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Cryopreservation of plant reproductive structures
Kryoprezervace reprodukčních struktur rostlin

BAKALÁŘSKÁ PRÁCE

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Poděkování:

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Prohlášení:

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Podpis:

Abstract

Cryopreservation is one of the methods to preserve plant tissue cultures, shoot-tips, seeds, pollen and other types of explants from plants. The conservation is important mainly for plant breeding and for the protection of rare and endangered species. It is a preservation of explants at very low temperatures around -196 °C, mostly using liquid nitrogen. This method includes many procedures such as slow cooling, vitrification, and vitrification-based procedures. Before cooling, samples are dehydrated to avoid the formation of intracellular ice during cooling and subsequent damage to the cell. For samples that are dehydration-sensitive, the use of cryopreservatives is required. This work is mainly devoted to the cryopreservation of seeds and pollen and its issues. Desiccation-tolerant and desiccation-sensitive seeds and pollen grains require specific cryopreservation protocols which have to be developed for each species. Furthermore, some plants do not produce seeds or they are reproduced mainly vegetatively and, thus, require specific preservation procedures.

Keywords

plants, pollen, seeds, cryopreservation

Abstrakt

Kryoprezervace je jednou z metod uchovávání kultur rostlinných pletiv, špiček výhonků, semen, pylu a dalších typů rostlinných explantátů. Konzervace je důležitá hlavně pro šlechtění rostlin a pro ochranu vzácných a ohrožených druhů. Jedná se o uchovávání explantátů při velmi nízkých teplotách okolo -196 °C většinou za použití tekutého dusíku. Tato metoda zahrnuje spoustu postupů jako je pomalé chlazení, vitrifikace a na vitrifikaci založené postupy. Před samotným chlazením vzorky musí být dostatečně dehydratované, aby nedošlo k tvorbě intracelulárního ledu a následnému poškození buňky. U vzorků, které nemůžou být dehydratovány tolik, je zapotřebí použití kryoprezervantů. Tato práce se věnuje především problematikou kryoprezervace semen a pylu. Existují více a méně k vysychání tolerantní semena a pylová zrna, proto musí být vyvinuty specifické kryoprezervační protokoly pro jednotlivé druhy. Další překážkou je, že některé rostliny semena netvoří nebo se množí převážně vegetativně. Jejich uchovávání proto vyžaduje jiné postupy.

Klíčová slova

rostliny, pyl, semena, kryoprezervace

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Introduction

Conservation of plants for various purposes like agriculture and forestry management has been done since late 18th century and it is considered that the conservation movement has its beginning in 1662 in book "Sylva or a discourse on forest trees and the propagation of timber in His Majesty's dominions" (Fig. 1) written by John Evelyn (Mason 2018). These days different methods are used to preserve plant specimens. However, one of the most efficient long-term methods of conservation is cryopreservation. Cryopreservation is a technique using very low temperatures and the samples are kept in the presence of liquid nitrogen (LN). Like this, the explant can be preserved for many years. However, some factors make this method difficult, especially a low tolerance to drying, because species have to have low water content (WC) when they are frozen to avoid the formation of intracellular ice. Thus, many samples have to be dehydrated first. Nevertheless, cryopreservation is used for preservation of many species and its procedures are constantly improved.

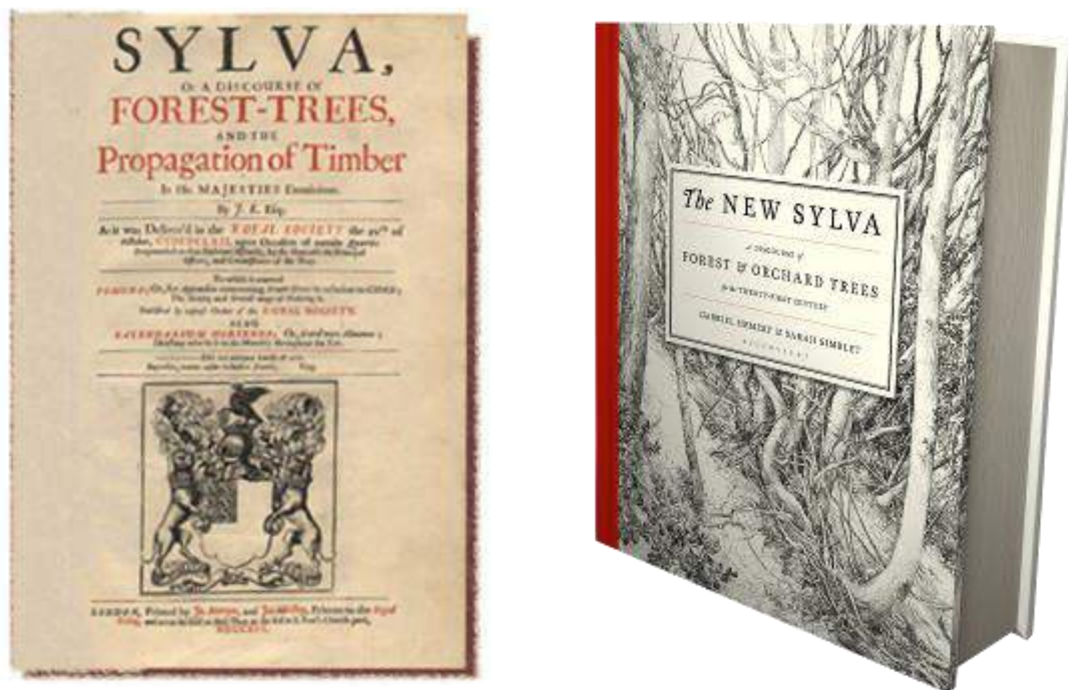

Fig. 1 – Sylva or a discourse on forest trees and the propagation of timber in His Majesty's dominions ('Sylva Foundation' n.d.)

1. Preservation of plants in Nature

All living organisms are dependent on water. Thus, the removal of intracellular water by drying or freezing from plant tissues and cells can cause osmotic stress, membrane disruption, and conformational changes resulting in disruption of ultrastructure and metabolism, as well as protein denaturation (Wolkers et al. 2021; Alpert 2000; Hoekstra et al. 2001). Nevertheless, some organisms like certain species of plants (Cawston 1929), yeasts (Rhodes 1950), fungal spores (Rhodes 1950), nematodes (Demeure et al. 1979), rotifers (Gilbert 1974), tardigrades (Wright 1989), prokaryotes (Rhodes et al. 1950), and crustaceans (Hengherr et al. 2008) have adapted to dry conditions and are able to survive in a dry state, where they can lose up to 99 % of water (Alpert 2000). These species are called "desiccation-tolerant" and they developed various mechanisms such as prevention of biochemical damage associated with desiccation or its repair according to the presence or absence of the bulk water (Alpert 2000), that allow the preservation of cellular structures under water shortage (Wolkers et al. 2021; Hoekstra et al. 2001; Crowe et al. 1992). This condition of surviving dryness and regaining normal function after rehydration is called anhydrobiosis. Some organisms can persist in it for decades (Crowe et al. 1992).

1.1. Types of tolerance to low water content

Two types of tolerance can be distinguished according to the critical WC in the cell (Hoekstra et al. 2001). The critical WC is a water level below which cells are damaged. This level can differ due to the chemical composition and structure of the cell (Walters et al. 2007). The first type is „drought tolerance", which is the tolerance of mild dehydration when no more volumetric cytoplasmic water is present in the cell. The second type is a „desiccation tolerance", which accepts further dehydration, gradually losing the hydration shell of the molecules. This type of tolerance involves the ability of cells to rehydrate upon re-access to water (Hoekstra et al. 2001).

However, species that are dehydrated in a relatively inactive phase of the life cycle, such as spores or seeds in dormancy, which is state preventing germination in unsuitable conditions leading to low probability of survival (Baskin and Baskin 2004), are not referred to as „desiccation-tolerant" species, but this name refers to species able to dehydrate in the adult active form, that means plants in vegetative stage of life (Alpert 2000). Today, many plant species are known to be able to tolerate dehydration in their active adult life cycle. Nevertheless,

tolerance of dehydration in the active stages of plant life is very rare. In contrast, desiccation tolerance is common among seeds and spores (Alpert 2000).

1.2. Desiccation-tolerant plants

Desiccation-tolerant organisms include mosses and lichens, which are the most common groups of desiccation-tolerant plants, ferns, very few flowering plants among which are five families of monocotyledons (*Boryaceae*, *Cyperaceae*, *Poaceae*, *Schizaeaceae*, and *Velloziaceae*) and three families of dicotyledons (*Gesneriaceae*, *Myrothamnaceae* and *Scrophulariaceae*), but no gymnosperms or trees. It appears that drying tolerance is more common in monocotyledonous plants than in dicotyledonous plants (Alpert 2000).

Dehydration-tolerant plants grow on all continents and occur in a large variety of taxa. However, they are very divers in morphology, areas of occurrence, and growth forms (Alpert 2000). Desiccation-tolerant plants grow in dry environment whereas desiccation-sensitive species do not grow in extremely dry habitats. However, species that withstand drought the most do not grow in the driest environment (Alpert 2000). Desiccation tolerance is lacking in gymnosperms, although they may have desiccation-tolerant pollen or seeds (Alpert 2000).

Most plants can tolerate dehydration at least in the inactive stage of the life cycle. There is also a significant difference in species diversity within taxa (Alpert 2000).

1.3. Damage through dehydration and tolerance mechanisms

Dehydration tolerance includes cellular mechanisms ensuring the prevention of biochemical damage associated with desiccation or its repair according to the presence or absence of the bulk water (Alpert 2000).

Biochemical damage includes oxidative damage caused by the accumulation of reactive oxygen species (ROS), which is most likely the result of metabolic imbalance (Alpert 2000). This dangerous situation is caused by reactions of plants to extreme environments. Active oxygen can be generated by exogenous toxic chemicals, physical factors influencing the plant metabolism, or other factors distorting the plant metabolism (Smirnoff 1993).

ROS can cause a lot of types of damage like denaturation of proteins, damage to nucleic acids and lipid peroxidation, which is connected with decomposition of lipids and damage to membrane function (Smirnoff 1993). Desiccation has its negative role when it distorts plant metabolism and cell structures and after that enzymatic catalytic reactions are stopped (Smirnoff 1993).

However, plants are equipped with protecting and repairing systems of two types. The first type of system reacts with ROS and keeps them on a low level. For example, superoxide dismutase (SOD), catalase, peroxidases, ascorbate, oc-tocopherol belong here. The second type of system includes glutathione (GSH), glutathione reductase (GR), ascorbate, mono- and dehydroascorbate reductases which help to regenerate oxidated antioxidants (Smirnoff 1993).

The second type of damage is the disruption of ultrastructure and metabolism caused by changes in the configuration of macromolecules and the disruption of membranes. The changes are caused by water shortages, so there can be no interaction with hydrophilic regions (Alpert 2000). Mechanisms preventing conformational changes during dehydration include the accumulation of protective proteins or nonreducing sugars (Alpert 2000). Therefore, organisms capable of anhydrobiosis commonly contain high concentrations of disaccharides, mainly trehalose (Alpert 2000; Crowe et al. 1992).

Desiccation tolerance can be triggered by dehydration and a plant hormone called abscisic acid. Mild dehydration in the vegetative tissues of green plants can lead to the launch of gene expression associated with desiccation tolerance (Hoekstra et al. 2001).

1.4. Seed desiccation tolerance

A lot of seeds are desiccation-tolerant to at least at certain level of WC (Towill 2002). As told above, mild dehydration can be responsible for the initiation of gene expression associated with desiccation tolerance (Hoekstra et al. 2001). This genes are present in orthodox seeds during maturation and make the seed embryos desiccation-tolerant (Hoekstra et al. 2001). The accumulation of dry matter is responsible for this reduction in the amount of water. Abscisic acid triggers a desiccation tolerance program in developing seeds while inhibiting premature germination. Premature, slow drying can also affect the development of seed desiccation tolerance. This can lead to the development of small orthodox embryos (Hoekstra et al. 2001).

2. The importance of plant conservation

The preservation of germplasm is important for plant breeding, the food industry, medicinal and forest crops, but also for the protection of rare and endangered species (Towill 2002). Further it is important for the preservation of plants that are multiplied vegetatively or have non-viable, recalcitrant or intermediate seeds (Pritchard 1995). The aim of preserving germplasm is to improve agricultural productivity thanks to discovery of the genetic bases of varietal storage ability, and preserve the genetic variability of plant species with the maximum possible genetic integrity (Finkeldey et al. 1993). Perhaps the most efficient way of long-term conservation of germplasm is storage in LN (Chin et al. 1989), but there are other methods like *in vitro* storage of cultured meristems, shoot-tips or plantlets, *in vitro* minimal growth storage or low temperature storage of seeds. However, these methods are just for short-term or medium-term storage for several month up to 2,5 years (Kartha 1985).

Cryopreservation is a method to preserve biological material at very low temperatures using LN (-196 °C) or its vapor phase (-160 °C) (Benelli 2021). At these temperatures, all cell divisions and metabolism are theoretically stopped, so the sample can be stored for a long time. The advantage of this conservation is that it does not take up much space (Engelmann 2004) in comparison to conservation in botanical garden or nature reservation (Engelmann 2000). Cryopreservation is an effective alternative way to store recalcitrant seeds and vegetatively propagated species compared to field and *in vitro* storage (Engelmann 2004). Other subjects for cryopreservation are somatic embryos, pollen and dormant buds. Cryopreservation can also be used to eliminate viruses from infected plants (Towill 2002).

3. Cryopreservation methods

In situ or *ex situ* preservation can be used to protect or preserve plants. *In situ* conservation involves maintaining selected species in their natural habitat, in parks, or nature reservations (Engelmann 2000). It allows plants to evolve and build, for example, resistance to diseases, but it is not suitable for domesticated lines (Towill 2002). However, *in situ* protection has many disadvantages, such as physiological, abiotic, and biotic stress, which can cause plant death (Towill 2002), high maintenance costs, and the impact of pathogens, pests, and natural disasters (Engelmann 2004) *Ex situ* conservation is the conservation of plant species outside their natural habitat. It includes seed and gene banks, often with usage of cryopreservation, and botanical gardens (Engelmann 2000). *Ex situ* conservation is used to preserve many endangered as well as commercially used plant species. Seeds and vegetative clones are the most common materials stored *ex situ*, for example by cryopreservation or by conventional storage (Towill 2002).

Cryopreservation is conservation of specimens at very low temperatures. But first, water has to be removed from the samples, because it could freeze and cause damage to the explant by forming ice crystals (Benelli 2021). There are two basic cryopreservation techniques, freeze-induced dehydration and solute vitrification. Both, freeze-induced dehydration and solute vitrification have several steps including sample pre-cultivation, cryoprotection, and subsequent regeneration (Engelmann 2004).

The success of cryopreservation is measured as survival, the state when the specimen remains viable (U.S. National Library of Medicine), and regeneration, a state when specimen have active repair mechanisms and resume normal physiological functions ('ScienceDirect Topics' n.d.). Survival and regeneration is influenced by methodological factors like the individual stages of cryopreservation (dehydration, cooling and warming) as well as biological factors like developmental stage, dehydration tolerance, and material size (Mazur et al. 1984).

Usually, plants grown *in vitro* are used because they are already in an aseptic state (Mazur et al. 1984). For higher rates of regenerated plants, cryopreservation techniques and their protocols are constantly improved (Mazur et al. 1984). When a new protocol is developed, it is necessary to pay attention to the selection of a suitable method of dehydration (air, cooling, osmotic dehydration), the type of cryopreservation (cooling, vitrification), and the usage of CPAs (none, single, combined) depending on the cryopreserved material (Faltus et al. 2021).

3.1. Classic cryopreservation – slow cooling

The oldest cryopreservation method was developed in 1970s (Kaviani 2011) and its first protocols are based on freeze-induced dehydration. This procedure involves many variables such as the substances applied, for example cryoprotectant solutions, the kinetics of exposure, and the rate of cooling and warming. A cryoprotectant is often added to protect the sample from damage through ice crystals formations (Towill 2002).

Before cooling, the sample is slowly cooled at a certain rate to a predefined temperature (approximately -40 °C) with the help of a programmable freezer (Fig. 2) and then immersed in LN (Towill 2002).

During cooling to a temperature of about 0 °C, the cell and the external medium undergo a state of hypothermia, a state when the temperature of cell is abnormally low, and extracellular ice begins to form in the medium. However, the ice does not get into the cell, because the cell wall and the cytoplasmic membrane work as a barrier (Engelmann 2004), so the inside of the cells remains hypothermic, but not frozen. As the temperature decreases further, the extracellular solution turns more and more into ice and the intracellular solutes are become concentrated. The cells remain in a hypothermic state. The pressure of water vapor in the cells is higher than the pressure in the extracellular environment. The difference is compensated by loss of water (Engelmann 2004).

Cells have to lose water to prevent intracellular ice formation, which would cause the death of the cell. However, the rate of water removal is influenced by several factors, including, for example, the temperature dependence of water permeability Lp. If the cooling rate is slow, solutes become concentrated inside the cell due to the loss of water from the cytoplasm. Thus, the balance between the chemical potential of intra- and extracellular water is maintained. The cell dehydrates and does not freeze. However, if the cooling rate is too fast, most of the water remains inside the cell, leading to intracellular ice crystal formation and cell death (Mazur et al. 1984). However, dehydration must not be too intense, so the cell is not damaged due to the high concentration of intracellular salts and changes in the cytoplasmic membrane (Engelmann 2004).

After the dehydration, the cell is immersed in LN. This step is the most critical point for cell survival depending on the avoidance of intracellular ice formation. Subsequently, the sample is stored in a special cooling box. During warming, recrystallization of the ice, in which the ice melts and turns into larger crystals, must be avoided. Therefore, it is necessary to warm

up the sample as quickly as possible to prevent this situation from happening (Engelmann 2004).

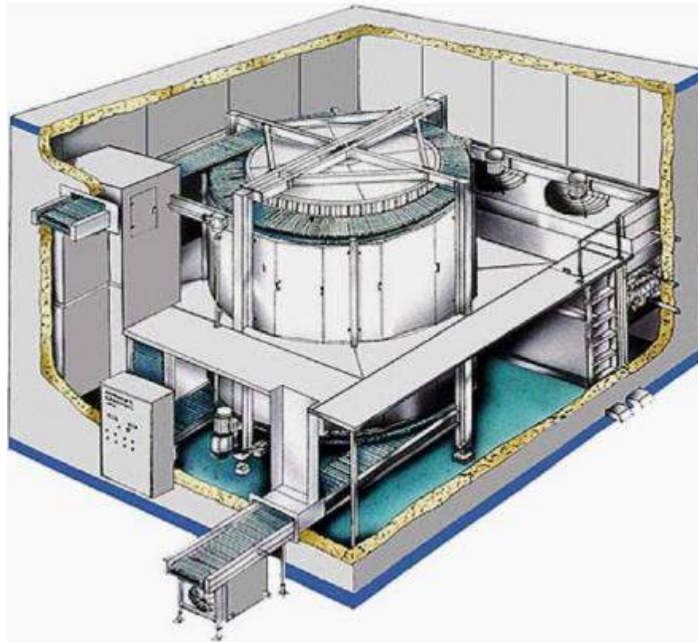

Fig. 2 – cryo-freezer (Khadatkar et al. 2004)

3.2. Encapsulation-dehydration

Encapsulation-dehydration is based on encapsulating the sample into gel capsules with sodium alginate (Mazur et al. 1984). Samples are pre-cultured in a high sucrose medium for about 1-7 days, dried to about 20 % of its fresh weight in an air stream with laminar flow, a fume hood, or with the help of silica gel, and frozen by immersion in LN. Warming occurs at a slow speed (Engelmann 2004).

This method guarantees a high probability of survival of the sample and there is a rapid recovery of the growth of the explant without callus formation (Engelmann 2004). Compared to the classical method, it has significant advantages, such as easy manipulation of small samples, simplification of cryoprotective media, no requirement of programmable freezers, and a larger number of samples survived exposure to LN. Also, the structure of the sample is more resistant thanks to its encapsulation inside the capsule (Mazur et al. 1984).

3.3. Vitrification

Vitrification has been used for a long time as well, since 1990, and was developed by Sakai and co-workers (Matsumoto 2017). It has become one of the main methods of cryopreservation

due to its applicability to many types of plants. The principle of the method is the formation of a glass structure, when water is transformed from the liquid phase directly into the amorphous phase, thus creating an unstable, glassy, solid state (Engelmann 2000). This prevents the formation of intracellular ice, the samples are not damaged and remain viable at temperature of -196 °C (Benelli 2021).

The vitrification procedure consists of a pre-cultivation of samples on a medium with cryoprotectants, an addition of loading solution, dehydration with highly concentrated vitrification solution or airflow, a rapid cooling, a subsequent warming, a removal of cryoprotectants, and a regeneration (Engelmann 2004). First of all, explants are pre-grown in a medium with sucrose and then treated with a loading solution, which is a mixture of sucrose and glycerol (O'Brien et al. 2021). Here, the water changes from a liquid state to an amorphous glassy phase, and a supersaturated highly viscous solution is formed, which, although no crystalline structure is formed, has the mechanical properties of a solid. It is not caused by chemical changes, but by physical changes in the viscosity of the liquid. By dehydrating the sample, vitrification of the cytoplasm occurs, and no freezable water remains in the cell. In this state, the sample can be immersed in LN. Dehydration is the most critical step in this method. If the sample is successfully dried without affecting its survival, the probability of sample survival after rewarming is very high (Engelmann 2004).

Dehydration is done with the usage of airflow or a highly concentrated solution of cryoprotective substances (DMSO, EG, methanol, glycerol, propylene glycol, and sugars) (Towill 2002), that is a so-called vitrification solution such as PVS2 or PVS3 (O'Brien et al. 2021). However, the disadvantage of this solution is that cryoprotectants can cause cytotoxicity and osmotic stress, which could lead to cell death or morphogenetic response in the culture (Engelmann 2000). Therefore, modified vitrification protocols are used to reduce the toxicity of the vitrification solution (Engelmann 2004).

Vitrification has many advantages compared to the classic dehydration technique. Among them belong a possibility of rapid cooling of the sample by immersion into the LN without the need for programmable freezers, an exclusion of the formation of intracellular ice due to the high speed when ice crystals do not have time to form, less complexity, and usability for many different complex plant organs and different types of cells with only minor modifications to the methods according to individual sample requirements (Engelmann 2004). During vitrification, the diffusion of substrate and products in the cell is also limited, so the cell remains metabolically quiescent, and diffusion-dependent chemical reactions do not occur. The glassy

state of the dehydrated cell also limits the loss of water and the crystallization of salts and proteins in the cytoplasm, protects the cell from pH changes during water removal. Thus, it ensures the stability of the cell when it is metabolically inactive and prevents cell death (Santos 2000).

Due to the success of this cryopreservation method, many methods based on vitrification have been developed (Tab. 1). They are encapsulation-dehydration, encapsulation-vitrification, dehydration, pre-growth, pre-growth-dehydration, droplet freezing (Engelmann 2004), and also more recent D- and V-cryo-plates (Benelli 2021).

3.4. Vitrification-based methods

3.4.1. Dehydration

This technique has one of the simpliest procedures. It only consists of dehydrating the sample and then its rapid cooling by immersion it in LN. Dehydration is achieved by the using a laminar flow air stream, sterile compressed air, or silica gel (Engelmann 2004). Dehydration using a stream of sterile compressed air is very fast enables cooling of a sample that initially contained a relatively large amount of water. By dehydration using the vitrification solution, it is possible to remove water from the intracellular space of the cells and transform the cytoplasmatic solution of a cell to an amorphous phase after the sample is rapidly frozen. Mixtures of cryoprotective substances, for example, PVS2 or PVS3, are mostly used as vitrification solutions. This prevents damage caused by over-drying. Ideally, the sample is frozen at about 10-20 % of the fresh weight (O'Brien et al. 2021). This method is used mainly with zygotic embryos or embryonic axes extracted from seeds (Engelmann 2004).

3.4.2. Encapsulation–vitrification

Encapsulation-vitrification is a technique based on a combination of encapsulation-dehydration and vitrification (Engelmann 2004). The samples are placed into alginate capsules and subsequently dehydrated in PVS solutions, for example in PVS2 or PVS3 solution, or by immersion in LN and frozen by the vitrification method. Although encapsulating is tedious, it helps to handling the samples (usually apices of a lot of plant species (Engelmann 2004)) (O'Brien et al. 2021). This method is much faster than encapsulation-dehydration because here is no time spent on air desiccation (Sakai et al. 2008)

3.4.3. Pre-growth

This method is based on the cultivation of the sample in the presence of defined solutions with cryoprotectants (Engelmann 2004). This will increase the likelihood of the sample, for example meristematic cultures (Engelmann 2004), surviving subsequent cooling by immersion in LN. It is also hypothesized that growing a sample in sugar may lead to a decrease in cell WC while simultaneously the intracellular concentration of sugars is increased. The sugars protect the membranes from drying out and help in the formation of glass during cooling (Towill 2002).

3.4.4. Pre-growth-dehydration

Pre-growth-dehydration is, like pre-growth, a method based on sample pre-cultivation using cryoprotectants. However, after that, it is not immediately immersed into LN, but dehydrated with a laminar airflow or silica gel (Engelmann 2004). Optimal conditions may vary depending on the type of plant (O'Brien et al. 2021). This method has been used to asparagus stem segments, oil palm polyembryonic cultures, and coconut zygotic embryos (Engelmann 2004).

3.4.5. Droplet freezing

During droplet freezing the samples (mostly apices) are first treated with a loading solution (Engelmann 2004) consisting of glycerol and sucrose (O'Brien et al. 2021) and then with a vitrification solution (PVS2 or PVS3) (Engelmann 2004; O'Brien et al. 2021). Then the samples are transferred on aluminium foil in small drops of the solution and a drop of cryoprotectants is added before cooling followed by rapid cooling in LN. The aluminium foil conducts heat well, which is also helpful in the warming of the sample. During warming, the cryoprotectant is removed by using an unloading solution with high sucrose content. Finally, the sample is transferred to the regeneration medium (O'Brien et al. 2021).

In this procedure, highly concentrated vitrification solutions are used for both rapid cooling and rapid warming. Sufficient dehydration using a vitrification solution is necessary for the survival of the sample so that it can be quickly frozen with LN(O'Brien et al. 2021). This method was used to asparagus, potato and apple apices (Engelmann 2004).

3.4.6. Cryo-plate methods

Cryo-plate methods are one of the newest vitrification-based cryogenic methods developed by Yamamoto (2011). Special aluminium plates with holes, V-cryo-plates and D-cryo-plates

are used here (O'Brien et al. 2021). The technique is based on vitrification dehydration of samples. For pre-culture of shoot tips a PVS2 solution (V-cryo-plates) is used or samples are dehydrated with laminar airflow or silica gel (D-cryo-plates) (Matsumoto 2017). A solution containing sodium alginate and sucrose is poured into the holes of the cryo-plate, samples are transferred into them, and covered with alginate solution. After that, samples in the plates are immersed in LN (O'Brien et al. 2021). Samples are placed in a sucrose solution for rapid warming, and transferred to the culture medium. The advantages of this method over slow cooling are easy and fast handling of the samples and rapid cooling, warming, and regrowth of the sample (Matsumoto 2017).

Tab. 1 – Some examples of cryopreservation method and applications adapted from (O'Brien et al. 2021)

Method	Application	Survival	Reference
Vitrification	Cocoa secondary somatic embryos	74.5 %	(Adu-Gyamfi et al. 2012)
Droplet-vitrification	*Hancornia speciosa* Gomes (rubber tree) shoot-tips	43 %	(de Oliveira Prudente et al. 2017)
Encapsulation-vitrification	*Olea europaea* (olive) somatic embryos	64 %	(Shibli et al. 2000)
Encapsulation-dehydration	*Prunus armeniaca* (apricot) shoots	40 %	(Soliman 2013)
Dehydration	*Juglans nigra* (walnut) embryo axes	100 %	(Ballesteros et al. 2019)
Pre-growth and Pre-growth-dehydration	*Garcinia mangostana L.* (mangosteen) shoot-tips	50 %	(Ibrahim et al. 2013)
V-cryoplate	*Morus alba* (mulberry) shoot-tips	87 %	(Yamamoto et al. 2012)
D-cryoplate	*Diospyros kaki* (persimmon) shoot-tips	87 %	(Matsumoto et al. 2015) **(Soliman 2013)**

4. Cryoprotective Agents

Cryoprotectants, or cryoprotective agents (CPAs) are substances that are used to support plant survival during cryopreservation (Elliott et al. 2017). However, after warming, the substance has to be removed again (Towill 2002). CPAs are used mainly in vitrification methods (droplet freezing, vitrification, encapsulation-vitrification), but also in slow-cooling (Faltus et al. 2021), because they prevent osmotically caused damage (Elliott et al. 2017). "Osmosis is the movement of water from a region of high water concentration or vapor pressure to an area of low water concentration or vapor pressure" (Fahy et al. 2015). This movement is important especially during vitrification methods when high concentrations of CPAs are used, which reduce the water concentration (Fahy et al. 2015). CPAs induce a glassy state in the cell by dehydrating the sample through binding water and, thus, prevent water crystallization during rapid cooling or rewarming (Faltus et al. 2021). CPAs are important especially for samples with a low tolerance to dehydration (Meryman 1971).

CPAs can be divided into non-penetrating substances and penetrating substances. The low molar concentration of non-penetrating CPAs (npCPAs) protects the sample during rapid cooling or warming (Meryman 1971). They operate outside the cells and osmotically dehydrate protoplasts. Penetrating CPAs (pCPAs) also have an osmotic effect on the cell (Faltus et al. 2021) and protect the cell from damage during slow cooling at their multimolar concentration (Meryman 1971). Penetrating CPAs have the colligative characteristic that makes them able to reduce formed ice (Meryman 1971). It is important for pCPA to enter the cell and to be present in the cell in a suitable concentration, otherwise CPA becomes dangerous to the cell – if it remain outside the cell, the cell will be osmotically dehydrated, which looks similar to cooling damage, or if the CPA has not an optimal concentration, it can be toxic to the cell (Faltus et al. 2021). Penetrating and non-penetrating CPAs have a different mechanism of protection. Penetrating CPAs help to create suitable conditions in the cell for reduction of WC at low temperatures reducing the damaging effect of concentrated intracellular solutes. Non-penetrating CPAs osmotically push the water out of the cell on the beginning of cooling at temperatures of about -20 °C while these npCPAs concentrate outside the cell (Fig. 3) (McGann 1978).

However, cells cannot be put in pCPAs alone because they would not be able to maintain their volume, pH, ion content or membrane integrity. Therefore, they are placed in a physiologically supportive medium, the so-called "carrier solution", where the CPAs, necessary for vitrification, are dissolved. This solution helps transport pCPAs into the cells and thus

prevents injury that is not related to CPAs. However, it is also important for cell survival without added CPAs. Also, the composition of the solutions can differ a lot (Fahy et al. 2015).

In order for both, pCPAs and npCPAs, to be sufficiently effective, they must be miscible or soluble in water, should not be in a toxic concentration and must withstand low temperatures (Fahy et al. 2015).

The carrier solution plays an important role in preventing the formation of ice inside the cell during cooling and may also influence the toxicity of CPAs (Fahy et al. 2015).

As said above, penetrating CPAs are toxic to plant cells when they are in incorrect concentration. Although cryopreservation protocols using CPAs are becoming more common and popular, they are not easy to be developed because they are formed empirically by testing the effects of CPAs on various samples (Faltus et al. 2021).

Cryoprotectants can also be divided into combined CPAs, such as PVS2 and PVS3, and single CPAs, which include ethylene glycol (EG), dimethyl sulfoxide (DMSO), glycerol, and sucrose (Faltus et al. 2021). DMSO, EG, and sucrose are commonly used in plants and are often combined with polyethylene glycol, proline, or glycine betaine. A plant cell is completely permeable to DMSO and EG, less permeable to proline and glycine betaine, and impermeable or only a little bit permeable to sucrose, glycerol, and polymers (Towill 2002).

Sugars, especially sucrose, trehalose, and glucose, are often used as cryoprotectants (Crowe et al. 1992), which serve as great vitrifying agents and are non-toxic to plant cells. In contrast to traditional cryoprotectants like DMSO, sugars play an important role in stabilizing membranes during cooling. Sugars can also act as external osmotic agents, removing excess water from the cell through an osmotic gradient (Walters 2015).

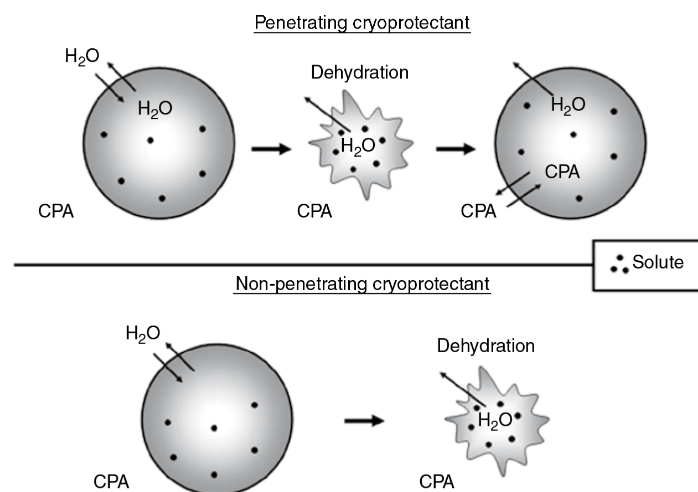

Fig. 3 – Function of which penetrating and non-penetrating cryoprotectants (CPAs) (Swain and Smith 2010)

5. Cryopreservation of seeds

Seed storage is probably the most efficient method of conserving plant resources due to its low cost and easy seed distribution. The seeds can be stored in the traditional "conventional" way at -18 °C or in a cryogenic way (Pritchard 1995). Criteria like cost of seed regeneration, viability at current storage temperature, seed size, and survival of LN exposure determine whether a method is suitable for seed (Towill 2002). Therefore, special protocols, storage techniques, and storage environments for different types of seeds had to be developed or modified (Chin et al. 1989).

There are 3 types of seeds based on the reactions of the seeds at full maturity: orthodox seeds which are tolerant to desiccation, recalcitrant seeds which are sensitive to desiccation, and intermediate seeds (Towill 2002; Walters 2015). Orthodox seeds are characterized by their ability to dry down to 5 % of fresh weight without loss of viability and they can be stored in this way for a long time at a temperature of about -18 °C (Pritchard 1995).

Orthodox seeds are mostly seeds of annual plant species growing in open fields and are present in agricultural crops like lettuce (*Lactuca sativa*), onion (*Allium cepa*), corn (*Zea mays*), tomato (*Lycopersicum esculentum*), peanut (*Arachis hypogeae*) and others (Walters et al. 2004), They are often smaller with low WC of about 30-50 % of fresh weight at physiological maturity when the seed achieves the maximum viability. After physiological maturity, they undergo drying, when the WC drops to 15-20 %. They have embryos that make up about 1,4 % of dry matter of the whole seed and their WC is constant. The embryos contain more moisture in comparison to the whole seed and can also be easily dried to a lower WC due to their smaller size (Chin et al. 1989). For orthodox seeds, cryopreservation may not have more advantage over conventional storage, due to their high probability of surviving dehydration (Pritchard 1995).

Recalcitrant seeds are mainly seeds of representatives of perennial trees from humid tropics (*Mangifera indica*, *Hancornia speciosa*, *Agathis robusta*, *Durio zibethinus*, *Dipterocarpus spp*., *Hevea brasiliensis*, *Nephelium lappaceum*, *Vochysia honurensis* (Farnsworth 2000)), temperate zone (*Acer spp*., *Castanea dentata*, *Lithocarpus densiflorus*, *Quercus spp*. (Farnsworth 2000)), and also of some aquatic plant species (*Ceratophyllum sp*., *Griselinia spp.*, *Crinum capense*, *Hymenocallis spp*., *Nymphaea sp.* (Farnsworth 2000)). Plants with recalcitrant seeds include also species of many economically important trees and shrubs from tropical and subtropical areas (*Elaeis oleifera Cortes*, *Cocos nucifera*, *Hevea brasiliensis M. Arg.*, *Theobroma cacao L.*) (Santos 2000).

The seeds are often larger and heavier compared to orthodox seeds, which is why they are not able to dehydrate as much as orthodox seeds and their WC at physiological maturity is 50-70 %. However, at physiological maturity, they do not undergo drying processes as in the case of orthodox seeds. Although recalcitrant seeds are usually large, their embryos make up only about 0,25 % of the dry matter of the whole seed, which is a much smaller ratio to the whole seed compared to orthodox seeds. Due to their high WC, they are very sensitive to drying and ice damage (Chin et al. 1989). Recalcitrant seeds would be damaged and would lose viability if they would be stored under the same conditions like orthodox seeds. They must be stored at least at 30 % WC of their fresh weight. Due to the high WC of the seeds damage is usually caused by the formation of ice in the sample at low temperatures (Chin et al. 1989). For this reason, they must be protected by cryoprotectants and also stored cryogenically with LN (Walters 2015).

Intermediate seeds have the properties of both orthodox and recalcitrant seeds, they can be dehydrated to relatively low WC, but temperatures below zero are already dangerous for them when the seeds are dried (Santos 2000). The examples of plants with this type of seeds are coffee (*Coffea arabica*), papaya (*Carica papaya*), hickory (*Carya spp.*) or citrus (*Citrus limon*) (Walters et al. 2004). The seeds differ from other seeds in their viability at temperatures between -10 and -30 °C, or they may be short-lived seeds regardless of their drying or cooling (Christina Walters 2015). Intermediate seeds can often tolerate more desiccation than recalcitrant seeds, but they are mostly handled like recalcitrant seeds. For storage, cryoprotective substances are sometimes used as well as cryopreservation (Towill 2002).

Despite the developing possibilities of seed preservation using conventional or cryogenic methods, it is not possible to preserve all seeds (Pritchard 1995). Plants with sterile genotypes as well as genotypes that reproduce vegetatively using clones like potatoes (*Solanum tuberosum*) and sugar cane (*Saccharum officinarum*), or that do not produce viable seeds like banana (*Musa acuminata*) and plantain (*Musa* x *paradisiaca*) (Pritchard 1995; Engelmann 2004) require an alternative preservation method including cryopreservation of meristems or the preservation of germplasm *in vitro* (Pritchard 1995).

6. Cryopreservation of pollen

6.1. Pollen types

Pollen can be divided into two types according to their number of cells (Towill 2002).

Bicellular pollen consist of one vegetative cell and one generative cell inside the pollen grain after the first mitotic division and it is not the final stage of the pollen maturity. The second type is tricellular fully matured pollen which finished second mitotic division at the time of dispersal, the division of generative cell into two sperm cells (Pacini et al. 2019). Pollen type often depends on taxonomic groups, where species mostly have the same type of pollen. For example, *Rosaceae* all have bicellular pollen, whereas *Compositae* have tricellular pollen. Some families may include both types of pollen species (Towill et al. 2000). The peculiarity is that species having desiccation-tolerant seeds can have desiccation-sensitive pollen and *vice versa* (Towill 2002).

Bicellular pollen usually can germinate *in vitro* (Towill et al. 2000), can survive extensive dehydration down to 5-10 % of fresh weight (Towill 2002), and can survive 1 to 5 years storage at temperatures of –20°C. Bicellular pollen often have a thick wall with the exine, of approximately 1,0 μm in thickness (Ariizumi et al. 2007), on the surface forming the outer layer of the pollen grain. Exine is composed of sporopollenin, a very resistant, hard-to-degrade, non-nitrogenous biopolymer of carotenoids and carotenoid esters and form the outer layer of the pollen grain (Bajaj 1987). Thanks to it, the pollen can withstand a higher level of dehydration, it can be stored at lower temperatures, and thus remains viable for a longer time (Bajaj 1987). An example of significant cryopreservation of bicellular pollen is the storage of tropical fruit tree pollen, which is very successful (Reed 2008). For example, kiwi pollen (*Actinidia deliciosa*) stored at temperature -196 °C stay viable for at least 1 year, also cryogenic storage of date pollen (*Phoenix dactylifera*) at the same temperature does not cause obvious loss of pollen viability (Ren et al. 2019).

Tricellular pollen is often more sensitive for desiccation than bicellular pollen and it is typical for species of the families *Compositae*, *Cruciferae*, *Poaceae*, and *Umbelliferae*. The WC varies between species and ranges between 40-60 % (Bajaj 1987), which is higher than WC of bicellular pollen (Pacini et al. 2019). Tricellular pollen has thinner walls compared to bicellular pollen and many species shed pollen that are sensitive to dehydration and storage (Bajaj 1987). Often storage can only be achieved at a water loss that ranges maximum between 50 % and 80% and the longevity is short, usually for few days to 1 year ('Pollen Storage in

Plants' 2016). At maturity, tricellular pollen have a more active metabolism than bicellular pollen (Towill et al. 2000).

6.2. Pollen viability and preservation

Pollen is collected from the shedding anthers of healthy flowers at a time when it is sufficiently mature and dry which is important for its viability (Bajaj 1987). Viability can be determined by staining with, for example, fluorescein diacetate, or by triphenyl tetrazolium chloride (TTC) test (Bajaj 1987). Usually, it is determined *in situ* (Towill et al. 2000) Enzymes as indicators, germination *in vitro*, and also the ability of pollen to fertilize egg cells, which subsequently become fruits and seeds, can also be used for viability determination (Bajaj 1987)

Pollen can be stored in different ways. It includes short-term storage by cooling to a temperature of 3-5 °C, freeze-drying and subsequent long-term storage at temperatures below 0 °C, vacuum drying of pollen and subsequent storage at temperatures of -20 °C, or storage using an organic solvent (Bajaj 1987).

Pollen conservation is important in many ways. The ultimate goal of pollen conservation is to preserve the viability and functionality of pollen grains (Reed 2008). However, depending on the subsequent use of the pollen, there are different demands on its longevity. The longest viability is required of pollen used for germplasm preservation and that is why mainly cryopreservation is used in this case (Towill et al. 2000). In plant breeding it can reduce greenhouse space (Reed 2008) and helps to overcome seasonal, geographical, and physiological limitation (Bajaj 1987). Furthermore, it is important for studying physiology, biochemistry, and fertility as well as biotechnological studies involving gene expression, transformation and fertilization of plants *in vitro* (Towill et al. 2000), allergen studies, and production of haploids through the embryogenesis of isolated pollen, for a constant supply of pollen for fertilization and finally for storage in pollen banks (Bajaj 1987).

Many pollen banks have been established (Bajaj 1987) for example for forest tree pollen (Towill et al. 2000). However, pollen must be replenished regularly in a pollen bank because pollen is a depletable resource. Furthermore, pollen preservation only complements the conservation of seeds or clones in gene bank and cannot be a substitute of them (Towill et al. 2000).

The viability and length of pollen storage are influenced by many biological and methodological factors. Biological factors include WC, pollen nuclear condition, pollen stage after full maturity, genotype, plant physiological condition, and pollen quality. Collection

method, dehydration, storage temperature, cooling and warming procedures, oxygen/air pressure are methodological factors affecting pollen survival and viability after preservation and rehydration (Bajaj 1987; Reed 2008). Pollen quality is difficult to be defined, but rate of pollen tube growth, metabolic activity (respiration rate), membrane semi-permeability, and pollen tube germination can be used (Towill et al. 2000). Water content is one of the most important factors. Pollen at different stages of development contains different amounts of water (Bajaj 1987). Immature pollen has vacuoles where the water is stored and the WC can be higher than 60 %, whereas mature pollen does not contain these vacuoles and the WC is below 40 % (Pacini et al. 2019). When water is reduced after shedding, there is a minimum critical WC below which pollen cannot survive. For example, pollen of *Pennisetum spp*. can contain down to 3 % WC when shed (Towill et al. 2000). Storage temperature is also an important factor affecting pollen survival after preservation. At -20 °C, desiccation-tolerant pollen can survive for 1-5 years, but it can survive longer at -196 °C (Bajaj 1987). Oil palm (*Elaeis guineensis Jacq.*) can be an example. Its pollen can be stored viable at temperature of -20 °C for 12 months or at temperature of -196 °C for 8 years with 54 % viability maintained (Tandon et al. 2007). Pollen collection and the physiological state of the plant also have a great influence on pollen viability (Bajaj 1987), because for certain species that have short flowering there can be a limited time to collect pollen in optimal conditions, which can make conservation a bit difficult (Towill 2002). The physiological state of the mother plant itself also has a great influence, as plants stressed by heat or drought often produce less fertile pollen (Towill 2002). Storage substances of pollen such as sugar, oils, and starch, as well as exine, also play an important role (Bajaj 1987). Starch can be used during creation of new cell wall during pollen development and then it is transferred into sugars (Stanley et al. 1962). Starch and sugars also serve as respiratory substrates (Stanley et al. 1962) and their deficiency leads to loss of viability in the tricellular pollen, whereas their increased usage leads to greater viability in the bicellular pollen (Hoekstra et al. 1975; 1980).

Bicellular pollen viability can be extended by drying and lowering the storage temperature. Although this pollen can be dried down to 5 %, the maximum pollen viability is between 15% and 25% RH (Towill et al. 2000). Due to the WC, usually no cryoprotectants are needed. The cooling rate of pollen does not particularly matter, so the samples can be directly immersed in LN and cryopreserved. Thus, cryopreservation of bicellular pollen is quite simple (Reed 2008). Cryopreservated highly desiccation-tolerant pollen often has the same longevity as an unrefrigerated sample of the same pollen (Towill et al. 2000). Although most bicellular pollen

grains can withstand extensive desiccation, there are some exceptions (Towill et al. 2000). For example, *Cucurbita pepo* has big pollen grains that are sensitive to desiccation and its viability decreases on 13 % of WC (Nepi et al. 2010)

In case of tricellular pollen, storage at temperatures down to -20 °C causes quick loss of viability and for most species with recalcitrant pollen, pollen cannot survive such procedures of preservation. The lower sugar content can also cause shorter viability (Towill et al. 2000), because sugars help to stabilize cell membranes (Walters 2015). Tricellular pollen, for example of *Zea maize*, can contain 5-12 % sucrose depending of pollen stage of dehydration (Hoekstra et al. 1989) There are also problems with desiccation-sensitive pollen during using cryopreservation, because due to the higher WC intracellular ice can form. However, the pollen can be partially dried in such a way that no ice crystals are formed, but WC remains above the levels at which drying damage cannot occur (Towill et al. 2000).

In addition to pollen, frozen microspores, pollen embryos, and anthers can also be stored (Bajaj 1987). However, for successful cryopreservation, it is necessary to have the correct cryopreservation protocols for a certain type of pollen as well (Fig. 4) (Reed 2008).

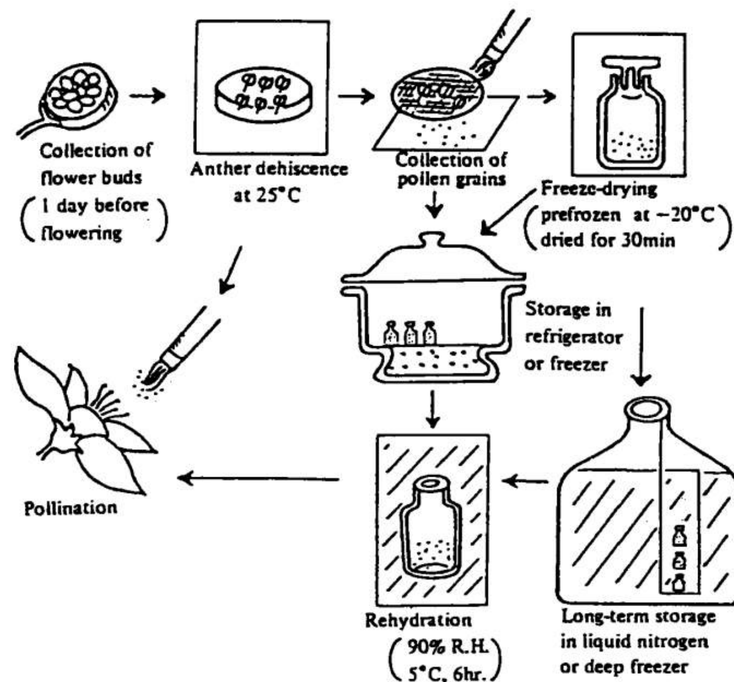

Fig. 4 – Protocol for the collection, storage, and utilization of pollen (Courtesy of Dr. A. Akihama, cit. by Bajaj 1987)

Conclusion

Cryopreservation of seeds and pollen is now a common method of conservation, and there are already many specially modified protocols for individual groups or species of plants, their seeds, and pollen. Desiccation-tolerant orthodox seeds are more often preserved conventionally than by cryopreservation, due to their high probability of survival when dehydrated down to 5 % of WC, and thus they are able to survive for several years in storage. Orthodox seeds can be found especially in annual plant species growing in fields, therefore also in many agricultural crops.

In contrast, recalcitrant, desiccation-intolerant, seeds require cryopreservation using cryoprotectants for long-term storage, as they will not survive drying below 30 % of their fresh weight. Plants with recalcitrant seeds include many economically important perennial trees and shrubs from tropical, subtropical (*Elaeis oleifera Cortes*, *Cocos nucifera*, *Hevea brasiliensis M. Arg*., *Theobroma cacao L.*) and temperate zones and aquatic plants.

Intermediate seeds undergo dehydration to relatively low WC, but they are damaged by exposure to sub-zero temperatures when they are dry. They are typical, for example, for *Coffea arabica* and *Citrus limon*.

Pollen can also be divided into 2 groups according to number of cells. There is bicellular pollen, which is mostly desiccation-tolerant, and tricellular usually desiccation-intolerant pollen. Bicellular pollen can survive for a long time even in non-cryogenic storage at a temperature of -20 °C, but cryopreservation extends its lifespan. No cryopreservation agents are needed due to the low WC of the pollen. Most angiosperms, tropical fruit trees, and all species of *Rosaceae* have bicellular pollen. For example, pollen of *Primulaceae*, *Saxiferaceae*, *Vitaceae,* and *Pinaceae* have a very long lifespan. *Cucurbita pepo* is one exception of species with bicellular pollen, because their large pollen grains are sensitive to desiccation.

Tricellular pollen, which is intolerant to desiccation, can also be stored conventionally, but its lifespan is very short, so cryopreservation is usually used. For example, *Compositae*, *Cruciferae*, *Poaceae*, *Umbelliferae*, *Araceae*, *Umbelliferae*, *Chenopodiaceae,* and *Caryophylacea* have this type of pollen, some pollen species of *Poacaea* are able to tolerate dehydration down to 3 % of WC.

In conclusion, reproductive structures of plants can be kept viable for a long time that by cryopreservation and then the samples can be used for many purposes. However, even though its methods and protocols are still being developed, there are many species of plants for which protocols do not exist and their conservation is difficult.

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