Avocado Conservation

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Avocado is sensitive to desiccation, chilling and freezing stress and is recalcitrant for seed banking. Field-based living germplasm collections are currently the most used conservation method to protect and preserve the genetic diversity of this species. Cryopreservation offers a secure long-term method to maintain avocado genetic resources in a space efficient and de-risked manner.

Keywords: long-term conservation ; embryogenic ; cryopreservation ; shoot tips

1. Background

Avocado (*Persea americana* Mill.), a high-value fruit found in almost all tropical and sub-tropical regions of the world [1][2] belongs to the plant family Lauraceae [3], genus *Persea* [4]. Mexico is thought to be the center of origin of the species [5]. The genus *Persea* has about 400 to 450 species consisting of the currently often recognized genera *Alseodaphne* Nees, *Apollonias* Nees, *Dehaasia* Blume, *Machilus* Nees, *Nothaphoebe* Blume, *Persea* Mill. and *Phoebe* Nees. There are eight sub-species of *P. americana* including *P. americana* var. nubigena (Williams) Kopp, *P. americana* var. steyermarkii Allen, *P. americana* var. zenymyerii Schieber and Bergh, *P. americana* var. floccosa Mez, *P. americana* var. tolimanensis Zentmyer and Schieber, *P. americana* var. drymifolia Blake, *P. americana* var. guatemalensis Williams, *P. americana* var. americana Mill. [2][6]. Genetic diversity within the genus *Persea*, the sub-genera *Persea* and *Eriodaphne* and the species *P. americana* is large and is threatened by the progressive loss of tropical and sub-tropical forests [6]. This genetic diversity can serve as a resource in crop improvement [7][8][9] and plays an important role both ecologically and culturally.

The three recognized ecological races of *P. americana* [10]; are the Mexican race, *P. americana* var. drymifolia, adapted to the tropical highlands; the Guatemalan race, *P. americana* var. guatemalensis, adapted to medium elevations in the tropics; and the West Indian race, *P. americana* var. americana, adapted to the lowland humid tropics [11]. 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 [12][13][14]. They have distinctive characteristics; e.g., plant habit, leaf chemistry, peel texture, fruit color, disease and salinity tolerance [15]. The Guatemalan and Mexican races and their hybrids are very important for conservation and future breeding programs [8]. 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 [8]. In Mexico and Central America, avocado trees grow under highly varied ecological conditions and natural selection over thousands of years has produced vast populations [8]. This serves as an essential source of varied attributes that are not among horticulturally available items [16].

The main avocado sold throughout the world, ‘Hass’, is a medium sized pear-shaped fruit with dark purplish black leathery skin [17]. Its commercial value is due to its superior taste, size, shelf-life, high growing yield, and in some areas, year-round harvesting [18]. The precise breeding history of ‘Hass ’ is unknown however, it is reported to be 61% Mexican and 39% Guatemalan [19]. This finding is supported by a study that analyzed the complete genome sequences of a ‘Hass’ individual and a representative of the highland Mexican landrace, *Persea americana* var. drymifolia; as well as genome sequencing data for other Mexican individuals, Guatemalan and West Indian accessions [19]. Analyses of admixture and introgression highlighted the hybrid origin of ‘Hass’, pointed to its Mexican and Guatemalan progenitor races and showed ‘Hass’ contained Guatemalan introgression in approximately one-third of its genome [19]. In Australia, ‘Hass’, represents 80% of total production [20] with 2019/20 producing 87,546 tonnes of avocados, an increase of 2% more than the previous season’s 85,546 tonnes [20]. This increased consumer demand is due to its popularity as a healthy food; often referred to as a superfood due to its beneficial nutrients, vitamins, minerals, fiber and healthy fats [21][22]. Consumer market value of Australian fruit sold domestically was worth ~$845 m in 2019/20 [20].

2. Avocado Conservation

2.1. Global Germplasm Repositories

Field living germplasm collections (Table 1) and (Figure 1), are currently the most used conservation method, but funding and threats from natural calamities; pest and diseases are a problem.

Figure 1. One of the 56 avocado accessions being maintained in The Huntington Botanical Gardens [in San Marino, California USA] living germplasm collection.

Table 1. Avocado germplasm maintained as field repositories throughout the world.

Country	Germplasm Repositories	No. of Accessions	References
USA	The Huntington San Marino CA	56 *Persea americana* accessions 4 wild *Persea* spp (6 accessions)	[23]
USA	Riverside University CA	~230 avocado scion accessions ~15 wild *Persea* spp. ~246 avocado rootstock accessions	[24] [24][25]
USA	National Genetic Resources Program, Miami, Florida	*P. americana* (167 accessions) and *P. schiedeana* (1 accession)	[26][27]
USA	The Sub-Tropical Horticulture Research Station, Miami, Florida	~400 avocado accessions	[28]
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*	[29]
Mexico	State of Mexico of the Fundación Salvador Sanchez Colin-CICTAMEX, S.C.	800 accessions of avocado and related species. Mexican, Guatemalan, West Indian races, *P. americana* var. costaricensis race materials.	[29]
Mexico	Coatepec Harinas and Temascaltepec; State of 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.	[29]

Country	Germplasm Repositories	No. of Accessions	References
Ghana	University of Ghana Forest and Horticultural Crops Research Centre	110 local land races and 5 varieties from South Africa ('Hass', 'Fuerte', 'Ryan', 'Ettinger' and 'Nabal')	[30]
Israel	Volcanic Centre in Bet Dagan	194 trees, propagated from 148 accessions	[7]
Spain	The Experimental Station 'La Mayora' in Malaga	75 avocado accessions	[28][31]
Cuba	N/A	210 genotypes	[28]
Chile	N/A	4 botanical breeds of *P. americana*: var. drymifolia, var. guatemalensis, var. costaricencis	[28]
Australia	Maroochydore Research Station	46 avocado accessions	[32]
Nigeria		8 avocado accessions	[33]
Brazil	Brasilia, in the Federal District, depending on the Embrapa Research Institute	30 avocado accessions	[34]
Brazil	Conceicao do Almeida and Juazeiro collections, both in the Bahia State	22 avocado accessions	[34]
Brazil	Piracicaba, in the Sao Paulo State	33 avocado accessions	[34]
Brazil	Jaboticabal, in the Sao Paulo State	7 avocado accessions	[34]

2.2. Cryopreservation of Avocado Somatic Embryos

To preserve global avocado diversity; development of improved technologies for avocado conservation, breeding/improvement and propagation is essential. In vitro somatic embryogenesis has direct importance to these objectives [35][36]. Somatic embryogenesis is the process by which somatic cells give rise to totipotent embryogenic cells capable of becoming complete plants [37]. Somatic embryogenesis can be a robust tool to regenerate genetically clonal plants from single cells chosen from selected plant material, or genetically engineered cells [38]. Somatic embryogenic cultures are generally highly heterogeneous since they consist of embryos at different developmental stages [39]. Though heterozygous in nature when regenerated using zygotic embryos as explants, cryopreservation of avocado somatic embryos offers an attractive pathway to conserve avocado germplasm. Recovery of plantlets from somatic embryos and clonal multiplication in vitro is an essential step for commercial application of this technology to crop improvement [40].

Somatic embryogenesis in avocado was first achieved using immature zygotic embryos of cv 'Hass' [41]. Studies have reported that the embryogenic capacity of avocado was highly genotype dependent [42]. To improve somatic embryogenesis previous studies have shown that several factors are vital for success, (1) composition of media, (2) hormone type and concentration, (3) type and concentration of gelling agent and (4) light intensity [43]. Morphogenic competence of somatic embryos has been reported to be lost 3–4 months after induction depending on the genotype [41][44]. In addition, the main factor limiting conversion of somatic embryos into plantlets is incomplete maturation [45]. Studies have found that there are two types of regeneration that occur after maturation; unipolar (only shoot apex or root) and bipolar (both shoot apex and root). Shoots regenerated from unipolar embryos can either be rooted or rescued using in vitro micrografting [46]. Studies have shown that the percentage of high-quality bipolar embryos from avocado somatic embryos was extremely low at 2–3% and was genotype dependent [41][46][47]. This low rate of somatic embryo conversion is currently the main bottleneck in avocado regeneration via somatic embryogenesis [40]. A study 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 [35]. Furthermore for one of the cultivars, cultivar 'Reed', a two-step regeneration system was developed that resulted in 43.3% bipolar regeneration [35].

Cryopreservation of avocado somatic embryos has been successful for various cultivars (Table 2). The effect of cryogenic storage on five avocado cultivars ('Booth 7', 'Hass', 'Suardia', 'Fuerte' and 'T362') using two cryopreservation protocols (controlled-rate freezing and vitrification) was investigated [48]. In terms of controlled-rate freezing, three out of five embryogenic cultivars were successfully cryopreserved with a recovery of 53 to 80%. Using vitrification, cultivar 'Suardia' showed 62% recovery whereas 'Fuerte' had only a 5% recovery. When the droplet-vitrification technique was used, two

‘Duke-7’ embryogenic cell lines showed viability ranging from 78 to 100% [49]. Protocols employed in both studies cannot be applied in general to multiple cultivars and optimization of loading sucrose concentrations and plant vitrification solution 2 (PVS2), temperature and times need more intensive research.

Table 2. 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	Cultivars	* Recovery Percentages
Vitrification	‘Suardia’	62%
	‘Fuerte’	5% [48]
	‘A10’	91%
	‘Reed’	73%
	‘Velvick’	86%
	‘Duke 7’	80% [40]
Slow freezing	‘Suardia’	60–80%
	‘T362’	4–53%
	‘Fuerte’	73–75% [48]
Droplet vitrification	‘A10’	100%
	‘Reed’	85%
	‘Velvick’	93% [40]
	Two lines of ‘Duke 7’	78–100% [49]

2.3. Shoot-Tip Cryopreservation of Avocado

Cryopreservation is a secure and cost-effective method for long-term storage of avocado. It provides a high degree of genetic stability in maintaining avocado collections for the long-term compared to other conservation methods. Shoot-tip cryopreservation conserves ‘true-to-type’ avocado plant tissue. It is ideal for preserving a core selection of avocado genotypes, for example, with superior characteristics, disease and pest resistance, rarity, drought and salinity tolerance. In one study, 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 was not achieved with the cryopreserved material [50]. Another study, showed that dehydration at 60 min with sterile air and 30 min in PVS4 at 0 °C produced normal plant development and 100% survival was obtained after 30, 45 and 60 days [51].

2.4. Critical Factors Identified for Successful Cryopreservation of Avocado Shoot-Tips

Although still cultivar-dependent, in vitro protocols have been established for multiple cultivars of avocado [22] advancing cryopreservation of avocado. Droplet vitrification can be considered as a “generic” cryopreservation protocol for hydrated tissues, such as in vitro cultures [52][53]. Vitrification-based procedures offer practical advantages in comparison to classical freezing techniques and are more appropriate for complex organs e.g., avocado shoot tips, which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration [54]. A problem associated with cryopreservation is formation of lethal ice crystals. To overcome this vitrification makes use of the physical phase called ‘vitrification’, i.e., solidification of a liquid forming an amorphous ‘or glassy’ structure [55] to avoid ice crystal formation of a watery solution. Glass is viscous and stops all chemical reactions that require molecular diffusion, which leads to dormancy and stability over time [56]. Samples can be vitrified and rapidly supercooled at low temperatures and form in a solid metastable glass with crystallization [57]. 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 [54]. It is important that cells are not damaged or injured during the vitrification process and are vitrified enough to sustain immersion in LN [58]. As a result, all factors that affect intracellular ice formation are avoided [54].

Oxidative stress is a common and often severe problem in plant tissue [59][60] of most woody plant species, such as avocado. Therefore, it is important to optimize regrowth conditions of extracted avocado shoot tips to prevent browning when developing an in vitro cryopreservation protocol. Browning of cell tissue takes place as the cytoplasm and vacuoles

are mixed and phenolic compounds readily become oxidized by air, peroxidase or polyphenol oxidase. Oxidization of phenolic compounds inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants [61]. The antioxidant ascorbic acid (ASA) or vitamin C (ASA) occurs naturally in plants, in plant tissue and meristems [62]. It has many roles in a plant's physiological processes but mainly in its defense against oxidative damage resulting from aerobic metabolism, photosynthesis, pollutants and other stresses caused by the environment [63]. Wounding of avocado tissue can lead to an increase in 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 [64]. The most common ROS species found in plants are superoxide (O_2^-), hydroperoxyl (OOH), hydroxyl radical (OH) and singlet oxygen (O_2) [64]. ASA has an important role in the detoxification of ROS species both enzymatically or non-enzymatically [65]. It can do this by scavenging a singlet oxygen, hydrogen peroxide, superoxide and hydroxyl radical [63].

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 [66][67][68][69][70][71]. By maintaining a higher antioxidant level protection improved post cryopreservation [68]. It has been reported that in *Actinidia* spp. (kiwifruit) the addition of ASA in regrowth media improved the survival after cryopreservation by reducing lipid peroxidation [66]. The addition of ASA to pre-culture media, loading solution, unloading solution and regrowth media significantly increased regrowth of shoot tips of *Rubus* spp. (raspberry) [70]. A recent study found treating *Persea americana* cv 'Reed' (avocado), with varying concentrations of different antioxidants (ASA, polyvinylpyrrolidone [PVP], citric acid and melatonin) reduced browning caused when extracting shoot tips. The type of antioxidant and concentration had an effect on viability, vigor and health of the shoots [72].

Avocado is highly susceptible to osmotic stresses imposed by cryoprotectants which are high in osmolarity. Cold sensitive species such as avocado are likely to be positively responsive to vitrification treatments during cryopreservation if optimizations are done carefully [73]. 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 [74]. Loading is achieved by incubating tissues for 10−20 min in solutions composed of glycerol and sucrose [64]. This loading step is particularly useful for plant species, that are sensitive to direct exposure to cryoprotectants due to dehydration intolerance and osmotic stresses [64]. However, use of loading solution alone for avocado shoot tips is not adequate to induce tolerance to cryoprotectants, and other pre-treatments/pre-culture such as osmotic conditioning with sugars and cold acclimatization are necessary [75].

Pre-culturing shoot tips with a high sugar enriched media has been reported previously by several authors [76][77][78] to increase the viability post-cryopreservation by better pre-conditioning the shoot. Also, time of incubation in pre-culture solutions was critical to ensuring survival and high regrowth rates [79][80]. There have been attempts to use alternative sources of sugar in pre-culture media, such as, sorbitol or mannitol [81][82][83][84], glucose and fructose; all have shown no negative effects on post-cryopreservation survival [85]. However, most researchers prefer to use sucrose as the sugar source when adding to pre-culture media [85]. 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 [86]. However, when 0.2 M sorbitol was combined with 5% DMSO it was an effective cryoprotectant for embryogenic tissue of *Pinus roxburghii* Sarg. (chir pine) [87]. Sucrose is an excellent glass former and is able to stabilize membranes and proteins [88]. Sucrose stimulates the production of other elements such as proline, glycine betaine, glycerol and polyamines, which have colligative as well as non-colligative effects [89][90]. Of the above-mentioned sugars [91], glycerol [92], proline [93] and glycine betaine [94] 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. It has also been shown that pre-culturing in high sucrose media enhances the acclimatization process to low temperature and stimulates osmotic dehydration [95].

Water availability and temperature are influenced by environmental variables and are major determinants of plant growth and development [96]. Most tropical and sub-tropical species have little to no freezing tolerance, however, temperate plant species have evolved some form of cold tolerance [96][97]. 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 [98]. Many plants can increase their tolerance to the cold by exposure to lower temperatures, generally with temperatures below 10 °C [98]. This process is referred to as cold hardening or cold acclimatization (CA) and requires days to weeks for full development [99][98][100]. Several biochemical, physiological and metabolic functions are altered in plants by low temperature as well as gene expression [101]. Expression of cold induced genes include those that control the function of cell membranes to stabilize and protect themselves against freezing injury [102]. 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 [98]. Cold acclimatization can help

improve the regrowth rates of in vitro plants, improve regeneration rates [103]. Cold acclimatization has been used as an in vitro pre-treatment on donor plants before shoot tip extraction [104] in developing cryopreservation protocols in plants such as *Malus domestica* Borkh (apple), *Malus sieversii* (Ledeb.) (wild apple) and *Phoenix dactylifera* (date palm) [105][106]. Cold acclimatization with or without ABA significantly improved the survival of *Rubus* spp. [107]. Abscisic acid (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 [107]. High sucrose (0.3 M) or low temperature (10 °C) incubation treatments primed in vitro plants of cvs ‘Reed’ and ‘Velvick’ shoot tips to tolerate cryoprotectant (PVS2) treatments but was cultivar-specific [108].

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