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INVITED REVIEW

# A practical framework for the cryopreservation of palm species

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## Abstract

Palms (*Arecaceae*) contribute remarkable economic and environmental values to human life. However, many elite and commercial palm species are becoming critically endangered and demand immediate action to preserve their genetic resources. Cryopreservation has established itself as the definitive *in vitro* conservation method for recalcitrant-seeded species, such as those within the *Arecaceae*. Progress in this area has moved steadily forward over the last three decades with the development of various techniques for different explants and palm genotypes and for molecular testing methods to ensure genetic fidelity is maintained in the regenerants. There remains a key challenge to properly identify the components that will enable the long-term conservation of palms using cryopreservation. This review methodically analyzes the state-of-the-art cryopreservation techniques developed for palms and places them within a practical framework. This framework encompasses four underlying components, namely the tissue culture approaches required, the recalcitrant nature of the palm seed, the cryobiology and cryogenic techniques required, and fidelity assessment after cryopreservation. Through a critical analysis of this framework, further optimization of palm cryopreservation protocols and more fundamental studies on the physiological and molecular changes in cryopreserved palm tissues are recommended. The present review helps to showcase a multi-decade global attempt to preserve these mostly recalcitrant species through *ex situ* collections. From a conservationist's perspective, this review hopes to stimulate awareness for further concerted efforts in the conservation of rare and endangered plant families. Meanwhile, from a managerial perspective, this work serves to inform decision-makers of the global research effort underway to improve key components of the cryopreservation program for palm species and to encourage funding bodies to appropriately allocate resources to these much-needed research areas.

**Keywords** Palm · Cryopreservation · Recalcitrance · Vitrification · Fidelity assessment

## Introduction

The palms (*Arecaceae*) belong to a plant family that is predominantly found in tropical and sub-tropical regions of the world (Hunter and Bystriakova 2004; Simpson 2010). For many countries, palms provide food and industrial materials, as well as valuable sources of bioenergy. Several domesticated palm species are regarded as the key drivers for multiple economies. As an example, oil palm (*Elaeis guineensis* Jacq.) is the primary source of vegetable oil, the most consumed palm oil across many regions, especially in Southeast Asia, South America, and sub-Saharan Africa (Ritchie and Roser 2021). Also, coconut (*Cocos nucifera* L.) is cultivated on 12 million ha and is contributing to the livelihoods of millions of smallholder farmers around the globe (Nguyen *et al*. 2015). However, both oil palm and coconut production are facing significant threats from palm senility, pests,

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diseases, natural disasters, climate change, and genetic erosion (Nguyen *et al*. 2015; Abul-Soad *et al*. 2017). Therefore, a conscious effort needs to be made to conserve and enhance genetic diversity of the major economically important palms. Any conservation plan should not only preserve palm varieties with high economic values, but also maintain those with undiscovered values. Fortunately, palms so far are the targets of international conservation efforts, which also require cross-sectoral collaborations (Coates 2016; Griffith *et al*. 2021). This ambitious goal can be achieved through *in situ* farmers' fields and through *ex situ* conservation collections.

*In situ* approaches often consist of storing germplasm in its natural habitat through on-farm conservation or using some other landscaping options. However, a major problem with many forms of *in situ* conservation is that they require significant financial support and labor for maintenance (Virchow 2005). Construction of more seed gardens is impossible due to the vast requirement for lands and the availability of so many varieties. Therefore, an alternative approach is to transport plant materials to designated facilities, such as field genebanks and *in vitro* laboratories. This method is called "*ex situ* conservation." However, one major disadvantage of *ex situ* field genebanks, similar to *in situ* conservation, is that they are under constant threat of abiotic and biotic stresses and the appearance of devastating pests and diseases (Panis and Nagel 2020; Engels and Ebert 2021). In addition, the recalcitrant nature of palm seeds prevents their dehydration and storage in *ex situ* genebanks. Alternatively, *in vitro* collections are believed to provide a cost-effective, safe, and sustainable long-term *ex situ* conservation approach for plant genetic resources, including the recalcitrant palm species (Bourdeix *et al*. 2020). Furthermore, with the presently developed *in vitro* culture protocols, maintenance of selected *in vitro*-conserved lines can be undertaken and, when needed for breeding and research purposes, can be rapidly produced and sustained over a long period of time. From an economic point of view, an *in vitro* collection is regarded to be a more logistically efficient process for the reintroduction of superior plant types back into the environment. At present, there exist two main *in vitro* approaches to conservation, that is, short- to medium-term conservation (Table 1) and cryopreservation. The former is a process of utilizing a limited source of nutrients or using growth suppressive conditions (for example, using low incubation temperatures, or a medium that induces an osmotic stress) with the aim of keeping the cultured tissues viable for a longer period of time (Panis and Nagel 2020). However, this method still involves repetitive subculturing due to nutrient depletion and is prone to contamination risk and genetic shift. On the other hand, cryopreservation method uses extremely low temperatures (− 196°C) to bring all metabolic activities of living cells to a halt, thereby enabling long-term maintenance (Panis *et al*. 2001; Engelmann 2014). This process requires an initial reduction of intra-cellular water molecules by using either a physical or a chemical dehydration process and aims to turn the internal cell solutes into a vitreous state where ice cannot be formed and cause irreversible damage. Compared to the short- to medium-term method, cryopreservation provides a long-term option for plant genetic diversity conservation. In addition, cryopreservation can also serve as a powerful tool to single out cold-tolerant varieties from a mixed collection (cryo-selection) and to eradicate viruses from culture stocks (cryo-therapy) (Watanabe *et al*. 1985; Brison *et al*. 1997; Helliot *et al*. 2002; Engelmann 2004; Wang and Valkonen 2009; Wang *et al*. 2018).

Over the past three decades, a range of cryopreservation techniques were developed and modified for various palm species. Most major palm cryopreservation studies focus on rapid cooling following either physical dehydration (N'Nan *et al*. 2008; Sisunandar *et al*. 2010a, 2010b) or chemical dehydration (Fki *et al*. 2013; Lédo *et al*. 2018; Wilms *et al*. 2019) techniques. For palm species, cryopreservation must be undertaken with careful consideration of the state-of-the-art techniques available. It should be mindful of the availability of explant types, species-specific biology, culture media, preculture conditions, cryoprotectants, and cooling and rewarming cycles and regrowth procedures. By using a proposed framework for palm cryopreservation, this paper aims to provide a more systematic approach to conducting cryopreservation techniques for palms and through which cryopreservation of palm tissues can be achieved efficiently and economically soon (Fig. 1).

In this review, the aim is to discuss four underlying elements to a framework for the cryopreservation of palm species, including (i) their response to tissue culture, (ii) the recalcitrant nature of their seed tissues, (iii) their cryobiology and responses to cryogenic techniques, and (iv) the fidelity assessment of regenerants. Together, these four elements form a basis to a practical framework for the long-term conservation of palm species using the core technology of cryopreservation.

**Presently developed tissue culture pathways for the major palm species - Tissue culture for material preparation and post-cryostorage recovery** The first cornerstone of the framework is built upon a sound understanding of plant cell totipotency, which is the capability of an individual cell or cell clusters to develop into a whole plant (Su *et al*. 2021). To date, biotechnological interventions are used to help overcome the recalcitrant nature (low responsiveness to *in vitro* culture) of various palm species. These interventions, therefore, have enabled the regeneration of some economically important palm species *via* somatic embryogenesis (SE) or direct organogenesis (Al-Khayri and Naik 2017; Lédo *et al*. 2019; Yarra *et al*. 2019). Given the diminishing nature of sample quantities after going through different

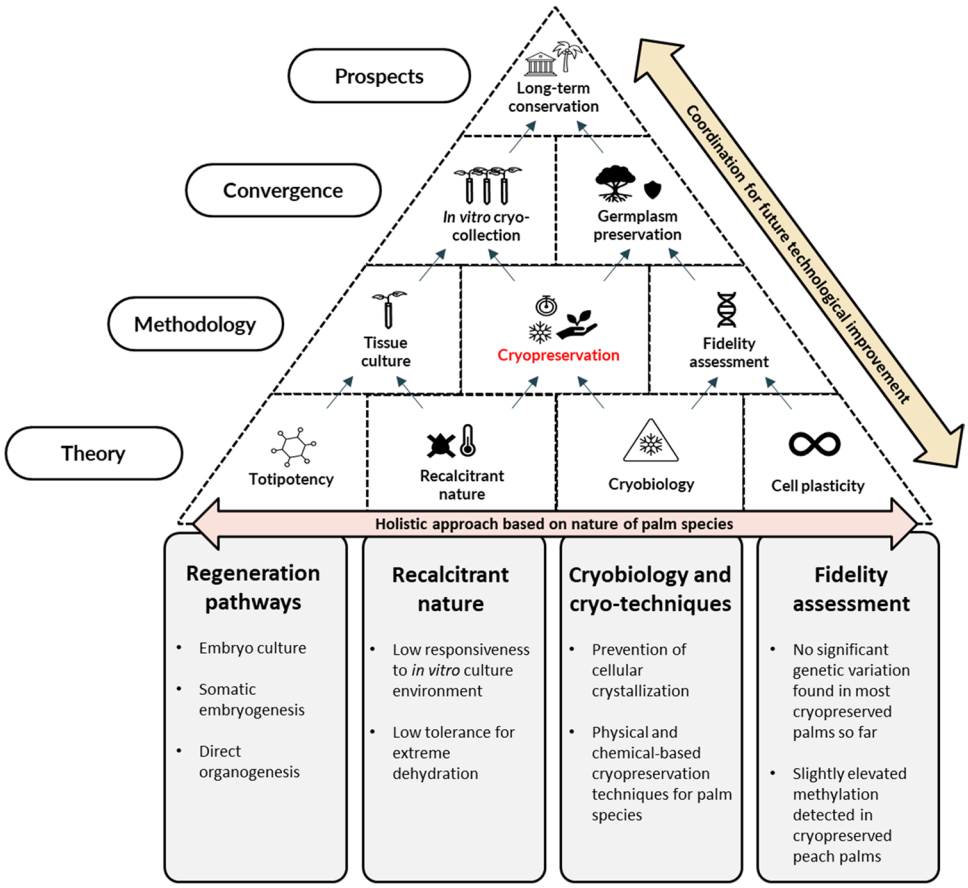


**Figure 1.** Proposed framework for long-term cryopreservation of *Arecaceae* species.

cryopreservation phases (Martinez-Montero and Harding 2015), it is necessary to multiply a surplus number of starting materials using the quickest method available, which is partially achieved by tissue culture. The process of SE involves a number of sequential steps, including the induction of callus, the formation, maturation and germination of somatic embryos, and the establishment of complete plantlets (Al-Khayri and Naik 2017). Meanwhile, direct organogenesis often starts with axillary shoot formation followed by shoot bud multiplication and plantlet establishment (Sidky 2017).

In oil palm, SE is reported to be a successful pathway for the multiple production of uniform plantlets, despite reported incidences of somaclonal variation (Weckx *et al*. 2019; Yarra *et al*. 2019). Also, SE is considered to be a fast and efficient pathway towards the large-scale propagation of date palm and coconut. Various explants (including zygotic embryos (Gomes *et al*. 2015; Monteiro *et al*. 2018), shoot tips (Al-Khayri 2010), immature leaves (Othmani *et al*. 2009; Gomes *et al*. 2017), adventitious bud and proximal leaf segments (Mazri *et al*. 2017), immature inflorescences (Teixeira *et al*. 1994; Jayanthi *et al*. 2015, anthers (Perera *et al*. 2008), plumules (Pérez-Núñez *et al*. 2006), and ovaries (Bandupriya *et al*. 2017)) were used as explants for the induction of callus in these palm species. On an industrial scale, it is speculated that advances in SE techniques may achieve a 10,000-fold multiplication (Pérez-Núñez *et al*. 2006; Sáenz *et al*. 2018).

On the other side, direct organogenesis was utilized in several regeneration protocols, especially for date palm (Bekheet 2013; Mazri and Meziani 2013; Meziani *et al*. 2015). In coconut, propagation of plant materials was mostly achieved through embryo culture (Assy-Bah *et al*. 1987; Rillo 1999; Sisunandar *et al*. 2015) and SE (Pérez-Núñez *et al*. 2006; Samosir and Adkins 2014) with a view towards conservation and germplasm exchange of coconut varieties (Nguyen *et al*. 2016; Oropeza *et al*. 2018). Very recently, coconut shoot tips have proven to be potential explants for both multiplication and cryopreservation, which completes the available repertoire of methodologies for regeneration of the most important palm species (Wilms *et al*. 2019, 2021). This new achievement has paved the way for *in vitro* establishment of regenerants in the post-cryopreservation stage.

**Explant choice** Since recalcitrant species cannot be conserved by cryopreservation using whole seeds, different explants must be used from which whole plants can be recovered. Each type of explant has different requirements

**Table 1.** Short- to medium-term *in vitro* conservation studies on *Cocos nucifera* L., *Phoenix dactylifera* L., and *Elaeis guineensis* Jacq.

| Species | Explant | *In vitro* method | Results | References |
|---|---|---|---|---|
| Coconut | ZE | Culture in a modified Y3 medium with half strength macro- and micro-nutrients | 53% of ZEs were able to produce normal seedlings after 2 mo | Karunaratne 1988 |
| | ZE | Storage in sterile water | 80% of embryos survived in recovery medium after 2 mo | Karun and Sajini 1994 |
| | ZE | Culture in MS medium without sucrose | 100% of embryos survived after 6 mo of storage | Assy-Bah and Engelmann 1993 |
| | ZE | Storage in freezer at − 20 and − 80°C | 12% of embryos were normally recovered after 3 wk of storage at − 20°C, and 28% were recovered after 26 wk at − 80°C | Sisunandar *et al*. 2012 |
| Date palm | Shoot buds | Culture in MS medium without PGRs | 100% of buds survived after 3 mo of storage | Bekheet *et al*. 2002 |
| | Callus | Culture in MS medium supplemented with 10 mg $L^{-1}$ 2,4-D and 3 mg $L^{-1}$ 2iP | Callus were viable with mild browning after 12 mo of storage at 5°C in the dark condition | Bekheet *et al*. 2002 |
| | Pollen | Storage in freezer at − 5°C | 71% of pollen germinated after 1 mo of storage | Ateyyeh 2012 |
| Oil palm | Polyembryoids | Storage in refrigerator at 5°C | 73.3% survived after 2 mo of storage | Palanyandy 2013 |
| | Pollen | Oven-drying at 37°C for 2–8 h and storage in deep freezer (− 15°C) | 100% of pollen germinated after 12 mo of storage | Ekaratne and Senathirajah 1983 |

**Abbreviations:** *2,4-D*, 2,4-dichlorophenoxyacetic acid; *2iP*, isopentenyl adenine; *MS*, Murashige and Skoog (1962); *PGR*, plant growth regulator; *Y3*, Eeuwens (1976); *ZE*, zygotic embryos

for cryopreservation (Supplementary Table 1). A broad range of explant types can be used for cryopreservation in major palm species, including pollen, zygotic embryos and isolated plumules, somatic embryos, friable embryogenic tissues (FET), embryogenic cell suspensions (ECS), nodular culture, polyembryonic masses (PEMs), and meristems (Table 2). Coconut cryopreservation was attempted on zygotic embryos, pollen, plumules, embryogenic callus, and recently vegetative shoot tips. However, cryopreservation of the zygotic embryo and pollen are considered the only complete protocols shown to be possible to date (Bourdeix *et al*. 2020). In date palm, attempts have been made on the palm's pollen, meristems, shoot tips, FET, somatic embryos, and ECS. For oil palm, advances have been made in using a wide array of explants, including intact seeds, whole kernels containing embryos, pollen, zygotic embryos, somatic embryos, and friable embryogenic callus.

When embryos are used, their maturity stage is a significant factor that affects the success of cryopreservation. Previous studies on orthodox species showed that mature embryo is the best stage for cryopreservation (Kermode and Finch-Savage 2002; Wen and Song 2007; Vineesh *et al*. 2015). However, the embryo stage for cryopreservation varies depending on the species in recalcitrant plants. In coffee (*Coffea arabica* L.) and jackfruit (*Artocarpus heterophyllus* L.), zygotic embryos harvested at an intermediate stage of maturity were more successfully used in cryopreservation as compared to immature or fully mature embryos while in tea (*Camellia sinensis* L.) and coconut, the mature embryos gave the highest survival rate after cryopreservation (Abdelnour-Esquivel *et al*. 1992; Chandel *et al*. 1995; Kim *et al*. 2002; Sisunandar *et al*. 2014.

For small explants, such as plumular tissues, embryogenic callus, or meristems, it is also essential to determine the appropriate development stage at which cryopreservation will be most successful, and to determine if any preculture of the tissues (for example, preculture using high sucrose solutions or osmoprotectants) can aid the cryopreservation process. Researchers should also be mindful that careful excision of the explants and their appropriate recovery will help reduce the impact of unavoidable stress. According to Sisunandar *et al*. (2010a), the highest post-cryotreatment survival rate, germination, and normal seedling production belong to embryos harvested at 11 mo of age. In cryopreservation of date palm meristematic cell aggregates, explants with sizes below 3 mm and displaying the meristematic-aggregate-bearing structures were precultured on 18% sucrose and incubated at low temperatures for several days before being subjected to different vitrification schemes. After cryotreatment, they were able to produce proembryos and adventitious buds, which later multiplied using a temporary immersion system (Fki *et al*. 2014). In cryopreservation of oil palm polyembryoids, selected cell suspension-derived aggregates must be larger than 500 µm and contain developing embryoids. Observation of haustorium and torpedo structures was the prerequisite for high regeneration rates in

**Table 2.** Cryopreservation protocols in notable economical *Arecaceae* species

| Species/genotypes | Explant type | Preconditioning treatment | Cryopreservation protocol | Fidelity assay | Results | References |
|---|---|---|---|---|---|---|
| *Cocos nucifera* L. | ZE | **Incubation pretreatment:** 60% sucrose and 15% glycerol (17 to 24 h) | **Method:** Air desiccation and modified vitrification<br>**Dehydration:** Up to 24 h (**WC:** 6.4%) → **LN storage:** 24 h | N/A | Survival: 6.6 to 92.8%<br>Further embryo development: 33 to 93% | Assy-Bah and Engelmann 1992 |
| *Cocos nucifera* L. | Plumule | **Sucrose preculture:** 34.2% sucrose (2 d) | **Method:** Encapsulation-dehydration<br>**Dehydration:** 6 to 16 h (**WC:** 32.9 to 43.8%, silica gel) → **LN storage:** 2 h | Histology observation | Survival: 60%<br>Regrowth rate: 20% | N'Nan *et al.* 2008 |
| *Cocos nucifera* L. | ZE | N/A | **Method:** Rapid dehydration and rapid freezing<br>**Dehydration:** 8 h (silica gel) → **LN storage:** 48 h | Morphological, cytogenetic analysis, DNA methylation and SSR profile analysis | Similar morphology, chromosome length and ploidy levels, allele makeup and global methylation rate; small, genotype-dependent differences in chromosome structures | Sisunandar *et al.* 2010a |
| *Cocos nucifera* L. | ZE | N/A | **Method:** Rapid dehydration<br>**Rapid dehydration**: 8 h (**WC:** 20%) → **LN storage**: 24 h | N/A | Regrowth rate: 64%<br>Soil establishment rate: 20 to 40% | Sisunandar *et al.* 2010b |
| *Cocos nucifera* L. | ZE | **Sucrose preculture:** 10.3% sucrose (3 d) → 20.5% sucrose (3 d) | **Method:** Vitrification (PVS3)<br>**PVS3:** 16 h (**WC:** 20%) → **LN storage:** 24 h | Viability testing with TTC and histology observation | Survival: 70 to 80%<br>Regrowth rate: 20 to 25%<br>Soil establishment rate: 22.5% | Sajini *et al.* 2011 |
| *Cocos nucifera* L. (var. Brazilian Green Dwarf) | ZE | **Sucrose preculture:** Y3, 20.5% sucrose (72 h) | **Method:** Vitrification (PVS3)<br>**PVS3:** 16 h (**WC:** 27.6%) → **LN storage:** 72 h | Histology observation | Survival: 72.5% | Lédo *et al.* 2018 |
| *Cocos nucifera* L. (var. Brazilian Green Dwarf) | Plumules | **Sucrose preculture:** Y3, 20.5% sucrose (72 h) | **Method:** Droplet-vitrification (PVS2 and PVS3)<br>**PVS2 and PVS3:** 15 min, T: 0°C → **LN storage:** 24 h | Histology observation | Survival: 90% | Lédo *et al.* 2020 |
| *Cocos nucifera* L. (var. MYD and WAT) | Meristem | N/A | **Method:** Droplet-vitrification<br>**PVS2:** 50 min, T: 0°C → **LN storage:** duration N/A | N/A | Survival rate: 80% | Wilms *et al.* 2019 |

**Table 2.** (continued)

| Species/genotypes | Explant type | Preconditioning treatment | Cryopreservation protocol | Fidelity assay | Results | References |
|---|---|---|---|---|---|---|
| *Cocos nucifera* L. | Embryogenic callus | **Sucrose preculture:** 25.7% sucrose (3 d) | **Method:** Encapsulation-dehydration **Dehydration:** 20 h (**WC:** 27 to 30%, silica gel) → **LN storage:** 2 h | SEM analysis and SSR marker | Survival: 45% Regrowth rate: 25% | Welewanni *et al*. 2020 |
| *Phoenix dactylifera* L. (cv. Zaghlool) | Offshoot | **Sucrose hardening:** 34.2% sucrose | **Method:** Encapsulation-vitrification **Dehydration:** 20 min (**WC:** 65%, laminar air flow) **PVS:** 80 min at 0°C, then 40 min at 0°C → **LN storage:** 48 h | Morphogenetic analysis and RAPD analysis | Survival: 80% Regrowth rate: 75% | Bekheet *et al*. 2007 |
| *Phoenix dactylifera* L. (cv. Barhee) | Pro-embryogenic masses (from juvenile leaves) | **Sucrose preculture:** 0.02% sucrose (3 d) | **Method:** Droplet-vitrification **Partial desiccation:** 75% **WC** **PVS2:** 30 min, T: 0°C → **LN storage**: 1 h | Histology analysis and viability analysis | Survival: 63.3% | Fki *et al*. 2011 |
| *Phoenix dactylifera* L. | Meristematic aggregates (smaller than 3 mm) | **Sucrose preculture:** (18% sucrose) and **cold hardening** | **Method:** (1) standard (tube) vitrification, (2) droplet-vitrification and (3) encapsulation-vitrification → **PVS2:** 30 min, T: 0°C → **LN storage:** 1 h | Histology observation and proline content analysis | Survival (1): 26.8%, (2): 53.5%, (3): 66.7% | Fki *et al*. 2014 |
| *Phoenix dactylifera* L. (cv. Sukkari and Sultany) | PEMs | **Sucrose preculture:** 17.1% sucrose (3 d) | **Method:** D-cryoplate **Dehydration:** 90 to 120 min → **LN storage:** 1 h | N/A | Survival: cv. Sukkari (74.6%) and cv. Sultany (95.8%) | Salma and Engelmann 2017 |
| *Phoenix dactylifera* L. | Embryogenic callus | **Sucrose preculture:** MS and 17.1% sucrose (2 d) | **Method**: (1) Encapsulation-dehydration, (2) Vitrification, (3) Encapsulation-vitrification **(1) Dehydration:** 2 to 4 h (**WC:** 49%, laminar air flow) → **LN storage:** ≥ 48 h **(2) and (3): PVS2:** T: 25°C (0, 15, 30, 60, or 120 min) → **LN storage** ≥ 48 h | N/A | Survival: (1): 80%, (2): 53.33%, (3): 86.67% Regrowth rate: (1): 53.33%, (2): 40%, (3): 46.67% | Alansi *et al*. 2019 |

**Table 2.** (continued)

| Species/genotypes | Explant type | Preconditioning treatment | Cryopreservation protocol | Fidelity assay | Results | References |
|---|---|---|---|---|---|---|
| *Phoenix dactylifera* L. | Embryogenic callus (from shoot tip) | **Sucrose hardening:** 34.2% sucrose | **Method:** Encapsulation-dehydration **Dehydration:** 20 min (**WC:** 65%, laminar air flow) → **LN storage:** duration N/A | Morphological analysis and RAPD analysis | Survival: 80% Regrowth rate: 70% | Solliman *et al*. 2019 |
| *Phoenix dactylifera* L. (cv. Magdoul) | Embryogenic callus (from shoot tip) | **Sucrose hardening:** precultured in MS and sucrose without PGR (25 ± 2°C), 16 h photoperiod, 75 µmol $m^{-2}$ $s^{-1}$ (24 h) → T: 4°C, dark (24 h) | **Method:** Encapsulation-dehydration **Dehydration:** 4 h (**WC:** 39.5%) → **LN Storage:** 6 wk | N/A | Survival: 74.4% Regrowth rate: 71.25% | Metwali *et al*. 2020 |
| *Phoenix dactylifera* L. (various) | Pollen | **Moisture equilibration** with $Ca(NO_3)_2$, $MgCl_2$ and 23% relative humidity room | **Method:** N/A (pollen are physically dehydrated) **WC:** 81.6 to 86.2% **Rehydration → LNV storage:** up to 9 mo | Viability analysis | Pollen viability: 62% to 77% | Araújo de Oliveira *et al*. 2021 |
| *Elaeis guineensis* Jacq. | SE | **Sucrose preculture:** 25.7% sucrose (7 d) | **Method:** Physical dehydration **Dehydration:** 4 to 10 h (**WC:** 29 to 63%, laminar air flow) or 0 to 18 h (**WC:** 24 to 80%, silica gel) → **LN:** ≥ 1 h | N/A | Survival: 53% | Dumet *et al*. 1993 |
| *Elaeis guineensis* Jacq. (cv. Deli x La Mé) | ZE and kernel | **Short hydration** (embryo) and **long hydration** (kernel) | **Method:** Physical dehydration **Embryo: Dehydration**: 5.25 h (**WC:** 10.7%, laminar air flow) → **LN**: 1 h **Kernel**: **Rehydration** (**WC:** 23.1%) → Held over LN (30 min) → **LN storage:** 1 h | N/A | **Short hydration:** Survival: 76.6%, regrowth rate: 63.3% **Long hydration:** Survival: 85%, regrowth rate: 65% | Engelmann *et al*. 1995 |
| *Elaeis guineensis* Jacq. | Polyembryoids | **Sucrose preculture:** 17.1% sucrose (12 h) | **Method:** Vitrification **PVS2**: 10 min (**WC:** 46%), T: 26 ± 2°C → **LN storage:** 1 h | TTC assay and SEM analysis | Survival: 45% | Suranthran *et al*. 2012 |

**Table 2.** (continued)

| Species/genotypes | Explant type | Preconditioning treatment | Cryopreservation protocol | Fidelity assay | Results | References |
|---|---|---|---|---|---|---|
| *Elaeis guineensis* Jacq. | Polyembryoids | **Sucrose preculture:** 17.1% sucrose | **Method:** Droplet-vitrification **PVS2:** 10 min (**WC:** 47%), T: 0°C → **LN storage**: 1 h | SEM analysis and PCR, RAPD or ISSR analysis | Survival: 68% | Gantait *et al*. 2015 |
| *Elaeis guineensis* Jacq. | ZE | N/A | **Method:** Silica gel desiccation **Embryo dehydration:** 1 to 5 h (**WC:** 10 to 20%, ideally 13%, silica gel) → **LN storage:** 16 h | N/A | Survival: 74.7% | Norziha *et al*. 2017 |
| *Elaeis guineensis* Jacq. | SE | **Sucrose preculture:** 25.7% sucrose | **Method:** Silica gel desiccation **Dehydration:** 16 h (**WC:** 19 to 35%, silica gel) → **LN storage:** up to 20 yr | Morphological analysis | Survival 19.1% (instant); 33.2% (after 20 yr) | Beulé *et al*. 2018 |
| *Elaeis guineensis* Jacq. | ZE and kernel/endosperm plug | N/A | **Method:** (1) Physical dehydration, (2) encapsulation-dehydration, (3) vitrification-dehydration **(1) Dehydration (for embryo/kernel/endosperm plug):** 4 h (silica gel) → **LN storage:** ≥ 24 h **(2) Dehydration (for embryo):** 6 h (laminar air flow) → **LN storage:** ≥ 24 h **(3) PVS2 (for embryo):** 20 min, T: 25°C → **LN storage:** ≥ 24 h | N/A | Endosperm plug: 20% Embryo: 22.5% Regrowth rate: 16.67% | Prakash *et al*. 2019 |
| *Elaeis guineensis* Jacq. | Polyembryoids | **Sucrose preculture:** transfer to increasing sucrose level (10.3%, 17.1%, 25.7% and 34.2%) (7 d) | **Methods:** Encapsulation-dehydration **Dehydration:** 9 h (**WC:** 23.3%, laminar air flow) → **LN storage:** 1 h | SEM analysis | Survival: 73.3% | Palanyandy *et al*. 2020 |

**Table 2.** (continued)

| Species/genotypes | Explant type | Preconditioning treatment | Cryopreservation protocol | Fidelity assay | Results | References |
|---|---|---|---|---|---|---|
| *Bactris gasipaes* Kunth. | ZE | **Sucrose preculture:** 10.3% sucrose | **Method:** Encapsulation-dehydration **Dehydration:** 4 h (**WC:** 20%, laminar air flow) → **LN Storage:** 24 h | Histology observation | Survival and soil establishment: 30% | Steinmacher *et al.* 2007 |
| *Bactris gasipaes* Kunth. | SE | **Sucrose preculture:** 10.3% sucrose (1 h) → 20.5% sucrose (1 h) | **Method:** Vitrification (PVS3) **PVS3:** 4 h → **LN storage:** 72 h | Histology observation | Regrowth rate: 37% | Heringer *et al.* 2013b |
| *Bactris gasipaes* Kunth. | SE (embryogenic cluster) | **Sucrose preculture:** 10.3% sucrose (1 h) → 20.5% sucrose (1 h) | **Method:** Droplet-vitrification (PVS3) and partial dehydration **Partial air dehydration** (1 to 3 h) → **PVS3** (60 to 240 min) → **Submerged** directly into LN (1 min) → **LN storage:** duration N/A | N/A | Embryogenic regrowth rate: 60 to 90% | Ree and Guerra 2021 |

**Abbreviations:** *CV*, cultivar; *DMSO*, dimethyl sulfoxide; *ISSR*, Inter simple sequence repeats; *LN*, liquid nitrogen; *LNV*, liquid nitrogen vapor; *LS*, loading solution; *MS*, Murashige and Skoog (1962); *MYD*, Malayan Yellow Dwarf; *NAA*, 1-naphthaleneacetic acid; *PCR*, polymerase chain reaction; *PEM*, polyembryonic masses; *PGR*, plant growth regulator; *PVS*, plant vitrification solution; *PVS2*, plant vitrification solution 2 (Sakai *et al.* 1990); *PVS3*, plant vitrification solution 3 (Nishizawa *et al.* 1993); *RAPD*, randomly amplified polymorphic DNA; *SE*, somatic embryos *SEM* scanning electron microscope; *SSR*, Simple sequence repeats *T* temperature; *TTC*, triphenyl tetrazolium chloride colorimetric assay; *VAR*, variety; *WAT*, Western African Tall; *WC*, water content (on fresh weight basis); *Y3*, Eeuwens (1976); *ZE*, zygotic embryos

cryopreserved oil palm polyembryoids (Gantait *et al*. 2015; Palanyandy *et al*. 2020).

**Insight to be gained** Successful establishment of regenerative cultures should be achieved prior to a start of any cryopreservation work, so it is essential to establish a solid tissue culture skillset. This crucial milestone will provide sufficient *in vitro* materials for preliminary and long-term cryostorage trials, as well as a means for recovery of plantlets post-cryopreservation. In addition, the input materials (also known as explant types) also dictate the cryopreservation procedures and outcomes. By comprehending the most efficient regenerative pathways and mastering the factors needed for successful regeneration (including physical conditions, medium composition, type of explants, and genotype), researchers would be able to conduct cryopreservation tasks on a wide choice of palm materials.

**Recalcitrant nature of palm species - Problem definition** Understanding the distinctive recalcitrant nature of a particular palm species is a crucial step towards developing an appropriate cryopreservation approach for that species. In the field of cryobiology, recalcitrance is mainly attributed to low tolerance of seed tissues to dehydration, and this has been extensively discussed in literature (Berjak and Pammenter 2013; Pammenter and Berjak 2014; Walters 2015; Ballesteros *et al*. 2021). A different response threshold to water removal and glassy state formation in the seed separates recalcitrant plant species from the rest of the plant kingdom (Walters 2015). It has been widely known that a 50 to 70% loss in total fresh weight due to dehydration is lethal to the seeds of most of recalcitrant plants (Engelmann 1997). This makes them highly vulnerable to extreme dehydration when compared with orthodox and intermediate plant species and must require specific dehydration approaches. Like other recalcitrant species, such as rubber (*Hevea brasiliensis* Müll.Arg.), avocado (*Persea americana* Mill.), and citrus (*Citrus* L.), the seeds of most palm species are unable to withstand excessive moisture loss and cannot tolerate the associated damage that is caused during inappropriate low-temperature storage (Umarani *et al*. 2015). Due to such limitations in drying, cryopreservation has been considered to provide a reliable method in place of traditional seed banks to conserve desiccation-intolerant seed crops, including most of palms (Engelmann 2004). Nevertheless, the *in vitro* culture and cryopreservation protocols of these recalcitrant-seeded species often take a great deal of time and effort to develop and yet are unable to provide a highly effective solution to the long-term conservation process.

**Dehydration approaches for recalcitrant palms - Physical dehydration** In cryopreservation, physical dehydration (also known as desiccation) is a method by which a laminar air flow hood or silica gel is used to dry the samples (Uragami *et al*. 1990; Sherlock *et al*. 2005). In the encapsulation-dehydration technique, explants can be further protected by being encapsulated in calcium alginate beads before sucrose preculture and drastic moisture reduction by physical means (Dereuddre *et al*. 1990). Physical dehydration approaches, especially encapsulation-dehydration, were used to prepare the tissues of many essential crops for cryopreservation (Gupta and Reed 2006; Halmagyi and Deliu 2006; Barraco *et al*. 2014; Pinto *et al*. 2016; Nakkanong and Nualsri 2018; AlMousa and Hassan 2021). In addition, the speed of dehydration can be modified to accommodate each specific species. For example, rapid dehydration methods using silica gel are found to be the most useful methods to dehydrate zygotic embryos of recalcitrant species (Berjak and Pammenter 2001; Sisunandar *et al*. 2010b). As the traditional slow dehydration approach used for orthodox seeds is unable to be used with recalcitrant species, the drying process for these species needs to be undertaken rapidly but only down to certain tissue water contents, then immediately rapidly cooled, which enables the cytoplasm to ultimately achieve the vitrified state (Sisunandar *et al*. 2010b). A preculture period in high sucrose can be beneficial for palm tissue undergoing physical dehydration. Sucrose acts as a non-penetrating osmotic agent to prime the explants into a suitable physiological state that can withstand subsequent moisture loss. However, at high concentrations, this compound can become toxic and must be gradually adjusted to accommodate each explant type (Sakai *et al*. 2008; Engelmann 2009).

**Chemical dehydration** Another cryopreservation approach is through chemical dehydration. This approach, which is shortened to "vitrification method" in many references and must be distinguished from physical dehydration methods that also aim at tissue vitrification, undertakes sample preparation using a mixture of cryoprotective agents, including dimethyl sulfoxide (DMSO), glycerol, and sucrose (Sakai *et al*. 1990; Panis and Lambardi 2005). Different plant vitrification solutions were developed by changing the amount and ratio of these components, but the major solutions used for palms are PVS1 (Uragami *et al*. 1989), PVS2 (Sakai *et al*. 1990), and PVS3 (non-DMSO containing) (Nishizawa *et al*. 1993). For horticultural and ornamental crops, which possess numerous vegetative shoot tips or buds, vitrification by chemical dehydration has become the technique of choice (Zamecnik *et al*. 2021) while physical approaches are still favored for palms with few vegetative shoot tips or buds (Table 2).

Over time, improved cryopreservation techniques that use vitrification solutions have been developed. Among those is encapsulation-vitrification, which, similar to

encapsulation-dehydration, utilizes alginate beads to prevent the plant tissues from coming into direct contact with the vitrification solutions thereby reducing the chances of tissues becoming intoxicated (Sakai and Engelmann 2007). Another method called droplet-vitrification involves using aluminum foil strips to hold the samples to achieve a faster cooling rate by direct and rapid exposure to liquid nitrogen (Panis *et al*. 2009). Indeed, droplet-vitrification has become the method of choice to cryopreserve the highly organized tissues of several species (Wang *et al*. 2021) while for palms it is mainly used for the cryopreservation of meristems or proembryonic cultures (Table 2). In the chemical approach, it is important to prevent damage to the tissues by controlling the concentration of the cryoprotectants and vitrification solutions used. Although DMSO-containing cryoprotective agents are often associated with high anti-freeze properties, they could be toxic to some members of the Palm family, as has been shown in peach palm (*Bactris gasipaes* Kunth.) (Heringer *et al*. 2013b; Awan *et al*. 2020). As the effect of cryoprotective chemicals can be species-dependent, future empirical research is of importance to determine appropriate use of them.

Based on the aforementioned technologies, two cryoplate approaches have been developed, namely the V-cryoplate and D-cryoplate techniques. The V-cryoplate technique was first developed from droplet-vitrification (Yamamoto *et al*. 2011) while the D-cryoplate technique was developed from encapsulation-dehydration (Niino *et al*. 2014).

**Potential approaches through molecular and oxidative responses** In temperate species, inducing the innate cold acclimation pathways before cryopreservation has long been applied so that the explants can better tolerate the extreme cooling process (Scottez *et al*. 1992; Wu *et al*. 2001; Senula *et al*. 2007; Kushnarenko *et al*. 2009; Bettoni *et al*. 2019), although efforts to improve cryopreservation on palms have not taken advantage of such molecular mechanisms. Empirical research showed that cold stress is likely to upregulate certain transcription factors (for example, the C-repeat binding factor (CBF)) that regulate the cold-related (*COR*) genes (Zhao and Zhu 2016; Mathivanan 2021). Despite the limited literature, some preliminary studies have shed some light on the cold-induced molecular pathways of palm species. For example, in oil palm, the CBF-binding capability is limited due to the lack of Dehydration Responsive Element (DRE) motifs in the *COR* genes, which leads to a lower tolerance to cold stress (Lei *et al*. 2014). One study on oil palm suggested that expression of certain *CBF* genes might be linked with tissue accumulation of sucrose or proline (Li *et al*. 2019). This prompts future investigators to conduct more basic research on similar pathways, which could be beneficial for understanding species-dependent responses to a particular cryogenic treatment (Volk 2010). However, even though desiccation-responsive and cold acclimation pathways can be universal among plant species, their expression levels substantially differ due to evolutionary adaptation (Lei *et al*. 2014; Subbiah *et al*. 2019). Therefore, practitioners should be considerate of any metabolic and proteomic differences that may be present between orthodox and recalcitrant plants.

Unlike physical damage, oxidative injury is induced only before and after the tissue is plunged into liquid nitrogen, and this is due to the stresses imposed by the dehydration and rewarming steps. Specifically, oxidative responses should be monitored when explants are recovered from cryostorage. In both orthodox and recalcitrant species, many studies have evidently shown a correlation between cryopreservation-induced oxidative stress and a direct uplift in antioxidant enzymes, lipid peroxidation, and an accumulation of reactive oxidative species (ROS) (Naidoo 2012; Ren *et al*. 2015; Pareek *et al*. 2017). Unfortunately, this is still a gap in cryopreservation research in palms. For instance, it is still elusive how ROS-induced oxidation correlates with palm explant viability after cryopreservation. In cryopreservation of Chinese fan palm, although plasma membrane destabilization and changes in antioxidant system were observed, induced oxidative stress was less likely to affect the viability of embryo explant (Wen *et al*. 2012). This implies that it is essential to identify stress tolerance threshold of explant types being used. In *Arabidopsis*, incorporation of dehydrins into vitrification solution can accelerate breakdown of ROS and promote expression of antioxidant enzymes, thereby enhancing survival of cryopreserved tissues (Yang *et al*. 2019). Hence, investigation of dehydrins, such as their expression and application, and similar antioxidative proteins (Uchendu *et al*. 2010) in palm cryopreservation should be given consideration.

**Insight to be gained** Storage of recalcitrant species by cryopreservation is much more challenging due to low tolerance for extreme moisture loss, either by long storage time or by artificial dehydration, and the survival rates often widely oscillate between 0 and 100%. Thus, choosing the most appropriate explants and developing a species-specific dehydration approach are necessary for successful cryopreservation of palms (see comparison among explants and approaches in Supplementary Table 1 and Supplementary Table 2), since many protocols, while reportedly working on other species, were proven infeasible for these recalcitrant plants. In addition, although basic research has pointed out some potential areas to investigate in terms of molecular and oxidative changes in palm under cold stress, cryopreservation studies utilizing these pieces of information continue to be extensively underdeveloped in palms and should be given priorities in the future.

**Cryobiology theory and responses of palm tissues to cryogenic techniques - The idea of achieving a vitreous state** The underlying principle of cryopreservation is to transform living tissues into a vitrified state without forming intra- or extra-cellular ice crystals, and then to store those tissues at ultra-low temperatures (Pegg 2007). This requires a clear understanding of several different fields of study, including plant physiology, cryobiology, and biochemical properties of water molecules (Martinez-Montero and Harding 2015). When water is frozen, ice crystals develop in a process known as "crystal seeding" (Huang *et al*. 2017). This can occur with water molecules in both intra-cellular and extra-cellular sites, and result in lethal damage to tissues when frozen (Pegg 2007). By coupling ultra-fast cooling with the addition of a physical or chemical dehydration approach, the cell cytoplasm can become vitrified without ice crystal formation (Wowk 2010). However, upon rewarming, further instability of the cryopreserved tissues can occur since unfrozen water molecules become remobilized within and out of individual cells and can reform ice crystals. The principle is to maintain a homogenous transition from normal to frozen state and *vice versa* to mitigate such instability and also cellular stress (Benson 2008). On the contrary, if the transition takes place heterogeneously, physical and biochemical states of the cells can be affected, leading to the so-called cryoinjury, or ruptures at intra- and extra-cellular sites (Gao and Critser 2000). The biology of recalcitrant species has in so far prevented them from being dehydrated and cryopreserved similarly to other plants and must follow specific procedural rules with regard to preculture, cryoprotectants, drying and cooling rate, and recovery (Pammenter and Berjak 2014; Roque-Borda *et al*. 2021).

**Developments of cryopreservation techniques for palm species** In recent years, a wide range of cryopreservation techniques have been attempted in palm species with some notable achievements. These optimizations are summarized in this review section and listed in Table 2.

**Oil palm** Among palm species, the cryopreservation of oil palm has been the most studied. Among physical dehydration techniques applied, silica gel-based desiccation is by far the most widely used (Norziha *et al*. 2017; Beulé *et al*. 2018; Prakash *et al*. 2019). In contrary to date palm, most of oil palm cryopreservation studies have been undertaken without a preculture treatment. There has been no clear explanation, except that the oil palm may not be a recalcitrant species as previously mentioned by Grout *et al*. (1983). Studies that actually used pretreatment reported that high-sucrose culture (17 to 26%) is important for cryopreservation of oil palm somatic embryos using both silica gel dehydration and vitrification techniques (Gantait *et al*. 2015; Beulé *et al*. 2018). There has been a variation in terms of physical dehydration duration, which could range from 4 to 6 h and towards a targeted water content, which varied between 10 and 63% (Table 2). In a recent study, other techniques have been tested, including encapsulation-dehydration on oil palm polyembryoids, which resulted in 73.3% survival rate (Palanyandy *et al*. 2020). A brief rehydration period following initial desiccation has also been successfully applied in oil palm, leading to improved survival and plantlet recovery (Norziha *et al*. 2017; Prakash *et al*. 2019; Araújo de Oliveira *et al*. 2021). Cryopreservation techniques that use vitrification solutions (PVS2) have been tested in oil palm with inconsistent results (Suranthran *et al*. 2012; Gantait *et al*. 2015; Prakash *et al*. 2019). Liquid nitrogen storage duration can also vary between 1 h and 20 yr time (Engelmann *et al*. 1995; Gantait *et al*. 2015; Norziha *et al*. 2017; Beulé *et al*. 2018). To the best of this review's knowledge, cryopreservation of oil palm has endured the longest-ever storage time among the Palm family (20 yr), albeit at a relatively low survival rate (19.1% after rewarming, 33.2% after 20 yr), with fifty shoots regenerated from one specific clone (Beulé *et al*. 2018).

As for recovery, after removing the explants from liquid nitrogen storage, most rewarming methods involve a water bath rewarming at 40°C (Suranthran *et al*. 2012; Beulé *et al*. 2018; Palanyandy *et al*. 2020) or at room temperatures (Norziha *et al*. 2017). Oil palm tissues can be then transferred onto recovery media containing high sucrose concentrations (10 to 41% sucrose) for up to 3 wk before being subcultured back onto normal propagation medium (Beulé *et al*. 2018; Palanyandy *et al*. 2020). This high sucrose medium can sometimes be referred to as unloading solution (Gantait *et al*. 2015; Prakash *et al*. 2019).

**Date palm** Both physical and chemical dehydration approaches have been applied for date palm cryopreservation (Metwali *et al*. 2020). PVS2 is a preferred solution for achieving chemical-based vitrification in date palm (Al-Bahrany and Al-Khayri 2012; Fki *et al*. 2013; Alansi *et al*. 2019). A high survival rate (up to 80%) was achieved with encapsulation-vitrification (chemical approach) (Bekheet *et al*. 2007; Alansi *et al*. 2019) and encapsulation-dehydration (physical approach) (Solliman *et al*. 2019). It is also important to note that droplet-vitrification in date palm requires a partial dehydration step to reduce the initial water content to 75% while the encapsulation-dehydration approach requires desiccation to 65% (Bekheet *et al*. 2007; Fki *et al*. 2011; Solliman *et al*. 2019). Physical dehydration using silica gel has also been demonstrated on date palm pollen; however, pollen moisture needs to be monitored and adjusted beforehand (Araújo de Oliveira *et al*. 2021). Data from other studies also indicated that cutoff moisture values can be a contributing factor for explant recovery, particularly

for heterogenous explants (Gonzalez-Arnao *et al*. 2008; Engelmann 2014).

Survival rates can also be enhanced by incorporating pretreatments, including preculture with sucrose, cryoprotective agents, and a brief dehydration, before being plunged into liquid nitrogen (Table 2). Pretreatment with higher sucrose concentrations (between 17 and 34%) was found suitable for pro-embryogenic masses, embryogenic callus, and isolated shoot tip (Fki *et al*. 2011, 2013; Salma and Engelmann 2017). It is also worth noting that duration of cryogenic treatment may range from 60 min to 6 wk. There has been no longer duration for cryopreservation of this palm species since the first study conducted three decades ago (Towill *et al*. 1989). The most recent advancement involves the use of D-cryoplate method, which produced a high survival rate (95.8%) (Salma and Engelmann 2017). Similar to other Palm members, during recovery, a medium containing normal sucrose concentration or similar with one used for tissue culturing the respective explant was used to recover date palm embryogenic callus (Alansi *et al*. 2019; Solliman *et al*. 2019; Metwali *et al*. 2020). For D-cryoplate method and vitrification, an initial high sucrose concentration (41%) was used for unloading; then, a lowered concentration (10 to 17%) was used for recovery (Fki *et al*. 2014; Salma and Engelmann 2017; Alansi *et al*. 2019).

**Coconut** Cryopreservation research in coconut has trailed behind that undertaken on other members of the Palm family. Due to a recent decline in coconut productivity around the globe and the rapid loss in diversity, there has been a concerted effort to develop such techniques (Karun *et al*. 1999, 2014; Sisunandar *et al*. 2010a; Malaurie *et al*. 2011; Sajini *et al*. 2011; Lédo *et al*. 2018; Kim *et al*. 2019). To date, most coconut germplasm is conserved in genebanks to be found in more than 30 countries around the world, including five International Coconut Genebanks located in Brazil, Côte d'Ivoire, India, Indonesia, and Papua New Guinea. The COGENT (The International Coconut Genetic Resources Network) is among the most active research organizations that facilitate global collaboration for coconut genetic resource conservation and utilization. It has released a 10-yr global strategy that includes a comprehensive analysis of the current coconut genetic diversity and the accompanying conservation methodologies (COGENT *et al*. 2017). Even though there are 39 country members that actively contribute to the network's visions, work into the cryopreservation of coconut has only been undertaken by research teams in Australia, Belgium, Brazil, Côte d'Ivoire, France, India, Korea, Malaysia, Sri Lanka, and the UK (Welewanni and Bandupriya 2017) (Table 2).

For coconut cryopreservation, zygotic embryos are often physically dehydrated either using a laminar air flow hood or by silica gel in a desiccator (Assy-Bah and Engelmann 1992; N'Nan *et al*. 2008; Welewanni *et al*. 2020). One method using rapid dehydration of zygotic embryos with a silica gel chamber, followed by rapid cooling and rewarming, resulted in a high number of seedlings of uniform morphology, cytology, and molecular makeup growing in soil (Sisunandar *et al*. 2010a). In order to achieve such a high rate of recovery to soil, the physical dehydration approach needed to reduce embryo water content to 20% in just a few hours (Sisunandar *et al*. 2010a). More recently, work has been undertaken on coconut vegetative shoots (Normah *et al*. 2019; Wilms *et al*. 2019). In these studies, a PVS2- or PVS3-based droplet-vitrification approach was used to overcome ice crystal formation (Sajini *et al*. 2011; Lédo *et al*. 2018; Wilms *et al*. 2019), yet no plants have been recovered to soil using these methods.

Recovery medium for encapsulated-dehydrated coconut tissues does not strictly require high sucrose concentrations and may be identical in composition to normal culturing media for the respective tissues (Assy-Bah and Engelmann 1992; Sisunandar *et al*. 2010b; Sajini *et al*. 2011; Lédo *et al*. 2020). However, for techniques using vitrification solutions, it is reported that a high-to-low sucrose recovery medium was used for up to 2 wk post-cryostorage (Lédo *et al*. 2018, 2020; Wilms *et al*. 2019).

**Insight to be gained** Due to the occurrences of lethal ice crystals during cooling and rewarming cycles, successful cryopreservation protocols for palms should be designed towards minimizing such events and achieving a homogenous vitreous state throughout the tissue. This state is called vitrification, which can be achieved with either physical or chemical dehydration approaches. Indeed, cryopreservation protocols have been refined for targeted palm species and varieties with a diverse array of techniques and explants used. The biggest gap in palm cryopreservation, however, is the lack of studies that adequately regenerate palm plantlets post-cryopreservation into field-grown plants. Taken together from past research, researchers should begin their studies by addressing a set of aspects: (i) explant choice and cryopreservation techniques; (ii) detailed procedures and chemicals; and (iii) clear outcomes and analytical measures (depicted in Supplementary Fig. 1).

**Fidelity assessment approaches for cryopreservation in palms-Morphological and physiological assessments** Several ways have been used to assess the viability of recovered cryopreserved plant tissues. It is worth mentioning that the term "survival" and "recovery" are sometimes used ambiguously in reports, although they should be clearly distinguished. While survival rates consider post-cryostorage recovered tissues to contain viable cells, recovery demands that those tissues be able to grow back into a fully functional

organ or a complete plantlet. Sometimes report may use the term "regrowth" to indicate the increase in size of recovered tissues with or without subsequent organ regeneration. In palms, the viability of zygotic embryos is determined by measuring certain phenotypic traits, such as shoot or root elongation or the increase in embryo biomass (Steinmacher *et al*. 2007; Sisunandar *et al*. 2010b). Cryopreserved callus tissues are considered to be viable when visible swelling or new callus formation occurs (Welewanni *et al*. 2020). Polyembryoids are considered to have survived if they show growth of meristematic regions or shoot apices (Gantait *et al*. 2015). However, since the final requirement for an effective cryopreservation protocol is the successful establishment of healthy plants in soil, using viability measurements alone cannot determine the efficiency of the protocol.

Morphological and physiological assessments of the recovering tissues should be monitored throughout the recovery process and to a point well after soil establishment. For coconut cryopreservation, only two studies have reported the successful soil establishment of seedlings coming from cryopreserved zygotic embryos (Sisunandar *et al*. 2010a; Sajini *et al*. 2011). The regenerated coconut seedlings produced after cryopreservation demonstrated normal morphological, cytological, and genetical character, and the only difference was in the rate of shoot growth, which was slower in plantlets coming from cryopreserved embryos (Sisunandar *et al*. 2010a). For oil palm, field observations carried out on plantlets coming from cryopreserved somatic embryos were reported to show normal morphology and floral conformity and identical to non-cryopreserved plantlets (Konan *et al*. 2006). In a similar study, long-term observations made on six oil palm clones coming from cryopreserved polyembryonic cultures showed an average recovery of 34% (Konan *et al*. 2007). The study revealed that the percentage of abnormal palms originating from cryopreserved polyembryonic cultures was considerably lower (5%) than those originating from non-cryopreserved culture (29%), suggesting the possibility of using cryopreservation as a tool to select highly proliferative and normally developing cultures of oil palms.

**Histological, ultrastructural, and cytological assessments** In a histological analysis, chromosomal changes of cryopreserved palms can be clearly observed by microsectioning and using various staining techniques (Table 2). However, the extent of nuclear damage observed by these methods is unlikely to give a true understanding of what may occur in the fully regenerated plant. In palm cryopreservation, osmotic damage can be detected by histological observations made on cell plasmolysis, damaged plasma membranes, retracted nuclei, and increasing periplasmic space, while dehydration damage can be detected by broken plasma membrane with condensed and poorly stained cytoplasm, decreasing nucleus and cytoplasmic ratio, and leaking intra-cellular soluble proteins. Furthermore, pyknotic nuclei and disappearing nucleoli observed through histological methods can predict chromatin contraction, which can be helpful when coupled with epigenetic assessments (Welewanni *et al*. 2020). Histological staining is used to observe the epidermal, protodermal, and procambium layer of cryopreserved coconut zygotic embryos (*var.* Brazilian Green Dwarf) although not at a comparable resolution to ultrastructural analyses (Lédo *et al*. 2018). In addition, it was reported on peach palm and date palm that detecting viability through a histological method can help draw conclusions about the correlation between the remaining meristematic regions and cellular vacuolation during a later stage of recovery (Steinmacher *et al*. 2007; Fki *et al*. 2013).

Ultrastructurally, technologies, such as Scanning Electron Microscopy (SEM), can assist in observing epidermal layers and specific contraction of cell regions (Welewanni *et al*. 2020). For example, in peach palm somatic embryo and oil palm polyembryoid cryopreservation, SEM helped visualize cell shape and configuration, cell and nucleus disruption, cell wall breakdown, mitochondria and middle lamella damage, and cellular space appearance (Heringer *et al*. 2013b; Gantait *et al*. 2015; ). Detecting such morphological damages, especially in similar minuscule tissues, can help accelerate the evaluation process for a particular treatment. Also, there is currently a lack of studies looking closely into gas exchange or leaf chlorophyll formation, which are indicators of self-sustaining capabilities of non-damaged tissues. In palm species, using these methods is less destructive and allows for more frequent sampling of plant material (Equiza and Francko 2010).

Cytological tests on coconut showed that the cryopreserved regenerants maintain the same gross chromosome arrangement (numbers and size) as non-cryopreserved plantlets. There was also no significant difference in the global methylation rates between coconut seedlings coming from cryopreserved and non-cryopreserved embryos (Sisunandar *et al*. 2010a) although some minor differences were detected among genotypes. This study also reported a high frequency of black banding (N-banding) in the cryopreserved seedlings, suggesting a possible relationship between the desiccation pretreatment with chromosomal protein denaturation and inhibition of certain quintessential functional genes (Sisunandar *et al*. 2010a).

**Molecular change assessments** It is believed that plant cells possess an exceptional ability, known as plasticity, which allows them to adapt and survive through stress events. However, during cryopreservation, plant genetic makeup has to encounter many impactful factors, for example, injury during cooling and rewarming transition, osmotic disruption, toxicity of cryoprotective agents, oxidative damage, and accumulation of secondary products (Martinez-Montero and Harding 2015). Therefore, genetic fidelity assessments are necessary to ensure that cryo-related stress does not exceed the limit of

cell plasticity as it would result in undesired mutations or malfunctioning gene expression. This kind of assessment is often carried out using molecular marker techniques (Khan *et al*. 2012), which were previously developed for gene mapping or studies of related species, and are now widely employed to ensure that targeted accessions are well-maintained in genebanks (Spooner *et al*. 2005). Still, the use of genetic markers in palm cryopreservation research has only been targeted at checking whether recovered progenies are of any significant difference from their source plants, while in fact, those assessments could have been serving a more integrative picture and applied consistently over an extended period.

In some other species, molecular marker methods detected a mixed result between some and no variation with some possible reasons. In *Lamprocapnos spectabilis* L., RAPD (Random amplified polymorphic DNA) and ISSR (Inter simple sequence repeat) methods detected 5% variation, mostly of non-encapsulated samples (Kulus 2020). Meanwhile, in *Chrysanthemum*×*morifolium* Ramat., RAPD and AFLP (Amplified fragment length polymorphism) methods helped conclude that other steps of the procedure, such as sucrose preculture, may have caused the variations (Martín *et al*. 2011). RAPD, ISSR, AFLP, and SSR (Simple sequence repeats) methods have also been used to detect instability in other species, although the results can be inconsistent between two different measurement periods, or between two methods used or two different studies on the same species (Kaity *et al*. 2008, 2013; Castillo *et al*. 2010; Martín *et al*. 2015; Bi *et al*. 2016; Ibáñez *et al*. 2019).

In contrast to aforementioned research, genetic variation has yet to be reported in different cryopreserved palm species, with different cryogenic techniques being used (Bekheet *et al*. 2007; Sisunandar *et al*. 2010a; Gantait *et al*. 2015; Alansi *et al*. 2017; Solliman *et al*. 2019; Welewanni *et al*. 2020). In coconut, all-round fidelity analyses were undertaken on cryopreserved regenerants and, thus far, no significant variation has been reported (Sisunandar *et al*. 2010a; Iroshini *et al*. 2017; Bandupriya *et al*. 2017). Among the methods used, RAPD and ISSR techniques are the most chosen for their low cost, high speed, and small amount of required DNA (Gantait *et al*. 2015; Alansi *et al*. 2017). Yet, one caveat to the use of RAPD and SSR markers to detect variations is that they can only perform screening on an extremely small portion of the DNA. Firm conclusions about the whole genome cannot be drawn if these methods detect no differences or detect differences that happen to be on non-coding DNA portions. Usage of fidelity assessment protocols on palms is further hindered by a lack of studies that aim to develop molecular markers based on recently completed genome sequences of palm species.

**Epigenetic change assessments** Plant living materials are known for their great adaptability and this capacity is closely linked to changes at transcriptional level. These changes are due to a phenomenon called DNA methylation (Finnegan and Kovac 2000). Scientists believe that assessing its rate can help understand how plant cell and tissue cope with cold-storage condition. This phenomenon was hypothesized to protect DNA regions from the stress-induced damages during cryopreservation process (Heringer *et al*. 2013a). Evidence suggested that global methylation profiles of cryopreserved plantlets return to a normal level after a period of elevation (Harding 2004). Global DNA methylation rate (often expressed in percentage) is usually the generic measure for this particular analysis, which determines the frequency of 5′- methylated deoxycytosines over the total amount of cytosines (Xie *et al*. 2017). In peach palm, global methylation rates of PVS3-treated peach palm embryogenic clusters increased temporarily after they had been subjected to cryopreservation treatment and eventually returned almost to the control level after several weeks of recovery (Heringer *et al*. 2013a). Similarly, no significant differences were found in the percentages of global DNA methylation rate between plants recovered from cryopreservation and non-cryopreservation on coconut (Sisunandar *et al*. 2010a). This might suggest that elevation of methylation rates can be beneficial for the survival and regrowth of cryopreserved materials in palms as it enables cells to temporarily hold up normal gene regulation and functionality. It is also worth combining these epigenetic studies with DNA change assessment to produce more robust fidelity assessment.

**Insight to be gained** Morpho-physiological assessments should involve consistent analysis of tissue viability and subsequent shoot and root growth upon rewarming until the plant is well established in the field. Major histological, ultrastructural, and cytological techniques should be applied to further observe gross changes which the cryogenic treatments can cause on the cryopreserved tissues of palms. These methods, however, demand destructive preparation of the tissues, and may not be suitable if there is low availability of materials. Finally, since the ultimate goal of palm conservation is to preserve true-to-type clones, an effective program should involve rigorous molecular and epigenetic evaluations of cryopreserved regenerants.

## Conclusion and prospects

Palms are an example of economically important species that demand immediate conservation efforts and also offer decades of illustrative cryopreservation research trials. The current review differs from previous palm reviews in that it encourages researchers and conservationists to base their cryopreservation research on the proposed framework and a series of consideration checkpoints (tissue culture,

recalcitrant nature, cryobiology, and fidelity assessments), and to progress to further topics created by the combination of two nearby foundations. Cryopreservation is a multifaceted process that requires careful construction of different research blocks and having a frail one can result in a futile research endeavor. In reality, even though considerable efforts have been made in cryopreservation of important palm crops to achieve a certain degree of regrowth rate, the goal of true regeneration of cryopreserved plantlets (also known as soil establishment) has not been met satisfactorily by the majority of reports. The long-term view of palm cryobank construction is yet to be fulfilled due to a number of factors, including the lack of a systematic effort and often times short duration of research projects. Indeed, most current palm collections are only serving the immediate consumer demands instead of creating broader and more collaborative collections to preserve the palm taxa. With a diversity of species-specific protocols and problems, it is easy to get lost in the minor details and forget about the big picture. Thus, this review provided the most important insights for each foundation of the framework. It is important to note that the approach used does depend on species recalcitrance and the availability of explant tissues. Looking ahead, technology transfer among research laboratories around the globe should be facilitated to ensure high reproducibility of cryopreservation protocols, and a fidelity assessment should be performed with robust and state-of-the-art methods.

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## Declarations

**Conflict of interest** The authors declare no competing interests.



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