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Cryopreservation for tree species with recalcitrant seeds: the avocado case

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Abstract

Recent developments in the cryopreservation space has increased the trend in germplasm collections established through cryopreserved *in vitro* material. Cryopreservation of recalcitrant seeds through embryos and embryonic axes, is not uncommon. Tropical and sub-tropical plants are not acclimated to the cold season, therefore have no in-built natural resilience to the cold. Also, larger seeds from trees, such as avocado (*Persea americana* Mill.), mango (*Mangifera indica*) and durian (*Durio zibethinus* L.) are sensitive to desiccation, chilling and freezing stress, making them unsuitable for seed banking or cryopreservation. Alternatively, as seeds do not carry the same genetic make-up as the mother plant, especially in the context of woody rainforest species of which the cross-pollination is dominant; seed conservation does not serve the purpose of germplasm preservation. Other plant material and methods are needed for these plants to be successfully stored in liquid nitrogen (LN). One such method commonly used is shoot-tip cryopreservation which ensures the clonal fidelity of germplasm. There are many problems when using shoot tips of tropical recalcitrant-seeded species. These include: 1) the toxic effects of cryoprotective agents towards structural integrity; 2) optimum developmental stage for success and 3) oxidative stress associated with excision injury leading to necrosis triggering cell death and hindering regeneration for the shoot tips in culture. A pre-requisite for any cryopreservation system is the availability of an established tissue culture regeneration platform. This review will outline conservation strategies for avocado with special emphasis on attempts and improvements made in the cryopreservation space for storing this horticulturally important crop 'avocado' at ultra-low temperatures.

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1. Introduction

Globally plants are recognized as a vital component of biodiversity and sustainability. An estimated 7000 species of plants provide food in the form of fibre, fuel, shelter, medicine [1]. Conservation and utilization of genetic resources requires many methods; however, the best results are often achieved when both traditional (*in situ* and *ex situ* conservation) and modern biotechnological tools (*in vitro* conservation and cryopreservation) are used in combination [2].

In situ conservation involves the designation, management and monitoring of target taxa where they are encountered [3]. It protects an endangered plant species in its natural habitat. It is especially appropriate for wild species and landrace material on farm [4]. *In situ* techniques are described as protected areas, e.g., genetic reserve, on-farm and home garden conservation. It is complementary to *ex situ* conservation particularly to conserve species that are difficult to conserve under *ex situ* conditions.

Ex situ conservation is advantageous as physical attributes and characteristics of the accessions such as plant habit, yield, tree height and disease resistance can be evaluated periodically [5]; however, there are several limitations posed; high maintenance cost, intensive labour and land requirements, pressure of natural calamities, risk of biotic and abiotic stresses as well as funding sources and economic decisions limiting the level of accession replication to maintain genetic diversity.

In vitro conservation is generally the maintenance of plant material under *in vitro* culture with growth retardants [6], reduced light [7] or reduced temperature [8] to achieve slow growth. Plant germplasm storage via these methods has been increased with more tissue culture protocols being developed for a vast number of plant species [9-11]. These methods allow for physiological evaluation of material and rapid multiplication and plant establishment when needed, still, very costly to maintain due to space, consumables and labour inputs.

Plant cryopreservation (storage at -196 ± 1 °C) is a technique whereby plant tissues are preserved at ultra-low temperatures without losing viability [12]. A wide range of plant tissue can be cryopreserved, e.g., pollen, seeds, shoot tips, dormant buds, cell suspensions, embryonic cultures, somatic and zygotic embryos and callus tissue [13-14]. The choice of material used, depends on the conservation goal, e.g., seeds and embryos capture species diversity; whereas shoot tips and dormant buds capture specific genotypes [15]. Cryopreservation offers a

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continual supply of material for long-term *in vitro* research involving genetic manipulations [16] and often combined with tissue culture techniques [17].

Recent uses of cryopreservation including cryotherapy to eradicate pathogens, such as bacteria, phytoplasmas and viruses in plants [18-19] is gaining a lot of attention [16]. Samples are normally given a short exposure to LN and surviving cells are regenerated from meristematic tissue which is pathogen-free [19].

Theoretically, cryopreserved materials can be stored indefinitely without any genotypic and phenotypic variations [16], offering an attractive approach to conserve germplasm of both shoot tips and somatic embryos [17]. Cryopreservation is currently the only available alternative for long-term storage of recalcitrant-seeded species such as avocado [12, 20].

2. Cryopreservation for plant species with recalcitrant seeds

During the time conservation methods have been used for storing plant tissue, many reviews have been carried out to determine success [21-24] and standards established for managing cryopreservation gene banks [25-26]. Due to the success of *in vitro* conservation techniques, many *in vitro* gene banks have been established nationally and internationally [27-28] with several cryopreservation germplasm repositories set-up for various plant species (Table 1).

Table 1 Some examples of germplasm repositories for various plant species.

Germplasm Bank	*Genus & Accessions held*
France • The International Network for the Improvement of Banana and Plantain • Institute of Research for Development	*Musa* – 882 [29] Coffea – approx. 500 [30]
Peru • International Potato Centre	Solanaceae – approx. 755 [31]
Costa Rica • Centre for Research and Higher Learning in Tropical Agriculture	Coffea – 80 [32]
Rep. Korea • National Institute of Crop Science, Rural Development Administration	Solanaceae – approx. 1223 [30]

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Japan • National Centre for Seeds and Seedlings • Shimane Agriculture Research Centre • National Institute of Agrobiological Sciences	Solanaceae – 130 [30] Wasabi – 40 [30] Mulberry – approx. 1000; Juncus – 50 [30]
Germany • Leibniz Institute of Plant Genetics and Crop Plant Research	Solanaceae – 2885 Mint – 97 Allium – 112 [30, 33]
India • Potato Research Institute, Shimla • National Bureau of Plant Genetic Resources, New Delhi	Solanaceae – 1500 [30, 34] Legumes – 67 274 [35]
USA • National Clonal Germplasm Repository	*Malus* – 6073 *Pyrus* – 131 *Rubus* – 57 *Vitis* – 1405 [36-37]

Cryopreservation is a safe and cost-effective option for long-term conservation of genetic resources of non-orthodox or recalcitrant seeded species [38] with seeds that cannot be stored long-term. A considerable number of species, mostly sub-tropical or tropical in origin have recalcitrant seeds, e.g., coconut, cacao, mango, avocado and many fruit and forest tree species [39-40]. These recalcitrant seeds are shed at relatively high moisture content, thus cannot undergo drying to facilitate long-term storage [4, 41]. The heterogenous nature of seeds produced through open pollination limits effectiveness of and aim of conservation [4, 42]. Over almost 100 different species of zygotic embryos/embryonic axes and somatic embryos of approximately 40 different species, from both tropical and temperate climates, consisting of fruit, forest trees, and crops with seeds of intermediate, orthodox and recalcitrant storage characteristics, have been successfully cryopreserved [16].

Many cryopreservation techniques/protocols have been developed for storing germplasm of various plant material e.g. seeds, pollen, somatic embryos, dormant buds and shoot tips [38, 43]. Plants cryopreserved using callus (unorganized) can be susceptible to

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genetic variation when regenerated [44]. Shoot tip cryopreservation is advantageous as it is clonal and true to the accession being preserved without any heterogeneity. Apical meristems, the most commonly used, are at less risk of genetic variations due to their organised structure. Meristems are made up of small unvacuolated cells generally having a small vascular system [44]. The earliest plant species studied for shoot-tip cryopreservation were potato (*Solanaceae* spp.) and cassava (*Manihot esculenta*) [45]. The early protocols used controlled-rate cooling, these were later refined to improve all steps of the protocols and applied to many plants, especially temperate species [46-47].

Shoot-tip cryopreservation protocols, developed following procedures for plant-cell suspensions [31], included the use of controlled-rate cooling (two-step cooling procedures) to freeze water in the intracellular spaces and dehydrate cells to the point where they would turn to a glassy state (vitrify) upon contact with LN [15, 48-49]. Rapid cooling usually results in intracellular ice formation, whereas slow cooling results in extracellular ice formation [50] responsible for osmotic shock injury [12]. The formation of intracellular ice damages cell membranes directly as the growing ice crystals push apart and rupture membranes causing cell damage [51]. Cryoprotectants are generally used in vitrification protocols; they are a chemical substance or mixture generally high in molarity that prevents intracellular ice formation, or prevents damage to cells during cooling [12, 52-53]. Cryopreservation has several steps: 1) initial excision of the germplasm; 2) desiccation or pre-culture on osmotic media to reduce water content; 3) cryoprotection through exposure to cryoprotective agents; 4) cryopreservation in LN; 5) re-warming; and 6) unloading of cryoprotective agents and recovery of germplasm after cryopreservation [20].

2.1 Cryopreservation methods to reduce water content

Concentrated intracellular solute is a pre-requisite for successful cryopreservation and can be achieved with the following methods (Table 2), either individually or in combination [54-57].

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Table 2 Methods to dehydrate/concentrate intracellular solute for cryopreservation.

Dehydration Method	*Uses*
• **Desiccation**	- Air drying of explants in laminar flow hood or using flow of compressed air. - Dehydration of explants in a desiccator with silica gel.
• **Cryoprotectants/ vitrification solutions**	- Penetrating cryoprotectants, e.g., dimethyl sulfoxide (DMSO) and glycerol have rapid penetrating power and act by replacing intracellular water. - Non-penetrating cryoprotectants, e.g., sucrose, polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG), dehydrate cells by exerting osmotic stress. - Commonly used cryoprotectant mixes are plant vitrification solution 2 (PVS2) [58] and plant vitrification solution 3 (PVS3) [59].
• **Freeze-induced dehydration**	- Preferential freezing of extracellular water by slow cooling at a rate of 0.5 – 2 °C per min creates a hypotonic surrounding for the cell, resulting in outflow of cellular water.
• **Pre-conditioning of donor plant or explant**	- Including DMSO, abscisic acid, sucrose, polyols or proline in the pre-culture medium or low temperature treatment to induce tolerance to dehydration and freezing.

Cryopreservation protocols using vitrification solutions typically involve a two-step cryoprotection process: (1) loading by incubation in loading solution; and (2) dehydration using vitrification solution [56]. Loading solutions are commonly used to improve permeation of the cryoprotectant through cell membrane, it also induces tolerance to dehydration, which will be imposed by vitrification solutions. A common loading solution used is 2 M glycerol + 0.4 M sucrose [56]. Vitrification solutions contain chemicals that are high in concentration, e.g., ethylene glycol, glycerol and DMSO which have been reported as toxic to plant tissue [60]. It is therefore important to establish minimum exposure time to vitrification solutions in order to dehydrate tissue sufficiently to undergo cryopreservation and avoid damage effects to plant tissue [61-62].

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Application of cryoprotectants is the most widely used method in cryopreservation protocols. Cryoprotectants are mainly classified into two categories: penetrating and non-penetrating [63]. Penetrating cryoprotectants pass through the plasma-membrane to equilibrate between extracellular solution and the cell interior [15]. Whereas non-penetrating cryoprotectants do not pass through the plasma membrane and accumulate in the extracellular solution [15]. Cryoprotectants that are penetrating in nature are able to reduce cell water at temperatures sufficiently low to minimize the damaging effect of the concentrated solutes on the cells [63]. Whereas non-penetrating cryoprotectants osmotically "squeeze" water from the cells during the initial phases of freezing at temperatures between -10 and -20 °C [63]. Cryoprotectants that are penetrating in nature are mainly comprised of DMSO, glycerol and amino acids [54] e.g., mixtures of DMSO and glycerol [58]. Non-penetrating cryoprotectants include various types of sugars, high molecular weight additives like PEG and alcohols [54]. Penetrating substances like, DMSO, are often preferred as cryoprotectants due to its ability to enter the cells rapidly, however, DMSO is considered as toxic to plant cells and often glycerol and sugar are substituted [54].

Many authors have developed mixtures of cryoprotectants since the discovery of their benefits in protecting cells during the cryogenic process [58-60, 64-65]. The most commonly used cryoprotectant for plant cells is PVS2 [58] and PVS3 [59]. Plants can be pre-treated with sucrose [60, 62, 66-67] to help acquire dehydration tolerance, thus usually enhancing survival after cryopreservation when applied with vitrification solution. Pre-culture is achieved by incubating shoot tips in high sugar containing media (usually by daily increasing concentration of sugar) causing dehydration [68]. Another method to improve tolerance to vitrification solutions is cold hardening in which the donor plants are exposed to a lower temperature usually around 4 °C [69-72].

Table 3 Some examples of cryoprotectants used for plant tissue.

Cryoprotectant	*Composition*
PVS1	30% w/v glycerol, 15% w/v EG, 5% w/v sucrose, 15% w/v DMSO [65]
PVS2	30 % (w/v) glycerol, 15 % (w/v) DMSO, 15 % (w/v) EG, and 15 % sucrose [58]
PVS3	50 % (w/v) glycerol and 50 % (w/v) sucrose [59]
PVS4	35 % (w/v) glycerol, 20 % (w/v) EG, and 20.5 % M sucrose [73]

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VSL+	20 % (w/v) glycerol, 10 % (w/v) DMSO, 30 % (w/v) EG, 15 % sucrose and 10 mM $CaCl_2$ [74]
VSL	20 % (w/v) glycerol, 10 % (w/v) DMSO, 30 % (w/v) EG, 5 % sucrose and 10 mM $CaCl_2$ [74]
Steponkus	50 % (w/v) EG, 15 % sorbitol, 6.0 % bovine serum albumin, 13.7 % sucrose [75]
Towill	35 % EG, 6.8 % (w/v) DMSO, 10 % PEG 8000, and 13.7 % sucrose [76]
Fahy	20 % DMSO, 20 % formamide, 15 % propylene glycol [77]

2.2 Cryopreservation techniques

Presently there is no one method of cryopreservation that can be applied to a diverse range of plant species. Many cryopreservation techniques (Table 4) have been developed for shoot tips and somatic embryos depending on the plant species used. Many cryopreservation techniques have been identified [9]; namely, vitrification, droplet-vitrification, encapsulation-vitrification, encapsulation-dehydration, dehydration, pre-growth, pre-growth-dehydration and the D and V plate method, a modification of the encapsulation-vitrification and droplet-vitrification [56, 78-79].

Table 4 Some examples of cryopreservation techniques and applications used.

Technique	*Application*
Vitrification. Pre-culture of cultures on basal medium supplemented with cryoprotectants, pre-treatment with loading solution, dehydration with PVS, rapid freezing and thawing.	*Cocoa* somatic embryos showed a 74 % survival rate with PVS2 treatment for 1 h at 0 °C [80].
Droplet-vitrification. Explants are placed individually in a droplet of PVS placed on alfoil strip and then placed in a cryo tube before immersion in LN.	Table, vineyard and rootstock grapes [17, 81-85]. Wild grape germplasm [17, 86]. Sugar-cane somatic embryos achieved 55 % viability [87]. Avocado embryogenic cultures with 77 – 100 % recovery [88].
Encapsulation-vitrification. Sodium alginate beads are formed and explants are	*Olea europaea* (olive) somatic embryos showed 64 % regrowth after 4-day pre-

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encapsulated in them and dehydrated in PVS before freezing.	culture in sucrose, PVS2 treatment for 3 hr treatment and rapid freezing [89].
Encapsulation-dehydration. Sodium alginate-encapsulated cultures are dehydrated osmotically with high concentrations of sucrose for 1 – 7 days or/and desiccated in an air current before slow cooling to -80 °C and then immersed in LN.	40 % regrowth of olive somatic embryos was achieved following 4 days of sucrose pre-growth, desiccation and freezing [89]. *Prunus armeniaca* shoots were recovered after being treated with 0.5 M sucrose for 2 days followed by air dehydration for 2 hr and frozen in LN [90]. *Vitis* spp. optimal survival of 60 and 40 % for cultivars LN33 hybrid and Superior was achieved when encapsulated shoots were dehydrated to 15.6 and 17.6 % water content respectively [91].
Dehydration. Samples are dehydrated by either air current, silica gels, or incubation with cryoprotectant, followed by rapid freezing or two-step freezing.	This technique gave good recovery with embryos of desiccation-tolerant species such as conifers, melon, *Brassica* and *Picea* [16]. Avocado somatic embryos showed 80 % recovery in one embryogenic line using DMSO as cryoprotectant [88].
Pre-growth and pre-growth-dehydration. Samples are cultured on media containing cryoprotectants such as DMSO, dehydrated and then frozen slowly or rapidly.	*Elaeis guineensis* (oil palm) somatic embryos required 7 days of pre-treatment before desiccation [92] whereas coffee embryos required a 2-week treatment [93], and citrus embryos only 1 day [94].
D and V-plate method. Is a modification of the encapsulation-vitrification and droplet-vitrification technique.	With the V cryo-plate method, high regrowth was obtained in *Solanum tuberosum* (potato) (average 99%, 17 lines), *Saccharum officinarum* (sugarcane) (average 70%, 12 lines),

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For the V cryo-plate method, dehydration is performed using the vitrification solution PVS2, while in the D cryo-plate method, dehydration is achieved using the air current of the laminar flow cabinet or silica gel [95].	*Fragaria* spp. (strawberry) (average 81%, 15 lines), *Mentha* spp. (mint) (average 89%, 17 lines) and *Morus alba* (mulberry) (average 87%, 13 lines) [95]. With the D cryo-plate method, high regrowth levels were obtained in *Juncus* spp. (mat rush) (average 86%, 20 lines), *Tanacetum cinerariifolium* (chrysanthemum) (average 88%, 7 lines), *Diospyros kaki* (persimmon) (average 87%, 10 lines), and *Vaccinium corymbosum* L. blueberry (average 80%, 10 lines) [95].

2.2.1 Vitrification

Vitrification technique can include the pre-culture of samples on medium supplemented with sucrose, then treated with a loading solution normally high in sucrose molarity [56] (e.g., a mixture of sucrose and glycerol), dehydration with a vitrification solution such as PVS2 or PVS3, rapid freezing, thawing, and plant recovery by removing cryoprotectants [96].

2.2.2 Droplet-vitrification

The droplet-vitrification technique is a modification of the vitrification technique [97]; treating explants with loading (usually 2 M glycerol and 0.4 M sucrose) and vitrification solutions; freezing them ultra-rapidly in a droplet of vitrification solution either PVS2 or PVS3 placed on an alfoil strip [98] with a droplet of cryoprotectant added before immersion in LN. The alfoil strip helps with the ultra-rapid cooling (about 4000 – 5000 °C min^{-1}) and re-warming (3000 – 4500 °C min^{-1}) of samples due to the good conductivity of thermal current of aluminium [99]. The removal of the cryoprotectant is achieved during re-warming stage by using an unloading solution usually with high level of sucrose 1.2 M, then transferred to recovery and regeneration media [13, 61]. The droplet vitrification protocol combines the use of highly concentrated vitrification solutions with ultra-fast cooling and re-warming rates [100] shown to be critical for survival [101]. For high success in survival and recovery of shoot tips after LN it is vital that samples are sufficiently dehydrated by the vitrification solution in order to vitrify while rapidly cooling in LN [70].

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2.2.3 Encapsulation-vitrification and encapsulation-dehydration

These two techniques have been successfully applied to cryopreserve shoot tips of woody species of crops, such as, *Malus* [102-103], *Pyrus*, *Morus* [102], *Vitis* [91] and *Poncirus trifoliata* × *Citrus sinensis* [104-105]. Dissected shoot tips or somatic embryos are suspended in a solution of sodium alginate. Beads (4 - 5 mm in size) are then formed using a truncated pipette tip and pipetting into a solution of $CaCl_2$ where they are allowed to set for 30 min [56]. For encapsulation-vitrification, once beads are formed with explant inside, they are then dehydrated in PVS solutions such as PVS2 or PVS3 prior to immersion in LN. Although encapsulation is time-consuming, it eases manipulation due to alginate beads being relatively large in size [56]. For the encapsulation-dehydration technique instead of dehydration with PVS solutions beads are dehydrated in a laminar flow hood or under silica gel before immersion in LN [56].

2.2.4 Dehydration

Of all the techniques explained, dehydration technique is the simplest procedure, as it involves just the dehydration of explants followed by direct immersion in LN. Embryonic axes or zygotic embryos extracted from seeds are mainly used for this technique. It produces good results with embryos that are of desiccation-tolerant species such as melon, *Brassica* and conifers [16]. Desiccation is usually achieved by the air current of a laminar airflow cabinet or over silica gel. Dehydration using a vitrification solution removes intracellular water from cells and permits intracellular solution to undergo phase transition from liquid phase into an amorphous phase upon rapid cooling [56]. Cryoprotectant mixtures are commonly used as vitrification solution, such as PVS2 and PVS3.

2.2.5 Pre-growth and pre-growth-dehydration

In the pre-growth and pre-growth-dehydration technique, explants are first exposed and grown on media containing cryoprotectants, dehydrated by air under a laminar flow cabinet or with silica gel, and then frozen rapidly. Depending on the plant species optimal conditions can vary greatly.

2.2.6 D and V-plate method

Both methods use special aluminium cryo-plates which have been developed (length 37 mm, width 7 mm and a thickness of 0.5 mm with 10 wells). An alginate solution containing 2% (w/v) sodium alginate in calcium-free MS basal medium with 0.4 M sucrose is poured over the cryo -plate. Samples are placed in wells and more sodium alginate solution is poured over

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the top to cover them. In the V cryo-plate method, dehydration is performed using the vitrification solution PVS2, while in the D cryo-plate method, dehydration is achieved using the air current of the laminar flow cabinet or silica gel [95]. After dehydration cryo-plates are immersed in LN. The main advantages of the V and D cryo-plate methods is that handling of specimens throughout the procedure is easy and quick because only the cryo-plates are manipulated [95].

3. The avocado case

3.1 Background

Avocado (*Persea americana* Mill.), a high-value fruit native found in tropical and sub-tropical regions of America belongs to the family Lauraceae, genus *Persea* [106]. It is a grafted crop and often cultivars for scions and rootstocks are chosen for important agronomical traits based on the growing region [107]. In Australia, avocados are produced almost all year round due to the vast range of climates and conditions in our eight major avocado growing regions [108]. The cultivar, 'Hass', represents 81 % of total production [108] with 2018/19 producing 85 546 tonnes of avocados, an 11 % increase on the previous season's 77 032 tonnes [108]. Consumer market value of Australian fruit sold domestically was worth ~$898 m [108]. Increased consumer demand is due to its popularity as a healthy eating alternative; often referred to as a superfood due to its beneficial nutrients, vitamins, minerals, fibre and healthy fats [109-110].

The main avocado sold throughout the world, 'Hass' is reported to be 61 % Mexican and 39 % Guatemalan [111]. Most of the new cultivars developed are 'Hass' doppelgangers such as 'Gem', 'Lamb Hass', 'Carmen' and 'Maluma' [112]. Many breeding programs have concentrated on the development of new rootstocks such as 'Dusa', 'Bounty' and 'Velvick' [113] to help the industry overcome abiotic threats and improve productivity.

3.2 Diversity of avocado

The diversity within the *Persea* genera can serve as a resource in crop improvement [114-116]; with approximately 90 species in tropical to sub-tropical America and the remaining species mostly distributed in the tropics to sub-tropics of Asia [106]. There are three recognised races of *P. americana* [117] with distinctive characteristics; e.g., plant habit, leaf chemistry, peel texture, fruit colour, disease and salinity tolerance [118]. The Mexican race, *P. americana* var. drymifolia is adapted to the tropical highlands; the Guatemalan race, *P. americana* var. guatemalensis is adapted to medium elevations in the tropics; and the West Indian race, *P.*

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americana var. americana is adapted to the lowland humid tropics [119]. Yet, there are at least 3 additional races suggested to exist [117, 120-121]. The ability of the three main races to withstand cold conditions varies; the West Indian race cannot tolerate temperatures below 15 °C, the Guatemalan race can tolerate cooler temperatures of -3 to -1 °C, and the Mexican race withstands temperatures as low as -7 °C exhibiting the highest cold tolerance [122-124]. Cultivars classified as pure Guatemalan and Mexican races and Mexican × Guatemalan hybrids have been shown to have more diversity than those of pure West Indian race and Guatemalan × West Indian hybrid cultivars [115]. The Guatemalan and Mexican races and their hybrids are very important for conservation and future breeding programs [115].

New cultivars are normally derived from chance seedlings or mutations due to the difficult nature of breeding programs, which are costly, time-consuming and under threat of abiotic and biotic stresses. The threat of Ambrosia beetle species and its symbiont fungus Laurel Wilt disease to the avocado field gene banks and commercial industry in Florida, California, and Israel is a glaring example of an abiotic stress that could destroy the industry [112]. In Australia the bushfires in 2019/2020 have seen natural forests, bushlands and genetic diversity lost. Many *Persea* spp. grow in these forest areas [120]. It has been estimated that we have already lost around 40 % of the forest cover in developing countries of the world through deforestation [125-126]. This has led to a loss of the genetic footprint of *Persea* spp. that has eroded potential useful genes for future breeding programs. This is devastating from a plant breeder's point of view, as the potential for new cultivar and rootstock development has been narrowed [125]. Also, genetic diversity plays an important role both ecologically and culturally.

Clonal rootstock propagation is a crucial step in avocado propagation. However, a problem exists in rootstocks with heavy reliance on seedling rootstocks due to several reasons 1) the long process needed to develop clonal rootstocks, 2) the price to produce clonal rootstocks and 3) difficulty in rooting mature cuttings of avocado [127], meaning the preferred choice is often seedling rootstocks. These rootstocks need to have good attributes, such as, salinity and resistance to diseases, e.g., *Phytophthora cinnamomi*, to be considered useful for avocado growers. An example of wild *Persea* spp. which can be taken advantage of is *Persea steyermarkii*, which grows adventitious roots from the main trunk when it is damaged [125] making it a dominant species in a forest in Chiapas and Mexico [128]. In Spain, a fungus called *Rosellina necatrix* is problematic, however, seedlings from the germplasm bank of the

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Fundación Salvador Sanchez Colin have shown a tolerance to this disease [129] with a potential to select accessions to combat the fungus.

3.3 Avocado conservation

3.3.1 Ex situ conservation – Global germplasm repositories

Avocado is an open-pollinated plant producing genetically different progeny of seeds, forcing germplasm to be conserved in field repositories (Table 5) instead of seed banking as the seeds are highly heterozygous and recalcitrant [130-131]. These field repositories (Fig. 1) are maintained at a high cost and are at risk, due to natural calamities; pest and disease threats.

Table 5 Germplasm sites throughout the world and avocado accessions held.

Location	*No. of Accessions*
USA • The Huntington San Marino CA	33 avocado accessions [132].
• Riverside University CA	~230 avocado scion accessions [133], ~15 wild *Persea* spp. [133], ~246 avocado rootstock accessions [134].
• National Genetic Resources Program, Miami, Florida	*P. americana* (167 accessions) and *P. schiedeana* (1 accession) [37, 135].
• The Sub-Tropical Horticulture Research Station, Miami, Florida	~400 avocado accessions [136].
Mexico • National Research Institute of Forestry and Livestock in Guanajuato	500 accessions belonging to *P. americana*: Mexican and Guatemalan races. Related species: *P. schiedeana, P. cinerascens, P. floccosa, P. nubigena* [137].
• State of Mexico of the Fundación Salvador Sánchez Colín-CICTAMEX, S.C	800 accessions of avocado and related species. Mexican, Guatemalan, West Indian races, *P. americana* var. costaricensis race materials.

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• Coatepec Harinas and Temascaltepec, State of Mexico, Mexico	Wild relatives: *Beilschmiedia anay*, *B. miersii*, *P. schiedeana*, *P. longipes*, *P. cinerascens*, *P. hintonni*, *P. floccosa*, *P. tolimanensis*, *P. steyermarkii*, *P. nubigena*, *P. lingue*, *P. donnell-smithii*, *P. parvifolia*, *P. chamissonis*, *Persea* spp. [137].
Ghana University of Ghana Forest and Horticultural Crops Research Centre	110 local landraces and 5 varieties from South Africa ('Hass', 'Fuerte', 'Ryan', 'Ettinger' and 'Nabal') [138].
Israel Volcanic Centre in Bet Dagan	194 trees, propagated from 148 accessions [139].
Spain The Experimental Station "La Mayora" in Malaga	75 avocado accessions [136, 140].
Cuba	210 genotypes [136].
Chile	4 botanical breeds of *P. americana:* var. drymifolia, var. guatemalensis, var. jacket and var. costaricencis [136].
Australia • Maroochydore Research Station	A small collection of 46 avocado accessions [141-142].
Nigeria	8 avocado accessions [143].
Brazil • Brasilia, in the Federal District, depending on the Embrapa Research Institute • Conceição do Almeida and Juazeiro collections, both in the Bahia State	30 avocado accessions [144]. 22 avocado accessions [144]. 33 avocado accessions [144].

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• Piracicaba, in the São Paulo State • Jaboticabal, in the São Paulo State	7 avocado accessions [144].

Fig. 1. One of the many avocado accessions being maintained in The Huntington Botanical Gardens [in San Marino, California USA] living germplasm collection.

3.3.2 Cryopreservation of avocado somatic embryos

Somatic embryogenetic cultures are generally highly heterogeneous since they consist of embryos at different developmental stages [145]. Recovery of plantlets from somatic embryos and clonal multiplication *in vitro* is an essential step for commercial application of this technology to crop improvement [146]. Previous studies have shown that the percentage of high-quality bipolar embryos from avocado somatic embryos is extremely low at 2–3 % and is genotype dependent [147-149]. This low rate of somatic embryo conversion is

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currently the main bottleneck in avocado regeneration via somatic embryogenesis [146]. A recent study (has described an *in vitro* induction and multiplication system for somatic embryos of avocado, across four cultivars, which remained healthy and viable for 11 months, on a medium used for mango somatic embryogenesis [150]. Furthermore for one of the cultivars, cultivar 'Reed', a two-step regeneration system was developed that resulted in 43.3 % bipolar regeneration [150].

Information about cryopreservation of avocado somatic embryos is limited (Table 6). The effect of cryogenic storage on five avocado cultivars ('Booth 7', 'Hass', 'Suardia', 'Fuerte' and 'T362') using two cryopreservation protocols (controlled-rate freezing and classical-vitrification) has been reported [151]. In terms of controlled-rate freezing, three out of five embryogenic cultivars were successfully cryopreserved with a recovery of 53 to 80 %. Using classical-vitrification, cultivar 'Suardia' showed 62 % recovery whereas 'Fuerte' had only a 5 % recovery. Using droplet-vitrification on two 'Duke-7' embryogenic cell lines gave promising results, ranging from 78 to 100 % recovery for both lines [88]. Protocols employed in both studies cannot be applied in general to multiple cultivars pointing to the need for more intensive research in this field.

Success with two cryopreservation protocols (cryovial and droplet-vitrification) for the conservation of avocado somatic embryos can be applied to multiple cultivars through the optimisation of loading sucrose concentrations and plant vitrification solution 2 (PVS2) temperature and times. Viability ranging from 59 to 100 % with cryovial and droplet-vitrification with somatic embryos of cultivars 'A10', 'Reed', 'Velvick' and 'Duke-7' after short and long-term LN exposure were achieved [146].

Table 6 Summary of successfully applied cryopreservation techniques to avocado somatic embryos. *Recovery is defined as any somatic embryo clump which was proliferating into new callus clumps.

Cryopreservation Technique	*Cultivar/s*	**Recovery Percentages*
Classical vitrification	'Suardia' 'Fuerte'	62 % 5 % [151]
Slow freezing	'Suardia'	60 - 80 %

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	'T362' 'Fuerte'	4 - 53 % 73 - 75 % [151]
Droplet vitrification	Two lines of 'Duke 7' 'A10' 'Reed' 'Velvick'	78 – 100 % [88] 100 % [146] 85 % [146] 93 % [146]
Cryovial vitrification	'A10' 'Reed' 'Velvick' 'Duke 7'	91 % 73 % 86 % 80 % [146]

3.3.3 Shoot-tip cryopreservation of avocado

Shoot-tip cryopreservation is a novel approach which can be used to conserve 'true-to-type' avocado plant tissue. It is clonal and true to the accession being preserved without any heterogeneity. Shoot tips from various accessions can be collected from the field without waiting for avocado fruit development. The conserved shoot tips can be tagged with the exact phenotypic parameters in the field serving as an excellent back-up source that can be maintained *in vitro*.

However, to date, very few studies have focused on cryopreservation of avocado shoot tips. It was shown that axillary buds of Mexican and Guatemalan races were viable through fluorescein diacetate staining after dehydration with sterile air and being treated with cryopreservation solutions; however, shoot regeneration has not been achieved with the cryopreserved material [152]. Another study, showed that dehydration at 60 mins with sterile air and 30 mins in PVS4 at 0 °C produced normal plant development and 100 % survival was obtained after 30, 45 and 60 days minus LN [153].

The development of a shoot-tip cryopreservation methodology is highly dependent on the availability of a reliable tissue culture multiplication and regeneration protocol for *Persea* spp. from physically mature plants. Although still cultivar-dependant, protocols have been established for multiple cultivars of avocado [110] advancing cryopreservation of avocado.

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3.4 Critical factors identified for successful cryopreservation of shoot-tips

3.4.1 Advantages of vitrification-based procedures

Vitrification-based procedures offer practical advantages in comparison to classical freezing techniques and are more appropriate for complex organs e.g., embryos and shoot tips, which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration [38]. The other advantage vitrification has over other cryopreservation techniques is that glass is viscous and stops all chemical reactions that require molecular diffusion, which leads to dormancy and stability over time [51]. Water is considered very difficult to vitrify as compared to glycerol, which is very viscous and can be supercooled to -70 °C without the formation of ice crystals [29]. Samples can be vitrified and rapidly supercooled at low temperatures and form in a solid metastable glass with crystallization [77]. For procedures that involve vitrification, cell dehydration occurs using a concentrated cryoprotective media and/or air desiccation and is performed first before rapid freezing in LN [38]. It is important that cells are not damaged or injured during the vitrification process and are vitrified enough to sustain immersion in LN [17]. As a result, all factors that affect intracellular ice formation are avoided [38].

3.4.2 Oxidative stress associated with excision injury

When cells are damaged during extraction, the cytoplasm and vacuoles are mixed and phenolic compounds can readily become oxidized by air, peroxidase or polyphenol oxidase. Oxidization of phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants [154].

Wounding can lead to an increase of reactive oxygen species (ROS) within the shoot therefore affecting the viability. ROS are highly reactive molecules and have been shown to cause damage in cells. Many molecules are considered as ROS, some of which include oxygen-free radical species and reactive oxygen non-radical derivatives [44]. The most common ROS species found in plants are superoxide (O2-), hydroperoxyl (OOH), hydroxyl radical (OH) and singlet oxygen (O2) [44]. Plants produce superoxide and singlet oxygen as by-products of photosynthesis and hydroxyl radicals and hydroperoxyl are commonly formed in a process called Fenton's reaction where hydrogen peroxide is converted into hydroxyl or hydroperoxyl radicals (1) [44]. ROS species are responsible for disrupting cellular activity through oxidative damage [155] through lipid peroxidation, which generate hydroxyl radicals that can damage vital cellular proteins and DNA [156]. This can be overcome by adding specific chelating agents which reduces the hydroxyl radical [49, 157-158].

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3.4.3 Role of antioxidants in reducing browning of cryopreserved shoot tips

It has been reported by several authors that the addition of antioxidants can help increase the viability of plants by suppressing browning which leads to shoot tip death [70, 155, 159-162]. By maintaining a higher antioxidant level protection improved post cryopreservation [159]. It was suggested that a higher antioxidant level was present in a cold tolerant *Ribes* genotype, whereas the cold sensitive genotype which had poor post cryopreservation tolerance had low antioxidant levels [70, 155, 159-162].

The antioxidant ASA or vitamin C (ASA) occurs naturally in plants, in plant tissue and meristems. ASA has many roles in a plant's physiological processes but mainly in its defence against oxidative damage resulting from aerobic metabolism, photosynthesis, pollutants and other stresses caused by the environment [163]. Thus, ASA has an important role in the detoxification of ROS species both enzymatically or non-enzymatically [164]. ASA can do this by scavenging a singlet oxygen, hydrogen peroxide, superoxide and hydroxyl radical [163]. It has been reported that in *Hordeum vulgare* (barley) and *Triticum aestivum* (wheat) the addition of ASA in regrowth media improved the survival after cryopreservation by reducing lipid peroxidation [165]. The addition of ASA to pre-culture media, loading solution, unloading solution and regrowth media significantly increased regrowth of shoot tips of *Rubus* spp. (blackberry) [161].

3.4.4 The toxic effects of cryoprotective agents

In order to improve on tolerance to cryoprotectants and increase permeation of the cryoprotectant through the cell membrane and induce tolerance to dehydration caused by vitrification solutions, a pre-step called 'loading' is used [56]. Loading is usually achieved by incubating tissues for 10−20 min in solutions composed of glycerol and sucrose [44]. This loading step is particularly useful for plant species that are sensitive to direct exposure to cryoprotectants due to dehydration intolerance and osmotic stresses [44].

However, in some cases, use of loading solution alone is not adequate to induce tolerance to cryoprotectants, and other pre-treatments/pre-culture such as osmotic conditioning with sugars and cold acclimatisation are necessary [166]. For success in cryopreservation of *Carica papaya* shoot tips, the critical parameters were duration of pre-culture, duration and temperature of cryoprotectant (PVS2) exposure and the type and concentrations of plant growth regulators in the regeneration media [167]; and optimizing the dehydration tolerance of samples in vitrifying solution [56, 168]. This tolerance can be improved by optimizing the pre-

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conditioning and loading treatments along with the standardization of concentration, time of exposure and temperature of vitrification solution. Re-warming and recovery steps are also crucial for success of cryopreservation protocols [56, 168].

3.4.5 The role of cold acclimitization

Water availability and temperature are influenced by environmental variables and are the major determinants of plant growth and development [169]. Most tropical and sub-tropical species have little to no freezing tolerance, however, temperate plant species have evolved some form of cold tolerance [169-170]. It has been shown in temperate plants that they have the genetic ability to increase cold tolerance significantly when exposed to environmental cues that signal the arrival of winter [171]. Many plants can increase their tolerance to the cold by exposure to lower temperatures, generally with temperatures below 10 °C [171]. This process is referred to as cold hardening or cold acclimatization (CA) and requires days to weeks for full development [54, 171-172]. Several biochemical, physiological and metabolic functions are altered in plants by low temperature as well as gene expression [173]. Expression of cold induced genes include those that control the function of cell membranes to stabilize and protect themselves against freezing injury [71]. Freezing tolerance can be increased by 2-8 °C in spring annuals, 10-30 °C in winter annuals and 20–200 °C in tree species [171]. CA has been used as an *in vitro* pre-treatment for donor plants in developing cryopreservation protocols in plants such as *Malus domestica* Borkh, *Malus sieversii* (Ledeb.) and *Phoenix dactylifera* [69, 174].

For temperate species and naturally occurring cold hardy plants, CA is very effective in the survival of cells after LN treatment [12] and the genotypes that do not cold acclimate well are difficult to cryopreserve [175]. This is because of the lipid and protein compositions of plasma membrane that dynamically alter during CA enhancing the cryo stability of the plasma membrane during ultra-cooling [176]. The cold acclimatization enhances the uptake of sugar from the media in sugar beet shoot tips, which safeguards the liquid crystalline state of membrane bilayers [177]. The decrease in the temperature increases the unsaturation of membrane lipids [178], which have a direct correlation in maintaining the fluid state in hydrocarbon chains at low temperature [179] and is crucial for the proper functioning and survival of cells [180]. It has been suggested that abscisic acid (ABA) can be substituted for the low temperature stimulus, provided there is also an adequate supply of sugars [181-182]. It has also been suggested that there may be ABA-dependent and ABA-independent pathways involved in the acclimatization process [72].

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There have been several proteins that have been found to accumulate in plants when exposed to cold conditions and thus associated with developing cold tolerance [183]. Cold tolerant proteins include dehydrins, which are glycine-rich, hydrophilic, D-II LEA (late embryogenesis abundant) and thermostable. These proteins are known to associate with the nucleus, in the cytosol, and with the plasma membrane [183]. Dehydrins are thought to be highly-conserved polar regions and bond to hydrogen regions of macromolecules, acting essentially as a surfactant and preventing coagulation when cells are exposed to dehydration or low temperatures conditions [183]. It has been shown that an acidic dehydrin localises in close proximity to the plasma membrane when plants are cold acclimatized, thus supporting the role of cryoprotection of the plasma membrane during dehydration and freezing stress [184].

Cold acclimatization can help improve the regrowth rates of *in vitro* plants, improve regeneration rates and is usually used on donor plants before shoot tip extraction [185]. Cold acclimatization with or without ABA significantly improved the survival of *Rubus* [175]. ABA pre-treatment alone could not increase the survival of plants grown under warm conditions after cryopreservation, but the survival tripled when cold acclimatization was combined with ABA pre-treatment [175].

3.4.6 The role of pre-culture with sucrose

Pre-culturing shoot tips with a high sugar enriched media has been reported previously by several authors [66-67, 186] to increase the viability post-cryopreservation by better pre-conditioning the shoot. It has also been shown that pre-culturing in high sucrose media enhances the acclimatization process to low temperature and stimulates osmotic dehydration [15]. In other studies, time of incubation in pre-culture solutions was critical to ensuring survival and high regrowth rates [61, 187]. However, periods of exposure to high sucrose concentrations can be detrimental to the recovery of shoot tips post-liquid nitrogen [61].

There have been attempts to use alternative sources of sugar in pre-culture media, such as, sorbitol or mannitol [188-191], glucose and fructose; all have shown no negative effects on post-cryopreservation survival [192]. However, most researchers prefer to use sucrose as the sugar source when adding to pre-culture media [192]. Sucrose has been found to be more beneficial in pre-culture as compared to sorbitol and mannitol as these two sugars were unable to support regrowth of olive somatic embryos [193]. Sorbitol was also shown not to have a positive effect on the regrowth of *Digitalis lanata* cell cultures [194]. However, when 0.2 M

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sorbitol was combined with 5 % DMSO it was an effective cryoprotectant for embryogenic tissue of *Pinus roxburghii* Sarg. [195].

Sucrose is an excellent glass former and is able to stabilise membranes and proteins [196]. Sucrose stimulates the production of other elements such as proline, glycine betaine, glycerol and polyamines, which have colligative as well as non-colligative effects [197-198]. Of the above-mentioned sugars [199], glycerol [200], proline [201] and glycine betaine [202] have proved their cryoprotectant ability, whereas polyamines are known for their antioxidant properties. Therefore, these compounds play a vital role in protecting the cells during cryopreservation.

In *Solanum* spp., genotype plays a critical role into which pre-treatment is best for regrowth of shoot tips after cryopreservation, i.e., sucrose or cold treatment [203]. An accumulation of sugar especially sucrose and the raffinose family oligosaccharides (RFOs) in potato shoot tips can be linked to increased tolerance towards cryopreservation [203]. A reduction in and a reduced water content was able to help prepare *Solanum* spp. for cryopreservation treatments [203]. Many other authors, [172, 204-206] have shown that sucrose pre-treatment and cold treatment prior to exposure to cryopreservation improves recovery results.

4. Avocado shoot tip conservation

There have been significant improvements within the cryopreservation platform to preserve *Persea* spp. germplasm [207-209]. Cryopreservation protocols can cause several stresses both osmotic and temperature related, i.e., exposure to low temperature, exposure to highly concentrated cryoprotectants and freezing injury [160]. This can lead to an increase of ROS production affecting viability [210]. The role of antioxidants such as ASA (100 mg L^{-1}) [208] in reducing browning of avocado shoot tips, the use of pre-treatments such as sucrose and cold treatment to improve regrowth of shoot tips exposed to cryoprotectants are key players for developing a shoot-tip cryopreservation protocol for avocado [207].

Results obtained for two avocado cultivars clearly showed that while pre-treatments are effective for avocado, the type of pre-treatment needed and the degree of effectiveness was cultivar-specific [207]. This can be directly linked to the genetics of the two cultivars which display varying tolerance to cold and salinity in their natural growing environments. Correctly treating avocado shoot tips with the ideal pre-treatment before LN is vital for a successful outcome during the development of cryopreservation protocols [207].

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The type of cryoprotectant and exposure time to the cryoprotectant was also vital in obtaining morphologically normal and vigorous plants [209]. Using the droplet vitrification technique combined with VSL produced positive results with two avocado cultivars belonging to different ecological races; cv 'Velvick' from West Indian race (no cold tolerance) and cv 'Reed' from Guatemalan race (moderate cold tolerance) [209]. Avocado shoots that survived LN grew into full plants ready for rooting after 24 weeks [209]. Cultivar 'Reed' shoots were successfully rooted [127] and after 8 weeks, plantlets were ready to be acclimatized in a glasshouse (Fig. 2). These plants will be screened for growth parameters and yield in a field trial at Duranbah, Queensland. Shoot tips from cv 'Velvick' are currently in the rooting stage.

Fig. 2. Shoot tips of cv 'Reed' treated with VSL and revived from LN growing in a glasshouse.

5. Conclusion

Natural disasters, such as fires, drought, the recent COVID-19 pandemic, are a real threat to conservation of biodiversity. The number of genotypes held in a field bank is restricted by human, financial and land resources, thereby limiting genetic diversity. Cryopreserving a core collection rather than duplicating germplasm at various sites requires less space; the higher the number of accessions, the lower the unit cost. Vitrification-based cryopreservation

techniques for both temperate and tropical plants species show potential for long-term conservation of plant diversity. Cryopreservation is an invaluable tool for securely preserving plant species with recalcitrant seeds that cannot tolerate desiccation to low moisture contents and only remain viable for a short time, e.g., avocado.

New advances in the tissue culture platform for avocado have enabled development of cryopreservation protocols. Based on the positive results, globally, this new cryopreservation platform provides the capacity to clonally cryopreserve this important crop. This is an immeasurably valuable tool to the avocado industry securely preserving valuable clonal rootstock with their specific genetic traits in a naturally outcrossing species for future breeding programmes. It is an important outcome particularly for elite novel varieties protected by Plant Breeders Rights when complete genetic conservation of characteristics is essential. The world's first germplasm repository to store a core collection of *Persea* spp. for true-to-type avocado shoot tip preservation is now an achievable goal.

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