CryoLetters **41 (3)**, 115-127 (2020)

PERSPECTIVE

POLLEN CRYOPRESERVATION FOR PLANT BREEDING AND GENETIC RESOURCES CONSERVATION

Naiana Barbosa Dinato[1]*, Izulmé Rita Imaculada Santos[2]; Bianca Baccili Zanotto Vigna[3], Ailton Ferreira de Paula[1] and Alessandra Pereira Fávero[3]

[1] Center for Biological and Health Sciences, Federal University of São Carlos, Brazil.
[2] Brazilian Agricultural Research Corporation, Embrapa Genetic Resources and Biotechnology, Brazil.
[3] Brazilian Agricultural Research Corporation, Embrapa Southeast Livestock, Brazil.
* Corresponding author email: naiana.dinato@gmail.com

Abstract

Pollen conservation is an important tool for the maintenance of plant genetic resources and can promote improved efficiency in breeding programs and germplasm conservation and exchange. This review aims to highlight the importance of pollen cryopreservation and how to use it for distinct species in order to encourage the use of this methodology in germplasm banks and plant breeding programs. Pollen from many plant species have already been successfully cryopreserved in liquid nitrogen. Analogous with other plant structures, to maintain pollen viability after storage at ultra-low temperatures it is necessary to adjust the water content so that at least the freezable is removed. Optimum pollen moisture levels for cryopreservation varies among species and different methods have been applied to control moisture content. Common methods to decrease pollen moisture content include exposure to saturated solutions of various salts (which have a well-defined relative humidity), silica gel, dry air or treatment with vitrification solutions. It is our understanding that pollen cryopreservation is a safe and practical alternative for conserving genetic material that is often neglected by potential users. The technique has the potential to overcome challenges of breeding programs, such as flowering asynchrony between different parent genotypes, and the production of insufficient pollen in nature. Generally, pollen cryopreservation techniques tend to be simple enough to be used routinely in research, plant breeding and germplasm conservation programs.

Keywords: germplasm conservation, hybridization, liquid nitrogen, plant cryopreservation, pollen grain.

INTRODUCTION

Pollen conservation is an important tool in the management of plant genetic resources. The creation of pollen banks and new methodologies aimed at maintaining the long-term viability of pollen are of interest to embryologists and geneticists (33). Pollen conservation improves the efficiency of breeding programs and enables the exchange of germplasm, e.g. for *Eucalyptus*, palms, sugarcane, yam and other species (19, 22, 25, 26, 38, 55). In addition, it is another tool for the preservation of genetic diversity (16). Furthermore, conserved pollen may also be used to support reproduction in species with inefficient, ineffective or non-existent pollinating agents (75); for example, by enabling crosses to be made between genotypes that

flower asynchronously, such as non-adapted materials and related species (16), or even to allow hybridization twice a year. In 1885, William King, referring to the requirements to learn how to store pollen, stated that "nothing would tend more toward the rapid termination of an experiment than control over the pollen supply, so that we may use it when and where convenient for ourselves" (36).

The success of pollen storage for genetic conservation purposes depends on many factors, and it is essential that the chosen procedure will ensure maintenance of high genetic integrity, vigour and germination percentages (84). Thus, it is essential to evaluate pollen viability before, during and after long-term conservation (26). This recommendation is also made by Dafni (20), who states that pollen viability evaluation is the first step to verify the germination changes on the flower stigma, a fundamental factor for fertilization (21). However, literature reports of preservation of pollen of several species do not always present a detailed description of the extraction, drying, conservation and thawing procedures tested, which make the replication of approaches somewhat difficult.

The objective of this review is to analyze and interpret the major findings on pollen cryopreservation in order to broaden the understanding of the subject and to encourage the use of this methodology in germplasm banks and plant breeding programs.

CRYOPRESERVATION: DEFINITION AND ITS IMPORTANCE ON POLLEN CONSERVATION

Cryopreservation consists of conserving biological material in liquid nitrogen (LN) at -196ºC, or in its vapour phase at -150ºC (45), such that the characteristics of the material are maintained after thawing. This technique has been shown to be efficient and practical for long-term conservation of plant genetic resources, especially for species that propagate vegetatively or have recalcitrant or intermediate seeds (67). Cryopreservation protocols have been developed for many plant species such as grasses, ornamental species, tropical and temperate fruit tree species, leguminous and oleaginous, medicinal and aromatic plants (67). This method is considered the most promising means for long term preservation of various plant parts, such as seeds, somatic and zygotic embryos, vegetative material (e.g., roots, bulbs, tubers, buds, meristematic apices), pollen and other cell systems (e.g. suspension cultures, callus). Success relies on interrupting cellular metabolism and maintaining the genetic stability and phenotypic characteristics of the samples. It allows storage for an undetermined period, using small spaces and requiring low maintenance (28, 76).

Cryopreservation can be performed by traditional or modern methods. Traditional approaches consist of freezing the material slowly or ultrafast. For slow freezing, a programmable freezer is used, at a pre-determined freezing rate down to a temperature range of -30 to -40°C, prior to exposure to the LN, allowing the removal of most of the water inside the cells by freeze-dehydration to the extra-cellular space. This type of cryopreservation protocol may involve the use of substances that interact and accelerate the distribution of water inside and outside the cell, besides promoting cell dehydration, and protecting biomembranes from potential injuries during cell freezing and thawing. These substances, which act as cryoprotectants, are derived from different chemical groups but share similar functions of reducing freezing depression (34). Two types of cryoprotectants are known in relation to their ability to penetrate membranes: diffusable, such as Me_2SO (dimethylsulfoxide), glycerol, propylene glycol, etc., are known as internal cryoprotectants; and non-penetrating, such as saccharose and starch, are known as external cryoprotectants (29, 49).

The second traditional method of plant cell cryopreservation is ultra-fast freezing. In this case, the biological material is dissected, to reduce mass / volume, and then exposed to dehydrating agents to avoid the formation of ice crystals inside the cells during rapid cooling by direct immersion in liquid nitrogen (28, 67). This technique tends to be used with embryonic axes of recalcitrant seeds.

The most modern cryopreservation methods combine rapid cooling with high concentrations of multiple cryoprotectants to ensure that the biological material produces a glass (vitrifies) on cooling, rather than crystalizes. Various methods of vitrification are known, including encapsulation-vitrification and droplet-vitrification. For example, encapsulation-vitrification described by Kaviani et al (44) for *Lilium ledebourii*, combined the advantages of a rapid vitrification procedure

(fast cooling in high concentrations of cryoprotectants), as well as the physical protection of encapsulation of the small explant in alginate beads. However, protocols usually have to be adjusted for each species.

In contrast, pollen cryopreservation is a much simpler, yet effective, method of prolonged storage. Pollen can be kept in LN for many years without loss of its essential pollination, fertilization and fruiting capabilities. Being able to store pollen is useful to plant breeding, e.g., for controlled pollination, and for plant genetic resource conservation (2, 79).

Pollen storage is essential, especially for species that have a long vegetative period or that bloom a few times a year, or for some plants that propagate vegetatively. Benefits include enabling: i) the crossing of plants that flower at different times; ii) the hybridization of plants that grow in different and distant locations; iii) the lowering the disease transmission when the vectors are pollinators. In addition, to conserving the male gene pool for long periods (7), pollen conservation is an useful way to overcome the temporal and spatial isolation of the parent species in breeding programs (10), overcoming the need to wait for the growth and flowering of the plant to obtain the male parent. In this way, pollen storage can make recurrent and breeding lines immediately available as needed, regardless of the response of material to flowering and planting date (37). Such work can be facilitated by the rapid exchange and use of stored pollen of specific or unique genotypes between scientists at national and international level.

For these many reasons, pollen conservation should be integrated into the conservation programs of germplasm banks, to avoid loss of the male parent's genetic material. Pollen conservation should be an additional means of conserving plant germplasm and not a substitute for the storage of seeds or clonal materials (77).

HOW CAN WE CRYOPRESERVE POLLEN?

The majority of the published pollen storage studies relate to fruit, floral or ornamental plants. Many studies describe storage in a freezer, with relatively few exploring the benefits of storing pollen under cryopreservation conditions (- 196ºC) (Table 1).

Storage can be classified into two types: short- and long-term. Generally, short-term storage is intended for genetic and breeding studies, and long-term storage for genetic resources conservation (70). To maintain the pollen viability as high as the fresh pollen during long-term storage, it is necessary to follow protocols of collection, drying, storage and viability pollen tests (71). Sousa (70) states that genetic alterations are common in long-term storage and can lead, after many years, to genetically different populations from the original ones.

Among the cryopreservation techniques cited, the most used method is the traditional approach of dehydration of the pollen grains before immersion in LN (Table 1). The most commonly used dehydrating agents are silica gel, saturated salt solutions, airflow cabinet and oven. Alternatively, some researchers have used modern methodologies, such as vitrification in the presence of the cryogenic plant vitrification solution (PVS) (Table 2).

Pollen dehydration

Knowledge of which species need to be conserved and for what purpose are important considerations when deciding which storage condition to use (62). Pollen storage success depends on environmental factors of humidity and storage temperature (41). Low temperatures and humidity are usually linked to pollen metabolism decrease, which allows greater longevity (12, 71). Assuming relatively ideal storage temperature and pollen moisture, viability is independent of the storage period (23, 51).

When the pollen moisture content is high, freezing will decrease its viability by inducing ice crystal formation and growth that can break the cell membranes (4). Indeed, ice crystal formation is one of the major problems encountered in cryopreservation (67). As the temperature decreases and falls just below 0°C, the cells supercool until ice is nucleated. In multicellular plant structures, ice crystals are formed in the extracellular space and, in effect, the cell wall and the plasma membrane act as barriers that prevent the formation of ice crystals in the intracellular spaces, thus reducing the likelihood of triggering the freezing of the cytoplasm. As pollen grains are single entities with only two or three nuclei, such extracellular freezing is likely limited. Therefore, the pollen hydration level must be reduced before cooling.

According to Sprague and Johnson (73), a pollen moisture content from 8 to 10% avoids the formation of ice crystals during the freezing process, regardless of the final cold storage method. Moreover, it is thought that successful long-term conservation requires pollen to be at moisture contents between 7 and 20% when using -80 to -196°C temperatures (16). Copes (17) and Towill (78) described analyses with desiccation-tolerant pollen of *Pseudotsuga menziesii* (Mirb.) Franco and *Solanum* sp., successfully stored at 5% and 7% moisture content, respectively. Although there is an indication that to achieve success in cryopreservation, the water content of pollen grains should be below 20%, there are still no studies setting the minimum moisture for the pollen to remain viable (16).

Beyond considerations of any upper and lower water contents for pollen cryopreservation, there are disagreements over what is the most favorable humidity level for pollen storage, even within the same crop (33). Barnabas et al (8) stated that the fertilization ability of corn pollen after liquid nitrogen storage was higher in samples with 13% water content, while Kerhoas et al (47) found that corn pollen viability drops dramatically below 15% moisture. Nonetheless, it is generally accepted that pollen moisture reduction improves long-term storage success, assuming that the pollen has the ability to fully tolerate the dehydration process (35, 41). For desiccation-sensitive pollen, specific protocols should be developed for their storage (16).

Pollen tolerance to dehydration is related to pollen morphology. Binucleate pollen is classified as tolerant and trinucleate pollen often as sensitive to drying (42). Also, many authors describe that binucleate pollen has greater viability when compared to trinucleate pollen (31, 51, 74). This occurs due to the fact that the second meiotic division in trinucleate pollen consumes enough reserves to negatively impact good longevity and germination (70). On the other hand, binucleate pollen present a greater amount of surface compounds on its wall and there is no second meiotic division in this type of pollen, preventing reserve losses (48). Therefore, a suitable drying methodology to trinucleate pollen is necessary, since the nuclear components can be damaged, reducing its viability. In general, Poaceae species have trinucleate pollen, which makes it difficult to store the male gametes of grass species (5). In addition to *Zea mays* L. (Poaceae), pollen of *Simmondsia chinensis* (Link) C.K. Schneid. (Simmondsiaceae) also lose viability with drastic drying. Williams and Brown (87) in their recent study with tricellular and bicellular pollen found that the water content and the number of pollen cells are positively correlated. Thirty species were studied at random and it was verified that tricellular pollen had a 30% higher hydration index than the bicellular pollen in a same range of pollen size. They concluded that in evolutionary terms bicellular pollen gave rise to tricellular pollen, and that the less variable the duration of dispersion, the less the pollen depends on dehydration as a mechanism to guarantee longevity in the dispersion.

Roberts (65) defined pollen that remain viable after drying as desiccation-tolerant, while pollen that loses viability during drying is called as desiccation-sensitive. Desiccation-tolerant pollen can be dehydrated to low water contents, ranging from 5 to 10%, using the same methods applied to the seeds, and then placed directly into LN and thawed at room temperature with success. Several species have pollen grains that tolerate such dehydration and freezing treatment and as such these pollen grains have storage behaviour that resembles that of orthodox seeds. It can be the case that plants with orthodox seeds can have desiccation sensitive pollen and vice versa (76).

Pollen desiccation is very similar to the seeds, except moisture equilibration in pollen is faster (38, 79). There are various methods that can be used to adjust the pollen water content. Drying can be achieved with silica gel, by vacuum desiccation (39), in liquid nitrogen vapour or by oven. However, it is preferable that the drying temperature does not exceed 28°C (6). For drying to 5-10% water (fresh weight basis), it is usual to use natural desiccation in air at room temperature or solutions of saturated salts (76).

It is possible to achieve distinct, well defined relative humidities by using particular salts; for example, sulfuric acid solutions in variable concentrations (50) and several saturated salt solutions (88, 89). These solutions keep the relative humidity constant in the atmosphere because any non-volatile solution in water will have a defined water vapour pressure at a given temperature when the vapour phase is in equilibrium with the liquid. Saturated solutions with an excess of solids maintain constant vapour pressure, even under the

variable humidity condition, because any water gain causes salt precipitation, while there is not much liquid on the solid. The diffusion that occurs inside the solution is very slow, and the humidity conditions will remain unchanged. Thus, a considerable amount of water can be gained or lost by the biological material without changing the vapor pressure in the humidity container (88).

For seeds there have been many water sorption studies carried out using solid substances, so as to create isotherms that show the relationship between moisture level and relative humidity under a given temperature; for example the studies of Eira et al. (27), Vertucci and Roos (81) and Walters et al (83). Often such studies are done to set the critical water content for seeds during storage at different temperatures (82, 83). As seeds are hygroscopic, when the seed water vapor pressure is lower than the air pressure, water absorption (adsorption) occurs and, in the opposite case, the seed loses water to the air (desorption). When the water pressure of the seed surface equals the vapour pressure of the ambient air (or that of the saturated salt solution in a closed container), equilibrium moisture is reached (59). The water relations of pollen are similar.

To control humidity, saturated salt solutions can be used for which the equilibrium relative humidity expected at a particular temperature is known. Tabulated data can be found in various reports (16, 88, 89). Some data are also presented on the equilibrium relative humidity of unsaturated salt solutions.

Overall, the use of saturated salt solutions to control relative humidity is a simple and economical method. Most reagents are readily available in reasonable purity, are safe to handle and are non-volatile, thus avoiding contamination of the specimen (89) (Table 2). Certain salts are unsuitable for this purpose because of their instability or irregular behavior. Winston and Bates (88) list several halides [ferric chloride ($FeCI_3$), aluminum bromide ($AlBr_3$), aluminum chloride ($AlCl_3$), and thorium (IV) chloride ($ThCl_4$)], which are prone to hydrolysis. Some others halides are sensitive to light. The halide $FeCl_3$, with a relative humidity of 5% at 25°C, is the only one that can be used with some confidence, despite differences among preparations. Buxton and Mellanby (13) pointed that NH_4Cl releases traces of NH_3 and that LiCl releases Cl_2 above 37°C. The extent and rate of pollen water content change will depend on the salt, the gradient between the relative humidity of the salt and the water content of the pollen, and the pollen type (16).

According to Connor and Towill (16), placing the desiccation-tolerant pollen above a saturated salt solution with a low relative humidity for approximately 2 h is sufficient to reduce the moisture level for storage purposes. Their study showed this time interval was enough for pollen to approach equilibrium above all solutions used, but that moisture content generally did not stabilize for 6-24 h.

Investigations on the influence of relative humidity and temperature on pollen longevity have already been conducted using saturated salt solutions (12, 41). Nonetheless, there is a general lack of information on the pollen moisture content and kinetics of moisture gain or loss at a certain relative humidity (16).

Cooling of pollen grains

After transferring the pollen to a sealable container, the most sophisticated cooling method is the use of liquefied gases and the easiest is the use of freezers and refrigerators. After adequate pollen grain dehydration, the material just needs to be put in the storage temperature required, generally without any specific cooling protocol. Many studies have shown the successful freezing of pollen of several species (Table 1).

Bhat et al. (10) showed that for *Pyrus* spp. pollen viability decreases according to the storage condition. The maximum loss in viability was observed in storage at -196°C (16.2% of viability), followed by -20°C (22.2%) and 4°C (46.5%), respectively. The lowest viability (0%) was observed after room temperature after 12-week storage over anhydrous calcium chloride.

Table 1. Cryopreservation in liquid nitrogen of pollen of different species.

Species	Cryogenic procedure	Storage duration (d, wk, mo, yr)
Aechmea bicolor L. B. Sm.(bromeliad). NB. Synonym of *Wittmackia bicolor* (L.B.Sm.) Aguirre-Santoro	Silica gel dehydration (desiccator) 3 h, WC*	365 d
Ananas spp.	Silica gel dehydration (desiccator) 6 h, 28% WC	24 h; 60 and 120 d
Brassica campestris var. purpurea. NB. Synonym of *Brassica rapa* L.	Silica gel dehydration + PVS 10-20% sucrose, 40% Me_2SO, 0°C, 20 min (mature pollen)	1 d
Brassica napus L.	Silica gel dehydration + PVS 10-15% sucrose, 35-40% Me_2SO, 0°C, 15 min (immature pollen)	1 d
Brassica chinensis. NB. Synonym of *Brassica rapa* L.	Silica gel dehydration + PVS 10-15% sucrose, 35-40% Me2SO, 0°C, 15 min (immature pollen)	1 d
Bromelia spp	Silica gel dehydration (desiccator) 4 h	6 mo
Capsicum spp	NR	47 d
Carya illinoinensis (Wangenh.) K. Koch (pecan)	NR	13 yr
Carya illinoensis (Wangenh.) K. Koch (pecan)	Saturated salt solution [$MgCl_2$ and $Mg(NO_3)$] dehydration, WC=7-10%	6 mo
Carica papaya L.(papaya)	Silica gel dehydration (desiccator) 24 h	6 mo
Citrus cavaleriei H.Lév. *ex* Cavalerie (Ichang lemon)	Air dehydration 16 – 24 h at 25°C, 5-14% WC	2 yr
Citrus maxima (Burm.) Merr. (pomelo)	Air dehydration 16 – 24 h at 25°C, 5-14% WC	2 yr

NR – not reported. *WC, water content or moisture content (%)

Table 1. continued

Species	Cryogenic procedure	Storage duration (d, wk, mo, yr)
Clianthus formosus (G. Don) Ford and Vickery (Sturt's desert pea). NB. Synonym of *Swainsona formosa* (G. Don) Joy Thomps;	Desiccant (Drierite or $CaCl_2$) dehydration 3 h, freezing -180°C (vapour phase of liquid nitrogen)	19 h or 2 d
Cocos nucifera L. (coconut)	Oven dehydration at 40ºC 24 h, WC*=7.5%	3 yr / 4 yr
Colocasia esculenta (L.) Schott (taro)	No dehydration (WC = 19.8 – 21.3%)	72 h 2 mo
Delphinium spp. (orchid)	Air dehydration at 20 °C, 3 h Silica gel dehydration (desiccator)	180 d
Dendrobium spp. (orchid)	Silica gel dehydration (desiccator) 24 h / PVS2 dehydration 0ºC or 27ºC, 1 and 4 h, initial WC= 8%, final WC= 5%	48 h
Hylocereus spp.	Silica gel dehydration (desiccator), WC= 5-10%	3 or 9 mo
Juglans regia L.	Air dehydration at room temperature 2 h, WC=5-7.5%	1 mo
Juglans regia L.	Dehydration, WC=4.6-12.1%	12 mo
Luisia macrantha Blatt. & McCann (epiphytic orchid)	Silica gel dehydration (desiccator) 120 min / PVS2 10 min Air dehydration (laminar flow cabinet) at 27ºC, 120 min	24 h / 668 d / 24 h
Litchi chinensis Sonn. (lichee)	Silica gel dehydration (desiccator) 1 h, freezing to -196ºC	4 yr

NR – not reported. *WC, water content or moisture content (%)

Table 1. continued

Species	Cryogenic procedure	Storage duration (d, wk, mo, yr)
Mangifera indica L. (mango)	Silica gel dehydration (desiccator) 1 h, freezing to -196ºC	4 yr
Olea europaea L. (olive)	Silica gel dehydration (desiccator) at 25°C for 24 h, freezing to -196ºC	1 yr
Ornamental plants?	Drying for 24 h at 22°C and 5% RH, freezing to -196°C	1 yr
Paspalum notatum Flűggé (Bahia grass)	Silica gel dehydration (desiccator) 120 min and Lithium chloride 30 min, freezing to -196ºC.	180 d
Picea pungens Engelm. (Blue spruce)	Saturated salt solution [$MgCl_2$ and $Mg(NO_3)$] dehydration , WC*=7-10%, freezing to – 196ºC	6 mo
Pinus ponderosa Douglas ex C.Lawson	Saturated salt solution [$MgCl_2$ and $Mg(NO_3)$] dehydration , WC=7-10%, freezing to -196ºC	6 mo
Prunus mume (Siebold) Siebold & Zucc.	NR	4 yr
Pseudotsuga menziesii (Mirb.) Franco (Douglas fir)	Air dehydration at room temperature, WC=4 to 7%, freezing to -196°C	3 yr
Pyrus spp.	NR, freezing to – 196°C	12 wk
Rosa spp. (rosa)	Silica gel dehydration (desiccator) 24 h, 12 h photoperiod, freezing to -196°C	48 h
Solanum spp.	No desiccation, freezing to -196°C	9 mo
Typha maxima Schur *ex* Rohrb. (cattail). NB. Synonym of *Typha domingensis* Pers	Saturated salt solution [MgCl2 and Mg(NO3)] dehydration , WC=7-10%, freezing to -196°C	6 mo
Vitis vinifera L. (grape vine)	Silica gel dehydration (desiccator), freezing to -196°C	64 wk
Zea mays L. (maize)	Room temperature dehydration 1 h, WC=30%, freezing to -196°C	1 yr
Zea mays L. (maize)	Flotation method (9) dehydration at room temperature, WC=13%, freezing to -196°C	7 d

NR – not reported. *WC, water content or moisture content (%)

Table 2. The relative humidity (RH) obtained from various saturated salt aqueous solutions incubated at 23 ± 2°C.

Salt	RH (%)
Phosphorus pentoxide (P_2O_5)	0.5
Sodium hydroxide (NaOH)	7
Lithium chloride hydrate ($LiCl.H_2O$)	12
Zinc chloride ($ZnCl_2$)	5.5
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	32
Calcium nitrate ($Ca(NO_3)2$)	50.5
Magnesium nitrate ($Mg(NO_3)_2$)	53
Ammonium nitrate (NH_4NO_3)	62.5
Sodium nitrate ($NaNO_3$)	64
Sodium chloride (NaCl)	75
Potassium chloride (KCl)	85
Copper sulphate pentahydrate ($CuSO_4.5H_2O$)	97

Thawing after cryopreservation

Thawing must be taken into account for a successful pollen storage in LN once this is directly related to pollen metabolism and the reactivation of post-conservation metabolic processes (18). There are a few papers describing the thawing methodology, although some of the studies listed in Table 1 do not clearly explain the process of pollen thawing prior to germination analysis.

Pollen thawing can be done slowly or quickly. In the quick technique, the tubes with pollen grains are put into a water bath at 37ºC for 5 min (69). The slow technique involves thawing the pollen grains for 30 min in the freezer (-20ºC), then 30 min in the refrigerator (4ºC) and then ambient conditions (c. 25ºC) for the same period (24) or leaving the cryotubes in running water at 25ºC for 15 min.

Vendrame et al (80) stored orchid pollen in LN and thawed the samples by keeping them at room temperature for 3 min. Xu et al (91), studying *Brassica* sp. pollen freezing, thawed the samples in a water bath at 40°C. Similarly, Chaudhury et al (14) also thawed mango and lychee pollen for 30 s in a water bath at 38 ± 2° C. In contrast, Marchant et al (54) placed the storage bottles containing rose pollen into sterile water at about 45ºC for 2 min. Wang et al (85) rewarmed lychee pollen samples at room temperature for 5 min and Xu et al (90) thawed ornamental plant pollen samples by rinsing in running water for 5 min. Therefore, the majority of the protocols used relatively fast thawing. Based on a review of ornamental species cryopreservation, Kulus and Zalewska (49) noted that slow thawing is generally less efficient than rapid warming, and is more time consuming.

POLLEN VIABILITY AFTER CRYOPRESERVATION

Inter-species variation in survival

There are many reports on pollen viability after cryopreservation (Table 1). Xu et al. (2014) reported that viability of pollen of 26 species/cultivars of ornamental plants decreased significantly after cryopreservation (90). Zhang et al. (2009) observed no significant difference in the viability of fresh and cryopreserved pollen of 51 species/cultivars of *Prunus mume* (92). Sparks and Yates (2002) found that the viability of pecan pollen was significantly higher after cryopreservation for 13 years than that of fresh pollen (72). Pollen from 12 different olive cultivars were stored for 1 year in liquid nitrogen at -196° C and the results of *in vitro* germinability, both before and after cryopreservation, showed a highly significant response among the 12 cultivars (3). Pollen of five grape cultivars stored in liquid nitrogen showed no significant decrease in germination percentage after 64 weeks of storage (32). In the case of maize pollen, Barnabas and co-workers report that they can be stored in liquid nitrogen without significant decrease in fertility (8, 9). According to Bhat et al. (2012) pollen of pear stored in liquid nitrogen showed better viability and germination percentages than pollen stored at room temperature and that cryopreservation could ensure pollen availability during the whole blooming season in hybridization programs by fruit breeders (10). Connor & Towill (1993) reported that pollen of different species were cryopreserved and retained viability after storage in liquid nitrogen after pollen water content was adjusted using saturated salt solutions (16). Douglas-fir pollen stored in liquid nitrogen for 1 year was nearly as fertile as fresh pollen when used in controlled pollination tests (17). Maize pollen quality determined after long-term storage in liquid nitrogen by a combination of viability tests and cytochemical methods remained stable,

indicating that storage in liquid nitrogen had no significant effect on pollen viability (33). In the case of *Clianthus formosus*, Hughes et al. (1991) reported that pollen retained high survival percentages (42). Luza & Polito (1988) observed that English walnut pollen with water content higher than 7.5% were killed by after freezing in liquid nitrogen, but that all pollen samples dried to water content between 4 and 7.5% survived cryopreservation (52). Marchant et al. (1993) also reported successful cryopreservation at -196° C of two cultivars of English rose cultivars and that the pollen retained the ability for fertilization (54). Metz et al. (2000) reported that the viability of pollen of two fruit crop cacti of the genus *Hylocereus* was maintained after cryopreservation after water content was reduced to 5 to 10%, and flowers pollinated with pollen stored for 3 to 9 months exhibited 100% fruit set (57).

Cryostored pollen was used for hybridization of taro plants in an attempt to overcome asynchrony in flowering and the results were positive ensuring fruit setting within weeks of pollination with cryopreserved pollen (Mukherjee et al. 2016) (58). Pollen of species and cultivars of bromeliads were stored in liquid nitrogen without significant loss of viability (Parton et al. 2002) (60). Viability of *Aechmea bicolor* pollen after storage in liquid nitrogen was investigated and a higher percentage of fruit set, as well as number of seeds, was obtained for pollen dehydrated and stored in liquid nitrogen (de Souza et al. 2014) (69). In vitro germination of pollen of pecan stored for 1, 10, 11, 12 and 13 years in liquid nitrogen showed that viability of pollen was not diminished in comparison to that of fresh pollen and that morphology of stored pollen and the germ tube was normal, confirming that cryopreservation is efficient means of haploid preservation of pecan (Sparks & Yates, 2002) (72). Cryopreservation of pollinia of *Dendrobium* hybrids was successfully accomplished either by direct freezing in liquid nitrogen without treatment with cryoprotectants or by using a PVS2 vitrification protocol, and pollinia showed over 80% germination after crosses were performed (Vendrame et al. 2008) (80). Potato pollen showed no significant decrease in percentage germination after 9 months of storage in liquid nitrogen, at -196°C (Weatherhead et al 1978) (86). In a study with 102 species/cultivars of diverse ornamental species, Ren et al. (2019) observed that after long-term storage in liquid nitrogen the viability of the pollen of 73 of them decreased, increased for 12 species/cultivars or remained the same for 17 species/cultivars) (64).

In some studies, the viability was higher than in others (Table 1). The low viability of pollen after cryopreservation observed in some studies may be related to oxidative stress induced by reactive oxygen species (ROS). In a recent study of cryopreservation of *Paeonia lactifora* and *Magnolia denudata* pollen, Jia et al (43) observed that the application of exogenous catalase (CAT) and malate dehydrogenase (MDH) can reduce oxidative damage through stimulation of antioxidant enzymatic activity and play a protective role for pollen during cryopreservation.

Nonetheless, these results overall confirm that conservation of pollen of many plant species and cultivars in liquid nitrogen is an important tool and can provide breeders with an alternative for carrying out crosses with asynchronous flowering species.

The importance of in vivo *growth*

Numerous studies have verified pollen viability after the cryopreservation using in vivo germination techniques, such to *Lycopersion esculentum* (66), *Dendrobium* (80), peppers (56), *Imperata cylindrical* (63), *Delphinium* spp. (40), *Cocos nucifera* (46), *Mangifera indica* (14) and *Ananas* spp. (68) (Table 1). However, obtaining pollen with viable germination in an in vivo test does not necessarily mean that the pollen is fully capable of fertilizing and producing seeds.

FINAL CONSIDERATIONS

Pollen viability depends on many factors and pollen longevity varies amongst species and can vary from a few days to years (30). It is common to hear reports from breeders about the non-synchronization of flowering in species to be crossed, or that when the female plant is fit, the male plant does not release enough pollen. This is why it is important to understand more about the storage response of biodiverse species' pollen, including to cryopreservation. The literature shows that cryopreservation is a feasible and efficient technique for pollen conservation, since the pollen can be stored for an indeterminate time when the adequate methodology is used. However, each species potentially behaves differently to the processes applied (i.e., drying, cooling, warming, viability

testing), so it is of paramount importance to conduct more research and to further develop optimal techniques for each species of interest (71).

Acknowledgements: Funding for this work was provided by Coordination for the Improvement of Higher Education Personnel (CAPES), Association for Promotion of Breeding Research in Tropical Forages (Unipasto) and Brazilian Agricultural Research Corporation (EMBRAPA), project SEG # 01.15.02.002.05.12.

REFERENCES

1. Ajeeshkumar S & Decruse WS (2013) *CryoLetters* **34**, 20-29.
2. Akihama T, Omura M & Kozaki I (1979) *Trop Agric Res* **13**, 238-241.
3. Alba V, Bisignano V, Alba E et al (2011) *Genet Resour Crop Evol* **58**, 977-982.
4. Almeida C, Amaral ALdo, Barbosa JF et al (2011) *Braz J Bot* **34**, 493–497.
5. Alvim PdeO, Pinho RGV, Pinho EVdeRV et al (2012) *Revista Agrarian* **5**, 206-211.
6. Argerich CA & Gaviola JC (1995) *Production de Semilla de Tomate, Primeira Edição*, Argentina: INTA-EEA la Consulta, Fascículo 6.
7. Bajaj YPS (1995) in *Cryopreservation of Plant Germplasm: I. Biotechnology in Agriculture and Foresty*, (ed) Bajaj YPS, Springer, Berlin, pp 3-28.
8. Barnabas B, Kovacs G, Abranyi A et al (1988). *Euphytica* **39**, 221-225.
9. Barnabas B & Rajki E (1981) *Annals of Botany*, *48*(6), 861-864.
10. Bhat ZA, Dhillon WS, Shafi RHS, et al (2012) *J Agric Sci* **4**, 128-135.
11. Bomben C, Malossini C, Cipriani G, et al et al (1999) *ActaHortic* **498**, 105-110.
12. Buitink J, Walters C, Hoekstra FA et al (1998) *Physiologia Plantarum*, **103(2),** 145–153.
13. Buxton PA & Mellanby K (1934) *Bulletin of Entomological Research* **25**, 171–175.
14. Chaudhury R, Malik SK & Rajan S (2010) *CryoLetters* **31**, 268-278.
15. Cohen E, Lavi U & Spiegel-Roy P (1989) *Sci Horticult* **40**, 317–324.
16. Connor KF & Towill LE (1993) *Euphytica* **68**, 77–84.
17. Copes DL (1985) *Forest Science* **31**, 569-574.
18. Cuchiara CC, Souza SAM, dos Anjos SD et al (2012) *Biotemas* **25**, 22-25.
19. da Silva JAT & Engelmann F (2017) *Cryobiology* **77**, 82-88.
20. Dafni A (1992) *Pollination Ecology: A Practical Approach*, IRL Press Ltd, New York.
21. Junior PCD, Pereira TNS & Pereira MG (2008) *Revista Ceres* **55**, 433-438.
22. Daniel IO, Ng NQ, Tayo TO et al (2002) *J Agric Sci* **138**, 57-62.
23. Dean CE (1965) *Crop Sci* **11**, 148-150.
24. Dinato NB, Santos IRI, Leonardecz E et al (2018) *Crop Sci* **58**, 1-8.
25. Duarte Filho LSC, da Silva EF, da Silva Ramos R et al (2017) *Int J Appl Microbiol Biotechnol Res* **5**, 88-94.
26. Einhardt PM, Correa ER & Raseira MC (2006) *Rev Bras Frutic* **28**, 5-7.
27. Eira MTS, Walters C, Caldas LS et al (1999) *Rev Bras Fisiol Veg* **11**, 97-105.
28. Engelmann F (2004) *In Vitro Cell Dev Biol Plant* **40**, 427–433.
29. Fornari DC, Ribeiro RP, Streit D et al (2011) *Zygote* **22**, 58–63.
30. França VL, Nascimento WM, Carmona R et al (2009) *Crop Breed Appl. Biotechnol* **9**, 320-327.
31. Frankel R & Galun E (1997) *Pollination Mechanisms, Reproduction and Plant Breeding*, Springer, New York.
32. Ganeshan S (1985) *Vitis* **24**, 169-173.
33. Georgieva ID & Kruleva MM (1994) *Euphytica* **72**, 87–94.
34. Gonzalez RAF (2004) *Efeito da Criopreservação Usando Diferentes Técnicas de Congelação e Crioprotetores Sobre Parâmetros Espermáticos e a Integridade de Membranas do Espermatozóide Bovino*, PhD thesis, Universidade de Sao Paulo Sistema Integrado de Bibliotecas – SIBiUSP, 94 pp.
35. Guilluy CM, Gaude T, Digonnet-Kerhoas C, et al (1990) in *Mechanism of Fertilization: Plants to Humans*, (ed) Dale B, Springer, Berlin, Heidelberg, pp 253-270.
36. Hanna W, Burton GW & Monson WG (1986) *Journal of Heredity* **77**, 361–362.
37. Hanna W (1990) *Theoretical Appl Genet* **79**, 605-608.
38. Hanna WW & Towill LE (2010) in *Plant Breeding Reviews*, (ed) Janick J, John Wiley & Sons Inc, New York, pp. 179-207.

39. Hanson CH & Campbell TA (1972) *Crop Sci* **12**, 874.
40. Honda K, Watanabe H & Tsutsui K (2002) *Euphytica* **126**, 315-320.
41. Hong TD, Ellis RH, Buitink J et al (1999) *Annals of Botany* **83**, 167–173.
42. Hughes HG, Lee CW & Towill LE (1991) *HortScience* **26**, 1411-1412.
43. Jia MX, Jiang XR, Xu J et al (2018) *Acta Physiologiae Plantarum* **40**, 37.
44. Kaviani B, Dehkaei MP, Hashemabadi D et al (2010) *Am Eurasian J Agric Environ Sci* **8**, 556-560.
45. Kartha KK (1985) in *Cryopreservation of Plant Cells and Organs*, (ed) Kartha KK, CRC Press Inc, Florida, pp. 115-134.
46. Karun A, Sajini KK, Niral V et al (2014) *CryoLetters* **35**, 407-417.
47. Kerhoas C, Gay G & Dumas C (1987) *Planta* **171**, 1-10.
48. Kirby E G & Smith JE (1974) in *Fertilization in Higher Plants*, (ed) Linskens HF, Proceedings of the International Symposium on Fertilization in Higher Plants, North-Holland, Amsterdam, pp. 127-130.
49. Kulus D & Zalewska M (2014) *Sci Hortic* **168**, 88-107.
50. Lanner RM & Forest PS (1962) *Silvae Genetica* **11**, 114-117.
51. Linskens HF (1964) *Annu RevPlantPhysiology* **15**, 225-226.
52. Luza JG & Polito VS (1988) *Euphytica* **37**, 141–148.
53. Luza JG & Polito VS (1985) *Sci Hortic* **27**, 303–316.
54. Marchant R, Power JB, Davey MR et al (1992) *Euphytica* **66(3)**, 235-241.
55. Maryam M, Fatima Bilques, Haider MS et al (2015) *Pak J Bot* **47**, 377-381.
56. Mathad RC, Vasudevan SN & Patil SB (2012) in *Prospects in Bioscience: Addressing the Issues*, (eds) Sabu A, Augustine A, Springer, India, pp.273-276.
57. Metz C, Nerd A & Mizrahi Y (2000) *HortScience* **35**, 199-201.
58. Mukherjee A, George J, Pillai R et al (2016) *Euphytica* **212**, 29-36.
59. Nellist ME & Hughes M (1973) *J Seed Sci* **1**, 613-643.
60. Parton E, Vervaeke I, Delen R et al (2002) *Euphytica* **125**, 155–161.
61. Pereira RC, Chamma LD, Patto MAR et al (2002) *Cerne* **8**, 60-69.
62. Pio LAS, Ramos JD, Pasqual M et al (2007) *Ciência e Agrotecnologia* **31**, 147–153.
63. Rather SA, Chaudhary HK & Kaila V (2017) *Cereal Res Commun* **45**, 525-534.
64. Ren R, Li Z, Li B et al (2019) *Cryobiology* **89**, 14-20.
65. Roberts EH (1973) *Seed Science and Technology* **1**, 499-514.
66. Sack EJ & Clai DAS (1996) *HortScience* **31**, 447-448.
67. Santos IRI (2000) *Rev Bras Fisiol Veg* **12**, 70-84.
68. da Silva RL, de Souza EH, de Jesus Vieira L et al (2017) *Sci Hortic* **219**, 326–334.
69. de Souza EH, Souza FVD, Rossi ML et al (2015) *Euphytica* **204**, 13–28.
70. Sousa VA (1990) *Boletim de Pesquisa Florestal* **21**, 15 - 19.
71. Sousa VA, Schemberg EA & Aguiar AV (2010) *Scientia Forestalis* **38**, 147-151.
72. Sparks D & Yates IE (2002) *HortScience* **37**, 176-177.
73. Sprague JR & Johnson VW (1977) in *Proccedings of 14th Southern Forest Tree Improvement Conference*, Gainsville, Florida, pp. 20-27.
74. Stanley RG & Linskens HE (1974) *Pollen: Biology, Biochemistry, Management*, Springer-Verlag, New York.
75. Tighe ME (2004) *Manual of Collection and Management of Tropical and Subtropical Pinus Pollen from Natural Stands*, 1st Edition, CAMOCORE, Carolina del Norte Estados Unidos, 20 pp.
76. Towill LE (2002) in *Cryopreservation of Plant Germplasm II, Biotechnology in Agriculture and Foresty, Volume 50*, (eds) Towill LE & Bajaj YPS, Springer, Berlin, pp 3-21.
77. Towill LE (2000) in *Plant Tissue Culture Concepts and Laboratory Exercises*, (eds) Trigiano RN & Gray DJ, CRC Press, Boca Raton, pp 337-353.
78. Towill LE (1981) *Plant Science Letters* **20**, 315–324.
79. Towill LE (1985) in *Cryopreservation of Plant Cells and Organs*, (ed) Kartha KK, CRC Press, Boca Raton, pp 171-198.
80. Vendrame WA, Carvalho VS, Dias JM et al (2008) *Hort Science* **43**, 264-267.
81. Vertucci CW & Roos EE (1990) *Plant Physiology* **94**, 1019-1023.
82. Vertucci CW, Roos EE & Crane J (1994) *Annals of Botany* **74** , 531–540.

83. Walters C, Rao NK & Hu K (1998) *Seed Science Research* **8**, 15-22.
84. Wang BSP (1975) in *Methodology for the Conservation of Forest Genetic Resources*, FAO, Rome, pp. 93-103.
85. Wang L, Wu J, Chen J et al (2015) *Scientia Horticulturae* **188**, 78–83.
86. Weatherhead MA, Grout BWW & Henshaw GG (1978) *Potato Research* **21**, 331–334.
87. Williams JH & Brown CD (2018) *Acta Bot Bras* **32**, 454-461.
88. Winston PW & Bates DH (1960) *Ecology*, **41**, 232–237.
89. Young JF (1967) *J ApplChem* **17**, 241–245.
90. Xu J, Li B, Liu Q et al (2014) *CryoLetters* **35**, 312-319.
91. Xu B, Han H, Zheng C et al (1997) *Wuhan University J Nat Sci Res* **2**, 120-123.
92. Zhang YL, Chen RD, Huang CJ, et al (2009) *CryoLetters* **30**, 165-170.
93. Zhang JM, Lu XX, Xin X et al (2017) *In Vitro Cell Dev Biol Plant* **53**, 318–327.