to cryobank important agricultural/horticultural/timber and threatened species. To improve cryopreservation efficiency, the effects of cryoprotectants and molecular processes need to be understood and the costs for cryobanking reduced. However, overall, the long-term costs of cryopreservation are low, while the benefits are huge.

Contents

INTRODUCTION

Cryobiotechnology: a multidisciplinary approach to support the cryopreservation of cells and organs, combining genetics, proteomics, biochemistry, and physiology

Cryobiotechnology can provide essential tools to prevent the extinction of plants on Earth and thus contribute to the maintenance of human well-being. Habitat loss due to land-use change, deforestation, and climate change; the hunting of herbivores and frugivores; and the use of modern varieties in farming are the main drivers of the globally recorded plant biodiversity loss (16). The need to protect our wild, as well as agronomic, biodiversity was recognized some 200 years ago, leading to the establishment of nature reserves and national parks (54). As a complementary approach, since the beginning of the twentieth century, plant genetic resources for food and agriculture have been collected and conserved in ex situ genebanks, while botanic gardens have focused on the conservation of wild species diversity. Since most plants on Earth produce desiccation-tolerant (orthodox) seeds, their long-term maintenance is realized under dry (15% seed water content) and low-temperature conditions (<0°C). For plants that produce no or

desiccation-sensitive (recalcitrant) seeds or that do not breed true, cryopreservation is the only alternative for long-term conservation of their species.

Cryopreservation exploits the effects of ultralow temperatures (commonly <-130°C), at which kinetic energy and molecular movements in biological systems are minimized or even arrested, thus attenuating transport, enzymatic reactions, and, hence, ageing rates. However, to preserve functionally intact cells, tissues, or complex organs, the concentration and location of water molecules required as plasticizers and for protein and membrane functions (6) must be manipulated (**Figure 1**). Otherwise, water molecules nucleate at temperatures between 0 and -40°C and form ice crystals that expand in volume (11), thus rupturing cell membranes. This results in the loss of their essential function, that is, semipermeability, leading to cell death. Ice formation is more critical intracellularly than in the extracellular space. However, the formation of lethal intracellular ice crystals can be prevented by (*a*) increasing the concentration of solutes, leading to more compounds interfering with water molecules, which hinders crystal formation and (*b*) ultrafast cooling, leading to a sudden stop in molecular motion. In this so-called glassy (vitrified) state, characterized by a highly viscous cytoplasm that balances the toxicity of the cellular solutes and inhibits intracellular ice formation, cell viability is maintained (99).

In other domains, cryobiotechnology has revolutionized reproductive medicine, cell therapy, and medical diagnostics by preserving and making available oocytes, spermatozoa, and embryos, as well as blood, stem cells, and other important human and animal tissues years after collection (97). Although some animals, for example, the wood frog and overwintering insects, can withstand freezing, most eukaryotic cells require additional cellular protection to survive ultralow temperatures. In 1949, the application of glycerol (106), and, a decade later, dimethyl sulfoxide (DMSO) (68), demonstrated the beneficial effects of penetrating cryoprotectants on the survival of sperm and red blood cells, leading to the establishment of one of the first human sperm banks in 1953 (97). Since then, numerous protectants, infiltration, cooling, and warming processes have been developed for clinical applications (97). However, the application of cryopreservation in plants has often been hampered by the fundamental differences between plant and animal/human cells. For example, the presence of stiff plant cell walls affects solute penetration, metabolic activity, and ice nucleation (126). Chloroplast activity may be responsible for excessive oxidative stress, especially during rewarming, when the photosynthesis apparatus and antioxidant systems have not yet fully recovered (127). Vacuoles are also exposed to extensive ice formation due to high water availability. In other cases, the natural adaptations of plants to dry and/or freezing environments have facilitated the development of cryopreservation protocols, as in dormant buds (118). The combined efforts of the international community were required to overcome the challenges, and the first cryobanks started their work in the 1980s.

With a desire to preserve plant genetic diversity for breeding and for future generations, scientists began cryopreserving plants over 60 years ago. Akira Sakai, known as the godfather of plant cryopreservation, was the first to report the survival of mulberry twigs after exposure to liquid nitrogen (LN) with a temperature of -196°C following a -30°C pretreatment (118). Later, in vitro cultured flax cells were successfully preserved by slow-cooling techniques that were introduced in 1968 (111). In this method, freeze dehydration is induced by exposing samples to a controlled cooling rate of 1 K h^{-1} until ~-40°C is reached; the samples are then immersed in LN. Since this method introduces a great deal of extracellular ice formation and plasmolysis, this approach has been efficiently employed mainly for unorganized tissues, such as cell suspensions and callus, as well as apices of cold-tolerant species (34, 42, 92). However, simpler protocols and wider applicability have been achieved with vitrification-based methods, the so-called one-step freezing techniques. These became the dominant approach after their successful application in 1989 to *Asparagus* embryos by Akira Sakai (34, 42, 135). During vitrification, an increased

Genebank: biorepository preserving genetic resources; plants are conserved as seeds, pollen, and/or clonal plants in field genebanks, in living collections, in vitro, or in cryostorage

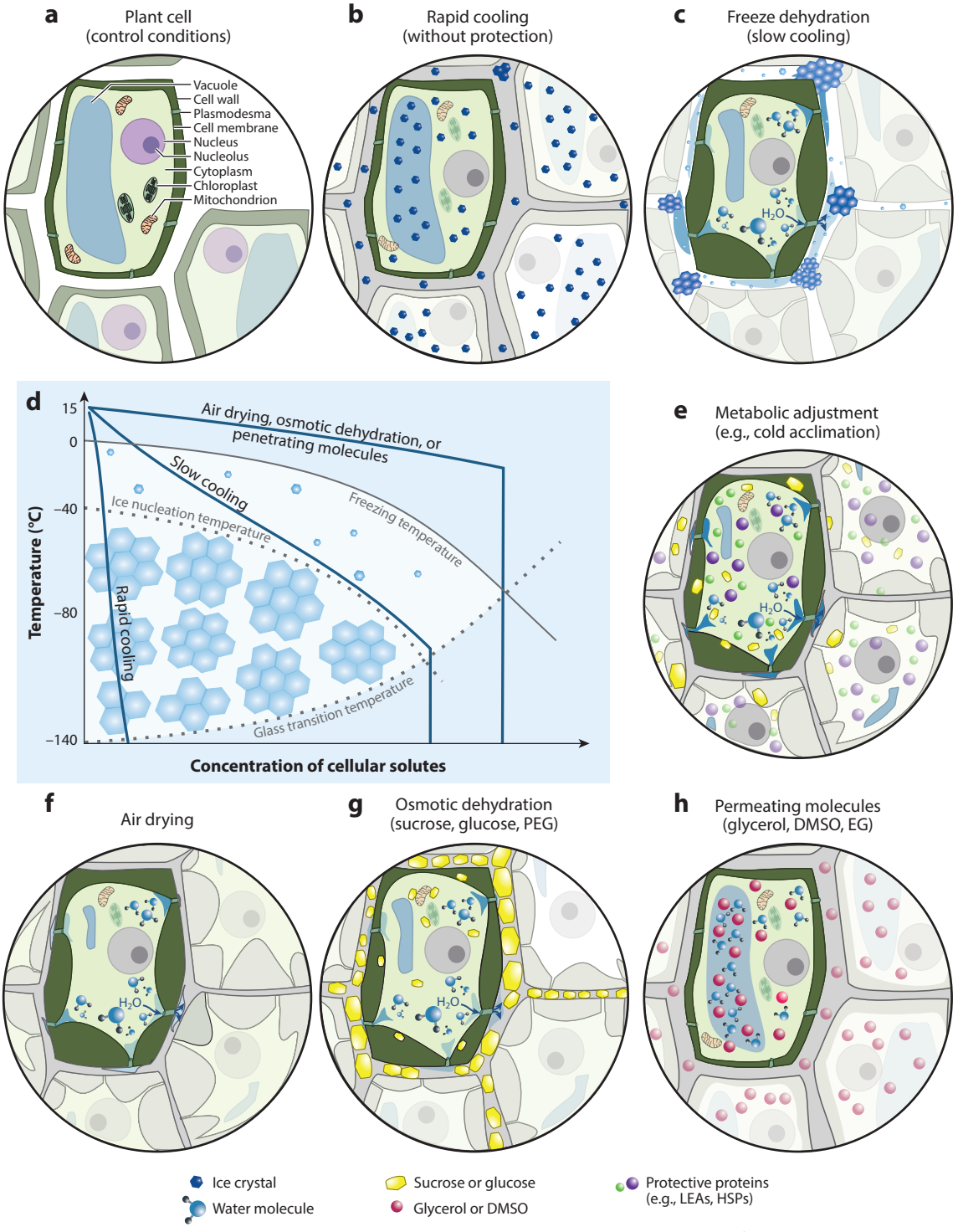
Cryoprotectant: a chemical that affects the physical state of intracellular solutes, inhibits ice nucleation, and stabilizes cell membranes and macromolecules during cryopreservation

Ice nucleation: stabilization (crystallization) of water molecules in an ice-like arrangement

Dormant buds: buds located along the shoots and older branches of temperate woody plants that enter a state of dormancy in winter

Dehydration: reduction of the explants' water content, commonly by exposure to relative humidities of 15–50% under ambient conditions

Cell suspension: a population of undifferentiated cells typically grown in liquid medium under controlled conditions



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Principles to increase cellular solute concentration and prevent lethal ice formation during cryopreservation. When hydrated plant cells (*a*) are exposed to rapid cooling without cellular protection (*b*), formation of intracellular ice (*blue hexagons*) occurs, triggering cell death. Freeze dehydration (*c*), metabolic adjustment (*e*), air drying (*f*), and application of osmotic dehydration (*g*) and/or permeating molecules (*h*) lead to an increase in the intracellular solute concentration, glass transition upon cooling, and thus the chance of cell survival after thawing. However, as a consequence of the lower water potential/osmotic pressure in the extracellular space, plasmolysis is shown in panels *c*, *e*, *f*, and *g*. Hypothetical phase diagrams (*d*) model various treatments passing through the zones of heterogeneous and/or homogeneous ice formation or directly transitioning to the glassy state. Abbreviations: DMSO, dimethyl sulfoxide; EG, ethylene glycol; HSP, heat shock protein; LEA, late embryogenesis abundant protein; PEG, polyethylene glycol.

concentration of cellular solution in the organized tissues is obtained by adding a mixture of penetrating compounds and osmotically active (nonpenetrating) compounds (34, 92). This is followed by (extremely) rapid cooling rates. Nowadays, a range of cryomethods (**Figure 2**) are available for the conservation of various explants from either in vitro culture, for example, shoot or root tips and embryogenic callus, or explants collected directly in the field, for example, seeds, pollen, dormant buds, bulbs, and bulbils (14, 57, 113, 144).

In this review, we summarize methods to cryopreserve plant diversity, describing the basic principles involved in freezing tolerance and cryoprotection. Further, we discuss challenges related to the cryobanking of agricultural species and woody and wild plants as well as new developments to increase the regrowth potential of material. To conclude, we outline gaps in our current knowledge.

PRINCIPLES OF PLANT FREEZING AND CRYOPROTECTION

Freezing of Plant Cells in Nature

At subzero temperatures, plants can be damaged or even die due to the nucleation of water molecules and their expansion (crystal growth), commonly termed freezing (55), or due to freezing-induced cellular dehydration. The initial formation and growth of crystals depend on the temperature and exposure time, the tissue size, the cooling rate, the amount of pure water available, and antifreeze factors such as ice-binding proteins (IBP) (see the sidebar titled Role of Antifreeze Proteins During Cryopreservation) or ice-nucleating active structures (3, 98). Pure water has an equilibrium freezing point at 0°C but may remain liquid (due to supercooling) until the homogeneous nucleation temperature is reached at −40°C (3). Under field conditions, plant tissues supercool only to a few degrees below 0°C (152). In woody species, mostly intrinsic plant-based ice-nucleating agents, for example, complexes of proteins, carbohydrates, and phospholipids (17, 153), trigger heterogeneous ice formation between −0.6 and −2.5°C (3, 98). In herbaceous species, extrinsic nucleating agents, for example, *Pseudomonas* bacteria, fungi (153), proteins, or other macromolecules (110), often facilitate ice formation between −2.5°C and −3.2°C (3, 152). However, the limited ability of plant tissue to supercool protects the tissue from spontaneous ice formation and damage at lower temperatures.

When a certain number of ice crystal clusters have formed, the rate of ice nucleation ranges between 4 and 100 mm s^{-1} (3, 98). At a slower cooling rate, ice nucleation is low, but crystals grow larger, and ice nucleation is higher at faster cooling rates (3) (**Figure 1**). During ice formation, water molecules reorganize from a tetrahedral coordination with fluxional and defective hydrogen bonds into hexagonal crystallization units with rigid hydrogen bonds and larger distances between molecules, leading to a 9% greater volume and lower density compared to liquid water (6). In dormant buds, water crystallizes extracellularly in discrete areas. As the water potential of ice is lower, the water moves to the extracellular space, the intracellular solute concentration increases, and the bud tissue dehydrates (126) and can vitrify during further fast cooling. In 1985, vitrification of the cytoplasm into an amorphous solid with high viscosity up to $>10^{-14}$ m s^{-1} (18) was

Vitrification: amorphous solidification of a liquid that occurs after rapid cooling of highly concentrated and viscous solutions to form metastable glasses

Supercooling: depression of the freezing temperature below its equilibrium freezing point

Ice-nucleating agents: macromolecules that trigger the formation of ice crystals by interacting with H and O atoms in the water molecules

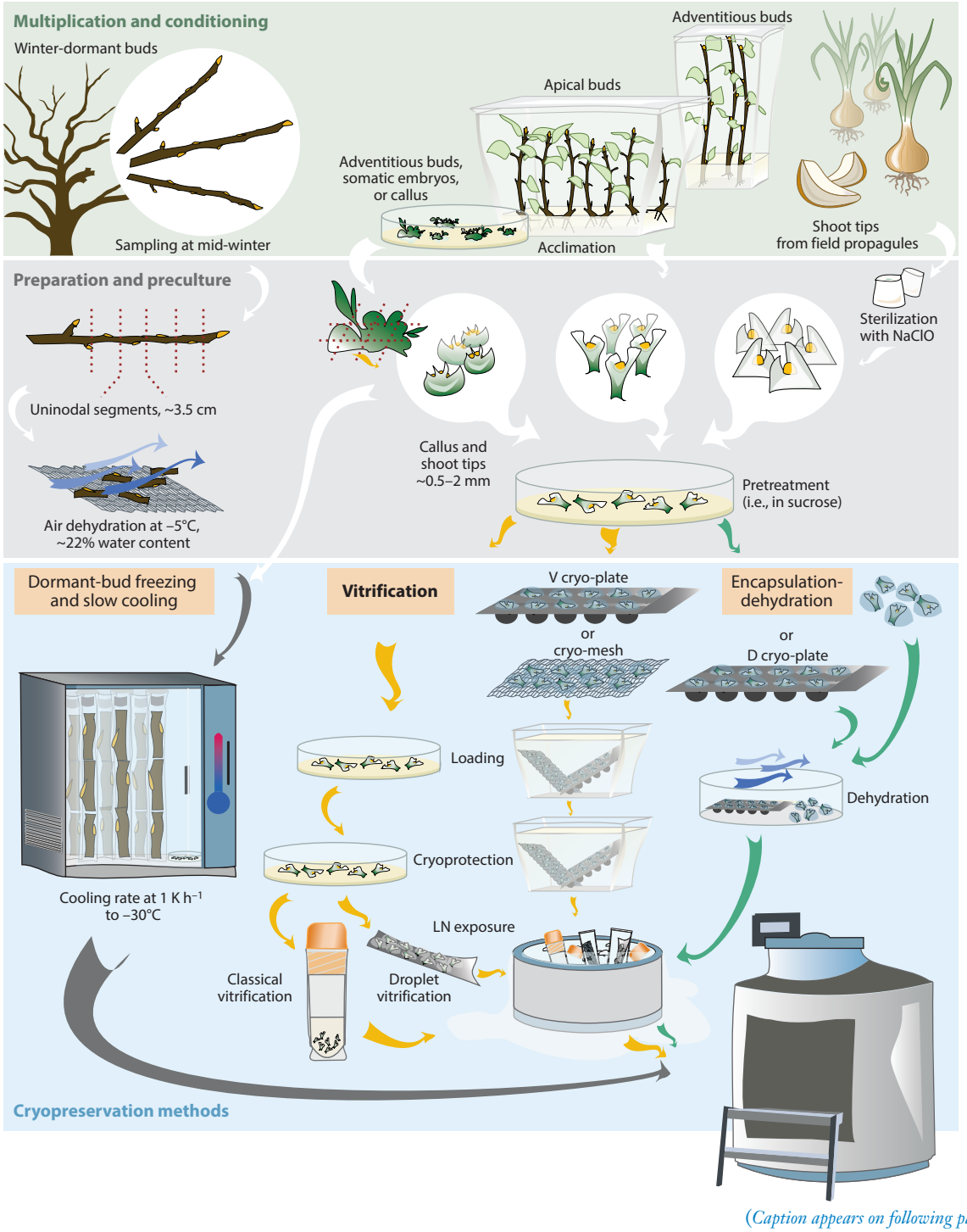


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Figure 2 (*Figure appears on preceding page*)

Schematic overview of the most common cryopreservation methods and modifications, that is, the droplet-vitrification, V and D cryo-plate, and cryo-mesh methods. Cryopreservation is often initiated with multiplication and conditioning of plants (*white arrows*), followed by preparation and preculture treatments and application of different cryopreservation methods such as slow cooling (*gray arrows*), vitrification (*yellow arrows*), or encapsulation-dehydration approaches (*green arrows*). Abbreviation: LN, liquid nitrogen.

identified as an important factor for maintaining survival of *Populus* twigs after cooling to below −70°C (49). Besides an increased solute concentration, a high ratio of cell wall thickness to cell size and a low relative volume of intercellular spaces (126) were identified as important traits for tolerating freezing.

When the temperature further drops, the decreasing water potential of ice facilitates the dehydration of the cell until cellular compartmentation is lost, electrolytes leak out, solutes precipitate, plasmolysis occurs, and bilayer membranes separate into one layer (96, 98). However, the size, the morphology, and the final location of the crystals are affected by the cooling rate (55). If the cooling rate is significantly elevated, that is, 3 K min^{-1}, cells fail to equilibrate, and ice is formed intracellularly (3). Intracellular ice is often lethal, as it damages cellular structures that may trigger programmed cell death (149).

Orthodox seeds: desiccation-tolerant seeds that are able to be dried to typically <0.10 g H_2O g^{-1} dry weight and stored at subzero temperatures

Recalcitrant seeds: desiccation-sensitive seeds that tend to die when water content drops to <0.20–0.30g H_2O g^{-1} dry weight and/or seeds are stored at <0°C

Mechanisms of Cryoprotection

The success of plant cryopreservation depends on the efficiency of the process at increasing the concentration of cellular solutes, the cooling rate, and the tolerance of the plants against dehydration and ice crystallization. In principle, five methods can be used alone or in combination to increase the cellular concentration of solutes (95) (**Figure 1**).

Air drying. Desiccation-tolerant orthodox seeds and pollen accumulate protective molecules during maturation drying, that is, sugars that ensure the integrity of membrane lipids by acting as osmotic spacers; late embryogenesis abundant (LEA) proteins and heat shock proteins (HSPs) function as molecular chaperones and maintain the three-dimensional structures of proteins and other cellular components (88). At ambient temperatures, the cytoplasms of orthodox seeds and pollen vitrify at a relative humidity of 40–60%, corresponding to water contents <10% (10, 29). In this glassy state, ice crystallization is unlikely, and organs resist freezing, which makes them highly suitable for cryostorage. By contrast, recalcitrant seeds and pollen are sensitive to

ROLE OF ANTIFREEZE PROTEINS DURING CRYOPRESERVATION

Protection of membranes and proteins against the damaging effects of ice formation can be achieved with antifreeze proteins (AFPs), often referred to in plants as ice-binding proteins (IBPs). IBPs bind reversibly to ice crystal facets (130) and force the ice to grow between molecules; they also prevent secondary nucleation leading to freezing point depression and inhibition of ice crystallization and recrystallization (55). IBPs were first discovered in marine fishes (28) and are widespread among insects, algae, plants, and microorganisms. They show significant structural diversity and are efficient at nano- to millimolar concentrations (30). After first being discovered in winter rye, they have been identified in 60 plant species (78). As supercooling in plants favors spontaneous, rapid ice nucleation, IBPs in plants do not prevent freezing but control crystal growth and prevent large ice crystal formation (44), which protects membranes from damage, as shown for *Arabidopsis* (15) and ryegrass (162). Ongoing research is attempting to substitute glycerol and DMSO with AFPs, especially in human sperm and blood (32). Potato shoot tips treated with IBPs showed greater regrowth after cryopreservation (124). However, as only a few studies have been published, the potential of IBPs for plant cryopreservation still needs to be elucidated.

desiccation, especially at water contents <20–30%, and dehydration causes cellular shrinking, damage to macromolecules and membranes, and eventually cell death. However, if they are partially dried and drying rate is sufficiently accelerated, for example, by a stream of ultradry air, the window during which damaging effects occur, caused by the reaction of reactive oxygen species (ROS) with proteins, lipids, and other macromolecules, can be shortened, and cells may retain viability after exposure to ultralow temperatures (19, 53).

Metabolic adjustment. Metabolic adjustment can be induced by exposure to temperatures between 0 and 5°C, or to elevated sugar concentrations and a short photoperiod, leading to down-regulation of photosynthesis (21) and accumulation of sugars, proline, and LEA proteins in the cytosol (96, 131). As a result, the cellular solute concentration increases, membrane proteins stabilize, and lipid composition alters (128), leading to an increase in membrane fluidity (137). In parallel, cell wall–modifying enzymes cause structural reorganizations such as changes in pore size and cell-wall thickness that affect the spread of extracellular ice into the intracellular solution (3, 96, 98). The various processes add up to an overall higher tolerance to low temperatures.

Freeze dehydration. Freeze dehydration takes advantage of the fact that cellular solute concentration increases as water moves toward the lower ice water potential in the extracellular space at low cooling rates. The more extracellular ice, the more concentrated the remaining solution and the more water leaves the cell. This can be achieved in simple freezing containers that result in cooling rates of 1 K min^{-1} (86), by one- or two-step approaches in freezers (121), or by programmable freezers. However, optimal cooling rates are defined empirically for different species, because rates that are too low may damage cells due to high (toxic) solute concentrations and dehydration, whereas rates that are too high may result in intracellular freezing (73). Freeze dehydration is especially successfully employed in undifferentiated cells and dormant buds, which can tolerate the presence of extracellular ice.

Osmotic dehydration. Osmotic dehydration is induced by exposure to nonpermeating cryoprotectant agents such as glucose, sucrose, and polyethylene glycol (PEG) with a high molecular weight (33). Sucrose is predominantly used, and it reduces water dynamics at concentrations >40% by interacting with the hydrogen bonds of water molecules (65). During cooling, sucrose distributes heterogeneously across the cell. Although nonfrozen sucrose solution can be detected intracellularly, it is primarily located extracellularly (160) with direct binding to the bilayer surface of the cell membrane (61), facilitating dehydration and membrane protection. Sucrose is also a signaling molecule that induces sugar-modulated gene expression and functions as a pro- and antioxidant to control oxidative stress (27). In plants, application of elevated concentrations of sucrose before (38) and during cryopreservation (62) can induce drought acclimation and increase survival after LN treatment.

Permeating molecules. Permeating molecules such as glycerol, DMSO, and low-molecular-weight ethylene glycol (EG) penetrate rapidly into the cell. In mint, DMSO enters meristematic cells of the shoot tips within 10 min (63) and interacts with the polar headgroups of phospholipids (158). Interestingly, DMSO preferentially accumulates in specific cell organelles (63). However, this may induce different effects, as DMSO applied in low concentrations promotes membrane thinning and enhances the fluidity of the hydrophobic core. At higher concentrations, DMSO induces water pores and can disrupt bilayer structures of membranes, which may lead to membrane fusion (46). Therefore, depending on the DMSO concentration and the temperature and composition of membranes, phytotoxic effects on explants (56, 158) and disruption of DNA methylation have been observed (136). Both DMSO and glycerol prevent rapid ice crystal formation. Glycerol

can substitute the hydrogen bonds of water molecules and segregate water into clusters, which prevents ice formation and cell damage due to volume expansion (133). Based on the different effects of various molecules, a combination of cryoprotectants is often used to increase cellular solute concentration and provoke vitrification during rapid cooling.

A second main requirement for vitrification, and thus cell survival, is the application of fast cooling and rewarming rates. At fast rates, there is no time for water molecules to organize into ice crystals or for crystals to grow to a damaging size during the cooling and rewarming processes. When sufficient dehydration is reached, explants are immersed in LN to prevent further ice crystallization. The application of cryoprotectants facilitating high detrimental solute concentrations requires fast cooling rates at >100 K min^{-1} to reduce the exposure to solutes and facilitate glass formation. The velocity of cooling is accelerated when explants are small and are directly exposed to LN. Speed also determines the occurrence of devitrification and recrystallization during rewarming. Biological barriers, that is, protein denaturation, limit warming temperatures to 37–40°C. However, when small volumes are used and explants exposed directly to warm solutions, warming rates of up to 250 K min^{-1} are achievable (119). In the future, the development of laser warming (79) may revolutionize the warming processes, thus increasing the regeneration capacity of cryopreserved explants, but so far, no such study has been reported in plants.

Slow cooling: samples are exposed to a controlled cooling rate of ~1 K min^{-1} until ~−40°C to induce freeze dehydration

Encapsulation: inclusion of organs or tissues from in vitro culture in capsules of calcium alginate to form artificial (synthetic) seeds

Chip budding: a grafting technique that involves joining a chip-shaped bud and a rootstock; used to control cryopreservation success in the dormant-bud technique and to regrow germplasm from cryobanks

Methods to Cryopreserve Plant Diversity

The first 60 years of plant cryopreservation have resulted in a wide range of methods and combinations thereof to protect tissue from ice formation, most of these empirically developed (**Figure 2**). A cryopreservation protocol process can be commonly separated into three phases: (*a*) plant multiplication and conditioning in vitro or in the field; (*b*) preculture, cryoprotection, cooling, and storage; and (*c*) rewarming and regeneration. During plant multiplication, young, healthy, viable, and high-quality material that is free of infections or other damages, which therefore has a high regrowth capacity, is made available for cryopreservation. In addition to field propagules, such as dormant buds that are directly collected from the field, in vitro shoot tips of 0.3–2 mm that contain the meristem can be excised from micropropagated shoots. For plants with low rates of in vitro reproduction, the production of many adventitious buds obtained from leaf discs or somatic embryos may be an alternative (91). However, prior to cryopreservation, some propagules benefit from a conditioning step involving cold and drought acclimation, which aims to protect plant membranes and cell walls against dehydration and ice nucleation (126). Depending on the sensitivity of the plant material to dehydration and freezing, a combination of cryoprotecting steps may be needed to gain full plant recovery after cryostorage. The most well-known cryopreservation methods (**Figure 2**) are dormant bud freezing, slow cooling, methods based on the application of vitrification solutions, and encapsulation-dehydration.

Dormant bud freezing. Dormant buds (budwood) from scions grown in the previous season are sampled in midwinter after trees have been exposed to temperatures between 0°C and −5°C for 3 to 4 days to achieve maximum desiccation tolerance (39). Twigs are cut into uninodal segments of approximately 3.5 cm and are further air-dried at approximately −5°C to a water content of 22–25%. After approximately 2 months, uninodal segments are packed in polyolefin tubes or cryovials and exposed to freeze dehydration by slow cooling from −5°C to −30°C at 1 K h^{-1} and then immersed in LN or stored in the LN vapor phase at −165°C to −190°C (64, 129). Thawing takes place in a cold room at 4°C. Afterward, segments are placed in moist peat to rehydrate for 2 weeks at 2°C. Finally, the buds are taken from the segments to make chip budding grafts on specially prepared rootstocks. This technique allows a marked reduction of time and labor (64) in comparison with the cryopreservation of germplasm using tissue culture–based methods.

Somatic embryogenic callus: a callus composed of dedifferentiated cells that is able to regenerate into somatic embryos and thereafter into plants

Slow cooling. Slow cooling combines freeze dehydration with chemical cryoprotection and was first introduced to cryopreserve somatic embryogenic cells (154); however, it has also been applied to somatic embryogenic callus, which consists of dedifferentiated cells and is able to develop into somatic embryos, and cell cultures that are of strategic importance for highly efficient propagation or genetic transformation (90). Somatic cells are commonly pretreated in a cryoprotectant solution, that is, 5–10% (w/v) DMSO with or without elevated sugar concentrations, for approximately 30–60 min. Afterward, they are slowly cooled at a rate of 0.5–1.0 K min^{-1} to −40°C and then immersed and stored in LN (89). Because freeze dehydration often results in severe plasmolysis, resulting in a loss of intercellular connections by means of plasmodesmata, this method is best suited for unorganized cell clumps where it is more important to maintain cell integrity than tissue integrity.

Vitrification methods. In 1990, two new plant cryopreservation techniques were introduced, the vitrification (120) and encapsulation-dehydration methods (36). To achieve cell protection by vitrification, plant vitrification solutions (PVSs) are used, including the first PVS developed, PVS2, which contains 30% (w/v) glycerol, 15% (w/v) EG, 15% (w/v) DMSO, and 0.4 M sucrose in a basal medium (120), or PVS3, which contains 50% (w/v) glycerol and 50% (w/v) sucrose in the basal medium (83). The most important characteristics of vitrification solutions are that the solutions themselves vitrify upon rapid cooling and they contain the necessary penetrating and osmotically active compounds facilitating cellular dehydration and vitrification of the cytoplasm. Additionally, this solution must not be too toxic.

The predominant method currently used is droplet vitrification, which depends on extremely rapid cooling of small explants exposed to droplets of vitrification solution placed on aluminum strips. This contrasts with classical vitrification, in which samples together with 1 mL vitrification solution are enclosed in 2 mL cryotubes, resulting in much slower cooling rates. As an example of the droplet-vitrification protocol, meristems from 4-week-old banana or potato shoots grown in vitro are excised and exposed to loading solution (LS), consisting of 0.4 M sucrose and 2 M glycerol prepared in basic nutrient medium, at room temperature for 20 min. Afterward, explants are transferred to PVS2 at 0°C for 30 to 50 min and finally transferred into one 15-µL PVS2 droplet on an aluminum foil strip that is then immersed in LN, ensuring rapid cooling at 130 K s^{-1}; the foil strips are subsequently transferred into cryotubes filled with LN that are used for storage (94). PVS2 is the most common cryoprotectant and produces high regrowth rates for propagules of many species. However, if regrowth results are not satisfactory, other vitrification solutions, for example, PVS3, can be an alternative.

PVS3 does not contain DMSO, which penetrates rapidly into the tissue. Therefore, longer incubation periods and often an additional overnight preculture on 0.3 M sucrose are essential for PVS3 droplet vitrification of potato (62). More recently, the V cryo-plate (156) and cryomesh (40) methods have been developed. Here, explants are transferred into wells or droplets of alginate sucrose solution and covered with calcium sucrose solution to fix encapsulated shoot tips as beads to the plate or mesh. The advantage is that the complete cryo-plates or -meshes, including explants, can be moved to reservoirs with LS and cryoprotectant solutions and then to LN (145), which reduces the mechanical stress during transfer. Overall, vitrification methods are highly successful, as they are easy, reproducible, and applicable to a wide range of species and explants including cell suspensions, embryogenic tissues, shoot tips, and seeds (119) of woody and herbaceous species from temperate and tropical zones (13).

Encapsulation-dehydration methods. Encapsulation-dehydration relies on the increase of solute concentration in the target organs by osmotic dehydration and osmotic protections. Commonly, explants are encapsulated using alginate calcium solution to form so-called synthetic or

artificial seeds. The alginate beads are treated with a high sucrose concentration and air dried under sterile airflow or using a desiccant with silica gel to achieve water contents of 20–30%. The beads are then immersed in LN and transferred for final storage (13, 119). As variants, the D and V cryo-plate methods (81) fix the alginate to wells, whereas the encapsulation-vitrification technique combines the encapsulation of explants with the application of a vitrification solution. However, as quite a number of complex steps are required, encapsulation methods are less frequently applied compared to other cryopreservation approaches.

Molecular Studies During Cryopreservation

Given the increasing loss of biodiversity, it is crucial to increase the number of species and accessions cryopreserved, and this requires a deeper understanding of the molecular processes that occur during cryopreservation. Plant cryopreservation combines different environmental stresses such as wounding provoked by excision, osmotic stress induced by the preculture and/or cryoprotection of the explants, and thermo-mechanical changes that occur during cooling and rewarming. These abiotic stresses induce the formation of ROS and affect survival after cryopreservation (45, 116). Although ROS generation and the resulting synthesis of antioxidant molecules seems to vary between species (41), ROS are also interpreted by cells as signaling molecules modulating the expression of a large set of genes and posttranslational modification of proteins (51). Cryogenic treatment with PVS2 increases the abundance of transcription factors, such as dehydration-responsive element–binding protein/C-repeat binding factors, oxidative signal inducible 1, and WRKY22 among others in *Arabidopsis* (45, 116, 127). As a result, changes to RNA processing, RNA methylation, protein synthesis, phytohormone-mediated defense, and lipid and primary metabolism are induced (52, 127). In particular, during LS treatment with sucrose and glycerol, enhanced lipid turnover, leading to increased concentrations of phosphatidic acid and phosphatidylglycerol, was observed (67), indicating severe membrane remodeling during cryopreservation. As the plants are expected to be maintained as true to type, the mechanisms underlying maintenance of (epi-)genetic integrity and metabolic stability need to be elucidated.

Changes in DNA methylation, histone modification, and the chromatin structure modulating gene expression are considered epigenetic changes (143). During cryopreservation, different methylation-sensitive markers showed that the methylation pattern may vary following different steps (70). However, this may depend on the species, the cryopreservation method, and the tissue culture regime. In all studies so far, comparisons of the morphology, growth, and reproductive capacity of cryopreserved versus noncryopreserved plants confirmed the maintenance of genetic integrity (143). Therefore, the role of epigenetic variation still needs to be elucidated and may benefit from genome-wide profiling methods with higher marker density.

APPLICATIONS OF PLANT CRYOPRESERVATION

Agricultural Plants

Maintaining the widest possible diversity of agricultural crops and their wild relatives is imperative for dealing with some of the most important agricultural challenges. These include climate change, food supply for the increasing world population, and the fight against pests and diseases that are causing increasing damage due to limited buffering genetic diversity. Cryopreservation is the ultimate long-term storage method for crops that do not breed true from seeds and crops that produce desiccation-sensitive or short-lived seeds (93). Hundreds of cryopreservation studies involving different methods and using various crop propagules have been published (**Figure 2**). Most of these, however, describe only post-cryopreservation survival or, at best, regeneration of one accession, which is far below the requirements for long-term cryobanking. In practice, the

number of viable explants needs to be predicted based on the number of explants processed and their post-cryopreservation regeneration rate, which is recommended to be ≥35% (138). Consequently, few cryopreservation protocols are widely applied, namely, PVS2 droplet-vitrification for the preservation of shoot tips of potato (140), banana (94), and strawberry (50); PVS3 (droplet) vitrification for shoot tips of potato, mint (123), garlic, and shallot (58, 123); and the recently developed V cryo-plate method for potato (157). Only two cryobanks [Bioversity International and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)] are systematically duplicating their cryopreserved material at a different location. However, as countries plan to store their important genetic resources for >100 years, secure genebank management involving data security, cryo duplicates or triplicates, and backup facilities should be considered for the future.

Field genebank: protected fields or orchards designed to maintain plant genetic resources in vivo

Supplemental Material >

So far, large-scale application of cryopreservation to collections remains limited to only a few species (**Figure 3**, **Supplemental Table 1**). A recent review revealed that 15 institutes together keep 9,650 accessions of 30 different crops under cryopreservation conditions (1). Only 11 institutes hold more than 100 cryopreserved accessions. The majority of material is still maintained in field genebanks (66%) and/or as in vitro cultures under slow growth (46%). In addition, according to Acker et al. (1), only 10% of the presumed 100,000 unique and distinct accessions of the species listed in Annex 1 of the Plant Treaty are cryopreserved. Currently, the largest cryopreserved collections include 1,258 banana accessions at Bioversity International, Belgium (B. Panis, unpublished data); 1,158 garlic accessions at the National Institute of Agricultural Sciences (NAAS), South Korea (58); 4,086 potato accessions at the International Potato Center (CIP), Peru (140); and 2,060 potato accessions at IPK, Germany (M. Nagel, unpublished data), where >50% of their genetic materials are kept in LN. The major challenge that hampers a broader utilization of cryopreservation is insufficient budget. The cost of transferring one accession into cryopreservation ranges between 42 and 1,500 USD, depending on tissue type (cryopreservation of dormant buds and seeds is much cheaper compared to in vitro shoot tips) and local labor costs. The low maintenance costs of cryopreserved collections, 1–2 USD per accession per year, however, make the investment of transferring materials into cryopreservation pay off after 10–15 years.

Beyond the storage of genetic resources, cryopreservation is important in clonal crop production systems. Increased contamination and somaclonal variation can be avoided by cryopreserving large amounts of clean in vitro cultures. Furthermore, cell lines that produce specific valuable metabolites in bioreactors or transformation-competent embryogenic cell lines that are difficult to initiate can be safely stored in LN (see the section titled Cell Cultures). An additional, equally important, application of cryopreservation, cryotherapy, enables the eradication of pathogens, such as viruses, phytoplasmas, and bacteria, from plants (146).

Woody Plants

A range of cryopreservation methods are available for the conservation of woody species. Explants are either directly collected from trees in the field or taken from in vitro cultures. For shoot tips, numerous vitrification-based protocols are available (13, 119). More recently, the newly proposed D and V cryo-plate methods showed promising results in persimmon (72) and *Prunus* spp. (141). In conifers, somatic embryogenesis has been repeatedly shown to be highly efficient for plant propagation (60, 76). Obtaining good quality embryogenic callus lines is a quite difficult and time-consuming procedure. For example, in conifers, embryogenic cells are generally produced from the few suspensor cells of zygotic embryos, which are excised and introduced in vitro during a specific and narrow period of immaturity (60, 89). After a proliferation period, embryogenic cell masses can be cryopreserved by slow cooling, which is still the most widely used method for the long-term conservation of embryogenic callus lines (90).

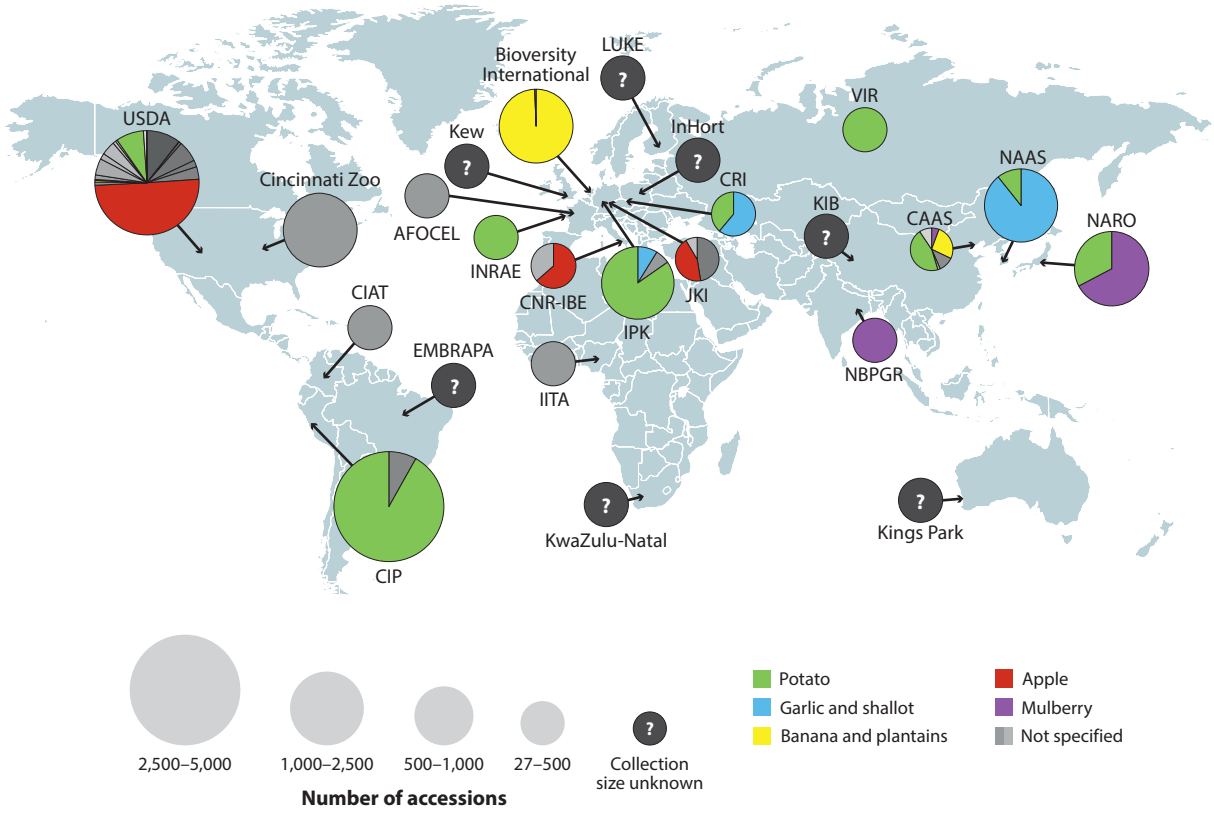

Figure 3

Overview of current global cryo collections showing major collections of potato, garlic, banana, apple, and mulberry genetic resources. Details are provided in **Supplemental Table 1**. Abbreviations: AFOCEL, Association Forêt Cellulose, France; CAAS, Chinese Academy of Agricultural Sciences; CIAT, International Center for Tropical Agriculture, Colombia; CIP, International Potato Center, Peru; CNR-IBE, Institute of BioEconomy, Italy; CRI, Crop Research Institute, Czech Republic; EMBRAPA, Brazilian Agricultural Research Corporation; IITA, International Institute of Tropical Agriculture, Nigeria; InHort, Institute of Horticulture, Poland; INRAE, National Research Institute for Agriculture, Food and Environment, France; IPK, Leibniz Institute of Plant Genetics and Crop Plant Research, Germany; JKI, Julius Kühn Institute, Germany; Kew, Royal Botanic Gardens, Kew, United Kingdom; KIB, Kunming Institute of Botany, China; LUKE, Natural Resources Institute Finland; NAAS, National Institute of Agricultural Sciences, South Korea; NARO, Research Center of Genetic Resources, Japan; NBPGR, National Bureau of Plant Genetic Resources, India; USDA, United States Department of Agriculture; VIR, Federal Research Center N.I. Vavilov All-Russian Institute of Plant Genetic Resources.

In addition to the cryopreservation of whole seeds and embryonic axes (see the section titled Recalcitrant-Seeded Species), an efficient cryopreservation technique to back up fruit genetic resources is the dormant-bud technique, first proposed by Forsline et al. (39) for the conservation of apple germplasm. Currently, more than 2,155 apple germplasm accessions are held by the National Plant Germplasm System (USA) (139). Smaller collections can be found at the Julius Kühn Institute (JKI; Germany; 180 accessions), Natural Resources Institute Finland (LUKE; 50 accessions) and the Institute of BioEconomy (CNR-IBE; Italy; 52 accessions) (**Figure 3**, **Supplemental Table 1**).

The current challenge to conserving fruit tree species is the need to contain the high costs arising from the large amount of land, pesticides, and fungicides necessary for the traditional maintenance of clonal tree collections in the field. For this reason, the duplication of in-field collections

Supplemental Material >

Embryonic axes: zygotic embryos consisting of the radicle, plumule, and hypocotyl, excluding cotyledons; used for cryopreservation of species with large nonorthodox seeds

in cryobanks will be increasingly necessary and, as a result, the number of trees needed for each accession in the field can be reduced. The cryopreservation of dormant buds will have an increasingly prominent role, allowing direct passages from field to cryobank to field, that is, avoiding the in vitro culture of accessions, before and after cryopreservation (64). Techniques based on the cryopreservation of shoot tips from in vitro culture and of embryogenic callus will continue to play an important role in all those tree species that cannot be propagated by bud grafting, for example, olive, kiwi, chestnut, and conifers, or do not produce dormant buds, for example, tropical and subtropical tree species.

Wild Species

Many wild plants, but also most clonally propagated crops, can be called exceptional species. This concept encompasses species that produce only limited numbers of viable seeds, desiccation-sensitive seeds, or seeds that are fully or partially desiccation tolerant but have a limited life span when frozen at −20°C. For these species, conventional seed banking is not possible (104), and living collections and cryopreservation are the primary options for ex situ conservation (142, 150). In the long term, cryobanking will be the most efficient, cost-effective method for germplasm storage, but protocol development for cryobanking is more resource intensive than seed banking. Given the prediction that thousands of species will need cryobanking for effective ex situ conservation (26) and the fact that 45% of all plant species, globally, are under threat of extinction (2), the need for improving methods and increasing efficiency and capacity is critical (101).

Research into cryopreserving tissues of wild species dates from the 1990s, including reports of protocol development and/or banking at Kings Park and Botanic Garden in Perth, Australia (132), the Lindner Center for Conservation and Research of Endangered Wildlife (CREW) at the Cincinnati Zoo & Botanical Garden in the United States (100), the Royal Botanic Gardens, Kew, United Kingdom (109), and the University of KwaZulu-Natal, South Africa (37). Since then, research on developing protocols for conserving wild exceptional species has multiplied.

While the feasibility of cryobanking tissues of wild species has been demonstrated and implemented on a small scale, it has not yet reached the scale of seed banking of wild species, as at the Millennium Seed Bank (United Kingdom) and the Germplasm Bank of Wild Species, Kunming Institute of Botany (China), or of tissue banking of crop species, as at the CGIAR crop centers (69). This is due to some particular challenges facing the conservation of wild species. The first challenge is to understand which species need tissue-based cryopreservation. A first attempt at creating a list of exceptional plants has been published (102). Of the 775 species listed as exceptional species, 98 are reported as having few or no seeds available for banking, while 389 are listed as desiccation sensitive. Some recalcitrant species can be conserved by cryopreserving excised embryo axes (see the section titled Recalcitrant-Seeded Species), but others, particularly some tropical species, require tissue banking. The current list represents 3% or less of the predicted number of exceptional plants, so further work is needed to identify these species.

The second challenge focuses on the cryobiotechnologies needed to accomplish the cryobanking of large numbers of wild exceptional species. For tissue cryopreservation, the primary approach is to apply a vitrification method (see the section titled Methods to Cryopreserve Plant Diversity) to either shoot tips or somatic embryos. Shoot cultures are often more straightforward to initiate with many species. However, once initiated, somatic embryo tissues do not require the labor involved in shoot tip dissection. In some systems, however, shoot meristematic tissues can be produced that require little or no dissection, such as buds regenerated from leaves, protocorm-like bodies from orchids, and green globular bodies from ferns (23, 82), and these can be handled easily, like somatic embryos.

An additional challenge is understanding the factors involved in tissue survival through the stress of cryopreservation. One of the most widely used protocols is that of droplet vitrification (see the section titled Methods to Cryopreserve Plant Diversity). This has proven to be successful in species with widely diverse adaptations and life forms, but these represent only a small percentage of the total species that require cryopreservation as a conservation tool. Many of the species that need attention are tropical trees (103), a group that has proven to be particularly challenging to initiate to in vitro culture and to cryopreserve (85) and one that is also especially under threat (103). As tropical woody species also represent a higher-than-average number of species with desiccation-sensitive seeds (134), methods for predictably culturing and cryopreserving tissues from tropical trees are urgently needed.

Intermediate seeds: relatively short-lived seeds that fall between the orthodox and recalcitrant seed limits; e.g., some tolerate some desiccation but are freezing sensitive

As tissue cryopreservation becomes more widely used for conserving wild species, it must be noted that the tissues used for banking are clonal lines. As a result, to capture a meaningful part of the natural diversity of a species, multiple lines must be initiated from a genetically representative group of individuals and subsequently cryopreserved. This can be a significant task, given the resources needed for the many genetic lines of clonal crop species that are being banked. Banking of multiple genetic lines has been reported for a few wild species (105) but has yet to be done widely for the many wild species in need of tissue cryopreservation.

While the challenges of banking wild exceptional species are significant, a recent positive development has been the increased awareness of the needs of these species. Articles defining and collating exceptional species (102, 104, 159), conferences focusing attention on the methods needed for their conservation, and network training workshops on methods for conserving exceptional plants are hopeful signs that these species will receive more attention in the future. Such species represent a significant proportion of the world's biodiversity, and meeting the challenge of conserving these plants will be a measure of the global community's commitment to maintaining that diversity into the future.

SPECIFIC APPLICATIONS

Recalcitrant-Seeded Species

For the cryopreservation of recalcitrant-seeded species, particularly when working with wild species or nonclonal crops, cryopreservation of the embryonic axes (zygotic embryos consisting of the radicle, plumule, and hypocotyl) or, alternatively, only plumules is preferred to the use of other plant tissues, such as shoot tips, somatic embryos, or dormant buds (8). This is due to the possibility of banking a large amount of the genetic diversity of the species, as each embryonic axis represents a seed, and a whole plant can be generated without complex tissue culture procedures (9). In addition, pollen storage has been suggested as a complementary germplasm source for recalcitrant-seeded species, in order to increase the number of alleles preserved, as pollen from recalcitrant-seeded species is often tolerant to desiccation and can undergo long-term dry preservation in freezers or at cryogenic temperatures (8).

In the last 40 years, research on the cryopreservation of embryonic axes of nonorthodox species has been performed on over 125 species, 36% of them of temperate origin and 64% of tropical origin (**Supplemental Table 2**); of these, 76 were recalcitrant seeded species, 40% were of temperate origin (largely in the genus *Quercus*), and 60% were from (sub)tropical areas. Based on this research, successful cryopreservation protocols with ≥30% regeneration rates (7, 35, 84) could be used for 41 recalcitrant and 41 intermediate-seeded species. Higher rates of success were often obtained for intermediate seeds compared to recalcitrant seeds (**Supplemental Figure 1**), independent of the species origin [temperate versus (sub) tropical].

Supplemental Material >

For over 93% of the embryos of nonorthodox species, the protocols developed are based on desiccation methods, with fast- or flash-drying methods producing higher percentages of normal

Supplemental Material >

plant growth from the embryonic axes of recalcitrant-seeded species (**Supplemental Figure 1*b***). The optimal water content for cryopreservation of embryonic axes in recalcitrant species peaked between 15% and 36% (dry weight basis), independent of the drying rate (**Supplemental Figure 1*c***). In relation to cooling rates, it seems that cooling rates >100 K s^{-1} provided better results when all optimal protocols are considered (**Supplemental Figure 1*d***). However, there is an interaction between water content, cooling rate, and species characteristics that may need to be considered (147, 148, 155). In addition, only 5% of intermediate-seeded species needed cryoprotection for successful embryo cryopreservation; however, in 34% of recalcitrant-seeded species some degree of additional chemical cryoprotection was needed (**Supplemental Figure 1*b***, **Supplemental Table 2**). Interestingly, cryoprotection of the embryonic axis of recalcitrant seeds is better achieved with single cryoprotective solutions than with mixtures such as PVS2 or PVS3 (125).

The availability of protocols obtained through fundamental research has not always been translated into the generation of long-term cryopreserved collections (1). Nonetheless, important collections of nonorthodox seeds or embryonic axes are held in diverse cryobanks globally, mainly for a few important crop, forestry, or commodity species. For example, 6,650 accessions of 804 *Citrus* species and diverse tropical underutilized fruits are cryopreserved at the National Bureau of Plant Genetic Resources (NBPGR), India (71). In addition, 63 genotypes of *Coffea arabica* from the Tropical Agricultural Research and Higher Education Center (CATIE), Costa Rica, are cryopreserved at the National Laboratory for Genetic Resource Preservation (NLGRP), USA, and at the Research Institute for Development (IRD), France (1, 71), as well as some of its wild relatives at the Millennium Seed Bank of the Royal Botanic Gardens, Kew, United Kingdom (**Supplemental Table 1**).

Cryopreservation of embryonic axes of recalcitrant seeds faces challenges at multiple steps of the cryopreservation process (8). For example, it is limited by differing responses between species and populations to desiccation and LN exposure (155), the quality and maturity of the explant (9, 43), physical damage to the embryo during isolation (43), oxidative stress associated with excision and cryopreservation procedures (117, 151), cryoprotectant toxicity (77), differential drying patterns and stress tolerances of shoot and root meristems (9, 47), and/or the need to improve in vitro growth and acclimation procedures (8). Many of these challenges have been addressed, and the findings have been translated into some improvements including optimization of the explant excision (9, 43) and protection procedures using vacuum infiltration vitrification (77), flash-drying, and optimization of the cooling procedures (7, 147).

Cell Cultures

A plant cell culture is a population of dedifferentiated cells grown in vitro as a callus or as a cell suspension. The absence of organismic control and tissue-specific differentiation make cell cultures a unique model system for physiological, biochemical, and molecular studies and a tool for the sustainable biotechnological production of heterologous proteins and phytochemicals of high pharmacological value (112). Despite high biotechnological potential, very few documented collections of plant cell strains have been reported, but such collections exist in the USA, Finland, Japan, Germany, Hungary, Czech Republic, and Russia (161). Most collections are maintained by periodic subcultures, an expensive and laborious method that may result in a reduction of vigor, genetic modifications (e.g., polyploidization), a shift in composition and quality of the desired metabolites, and culture loss due to contamination or mislabeling (31, 74, 161). These concerns prompted the development of cryopreservation protocols as the only reliable method to preserve plant cell culture traits for the long term.

Since the first successful experiments in the 1960s and 1970s, cell strains of over 50 species of both dicotyledonous and monocotyledonous plants were cryopreserved (48, 80, 115). Cultured cells at the exponential growth stage were preferentially cryopreserved due to their high proliferation capacity, smaller cells and vacuoles, and higher viability after cryopreservation. Cell cultures of several species also benefited from additional treatments such as cold acclimation (20, 25), heat shock (115), and/or preculture with abscisic acid (ABA) (12, 115). However, the response to cold acclimation, ABA treatments, and, hence, the freezing tolerance of suspension cells seems to be affected by their growth stage (66).

Cold acclimation: induction of freezing tolerance in (winter-hardy) plants by accumulation of protective compounds and membrane and cell wall reorganization

Currently, slow cooling is the most efficient method for cryopreserving cell cultures, resulting in cell survival between 20% and 100% depending on the strain (80). Encapsulation-based protocols were efficient for cell lines of *Arabidopsis* and tobacco (4, 87, 122), while vitrification worked well for cell cultures of tobacco, carrot, *Doritaenopsis*, and *Gentiana tibetica* (75, 80, 114). Moreover, most cell cultures benefit from preculture with sucrose, mannitol, or amino acids (20, 108, 115), followed by cryoprotection using various combinations of glycerol, DMSO, sucrose, mannitol, sorbitol, and trehalose for slow cooling or PVS2 for vitrification procedures or air dehydration (80). After cryopreservation, both callus and suspension cell cultures generally recover better on (semi)solid medium or on filter paper placed on the surface of a liquid medium (4, 59, 75, 107, 115), which helps cluster and concentrate the surviving cells, thus leading to a greater ability to proliferate and sustain growth (80). The most serious challenges remaining are the absence of a uniform cryopreservation protocol applicable to most cell lines and the genotype-specific responses of cell lines to pretreatment and cryoprotection conditions.

The majority of studies confirmed the stability of growth parameters, genetics, and secondary metabolite production in cell cultures after cryopreservation. The profiles and content of bioactive compounds were retained in cell cultures of species recovered after cryopreservation such as *Taxus chinensis* producing paclitaxel, *Dioscorea deltoidea* producing steroidal glycosides, or *Thalictrum minus* producing protoberberine alkaloids (12, 20, 59). Accordingly, metabolome analysis revealed no marked difference between pre- and postcryopreserved *Arabidopsis* T87 cells (87). Furthermore, the ploidy level was not affected, and peroxidase activity and cell responses to hypothermia and osmotic stress were fully restored in *Medicago sativa* cell culture after 27 years of cryopreservation. Cell culture of *Polyscias filicifolia* recovered after 5 years of cryopreservation was successfully scaled up to 630-L bioreactors (161). These and other studies demonstrate the high efficiency of cryopreservation as a reliable method for long-term conservation of cell cultures, which ensures stability of their growth and biosynthetic characteristics, genetic integrity, and the ability for bioreactor cultivation.

CHALLENGES

Discrepancy Between Cryo Research and Cryobanking

The availability of cryopreservation protocols obtained through empirical and fundamental research has not always been translated into the generation of long-term cryopreserved collections. Often cryopreservation research is performed on a limited number of explants, and typically, due to funding or staff limitations or the needs of career development, once the fundamental research is concluded for one species, researchers move on to other species. Sometimes, if enough explants were obtained for the basic research, a few samples may be left stored in LN for the long term, which tends to constitute the core collection for some of these species. Moreover, research is conducted only occasionally in labs with long-term LN storage facilities, and the cryopreserved material cannot be maintained after the researcher moves to another lab.

Cryobanks and Economy

Although many cryopreservation protocols have been developed over the last 60 years for a wide range of species, only a few protocols are used in the few cryobanks that exist. Although the need for cryopreservation is realized in major national (China, Japan, Germany, USA) and international genebanks (CIP, Bioversity International), overall, very few are capable of cryobanking plant genetic resources. With the loss of biodiversity increasing, it is critical to increase the number of species and accessions, especially wild species, preserved in cryobanks. This development is hampered by the high cost of placing plant material into cryostorage. However, the costs of keeping the material in LN are low and cheaper than field maintenance. Furthermore, once the material is in cryostorage, the tanks and temperature stability only have to be checked periodically, which is advantageous in case of pandemics or other exceptional situations that restrict access to the storage area.

Data Security and Standardization

Although the number of active plant cryobanks is limited, the number of cryopreserved accessions is increasing daily. The cryopreserved plant material will be banked for decades or even longer, and it is very unlikely that most accessions will be recovered before the responsible technicians retire. Therefore, standards for hygiene (5) and cryopreservation should be developed to ensure that genetically valuable (unique), high-quality material is preserved under adequate conditions for the maximum length of time and can be regrown as true to type. Here, standard operating procedures must include the documentation of essential information about cryopreservation and rewarming procedures and ensure that mislabeling is avoided, for example, by tracking accessions with barcodes. In addition, information about accessions and storage positions must be securely stored in data management systems and/or ideally stored side by side with the accessions in LN as proposed by the Norwegian company GeneEver (**https://geneever.com**) and currently implemented at IPK.

Duplicate Storage and Storage Security

Very few cryobanks store duplicates or even triplicates because it is very costly to introduce material into cryostorage. However, due to rising costs, strategic decisions may result in the removal of cryopreserved accessions from the field or in vitro collections. This increases the need for higher security standards such as the use of active, base, and safety (A-B-S) replicates to ensure the secure long-term survival of accessions in cryostorage. To date, however, no central backup facilities have been established, as has been done for orthodox seeds at the Svalbard Global Seed Vault, but this will be required in future.

New Technologies and Cryoprotectants

The acceleration of biodiversity loss and the rapid development of biotechnological tools emerging from clinical applications challenge the limited capacities of the small community of plant cryobiologists. Application of developments in the cryopreservation of mammalian cells is hampered by the morphological differences between plant cells and animal or human cells and requires specific testing of new technologies for plants. However, new technologies that modulate warming rates by inhibiting recrystallization and devitrification through laser warming (79), nanowarming with magnetic iron oxide nanoparticles (24), ultrasound-assisted freezing, or magnetic fields affecting the movement of water molecules are of great interest (22). Similarly, the publication of results from studies using ice-inhibition molecules such as IBPs (124), synthetic polymers,

nanomaterials, or hydrogels are highly desirable. However, studies should also include a risk assessment to prevent unwanted effects on the genetic makeup or storability of the accessions.

Robotics

Shoot tips, embryonic axes of recalcitrant-seeded species, and somatic embryogenic callus all require the fine excision of specific plant parts. Well-trained technicians are able to prepare up to 450 uniform and vigorous shoot tips per day, which comprise one or two accessions, depending on the cryobank standards. However, this is a serious limitation in efficiency for all cryobanks. As robots have started transplanting tissue-culture plantlets, it will be interesting to explore the possibility of applying robotics to explant excision to accelerate the process of cryobanking.

Longevity of Cryopreserved Explants

To date, there are only a limited number of studies on the long-term viability of plant material stored in LN. Because ultralow temperatures minimize or stop metabolism, and thus ageing processes, ionizing background radiation or cosmic rays are speculated to be the only rare cause of radical formation and macromolecule damage (73). However, it is likely that molecules move or vibrate to some degree under ultralow temperatures, and ageing processes may occur slowly, as noted in dry seeds, pollen, and fern spores stored in LN (10). To evaluate these effects on explant viability, long-term storage experiments are required to control viability over an extended period.

Potential of Endophytes

For a long time, in vitro cultures were believed to be free of microbes. Nowadays, it is known that endophytes are common and can affect the growth pattern of their host plants. Depending on environmental conditions, some of these microbes are able to switch between parasitism, commensalism, and mutualism. Often, unfavorable microbes reduce the survival of explants after cryopreservation (5). However, the effect of mutualistic endophytes has not yet been investigated and may open the possibility of increasing survival.

Systematic Storage of Fundamental and Cryopreservation Data

Cryopreservation success depends on knowledge of the (epi)genetic, molecular, and biochemical processes during different (pre)treatment steps. Therefore, there is a general call to gather more fundamental knowledge about molecular processes, and this information should be systematically stored together with all relevant treatment data and regrowth results. As cryopreservation is complex and time consuming, it needs to be possible to predict a cryopreservation protocol for new species, lines, and organs based on the available knowledge for other such specimens. To enable this prediction, the global cryobiology community needs to initiate large-scale experiments guided by statisticians and machine learning to explore key molecular pathways for various tissues. This knowledge could enable predictions about the probability that a specific protocol will be successful in a given species.

SUMMARY POINTS

1. At temperatures below the freezing point, plants can be damaged or die due to nucleation of water molecules and their expansion, with intracellular ice formation being particularly harmful.
2. Plants in nature protect their tissues by cold acclimation, including changes in membrane and cell wall composition and by supercooling to approximately −3°C, which facilitates

cellular dehydration and increases in cellular solute composition, leading to vitrification once temperatures drop further.

3. For the past 60 years, basic principles to increase the concentration of cellular solutes, that is, air drying, metabolic adjustment, freezing and osmotic dehydration, and permeation of molecules, have been used to develop a number of efficient cryopreservation protocols; most of these focus on freezing dormant buds and PVS2 droplet vitrification of shoot tips, but only a few are implemented at large scale for species banking.
4. The largest cryobanks use established protocols for storing agri-/horticultural species, for example, potatoes, bananas, apples, and garlic, among others, at genetic resource centers such as CIP (Peru), Bioversity International (Belgium), USDA (USA), IPK (Germany), and NAAS (South Korea).
5. Thousands of wild species have yet to be cryopreserved, but few protocols have been developed. Cryobanking of wild species has not been used on a large scale but has been demonstrated at a few locations, such as Kings Park and Botanic Garden (Australia), Cincinnati Zoo & Botanical Garden (USA), and the Royal Botanic Gardens, Kew (United Kingdom).
6. Cryopreservation of embryonic axes avoids prolonged tissue culture procedures and provides an easy way to store nonorthodox seed species, e.g., for *Citrus*, *Coffea*, and *Cocos* species; some have been stored at NBPGR (India) and CATIE (Costa Rica).
7. Important cell cultures used as model systems or for biotechnological production of heterologous proteins and plant bioactive compounds require cryopreservation, usually by slow cooling, but few lines are successfully conserved because development is hindered due to high sensitivity of cultured undifferentiated cells to osmotic and freezing injury and the need for genotype-specific modifications for cryopreservation.
8. More basic research is needed to develop widely applicable protocols to increase the number of species cryopreserved in the world's cryobanks; in parallel, new technological tools and cryoprotectants must be explored and standards developed to safely safeguard our plant genetic diversity.

FUTURE ISSUES

1. Do new biotechnological tools from clinical research, that is, nanomaterials, polymers, and robots, have the capability to revolutionize plant cryopreservation and speed up the banking of agricultural and wild species?
2. The size of biological materials is a serious limitation at the moment; is it realistic to reach the higher cooling and rewarming rates essential to obtaining a vitrified intracellular liquid and to prevent lethal ice crystal formation during cryopreservation of tissues, such as shoot tips or somatic embryos, of all sizes?
3. Molecular processes underlying stress adaptation in plants are complex and tissue specific. With the new advances in omics technologies such as proteomics, methylome profiling, transcriptomics, and chromatin immunoprecipitation sequencing, could we identify specific molecular markers that can be used to predict the sensitivity or tolerance of plants to cryopreservation?

4. Will plant cryopreservation allow us to minimize the costs and land necessary for the maintenance of fruit and timber tree species in traditional clonal-orchard germplasm banks?
5. After surviving exposure to liquid nitrogen temperatures, is life maintained forever during cryogenic storage?
6. Cryopreservation protocols are very specific; will it be possible to develop widely applicable protocols based on a systematic evaluation of material sensitivity to osmotic, temperature, and chemical stresses?
7. How can the successes achieved by fundamental research on plant cryopreservation be efficiently translated into the implementation of plant genetic resource conservation?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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