Avocado Conservation

Subjects: Plant Sciences

Contributor: Chris O'Brien

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.

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	[24]
		~15 wild *Persea* spp.	
		~246 avocado rootstock accessions	[24][25]
USA	National Genetic Resources Program,	*P. americana* (167 accessions) and *P. schiedeana* (1 accession)	[26][27]

Country	Germplasm Repositories	No. of Accessions	References
	Miami, Florida		
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]
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]

2.2. Cryopreservation of Avocado Somatic Embryos

Country	Germplasm Repositories	No. of Accessions	References
Brazil	Piracicaba, in the Sao Paulo State [35][36]	33 avocado accessions	[34]
Brazil	Jaboticabal, in the Sao Paulo State [37]	7 avocado accessions	[34]

servation, e to these bryogenic egenerate 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].

References

1. Kopp, L. A taxonomic revision of the genus Persea in the Western Hemisphere (Persea: Lauraceae). Revisión taxonómica del género Persea en el hemisferio occidental (Persea: Lauraceae). Garden 1966, 14, 1–120.
2. Scora, R.W.; Bergh, B.O. Origin of and Taxonomic Relationships within the Genus Persea. In Proceedings of the Second World Avocado Congress, Orange, CA, USA, 21–26 April 1992; Volume 2, pp. 505–574.
3. Bergh, B.; Ellstrand, N. Taxonomy of the avocado. Calif. Avocado Soc. Yearb. 1986, 70, 135–145.
4. Rohwer, J.G.; Li, J.; Rudolph, B.; Schmidt, S.A.; van der Werff, H.; Li, H.-w. Is Persea (Lauraceae) monophyletic? Evidence from nuclear ribosomal ITS sequences. Taxon 2009, 58, 1153–1167.
5. Storey, W.; Bergh, B.; Zentmyer, G. The origin, indigenous range and dissemination of the avocado. Calif. Avocado Soc. Yearb. 1986, 70, 127–133.
6. Pliego-Alfaro, F.; Palomo-Ríos, E.; Mercado, J.; Pliego, C.; Barceló-Muñoz, A.; López-Gómez, R.; Hormaza, J.; Litz, R. Persea americana avocado. Biotechnol. Fruit Nut Crop. 2020, 258–281.
7. Ben-Ya’acov, A.; Bufler, G.; Barrientos-Priego, A.; De La Cruz-Torres, E.; López-López, L. A Study of Avocado Germplasm Resources, 1988–1990. I. General Description of the International Project and its Findings. In Proceedings of the Second World Avocado Congress, Orange, CA, USA, 21–26 April 1992; Volume 2, pp. 535–541.
8. Ge, Y.; Zhang, T.; Wu, B.; Tan, L.; Ma, F.; Zou, M.; Chen, H.; Pei, J.; Liu, Y.; Chen, Z.; et al. Genome-wide assessment of avocado germplasm determined from specific length amplified fragment sequencing and transcriptomes: Population structure, genetic diversity, identification, and application of race-specific markers. Genes 2019, 10, 215.
9. O’Brien, C.; Hiti-Bandaralage, J.C.H.; Hayward, A.; Mitter, N. Avocado (Perse, americana Mill.). In Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants; Jain, S.M., Gupta, P.K., Eds.; Springer Cham: cham, Switzerland, 2018; Volume 85, pp. 305–328. ISBN 978-3-319-79086-2.
10. Furnier, G.; Cummings, M.; Clegg, M. Evolution of the avocados as revealed by DNA restriction fragment variation. J. Hered. 1990, 81, 183–188.
11. Popenoe, W. The avocado—a horticultural problem. Trop Agric. 1941, 18, 3–7.

12. Crane, J.H.; Balerdi, C.F.; Maguire, I. Avocado growing in the Florida home landscape. Hort. Sci. Dept. Fla. Coop. Ext. Serv. Inst. Food Agric. Sci. Univ. Florida. Circ. 2007, 1034, 1–12.
13. Krezdorn, A. Influence of rootstock on cold hardiness of avocados. Proc. Fla. State Hort. Soc. 1973, 86, 346–348.
14. Mickelbart, M.V.; Arpaia, M.L. Rootstock influences changes in ion concentrations, growth, and photosynthesis of ’Hass’ avocado trees in response to salinity. J. Am. Soc. Hortic. Sci. 2002, 127, 649–655.
15. Chen, H.; Morrell, P.L.; Ashworth, V.E.; de La Cruz, M.; Clegg, M.T. Tracing the geographic origins of major avocado cultivars. J. Hered. 2009, 100, 56–65.
16. Barrientos-Priego, A.F.; López-López, L. Historia y genética del aguacate. Télizd. Y Moraa. (Comps.). El Aguacate Y Su Manejo Integrado. 2ª (Ed.) Ed. Mundi-Prensa. Df México 2000, 19–31. Available online: (accessed on 12 February 2021).
17. Ayala-Silva, T.; Ledesma, N. Avocado history, biodiversity and production. In Sustainable Horticultural Systems; Nandwani, D., Ed.; Springer: Cham, Switzerland, 2014; pp. 157–205. ISBN 978-3-319-06903-6.
18. Köhne, S. Selection of Avocado Scions and breeding of rootstocks in South Africa. In Proceedings of the New Zealand and Australia Avocado Grower’s Conference, Tauranga, New Zealand, 20–22 September 2005.
19. Rendón-Anaya, M.; Ibarra-Laclette, E.; Bravo, A.M.; Lan, T.; Zheng, C.; Carretero-Paulet, L.; Perez-Torres, C.A.; Chacón-López, A.; Hernandez-Guzmán, G.; Chang, T.-H. The avocado genome informs deep angiosperm phylogeny, highlights introgressive hybridization, and reveals pathogen influenced gene space adaptation. Proc. Natl. Acad. Sci. USA 2019, 116, 17081–17089.
20. Avocados Australia. Facts at a Glance 2019/20 for the Australian Avocado Industry. Available online: (accessed on 11 February 2021).
21. Dreher, M.L.; Davenport, A.J. Hass avocado composition and potential health effects. Crit. Rev. Food Sci. Nutr. 2013, 53, 738–750.
22. Hiti-Bandaralage, J.C.; Hayward, A.; Mitter, N. Micropropagation of avocado (Persea americana Mill.). Am. J. Plant Sci. 2017, 8, 2898–2921.
23. Folgado, R.; (The Huntington Library, Art Museum, and Botanical Gardens, San Marino CA, USA). Personal Communication, 2020.
24. Arpaia, M.L.; Focht, E.; (Department of Botany and Plant Sciences, University of California, Riverside, CA, USA). Personal Communication, 2020.
25. Manosalva, P.; (Department of Microbiology and Plant Pathology, University of California, Riverside, CA, USA). Personal Communication, 2020.

26. Gutierrez, B.; (The Agricultural Research Service United States Department of Agriculture, Geneva, NY, USA). Personal Communication, 2020.
27. Goenaga, R.; (Agricultural Research Service, United States Department of Agriculture, Miami, FL, USA). Personal Communication, 2020.
28. Álvarez, S.P.; Quezada, G.Á.; Arbelo, O.C. Avocado (Persea americana Mill). Cultiv. Trop. 2015, 36, 111–123.
29. Barrientos-Priego, A.; (Departamento de Fitotecnia, Texcoco de Mora, Mexico). Personal Communication, 2020.
30. Nkansah, G.; Ofosu-Budu, K.; Ayarna, A. Avocado germplasm conservation and improvement in Ghana. In Proceedings of the VII World Avocado Congress 2011, Cairns, Australia, 5–9 September 2011.
31. Alcaraz, M.; Hormaza, J. Molecular characterization and genetic diversity in an avocado collection of cultivars and local Spanish genotypes using SSRs. Hereditas 2007, 144, 244–253.
32. Dann, E.; (Centre for Horticultural Science Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Queensland, Australia). Personal Communication, 2020.
33. Borokini, T.I. Conservation Science. Int. J. Conserv. Sci. 2013, 4, 197–212.
34. Cantuarias-Avilés, T.; (Department of Plant Production, University of Sao Paulo, Piracicaba SP, Brazil). Personal Communication, 2020.
35. Encina, C.L.; Parisi, A.; O'Brien, C.; Mitter, N. Enhancing somatic embryogenesis in avocado (Persea americana Mill.) using a two-step culture system and including glutamine in the culture medium. Sci. Hortic. 2014, 165, 44–50.
36. Guan, Y.; Li, S.-G.; Fan, X.-F.; Su, Z.-H. Application of somatic embryogenesis in woody plants. Front. Plant Sci. 2016, 7, 938.
37. Kulkarni, V.; Suprasanna, P.; Bapat, V. Plant regeneration through multiple shoot formation and somatic embryogenesis in a commercially important and endangered Indian banana cv. Rajeli. Curr. Sci. 2006, 842–846. Available online: (accessed on 4 April 2021).
38. Márquez-Martín, B.; Barceló-Muñoz, A.; Pliego-Alfaro, F.; Sánchez-Romero, C. Somatic embryogenesis and plant regeneration in avocado (Persea americana Mill.): Influence of embryogenic culture type. J. Plant Biochem. Biotechnol. 2012, 21, 180–188.
39. Jain, S.M.; Ishii, K. Micropropagation of Woody Plants and Fruits; Kluwer Academic Publishers: Amsterdam, The Netherlands, 2003; Volume 75, ISBN 1-4020-1135-0.

40. O’Brien, C.; Constantin, M.; Walia, A.; Yiing, J.L.Y.; Mitter, N. Cryopreservation of somatic embryos for avocado germplasm conservation. Sci. Hortic. 2016, 211, 328–335.
41. Pliego-Alfaro, F.; Murashige, T. Somatic embryogenesis in avocado (Persea americana Mill.) in vitro. Plant Celltissue Organ Cult. 1988, 12, 61–66.
42. Litz, R.; Litz, W. Somatic embryogenesis of avocado (Persea americana) and its application for plant improvement. Int. Symp. Trop. Subtrop. Fruits 2000, 575.
43. Mujib, A.; Šamaj, J. Somatic Embryogenesis; Springer Science & Business Media: Berlin/Heidelberg, Germany, 2006; Volume 2, p. 3540287175.
44. Mooney, P.; Staden, J.V. Induction of embryogenesis in callus from immature embryos of Persea americana. Can. J. Bot. 1987, 65, 622–626.
45. Ammirato, P.V. Organizational events during somatic embryogenesis. Plant Biol. 1986. Available online: (accessed on 7 January 2021).
46. Raharjo, S.; Litz, R.E. Rescue of Genetically Transformed Avocado by Micrografting. In Proceedings of the V World Avocado Congress (Actas V Congreso Mundial del Aguacate), Granada-Málaga, Spain, 19–24 October 2003; pp. 119–122.
47. Witjaksono, Y.; Litz, R. Maturation and germination of avocado (Persea americana Mill.) somatic embryos. Plant Cell Tissue Organ Cult 1999, 58, 141–148.
48. Efendi, D.; Litz, R.E. Cryopreservation of Avocado. In Proceedings of the V Congreso Mundial del Aguacate, Actas; Consejería de Agricultura y Pesca, Junta de Andalucía, Sevilla, Spain; 2003; Volume 1, pp. 111–114.
49. Guzmán-García, E.; Bradaï, F.; Sánchez-Romero, C. Cryopreservation of avocado embryogenic cultures using the droplet-vitrification method. Acta Physiol. Plant. 2013, 1–11.
50. Vargas, V.M. Efecto Fisiológico de Brasinoesteroides y Crioprotectores Sobre Yemas Axilares de Aguacate Criollo Producidas in Vitro. 2008. Available online: (accessed on 24 February 2021).
51. Vidales-Fernandez, I.; Larios-Guzman, A.; Tapia-Vargas, L.M.; Guillen-Andrade, H.; Villasenor-Ramirez, F. Criopreservación de germoplasma de aguacate. In Proceedings of the VII World Avocado Congress, Cairns, Australia, 5–9 September 2011.
52. Streczynski, R.; Clark, H.; Whelehan, L.M.; Ang, S.-T.; Hardstaff, L.K.; Funnekotter, B.; Bunn, E.; Offord, C.A.; Sommerville, K.D.; Mancera, R.L. Current issues in plant cryopreservation and importance for ex situ conservation of threatened Australian native species. Aust. J. Bot. 2019, 67, 1–15.
53. Panis, B.; Piette, B.; André, E.; Van den houwe, I.; Swennen, R. Droplet Vitrification: The First Generic Cryopreservation Protocol for Organized Plant Tissues? International Society for Horticultural Science (ISHS): Leuven, Belgium, 2011; pp. 157–162, 2406–6168.

54. Engelmann, F. Plant cryopreservation: Progress and prospects. Vitr. Cell. Dev. Biol. Plant 2004, 40, 427–433.
55. Panis, B.; Nagel, M. Challenges and Prospects for the Conservation of Crop Genetic Resources in Field Genebanks, in In Vitro Collections and/or in Liquid Nitrogen. Plants 2020, 9, 1634.
56. Burke, M.J. The glassy state and survival of anhydrous biological systems. In Membranes, Metabolism and Dry Organisms; Cornell University Press: Ithaca, NY, USA, 1986; pp. 358–363. ISBN 978-0801419799.
57. Fahy, G.M.; MacFarlane, D.; Angell, C.; Meryman, H. Vitrification as an approach to cryopreservation. Cryobiology 1984, 21, 407–426.
58. Bi, W.L.; Pan, C.; Hao, X.Y.; Cui, Z.H.; Kher, M.; Marković, Z.; Wang, Q.C.; Teixeira da Silva, J. Cryopreservation of grapevine (Vitis spp.)—A review. Vitr. Cell. Dev. Biol. Plant 2017, 53, 449–460.
59. Krishna, H.; Sairam, R.; Singh, S.; Patel, V.; Sharma, R.; Grover, M.; Nain, L.; Sachdev, A. Mango explant browning: Effect of ontogenic age, mycorrhization and pre-treatments. Sci. Hortic. 2008, 118, 132–138.
60. Uchendu, E.E.; Paliyath, G.; Brown, D.C.; Saxena, P.K. In vitro propagation of North American ginseng (Panax quinquefolius L.). Vitr. Cell. Dev. Biol. Plant 2011, 47, 710–718.
61. Preece, J.; Compton, M. Problems with Explant Exudation in Micropropagation. In High-Tech and Micropropagation I. Biotechnology in Agriculture and Forestry; Bajaj, Y.P.S., Ed.; Springer: Berlin, Germany, 1991; Volume 17, pp. 168–189. ISBN 978-3-642-76417-2.
62. Akram, N.A.; Shafiq, F.; Ashraf, M. Ascorbic Acid-A Potential Oxidant Scavenger and Its Role in Plant Development and Abiotic Stress Tolerance. Front. Plant Sci. 2017, 8, 613.
63. Shao, H.-B.; Chu, L.-Y.; Lu, Z.-H.; Kang, C.-M. Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. Int. J. Biol. Sci. 2007, 4, 8.
64. Kaczmarczyk, A.; Funnekotter, B.; Menon, A.; Phang, P.Y.; Al-Hanbali, A.; Bunn, E.; Mancera, R. Current issues in plant cryopreservation. In Current Frontiers in Cryobiology; Katkov, I.I., Ed.; In Tech: Rijeka, Croatia, 2012; pp. 417–438. ISBN 978-9535101918.
65. Smirnoff, N.; Wheeler, G.L. Ascorbic acid in plants: Biosynthesis and function. Crit. Rev. Plant Sci. 2000, 19, 267–290.
66. Mathew, L.; McLachlan, A.; Jibran, R.; Burritt, D.J.; Pathirana, R. Cold, antioxidant and osmotic pre-treatments maintain the structural integrity of meristematic cells and improve plant regeneration in cryopreserved kiwifruit shoot tips. Protoplasma 2018, 255, 1065–1077.
67. González-Benito, M.E.; Kremer, C.; Ibáñez, M.A.; Martín, C. Effect of antioxidants on the genetic stability of cryopreserved mint shoot tips by encapsulation–dehydration. Plant Celltissue Organ

Cult. 2016, 127, 359–368.

68. Johnston, J.W.; Harding, K.; Benson, E.E. Antioxidant status and genotypic tolerance of Ribes in vitro cultures to cryopreservation. Plant Sci. 2007, 172, 524–534.
69. Reed, B.M. 4. Are antioxidants a magic bullet for reducing oxidative stress during cryopreservation? Cryobiology 2012, 65, 340.
70. Uchendu, E.E.; Leonard, S.W.; Traber, M.G.; Reed, B.M. Vitamins C and E improve regrowth and reduce lipid peroxidation of blackberry shoot tips following cryopreservation. Plant Cell Rep. 2010, 29, 25.
71. Wang, Q.; Laamanen, J.; Uosukainen, M.; Valkonen, J. Cryopreservation of in vitro-grown shoot tips of raspberry (Rubus idaeus L.) by encapsulation–vitrification and encapsulation–dehydration. Plant Cell Rep. 2005, 24, 280–288.
72. O’Brien, C.; Hiti-Bandaralage, J.C.A.; Folgado, R.; Lahmeyer, S.; Hayward, A.; Mitter, N. Developing a Cryopreservation Protocol for Avocado (Persea americana Mill.) Apical Shoot Tips Using Different Antioxidants; International Society for Horticultural Science (ISHS): Leuven, Belgium, 2020; pp. 15–22, 2406–6168.
73. Sharma, S.D. Cryopreservation of Somatic Embryos—An Overview. 2005. Available online: (accessed on 12 January 2021).
74. Sakai, A.; Engelmann, F. Vitrification, encapsulation-vitrification and droplet-vitrification: A review. CryoLetters 2007, 28, 151–172.
75. Chang, Y.; Reed, B.M. Pre-culture conditions influence cold hardiness and regrowth of Pyrus cordata shoot tips after cryopreservation. HortScience 2001, 36, 1329–1333.
76. Feng, C.-H.; Cui, Z.-H.; Li, B.-Q.; Chen, L.; Ma, Y.-L.; Zhao, Y.-H.; Wang, Q.-C. Duration of sucrose pre-culture is critical for shoot regrowth of in vitro-grown apple shoot-tips cryopreserved by encapsulation-dehydration. Plant Celltissue Organ Cult. 2013, 112, 369–378.
77. Kaczmarczyk, A.; Shvachko, N.; Lupysheva, Y.; Hajirezaei, M.-R.; Keller, E.R.J. Influence of alternating temperature pre-culture on cryopreservation results for potato shoot tips. Plant Cell Rep. 2008, 27, 1551–1558.
78. Park, S.U.; Kim, H.H. Cryopreservation of sweet potato shoot tips using a droplet-vitrification procedure. CryoLetters 2015, 36, 344–352.
79. Azimi, M.; O’Brien, C.; Ashmore, S.; Drew, R. Cryopreservation of papaya germplasm. Acta Hortic. 2005, 692, 43–50.
80. Ashmore, S.E.; Azimi, M.; Drew, R.A. Cryopreservation trials in Carica papaya. Acta Hortic. 2001, 560, 117–120.

81. López-López, L.; Barrientos-Priego, A.; Ben-Ya'acov, A. Variabilidad genética de los bancos de germoplasma de aguacate preservados en el Estado de México. Rev. Chapingo Ser. Hortic. 1999, 5, 19–23.
82. Mikuła, A. Comparison of three techniques for cryopreservation and reestablishment of long-term Gentiana tibetica suspension culture. CryoLetters 2006, 27, 269–282.
83. Mikuła, A.; Tykarska, T.; Kuraś, M. Ultrastructure of Gentiana tibetica proembryogenic cells before and after cooling treatments. CryoLetters 2005, 26, 367–378.
84. Pritchard, H.; Grout, B.; Short, K. Osmotic stress as a pregrowth procedure for cryopreservation: 1. Growth and ultrastructure of sycamore and soybean cell suspensions. Ann. Bot. 1986, 57, 41–48.
85. Panis, B.; Strosse, H.; van den Hende, S.; Swennen, R. Sucrose pre-culture to simplify cryopreservation of banana meristem cultures. CryoLetters 2002, 23, 375–384.
86. Lynch, P.T.; Siddika, A.; Johnston, J.W.; Trigwell, S.M.; Mehra, A.; Benelli, C.; Lambardi, M.; Benson, E.E. Effects of osmotic pre-treatments on oxidative stress, antioxidant profiles and cryopreservation of olive somatic embryos. Plant Sci. 2011, 181, 47–56.
87. Malabadi, R.B.; Nataraja, K. Cryopreservation and plant regeneration via somatic embryogenesis using shoot apical domes of mature Pinus roxburghii sarg, trees. Vitr. Cell. Dev. Biol. Plant 2006, 42, 152.
88. Crowe, L.M. Lessons from nature: The role of sugars in anhydrobiosis. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 2002, 131, 505–513.
89. Antony, J.J.J.; Keng, C.L.; Mahmood, M.; Subramaniam, S. Effects of ascorbic acid on PVS2 cryopreservation of Dendrobium Bobby Messina's PLBs supported with SEM analysis. Appl. Biochem. Biotechnol. 2013, 171, 315–329.
90. Hirsh, A.G. Vitrification in plants as a natural form of cryoprotection. Cryobiology 1987, 24, 214–228.
91. Herbert, R.; Vilhar, B.; Evett, C.; Orchard, C.; Rogers, H.; Davies, M.; Francis, D. Ethylene induces cell death at particular phases of the cell cycle in the tobacco TBY-2 cell line. J. Exp. Bot. 2001, 52, 1615–1623.
92. Williams, W.P.; Quinn, P.J.; Tsonev, L.I.; Koynova, R.D. The effects of glycerol on the phase behaviour of hydrated distearoylphosphatidylethanolamine and its possible relation to the mode of action of cryoprotectants. Biochim. Biophys. Acta Bba Biomembr. 1991, 1062, 123–132.
93. Burritt, D.J. Proline and the cryopreservation of plant tissues: Functions and practical applications. In Current Frontiers in Cryopreservation; Katkov, I.I., Ed.; InTech: Rijeka, Croatia, 2012.

94. Cleland, D.; Krader, P.; McCree, C.; Tang, J.; Emerson, D. Glycine betaine as a cryoprotectant for prokaryotes. J. Microbiol. Methods 2004, 58, 31–38.
95. Reed, B.M. Plant Cryopreservation: A Practical Guide. Reed, B.M., Ed.; Springer New York: New York City, NY, USA, 2008; ISBN 978-0-387-72275-7.
96. Janská, A.; Maršík, P.; Zelenková, S.; Ovesná, J. Cold stress and acclimation—What is important for metabolic adjustment? Plant Biol. 2010, 12, 395–405.
97. Arora, R. Freezing Tolerance and Cold Acclimation in Plants; Deparment of Horticulture, Iowa State University: Ames, IA, USA, 2010.
98. Gusta, L.; Trischuk, R.; Weiser, C.J. Plant cold acclimation: The role of abscisic acid. J. Plant Growth Regul. 2005, 24, 308–318.
99. Panis, B.; Lambardi, M. Status of cryopreservation technologies in plants (crops and forest trees). In The Role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources; Ruane, J., Sonnino, A., Eds.; Food & Agriculture Organization: Rome, Italy, 2006; pp. 61–78. ISBN 92-5-105480-0.
100. Reed, B. Pre-treatment strategies for cryopreservation of plant tissues. In In Vitro Conservation of Plant Genetic Resources; Normah, M.N., Narimah, M.K., Clyde, M.M., Eds.; Universiti Kebangsaan: Bangi Selangor, Malaysia, 1996; pp. 73–87.
101. Janmohammadi, M.; Zolla, L.; Rinalducci, S. Low temperature tolerance in plants: Changes at the protein level. Phytochemistry 2015, 117, 76–89.
102. Thomashow, M.F. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Biol. 1999, 50, 571–599.
103. Dumet, D.; Chang, Y.; Reed, B.M.; Benson[1], E.E. Replacement of cold acclimatization with high sucrose pretreatment in black currant cryopreservation. Satoshi Katomasaya Ishikawamiwako Ito Tatsuo Matsumoto 338 2000, 17, 393.
104. Coelho, N.; González-Benito, M.E.; Martín, C.; Romano, A. Cryopreservation of Thymus lotocephalus shoot tips and assessment of genetic stability. CryoLetters 2014, 35, 119–128.
105. Fki, L.; Bouaziz, N.; Chkir, O.; Benjemaa-Masmoudi, R.; Rival, A.; Swennen, R.; Drira, N.; Panis, B. Cold hardening and sucrose treatment improve cryopreservation of date palm meristems. Biol. Plant. 2013, 57, 375–379.
106. Kushnarenko, S.V.; Romadanova, N.V.; Reed, B.M. Cold acclimation improves regrowth of cryopreserved apple shoot tips. CryoLetters 2009, 30, 47–54.
107. Reed, B.M. Responses to ABA and cold acclimation are genotype dependent for cryopreserved blackberry and raspberry meristems. Cryobiology 1993, 30, 179–184.

108. O’Brien, C.; Hiti-Bandaralage, J.; Folgado, R.; Lahmeyer, S.; Hayward, A.; Folsom, J.; Mitter, N. A method to increase regrowth of vitrified shoot tips of avocado (Persea americana Mill.): First critical step in developing a cryopreservation protocol. Sci. Hortic. 2020, 266, 109305.