

**Cryopreservation of somatic embryos and
shoot tips towards development of a
Cryo-Bank for Avocado Germplasm
(CBAG)**

Victoria Lane
The Department of Agriculture, Fisheries and
Forestry, Qld

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Cryopreservation of somatic embryos and shoot tips
towards development of a Cryo-Bank for Avocado
Germplasm (CBAG)

Neena Mitter *et al.*

Horticulture and Forestry Science, Department of Agriculture,
Fisheries and Forestry, St Lucia, QLD, 4067.

AV11025 - Cryopreservation of somatic embryos and shoot tips towards development of a Cryo-Bank for Avocado Germplasm (CBAG)

Ass/Prof Neena Mitter
Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Queensland, 4067, Australia.
Ph: (07) 3346 6513

Chris O'Brien
Roger Mitchell
Jamie Lim
Amitoj Walia
Dr Myrna Constantin
Garry Fullelove
Victoria Lane
Rod Edmonds

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Contents

Media Summary.....	3
Technical Summary	4
Background.....	6
Chapter 1.....	14
Avocado somatic embryo cryopreservation.....	14
Materials and Methods.....	14
Plant materials.....	14
Culture conditions.....	14
Induction of embryogenic cultures	14
Maintenance of embryogenic culture.....	15
Pre-treatment of SE.....	15
Vitrification optimization with PVS2	15
Vitrification with PVS3	15
Cryopreservation techniques for SE	15
1. Encapsulation-dehydration of SE	15
2. Encapsulation-vitrification of SE.....	17
3. Cryovial-vitrification of SE using PVS2	19
4. Droplet-vitrification of SE using PVS2	20
Assessment of viability	21
FDA staining.....	22
Long-term cryopreservation storage.....	23
Results.....	23
Induction and maintenance of SE	23
Effect of loading treatments on SE	24
Effect of PVS2 exposure time and temperature on SE.....	26
Effect of PVS3 exposure time and temperature on ‘Reed’ SE.....	27
Cryopreservation techniques for SE	27
1. Encapsulation-dehydration of SE	27
2. Encapsulation-vitrification of ‘A10’ SE	29
3. Cryovial-vitrification of SE using PVS2	31
4. Droplet Vitrification.....	35
FDA staining.....	37
Long-term storage of ‘Reed’ and ‘Velvick’ SE.....	38
Discussion.....	41
1. Encapsulation-dehydration of SE	42
2. Encapsulation-vitrification of SE.....	43
3. Cryovial-vitrification of SE	43
4. Droplet-vitrification of SE	45
Chapter 2.....	46
Shoot tip cryopreservation	46
Introduction.....	46
Materials and methods	47
Plant Materials and culture conditions.....	47
Establishment and multiplication of in vitro ‘Reed’ avocado plantlets.....	47
Dissection of shoot tips.....	49
Optimisation of regeneration medium compositions.....	49
Optimization of cryoprotectant for shoot tips.....	50
Cryopreservation techniques for shoot tips	51
1. Cryovial-vitrification of shoot tips.....	51

2. Encapsulation-vitrification of shoot tips.....	53
3. Encapsulation-dehydration of shoot tips.....	54
Assessment of Regrowth.....	54
Results.....	55
Shoot multiplication to generate materials for cryopreservation.....	55
Explant size, structure and plant regeneration media	55
Optimization of cryoprotectant.....	58
1. Cryovial-vitrification of shoot tips.....	59
2. Encapsulation-vitrification of shoot tips.....	60
3. Encapsulation-dehydration of shoot tips.....	62
Discussion for cryopreservation of shoot tips.....	63
Optimisation of regeneration conditions.....	63
Explant size.....	63
Regeneration media	64
Sucrose pre-culture of shoot tips.....	64
Cold-acclimation of avocado plantlets.....	64
1. Cryovial vitrification of shoot tips.....	65
2. Encapsulation-vitrification of shoot tips.....	65
Technology Transfer.....	66
Summary and conclusions	66
Recommendations.....	67
Acknowledgements.....	68
References.....	68

Media Summary

Presently avocado germplasm is conserved *ex situ* in the form of field repositories across the globe including Australia. The maintenance of germplasm in the field is costly, labor and land intensive, exposed to natural disasters and always at the risk of abiotic and biotic stresses. The aim of this project was to overcome these problems using cryopreservation (storage at -196°C) to store avocado somatic embryos and shoot tips.

In our laboratory we now have an optimised system for generation of avocado somatic embryos (SE) of 'Reed', 'A10', 'Velvick', 'Hass' and 'Duke 7', which are routinely subcultured every 4-5 weeks to fresh maintenance medium. We have more than 100,000 SE of these cultivars in our repository.

Two methodologies (cyro-vial and droplet vitrification) have been established for cryopreservation of SE of four avocado cultivars ('Reed', 'A10', 'Velvick' and 'Duke 7'). SE have been successfully multiplied on maintenance medium after 1 week, 3, 6 and 12 months of liquid nitrogen storage with >60% viability.

We have also improved the efficiency of regeneration of the SE from 5% to 58% for the cultivar 'Reed' and work is underway to apply this system to other avocado cultivars.

Shoot tips have been initiated *in vitro* for shoot tip cryopreservation. We have optimized shoot tip re-growth from a small size of 2-3mm. These excised shoot tips have also been optimized for survival after different sucrose and vitrification treatments. We are still currently investigating the best combinations for successful recovery of shoot tips post liquid nitrogen storage.

The outcome from this work will offer avocado growers an unlimited source of disease-free, genetic material that can be supplied upon demand for the establishment of orchards in Australia. The generation of a cryo-bank for avocado and other horticultural crops will be a valuable source for growers and industry worldwide. We aim to start a global initiative to establish the cryo-bank for germplasm conservation with international collaboration and funding at national, international, industry and government level.

Technical Summary

Avocado genetic resources across the globe are currently being maintained *ex situ* in field repositories. The field repositories have the advantage that one can physically evaluate and characterise the accessions for parameters like yield, tree height, disease resistance etc. However, the limitations posed by the avocado field gene banks are: 1) costly to maintain 2) prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance and even their very survival in times of economic stringency 3) require considerable inputs in the form of land (often needing multiple sites to allow for rotation), labour, management and materials 4) capacity to ensure the maintenance of much diversity is limited 5) exposed to natural disasters, pest and diseases and sometimes even vandalism 6) exchange of materials is limited by cost, certification of diseases free status and Quarantine restrictions 7) heterozygous nature of the seedling accessions and time consuming and expensive clonal propagation methods.

Cryopreservation that is storage at ultra low temperature (liquid nitrogen, -196°C) coupled with advancement in tissue culture and marker technologies to tag the germplasm offers an attractive alternate approach to conserve germplasm. The current proposal focuses on conservation of Avocado genetic resources and is aimed at 1) Cryo-preservation of avocado SE 2) Cryo-preservation of avocado shoot tips (novel approach for avocado).

Major Outcomes:

1. Avocado SE has been reported to lose their viability on repeated within 3-4 months after induction in culture, depending on the genotype. We have optimized a medium on which the SE of five different cultivars ('Reed', 'A10', 'Velvick', 'Hass' and 'Duke-7') remain viable for greater than 12 months.
2. We now have more than 100,000 SE in our repository for the above mentioned cultivars.
3. We have also improved the efficiency of regeneration of the SE from 5% to 58% for the cultivar 'Reed' and work is underway to apply this system to other avocado cultivars (published in *Scientia Horticulturae*).
4. We have successfully applied two methods for cryopreservation of avocado SE *in vitro* using a cryovial and droplet-vitrification technique.
5. In case of cryovial-vitrification for short-term (one hour) liquid nitrogen exposure, 'Reed', 'A10', 'Velvick', and 'Duke-7' SE showed a viability of 75%, 91%, 86%, and 93%, respectively.
6. Using the alternative droplet-vitrification method, we observed viability for 'Reed', 'A10', and 'Velvick' SE of 85%, 100%, and 48%, respectively, after 'short-term' liquid nitrogen exposure.
7. In case of 'long-term' cryopreservation studies, 'Reed' and 'Velvick' SE were immersed in liquid nitrogen for 3, 6, and 12 months Both 'Reed' and 'Velvick' SE gave >60% viability after 'long term' storage.
8. 'Reed' shoot tips were initiated (induced to grow as excised sections) *in vitro* prior to cryopreservation and important parameters for regenerating

excised shoot tips have been optimised. These parameters include initial shoot tip size, culture medium, growth hormones, and the choice of dissection method (v-wedge cut versus entire nodal section cut).

9. Shoot tips regrowth has been established from small size of 2-3mm.
10. The cryopreservation of avocado shoot tips has not yielded successful results. However, we have developed strategies to address the issue that will be investigated in future.

Background

The current project focused on cryopreservation techniques for *in vitro* conservation of avocado germplasm with the objective of:

- 1) Cryopreservation of avocado somatic embryos
- 2) Cryopreservation of avocado shoot tips

Cryopreservation is a safe and cost-effective option for long term conservation of genetic resources on non-orthodox seed species. In contrast to orthodox seeds which can be stored easily, a considerable number of species, predominantly tropical or sub-tropical in origin are recalcitrant seeds, examples of these are coconut, cacao, mango, avocado and many forest and fruit tree species. These recalcitrant seeds do not undergo maturation drying and are shed at relatively high moisture content and are therefore not amenable to be stored. Another disadvantage is that these seeds may be highly heterozygous as is the case in avocado, and therefore, of limited utility for the conservation of particular genotypes.

Traditionally, the *ex situ* storage of germplasm as field repositories is the method of choice for fruit trees including avocado which are regarded as ‘problem materials’ and around 527,000 accessions are maintained in field gene banks (FAO, 1996). In some ways, this method offers a satisfactory approach to maintain the germplasm as the accessions are readily available, and can be observed for various phenotypic and genotypic parameters of value to the growers. The field germplasm storage banks therefore allow detailed evaluation. However, there are certain drawbacks that limit its efficiency and threaten its security. The genetic resources are exposed to pests, diseases, and other natural hazards such as drought, weather damage, human error, and vandalism. In addition, they are not in a condition that is readily conducive to germplasm exchange because of the great risks of disease transfer through the exchange of vegetative material. Field gene banks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance, and even their very survival in times of economic stringency. Even under the best circumstances, field gene banks require considerable inputs in the form of land (often needing multiple sites to allow for rotation), labour, management and materials, and, in addition, their capacity to ensure the maintenance of much diversity is limited.

Avocado germplasm: Current Status

Avocado genetic resources across the globe are currently being maintained *ex situ* in field repositories at great cost and are exposed to the constant threat from weather, pest and disease. The recent threat posed by Laurel Wilt to the Avocado germplasm in US is a glaring example (Tropical research and education center, 2009). According to some predictions we have already lost 40% of the forest cover in developing countries of the world. Unfortunately, much of the wild avocado germplasm resources are located in these areas. This genetic erosion of avocado germplasm is devastating since possible useful genes that could be used for future plant breeding for cultivars and rootstocks are lost (Barrientos-Priego *et al*, 2000). Wild avocado germplasm can serve as an important resource for characteristics like resistance to

diseases, tolerance to soil stress and ability to form adventitious roots. For example *Persea steyermarkii* grows adventitious roots from the main trunk when it is damaged. This makes this tree a dominant species in a forest in Chiapas and Mexico (Barrientos-Preigo *et al.*, 1992). Spain is trying to solve the problem caused by a fungus *Rosellina necatrix* and seedlings obtained from the germplasm bank of the Fundacion Salvador Sanchez Colin- CICTAMEX, SC have shown a tolerance to this disease (Lopez- Herrera, 1999; Benz *et al.*, 2012).

Some examples of field germplasm repositories for Avocado:

USA

In 1990, the U.S. Congress authorized establishment of a National Genetic Resources Program (NGRP). It is the NGRP's responsibility to: acquire, characterize, preserve, document, and distribute to scientists, germplasm of all life forms important for food and agricultural production. The National germplasm repository in Miami Florida currently has *Persea americana* (263 accessions), *Persea americana var. nubigena* (2 Accessions) and *Persea schiedeana* (1 accession) (<http://www.ars-grin.gov>). The Subtropical Horticulture Research Station (SHRS) in Miami, FL maintains ~400 avocado accessions. Laurel wilt has become a potential threat to commercial avocado production in Florida, as well as the National Germplasm Repository for avocado in Miami (USDA-ARS)(FDACS, 2012). Steps are now being taken to establish a backup Germplasm collection at sites in Hawaii and California, in collaboration with university and ARS co-operators. All accessions will be grafted onto rootstock and maintained under quarantine conditions prior to transport, ensuring that plant material is free of pests and pathogens. This is a glaring example of why we need *in vitro* germplasm conservation methods as proposed in the current application to complement the field repositories.

Mexico

Barrientos-Priego *et al.* (1999) collected 143 accessions of Avocado and established these at the high and low elevation germplasm banks located in the State of Mexico of the Fundación Salvador Sánchez Colin-CICTAMEX, S.C. (López *et al.*, 1994). The high elevation Bank is located in "El Potrero", Coatepec Harinas, State of Mexico; the soil is sandy clay loam, deep, with pH about 6.5. In this place are kept Mexican race and Guatemalan materials and some wild relatives. The low elevation bank is located in "El Salitre", Ixtapan de la Sal, State of Mexico, the soil is a little deep with a pH between 7.2 to 7.8, classified as slightly alkaline, the sodium level in the irrigation water is about 375 mg/L, and the electrical conductivity is 761.5 mmhos. This houses materials of the West Indian race and wild relatives like *Beilsmedia* and *Persea schiedeana*. A vast variability has been found in the 3 races of avocado, with special characteristics under their natural conditions, like tolerance to high lime content of the soil (West Indian race in Yucatán), drought conditions (Guatemalan race in Chiapas), good fruit quality (Guatemalan race in Chiapas), tree longevity (Mexican race in Veracruz), fruit peel thickness of 0.5 cm (Guatemalan race in Chiapas), high oil content (Mexican race in Veracruz), two productions a year (West Indian race in Yucatán), resistance to moth borer (West Indian race in Tamaulipas), production of adventitious roots on the trunk (*Persea steyermarkii* in Chiapas), among others. Once again this highlights the specialised requirements to maintain variable avocado germplasm on land and limitation of space and cost for such endeavours.

Ghana

Avocado (*Persea americana* Mill) is a very important crop in Ghana and is one of the future promising export crops. The crop is widespread in the forest regions of Ghana. Almost Ghana's entire avocado is grown by smallholders. Avocado fruits of different shapes, sizes and colours can be seen displayed for sale all year round when travelling along the main road networks in the avocado producing regions, an indication of a large gene pool in the country. Commercial avocado plantations are not available and there are no known certified avocado nurseries to supply the growers with grafted material except materials that are supplied by the Forest and Horticultural Crops Research Centre-Kade of the University of Ghana (Nkansah unpublished). George Nkansah has started work on collecting various germplasm accessions and currently maintains 110 local landraces and five varieties obtained from South Africa ('Hass', 'Fuerte', 'Ryan', 'Ettinger' and 'Nabal') at the University of Ghana Forest and Horticultural Crops Research Centre (FOHCREC), Kade in the Eastern Region of Ghana. He is very keen to collaborate with us on the *in vitro* Avocado germplasm bank.

Israel:

The introduction of the avocado into Israel commenced in the 1930's. Seeds from different sources, graft wood and even grafted plants were introduced, and this became the grain of a successful industry, distributed all over the country in the last 30 years. More recently, it has become necessary to enlarge the genetic base of the propagation material, to ensure the survival of the industry. After a wide survey in countries of origin, followed by collection work, a germplasm bank was established in Israel. The rapid disappearance of many native populations was the catalyser for this project. The Israeli gene pool is grown as a regular orchard in Volcani Center in which 200 trees are included. Most of the trees are grafted on West Indian rootstocks, even if the introduction material was from seed. In some cases the original seedling, grown from the introduced seed, is the one planted in the gene pool. The Bet-Dagan gene pool orchard now includes 194 trees, propagated from 148 accessions (Ben Ya'acov *et al*, 2003). Once again the expansion of the gene pool base is constrained due to land and resource limitations.

In Australia, we do not have any dedicated land leased site for avocado germplasm except for a site in Maroochy which is running into management issues. Discussions with various growers and Nurseries have indicated that the idea has been explored few times but never went to fruition because of various constraints. Thus, at present the Avocado germplasm in Australia is being maintained by various growers as per their requirement.

Cryopreservation of germplasm:

Tissue culture techniques are of great interest for collecting, multiplication and storage of plant germplasm (Engelmann, 1997; George *et al*. 2008). Tissue culture systems allow propagation of plant material with high multiplication rates in an aseptic environment. During the last 40 years, *in vitro* propagation techniques, mainly based on micropropagation and somatic embryogenesis, have been extensively developed and applied to well over 1,000 different species. *In vitro* conservation comprises two independent techniques, tissue culture and cryogenic storage. Cryopreservation is the storage of living cells and tissues in liquid nitrogen at an ultra

low temperature (-196°C). Cryopreservation protocols have been developed for various materials including seeds, dormant buds, cell suspensions, calli, apices, zygotic, and somatic embryos of numerous plant species. Zygotic embryos or embryonic axes of almost 100 different species and somatic embryos of almost 40 different species from both temperate and tropical climates, comprising crops, fruit, and forest trees as well as wild species, whose seeds displayed orthodox, intermediate, and recalcitrant storage characteristics, have been successfully cryopreserved. More recently, new cryopreservation techniques viz. encapsulation-dehydration and vitrification have been employed, leading to generally improved results.

Cryopreservation of Somatic Embryos

Somatic embryo cultures are generally highly heterogeneous since they consist of embryos at different development stages (Jain *et al.*, 2000). However, embryos at one particular stage are selected for freezing and as a consequence it becomes homogenous in terms of size, water content and composition. Classical cryopreservation techniques involve slow cooling down to a defined pre-freezing temperature, followed by rapid immersion in liquid nitrogen. With slow cooling the cell membrane acts as a physical barrier and prevents ice formation in the cell interior. In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation upon immersion in liquid nitrogen. Classical freezing procedures include pre-growth of samples, immersion in a cryoprotectant; slow cooling (0.5– 2.0°C/min) to a determined pre-freezing temperature (usually around -40°C), rapid immersion of samples in liquid nitrogen, storage, rapid re-warming, and recovery. Success has been achieved with coffee somatic embryos (Bertrand-Desbrunais *et al.*, 1998) and *Citrus sinensis* embryos (Marin *et al.* 1993) when treated with a sucrose/DMSO cryoprotectant prior to freezing.

Depending on the plant material different cryopreservation techniques have been used for the freezing of somatic embryos. For procedures that involve vitrification, cell dehydration is performed prior to freezing by exposure of samples to concentrated cryoprotective media and/or air desiccation followed by rapid cooling. As a result, all factors that affect intracellular ice formation are avoided. Vitrification-based procedures offer practical advantages in comparison to classical freezing techniques and are more appropriate for complex organs (shoot tips, embryos) which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration.

Eight different cryopreservation procedures can be identified: (1) encapsulation-dehydration, (2) vitrification, (3) encapsulation-vitrification, (4) dehydration, (5) pre-growth, (6) pre-growth dehydration, (7) droplet freezing, and (8) droplet-vitrification.

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pre-treated in liquid medium enriched with sucrose for 1–7 days, combined with air desiccation then frozen rapidly. This technique has been applied to apices of numerous species from temperate and tropical origin as well as to cell suspensions and somatic embryos of several species. Encapsulation-dehydration was first applied to carrot somatic embryos (Dereuddre *et al.*, 1991; Lecouteux *et al.*, 1992). After

treatment for 4 days with sucrose followed by air desiccation and rapid freezing, 40% survival of olive somatic embryos was achieved (Shibli, 2000). In the case of *Theobroma cacao*, embryos were treated for 7 days on sucrose and following freezing, regrowth between 25% and 72% was obtained with the four genotypes tested (Fang *et al.*, 2009).

Vitrification includes the pre-culture of samples on medium enriched with sucrose, treatment with a loading solution (e.g. a mixture of glycerol and sucrose), dehydration with a vitrification solution such as PVS2 solution rapid freezing and thawing, and removal of cryoprotectants and recovery. In the case of *Castanea* (Corredoira *et al.*, 2004), 68% re-growth of somatic embryos after cryopreservation was achieved by pre-treatment with sucrose for 3 days followed by dehydration with PVS2 for 60 min. A recent study performed on *Theobroma cacao* showed that high survival (74%) of somatic embryos was achieved after treatment with sucrose, loading, PVS2 treatment for 60 min at 0°C, and rapid freezing (Adu-Gyamfi and Wetten, 2009).

Encapsulation-vitrification is a combination of encapsulation dehydration and vitrification procedures, where samples are encapsulated in alginate beads, then subjected to freezing by vitrification. Encapsulation-vitrification has been successfully applied to olive (Shibli, 2000) and sugarcane somatic embryos (Martinez-Montero *et al.*, 2008). With olive, 64% survival was achieved after pre-culture of encapsulated embryos for 4 days in sucrose, 3 h treatment with PVS2 and rapid freezing. With sugarcane, somatic embryos were loaded in glycerol and sucrose for 20 min, treated with PVS2 solution for 80 min at 0°C and frozen rapidly, ensuring 30% recovery after cryopreservation.

Dehydration is the simplest procedure since it consists of dehydrating explants, then freezing them rapidly by direct immersion in liquid nitrogen. This technique is mainly used with zygotic embryos or embryonic axes extracted from seeds. The desiccation technique produces good results with embryos of desiccation-tolerant species such as conifers, melon and *Brassica*. Desiccation is usually performed in the air current of a laminar airflow cabinet or over silica gel. In the case of melon somatic embryos, equilibration in 60% relative humidity resulted in 65% survival after cryopreservation (Marin *et al.*, 1993).

In the pre-growth and pre-growth-dehydration procedure, explants are pre-grown in the presence of cryoprotectants, dehydrated under the laminar airflow cabinet or with silica gel, and then frozen rapidly. Optimal conditions vary greatly depending on the material. Oil palm somatic embryos required 7 days pre treatment before desiccation (Dumet *et al.*, 1993) where as coffee embryos required a 2-week treatment (Tessereau *et al.*, 1994), and citrus embryos only 1 day (Gonzalez-Arno *et al.*, 2003; Perez, 2000).

Finally the newly developed droplet-vitrification technique consists of treating explants with loading and vitrification solutions and freezing them ultra-rapidly in a droplet of vitrification solution placed on an aluminum foil. Droplet-vitrification has been successfully applied to sugarcane somatic embryos with 55% viability achieved (Martinez-Montero *et al.*, 2008). Embryos were loaded with glycerol and sucrose, dehydrated with PVS2 vitrification solution for 20–40 min, and cooled rapidly.

In terms of avocado little information has been published on its successful cryopreservation and regeneration. Efendi and Litz (2003) reported they were able to obtain plants from avocado somatic embryos derived from cryopreserved embryogenic cultures. The frequency at which plants were obtained from cryopreserved embryos was similar to that reported for other studies involving non cryopreserved avocado somatic embryos. They tested slow as well as rapid cooling. For the slow cool method embryogenic cultures were immersed in a cryoprotectant solution of DMSO and glycerol for 15 min, cooled to -80°C at a rate of 1°C per min, and plunged into liquid nitrogen (-196°C). In order to restore growth to cryopreserved cultures, they are warmed to room temperature, washed thoroughly with avocado plant growth medium and re-plated on plant growth medium. For rapid cooling embryogenic cultures were suspended in PVS2 solution for 15mins prior to rapid cooling by plunging into liquid nitrogen. Samples were rapidly re-warmed to room temperature and thoroughly washed prior to re-plating on plant growth medium. However, not much data is provided in the publication.

Thus, there are very good prospects for the future development and application of cryopreservation to somatic embryos. They have been developed for a broad range of species and there are already examples of their routine application, as in the case of coffee (Florin *et al*, 2008). The information published indicates indeed that, for any new material, there is at least one technique which produces positive results among the techniques tested. Today, the main bottleneck seems to lie more with the establishment of somatic embryo cultures from any new material than with the development of a cryopreservation protocol for this material.

As mentioned in the previous work done we have established methods for somatic embryo generation, multiplication and regeneration for different avocado cultivars in our laboratory. We have also done some preliminary investigations on cryopreservation using the encapsulation method and obtained promising indications. Thus, we are every well placed to successfully deliver the objectives of the current proposal to look into short and long term cryopreservation of avocado somatic embryos.

Cryopreservation of Shoot tips

Shoot-tip cryopreservation is the ability to regenerate plants from cryopreserved, meristematic shoot tissue. This technique is especially important for vegetatively propagated species, or, for plants that produce recalcitrant seeds such as fruit trees and many root and tuber crops (Panis and Lambardi, 2005). Current materials used for shoot tip cryopreservation are meristematic tissues (apical and axillary shoot-tips, nodes, and buds) that have the potential to develop new shoots and regenerate into plants after cryogenic storage. The shoot meristem is anatomically defined as a structure that contains the apical dome, and the youngest, unexpanded leaf primordia directly associated with the dome meristem (Benson *et al.*, 2007). Meristems are often preferred over non-organized tissues like calli and cell suspensions. Shoot-tip size and origin (e.g., apical or axillary) are critical factors in influencing the ability of tissues to survive cryopreservation (Panis and Lambardi, 2005). It is also possible to freeze nodal stem cuttings containing an axillary meristem and the dormant, and/or cold hardened buds of woody perennial species.

The majority of reported literature deals with encapsulation/dehydration or vitrification methods for preservation of shoot tips. A major advantage of these techniques is that tissues may be plunged directly into liquid nitrogen, circumventing the need for expensive programmable freezing equipment. However, these methods will require optimizing for a particular plant species. Minor modifications to a protocol (e.g., duration and temperature of exposure to vitrification solutions, level of tissue dehydration) may significantly enhance post-thaw survival. Thus, the development of successful cryogenic storage protocols depends on the ability to combine optimized component steps (pre-growth, cryoprotection, freezing, thawing, and recovery) in such a way as to maximize post-freeze regeneration.

Presently there is no one method of shoot-tip cryopreservation that may be applied to a variety of plant species. However, there are several approaches that have been shown to be successful for a wide range of species including *Carica papaya* (Kaity *et al.*, 2008), and *Malus domestica* Borkh cv. Fuji *Pyrus pyrifolia*, *Morus bombysis* (Niino and Sakai, 1992) just to name a few.

Ashmore *et al.*, 2007 reported that the critical parameters for success in cryopreservation of shoot tips of *Carica papaya* are (i) duration of pre-culture, (ii) duration and temperature of PVS2 exposure and (iii) the type and concentrations of plant growth regulators in the regeneration media. Optimisation of these particular steps in the cryopreservation protocol have similarly been found to be critical for some other species including: Apple (Wu *et al.*, 1999); Garlic (Baek *et al.* 2003), Persimmon (Matsumoto *et al.* 2001), Papaya (Wang *et al.* 2005), Mint (Volk *et al.* 2006) and Sweet Potato (Pennycooke and Towill 2000).

There is no existing report in the literature for cryopreservation of avocado shoot tips. Thus, the current proposal will be the pioneering work in this area.

Cryopreservation of Avocado Shoot Tips- an innovative approach

In the new proposal, in addition to cryopreservation of somatic embryos, we will be investigating the new approach of cryopreservation of avocado ‘shoot tips’ as a method for conservation of ‘true to type plant tissue’. In our laboratory we have established protocols to obtain shoot tips and multiply them in tissue culture. We have been successful in getting cuttings from glass house grown avocado plants and multiplying them in tissue culture as clean material. Thus, we are in a good position to work on this novel approach.

It is clear that avocado germplasm maintained in the field (*ex situ*) is currently the most used method, but the challenge for many gene banks of avocado is to get sufficient funding to continue in the preservation of invaluable gene pools. In addition as mentioned above it has the disadvantage of being exposed to fire, drought and disease and is limited by available land and labour resources. The Cryo-Bank for Avocado Germplasm proposed by us will generate many possibilities for the world avocado industry. The project data generated will serve as a major push and impetus towards direct participation of the International Avocado Society as only international cooperation can make this dream a reality.

Shoot tip cryopreservation, if successful, will be extremely useful for avocado as it will be true to the accession. Somatic embryos of avocado originate from zygotic tissue and are therefore heterozygous in origin. The shoot tips will be clonal and will represent the accession without any heterogeneity. *In the Cryo-Bank for Avocado Germplasm, one will be able to tag the conserved shoot tips with exact phenotypic parameters in the field. They will be perfectly complementary to the field germplasm and will therefore serve as an excellent backup source that can be maintained in situ with the advantage of requiring minimal space, protected from pests, diseases and climate change.* It will make it possible to collect shoot tips without waiting for the development of fruits from various accessions. Another major advantage will be that shoot tips, once revived from the frozen state, could be easily multiplied in tissue culture and grafted on to suitable seedling stocks. In fact, some of our work on inducing roots in tissue culture will add to the benefits of 'shoot tip' preservation.

Cryopreservation will be much more cost effective as compared to field gene banks. In addition to cost, cryopreservation has many other advantages over field collections. It can be regarded as clean environmentally friendly technology. The space required is much smaller as compared to a field gene bank. For example, conservation of 1992 coffee accessions in the field required 9 ha of land while the same number of accessions could be cryopreserved within 10m² (Dulloo *et al*, 2009). The number of genotypes that a field bank can hold is restricted by human, financial and land resources, thereby limiting the genetic diversity it can conserve. The opposite is true for cryopreservation: the higher the number of accessions, the lower the unit cost. In a field collection, accessions are grown in a monoculture making them particularly susceptible to pests and diseases. Local climate and other environmental conditions represent strong selection pressures on individuals in field gene banks. These problems can be mitigated by cryopreserving a core collection rather than duplicating the germplasm at another site.

There is an urgent need for collection and conservation of avocado germplasm to realize the future of avocado crop improvement from various perspectives, be it disease resistance or rooting system, or better yield or tree architecture or alternate bearing or improved shelf life of the fruit. I have already championed this cause at the World Congress and have developed active communication with George Nkansah (Ghana), Mary Lu Arpaia and Greg Douhan (California), Alejandro F. Barrientos-Priego (Mexico), Colin Partridge (New Zealand.) as well as California Avocado Commission. The funding provided by the Avocado Industry Australia for the proposed project has served as the first most important step to move in this direction. The funding for taking this work further will be sought from National, International, Government and Industry resources. A fee for service arrangement could also be explored. Government of NSW has already committed \$15.5 million to the development of a Plant Bank at the Australian Botanic Garden with the objective of safeguarding the future of our precious plant species through 'seed-banking', research and information-sharing (<http://www.rbg Syd.nsw.gov.au/annan/plantbank>)

Chapter 1

Avocado somatic embryo cryopreservation

Materials and Methods

Plant materials

The four cultivars used in this project were ‘Duke 7’, ‘A10’, ‘Reed’ and ‘Velvick’. A brief overview of their origin described below:

- ‘Duke 7’ belongs to the Mexican race which is both ‘tropical highland’ and borderline ‘cool subtropical’ in adaptation.
- A10’ is a hybrid of the Guatemalan and Mexican race avocados.
- ‘Reed’ belongs to the Guatemalan race which evolved in upland montane cloud forests.
- ‘Velvick’ belongs to the West Indian race and its origin is of ‘tropical lowland’.

Culture conditions

Approximately 100 mg of globular SE that were actively multiplying 3-4 weeks after subculture were used in all cryopreservation experiments. The pH of all culture media and cryoprotective solutions mentioned in this report were adjusted to 5.65 prior to autoclaving at 121°C for 15 min or in the case of PVS2 and PVS3 they were filter-sterilised using 0.22 µm filter.

Induction of embryogenic cultures

Embryogenic cultures were induced from immature zygotic embryos. Immature fruits of avocado (5 – 15 mm in size, Fig 1) were surface-sterilised in 2% sodium hypochlorite solution supplemented with 500 µL/L of Tween® 20 (Sigma-Aldrich, Missouri, USA) for 20 min. After 20 min, the sodium hypochlorite solution was drained and fruits were rinsed three times with sterile deionised water in a laminar flow cabinet. These fruits were then bisected longitudinally to extract the zygotic embryos. Zygotic embryos were then placed in 90 x 15 mm Petri dishes containing solid B5P induction medium (Witjaksono and Litz, 1999). Petri dishes were sealed with Parafilm® (Pechiney, Illinois, USA) and cultures were maintained in the dark at 27±1°C.



Figure 1. Immature avocado fruitlets of different sizes (Mitter et al., 2012).

Maintenance of embryogenic culture

SE generated were transferred to MMSE (30 – 40 mL in each 90 x 15 mm Petri dish). The cultures were maintained as previously described and subcultured every 4 – 5 weeks.

Pre-treatment of SE

Before cryopreservation techniques could be compared the effect of sucrose pre-treatment on SE was trialled with cultivars ‘A10’, ‘Reed’ and ‘Velvick’. SE were incubated in the dark at $24 \pm 1^\circ\text{C}$ for 0, 10, 20, 40, 60 or 90 min in various loading solutions which contained 2M glycerol and sucrose concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1 or 1.2M. SE were then transferred to MMSE medium in clumps of 5 and grown as previously described and replicated three times.

Vitrification optimization with PVS2

SE of cultivars ‘A10’, ‘Reed’ and ‘Velvick’ were dehydrated with 30 mLs of PVS2 vitrification solution (30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v DMSO, 0.4M sucrose and MMSE basal salts) at either $24 \pm 1^\circ\text{C}$ or $0 \pm 1^\circ\text{C}$ for 0, 30, 60, 120 or 180 min. SE were then transferred to MMSE medium in clumps of 5 and grown as previously described and replicated three times.

Vitrification with PVS3

SE of ‘Reed’ were dehydrated with 30 mLs of PVS3 vitrification solution (50% w/v glycerol, 50% w/v sucrose w/v and MMSE basal salts) at either $24 \pm 1^\circ\text{C}$ or $0 \pm 1^\circ\text{C}$ for 0, 15, 30, 45, 60, 90, 120, 150 or 180 min. SE were then transferred to MMSE medium in clumps of 5 and grown as previously described and replicated twice.

Cryopreservation techniques for SE

1. Encapsulation-dehydration of SE

SE of ‘A10’, ‘Reed’ and ‘Duke 7’ were first suspended into a Na-alginate solution (Table 1a) and mixed well in separate Petri dishes for each cultivar. A 1000 μL pipette that had the bottom 1/3 of the tip truncated was used to pipette SE into a calcium solution (Table 1b). Beads which formed were left in the calcium solution for 30 min to solidify (Fig 2).

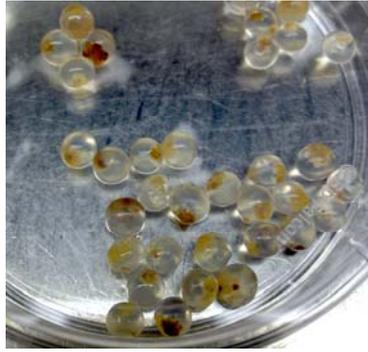


Figure 2. Alginate-encapsulated beads containing SE.

The beads were incubated in dehydration solution (Table 1c) for 0 – 3 h. They were then surface dried by spreading out on uncovered Petri dishes and air-dried in a laminar flow cabinet for 1 - 2 h. The dehydrated beads were transferred into 2-mL cryovials (15 beads per cryovial) and slowly cooled to -80°C using a controlled rate freezer at a rate of -1°C per min and subsequently plunged into LN. For thawing, cryovials were allowed to sit at room temperature for 10 min before the beads were rehydrated in liquid MMSE medium for 30 min. Rehydrated beads were plated onto MMSE medium and placed in the dark as previously described. Samples were taken at each stage in order to determine viability, as outlined in figure 3.

Table 1. Composition of encapsulation and cryoprotective solutions used in encapsulation-dehydration experiment for cryopreservation of SE.

Solutions for encapsulation-dehydration experiment

(a) Na-alginate solution

Calcium-free liquid basal MMSE medium
 5.6% (w/v) alginic acid-sodium salt (Viscosity: 66.5 mPas)
 (*Phyto*Technology Laboratories® A108, Kansas, USA)
 0.4M sucrose

(b) Calcium solution

Calcium-free liquid basal MMSE medium
 0.1M calcium chloride
 0.4M sucrose

(c) Dehydration solution

Liquid basal MMSE medium
 1M glycerol
 0.8M sucrose

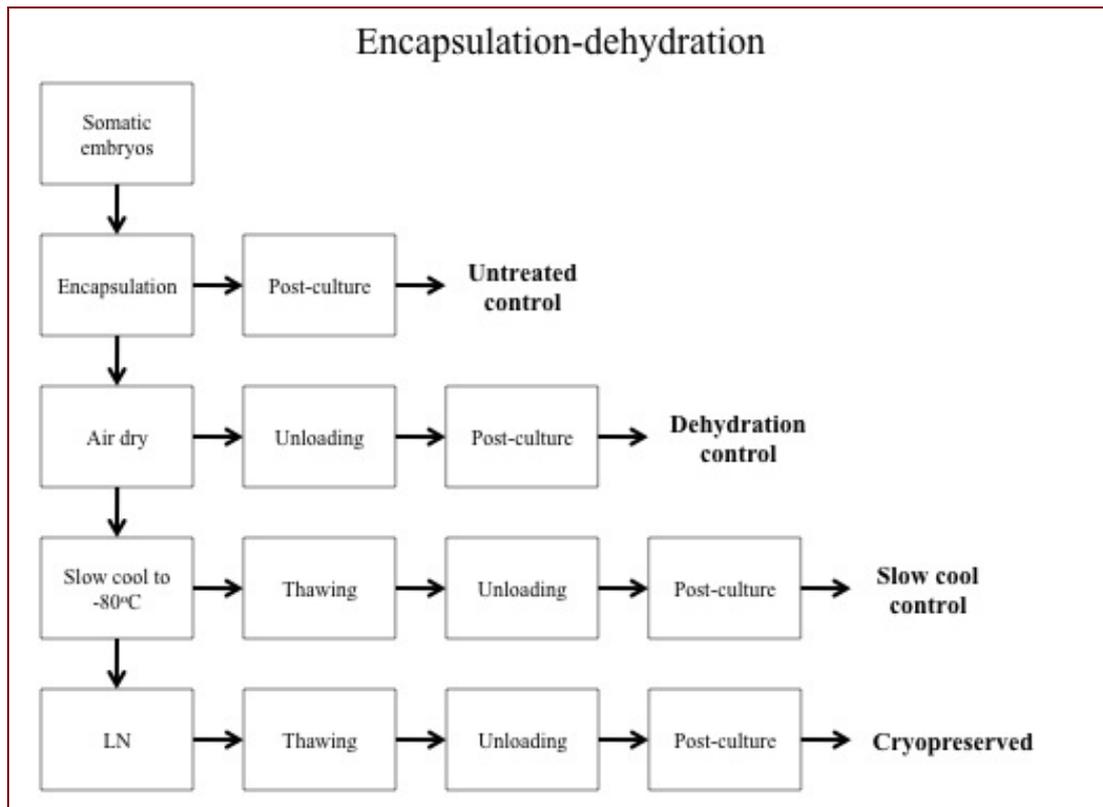


Figure 3. Flow chart of SE cryopreservation by encapsulation-dehydration. Viability was tested at every stage.

2. Encapsulation-vitrification of SE

SE were encapsulated in Na-alginate and the effect of different loading times and PVS2 exposure was tested.

i) The effect of sucrose on encapsulated 'A10' SE

SE of 'A10' were first encapsulated with the same procedures previously described in encapsulation-dehydration method. Beads formed were transferred to the loading solutions that consisted of MMSE medium supplemented with 2M glycerol + 0.2, 0.4, 0.8 or 1.2M sucrose (Table 2a) at room temperature for 20 min. The beads were rapidly surface dried and exposed to PVS2 at 0°C for 60 min. Subsequently dehydrated beads were transferred into 2-mL cryovials (15 beads per cryovial) and frozen in LN. After cryopreservation, cryovials were rapidly thawed in water bath at 37°C for 3 min and beads were incubated in unloading solution (Table 2b) for 30 min. Thereafter beads were plated onto MMSE medium and placed in the dark as previously described. Samples were taken at each stage in order to determine viability, as outlined in figure 4.

ii) The effect of loading time on encapsulated 'A10' SE

Beads which contained SE of 'A10' were made as previously described. The beads which formed were blotted dry and treated with loading solution 2M glycerol + 0.4M sucrose at room temperature for 10, 30 or 60 min. The beads were rapidly surface dried and exposed to PVS2 at 0°C for 60 min. Beads were frozen in LN and samples were taken at each stage as previously described.

Table 2. Composition of cryoprotective solutions used in encapsulation-vitrification experiment for cryopreservation of SE.

Solutions for encapsulation-vitrification experiment	
(a) Loading Solutions	
Liquid basal MMSE medium	
2M glycerol	
0.2, 0.4, 0.8 or 1.2M sucrose	
(b) Unloading Solution	
Liquid basal MMSE medium	
1.2M sucrose	

iii) The effect of PVS2 exposure on encapsulated 'A10' SE

SE of 'A10' were first encapsulated with the same procedures previously described. Beads which formed were transferred to the loading solution that consisted of MMSE medium supplemented with 2M glycerol + 0.4M sucrose at room temperature for 20 min. The beads were rapidly surface dried and exposed to PVS2 at 0°C for 0-5 h. Beads were frozen in LN and samples were taken at each stage as previously described.

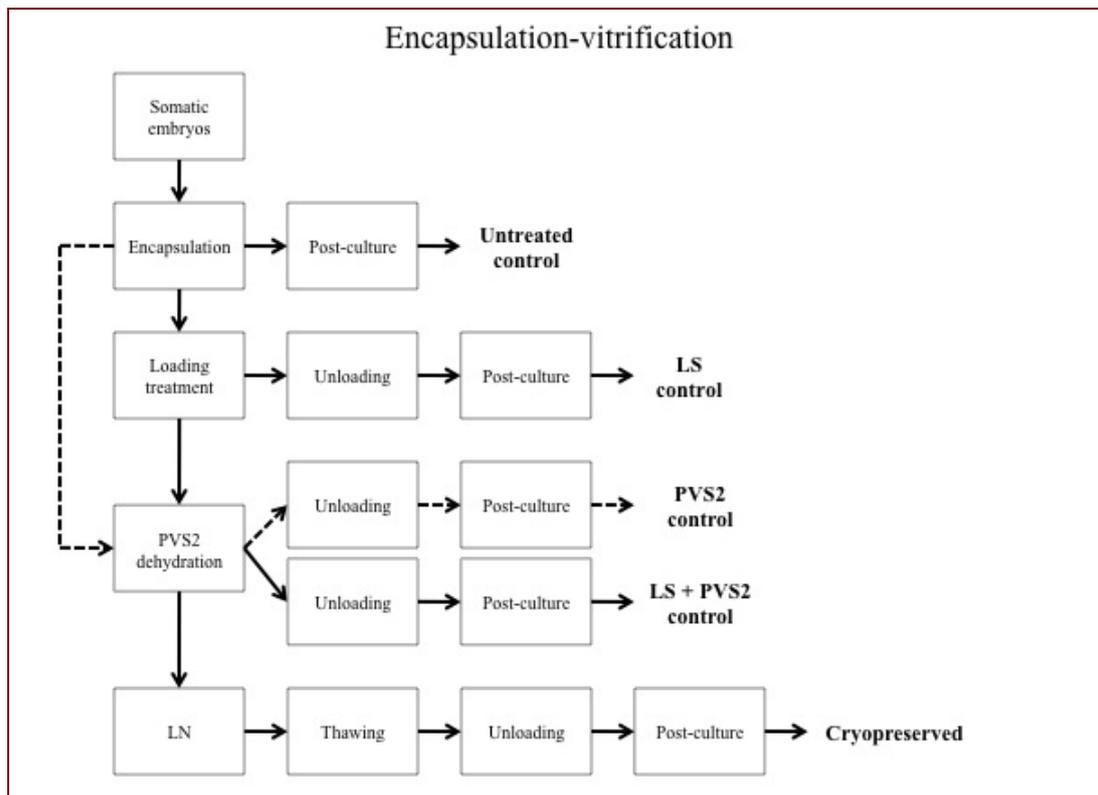


Figure 4. Flow chart of SE cryopreservation by encapsulation-vitrification. Viability was tested at every stage.

3. Cryovial-vitrification of SE using PVS2

i) Vitrification-based techniques were based on the procedures developed by Guzmán-García et al. (2012) with some modifications. SE of each cultivar were first incubated at room temperature for 20 min in loading solution 2M glycerol + 0.4M sucrose. Loading solution was then replaced by PVS2 at 0°C and incubated for either 30 or 60 min. Before incubation time had expired dehydrated SE were transferred into 2-mL cryovial (100 mg SE per cryovial) containing 1 mL of fresh ice cold PVS2 and plunged directly in LN. After cryopreservation, cryovials were thawed in water bath at 37°C for 80 s. PVS2 was replaced by unloading solution (Table 2b) and incubated for 30 min. Subsequently, SE were transferred MMSE medium and placed in the dark as previously described. Samples were taken at each stage in order to determine viability, as outlined in figure 5 and replicated twice.

ii) A second experiment was setup using the cryovial-vitrification method with optimal loading solutions and times as stated in table 3. PVS2 times for ‘A10’ and ‘Velvick’ were 60 min and for ‘Reed’ 30 min. All experiments were plated on MMSE media and grown in the dark as previously described and replicated twice.

Table 2. Optimal loading solutions and times selected from previous experiments for each cultivar in vitrification-based experiments.

Cultivar	Loading Solution sucrose concentration (M)	Time in loading solution (min)
‘A10’	0.2M	20
	0.8M	20
‘Reed’	0.6M	20
‘Velvick’	0.2M	20
	0.8M	20

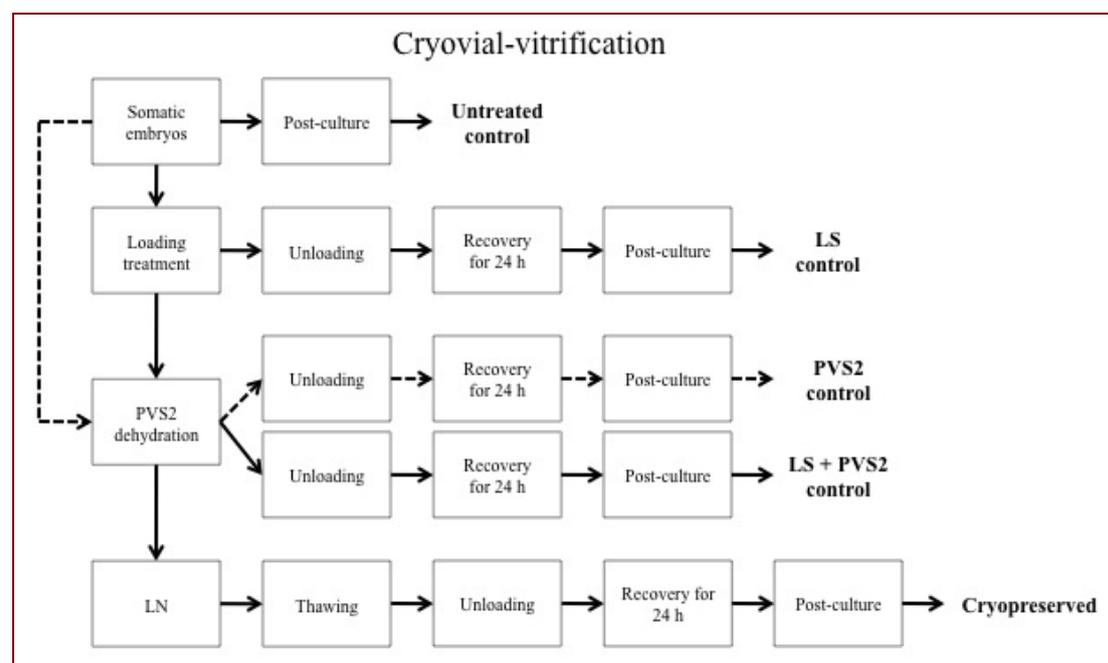


Figure 5. Flow chart of SE cryopreservation by cryovial-vitrification. Viability was tested at every stage.

4. Droplet-vitrification of SE using PVS2

i) In the droplet-vitrification experiment, SE were first incubated in loading solution 2M glycerol + 0.4M sucrose at room temperature for 20 min. After loading solution was removed SE were dehydrated in PVS2 at 0°C for 30 or 60 min. 10 min before the completion of PVS2 incubation, dehydrated SE were placed individually into a 15µL droplet of pre-chilled PVS2 on sterile aluminium foil strips (35 x 25 mm). These manipulations were carried out on top of a cooling block (Fig 6) and the aluminium strip was placed into a 2mL cryovial before being plunged directly into LN. For thawing, 1mL of 37°C unloading solution (Table 2b) was added to the cryovial before strip and SE were poured in petri dishes containing 15mLs of the same unloading solution. SE were left in unloading solution for 30 min and were plated in the same manner as described in cryovial-vitrification. Samples were taken at each stage in order to determine viability, as outlined in figure 7.

ii) A second experiment was setup using the droplet vitrification method with optimal loading solutions and times as stated in table 3. PVS2 times for ‘A10’, ‘Reed’ and ‘Velvick’ were 60 min. All experiments were plated on MMSE media and grown in the dark as previously described and replicated twice.



Figure 6. SE suspended in droplets of PVS2 on aluminium foil strips.

Table 3. Optimal loading solutions and times selected from previous experiments for each cultivar in vitrification-based experiments.

Cultivar	Loading Solution sucrose concentration (M)	Time in loading solution (min)
‘A10’	0.2M	20, 40
	0.8M	20
‘Reed’	0.2M	20
‘Velvick’	0.2M	20
	0.4M	20
	0.8M	20

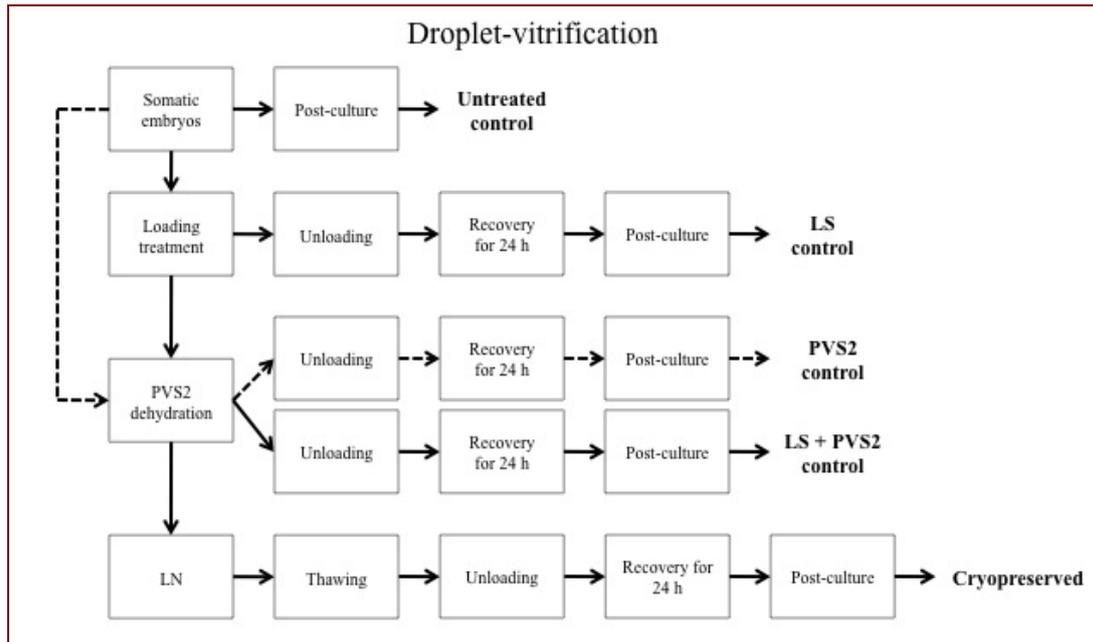


Figure 7. Flow chart of SE cryopreservation by droplet-vitrification. Viability was tested at every stage.

Assessment of viability

Cryopreserved SE were plated onto MMSE medium to encourage new growth/proliferation. For encapsulation-based experiments, fifteen beads were plated onto each Petri dish. For cryovial and droplet-vitrification experiments, cryopreserved SE were divided into 5 clumps (approximately 100 mg per clump) on each Petri dish. For encapsulation-based experiments, the beads were dissected using a scalpel blade (no. 22) upon subculture. The bead halves were laid on fresh MMSE medium with the SE in contact with the medium (Fig 8).

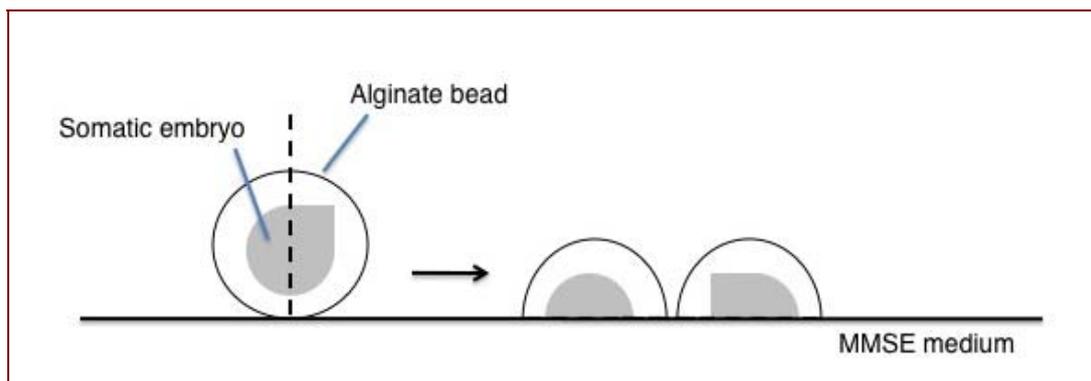


Figure 8. Dissection of alginate-encapsulated SE to promote growth.

Embryo viability was recorded and expressed as percentage viability. Viability corresponds to any manifestation of growth of SE (Fig 9 and 10) and percentage viability is calculated as the percent of proliferating beads or clumps out of the total number of beads or clumps.

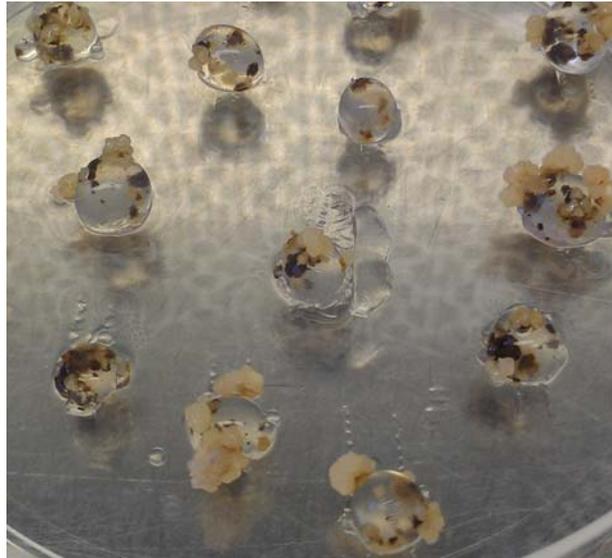


Figure 9. Proliferation of encapsulated SE.

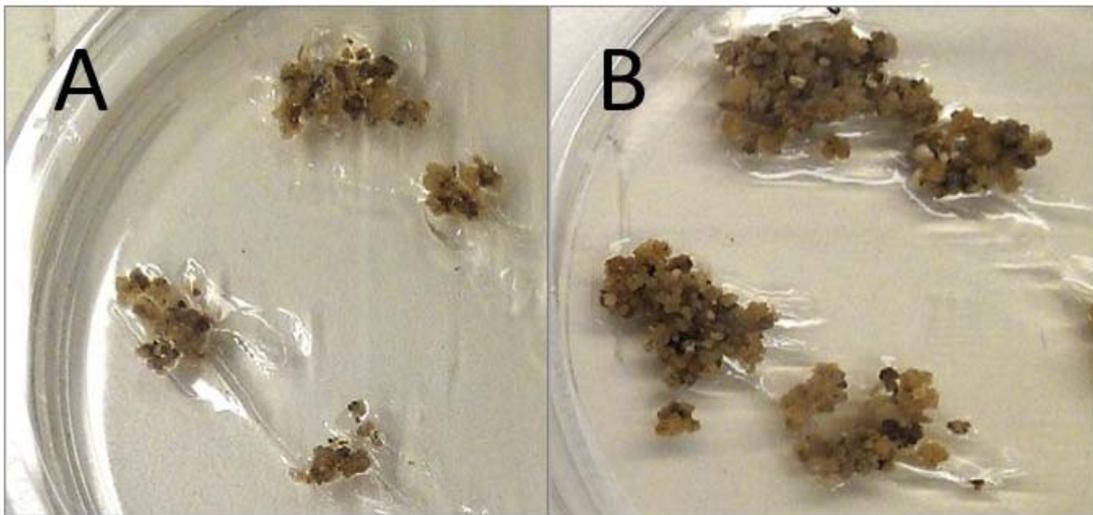


Figure 10. Proliferation of SE clumps after inoculation (A) and 14 days later (B).

FDA staining

Fluorescein diacetate (FDA) staining (Sigma-Aldrich) was carried out to determine the cell viability of SE after 5 weeks post liquid nitrogen. To prepare a final 2% (v/v) working solution a 1% (w/v) acetone stock of FDA was added to water. The stain was added directly to the SE and incubated at room temperature for 5 min. Green fluorescence was observed under UV illumination. Samples of SE were taken from dead material for the negative control, as well as from the LS + PVS2 treatment -LN and the LS + PVS2 treatment +LN either from the cryovial or droplet vitrification technique. Photographs were taken under UV light.

Long-term cryopreservation storage

Based on the most successful cryopreservation storage method, SE for the relevant cultivars will be setup for long-term storage with assessment at the 3, 6 and 12 months storage. SE will be assessed as previously described.

Results

Induction and maintenance of SE

As a first step towards cryopreservation, SE of ‘Duke 7’, ‘A10’, ‘Reed’ and ‘Velvick’ were successfully generated from immature fruitlets. In our laboratory we have optimized the maintenance medium used for avocado SE proliferation by using a medium which is used for mango somatic embryos maintenance (MMSE). We and previous authors had used MSP medium for maintenance of SE which shows lower proliferation rates and SE die out after 11 months *in vitro* for some cultivars. The SE obtained were routinely subcultured every 4-5 weeks to maintain viability. The main differences between MSP and MMSE medium are listed below in table 5.

Table 5. Difference between MSP and MMSE medium

Medium composition	MSP	MMSE
Basal medium	Murashige and Skoog	Gamborg
Gelling agent	Agar	Phytigel
Coconut water	No	Yes
Auxin used	Picloram	2-4D

SE proliferated well on MMSE medium and SE of all four cultivars differed in their size, as shown in figure 11. ‘Duke-7’ and ‘A10’ SE were noticeably the smallest and largest in size, respectively. The size of ‘Reed’ SE ranged from medium to large and ‘Velvick’ SE showed maximum variation from small to large size.

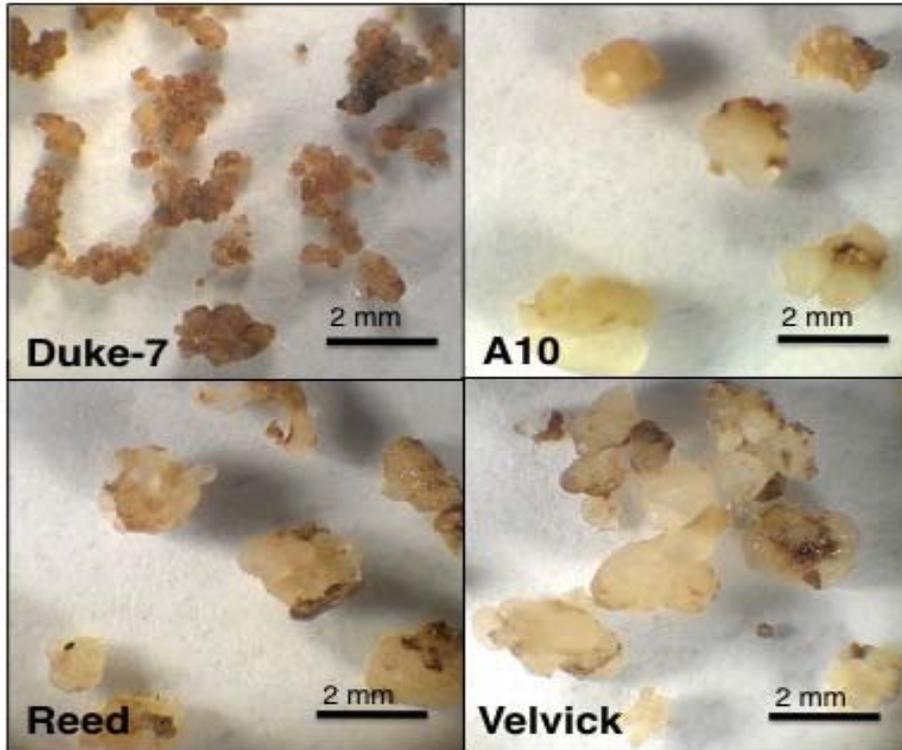


Figure 11. Comparisons of SE size of ‘Duke-7’, ‘A10’, ‘Reed’ and ‘Velvick’ on MMSE medium. Data was taken after 2 weeks of subculture.

We now have an optimised system for generation of avocado SE of ‘Duke-7’, ‘A10’, ‘Reed’ and ‘Velvick’ which are routinely subcultured every 4-5 weeks to fresh MMSE medium. We have in our repository more than 100,000 SE of these cultivars.

Effect of loading treatments on SE

Cultivar ‘A10’ SE demonstrated consistent results in viability for all the testing conditions. The best loading treatments were 2M glycerol + 0.2M sucrose as well as 1M sucrose which attained maximum viability up to 90 min (fig 12a) after exposure. Other treatments such as 2M glycerol + 0.8M sucrose were fairly consistent with only a slight decline in viability at the 20 min time point.

For cultivar ‘Reed’, the best loading treatment was found to be 2M glycerol + 0.2M sucrose as maximum viability was attained up to 90 min (Fig. 12b) after exposure, followed by 0.4M and 0.6M sucrose which also had 100% viability for 10 – 20 min. Overall cultivar ‘Reed’ had the greatest variability across all treatments.

SE of cultivar ‘Velvick’ achieved a maximum viability after exposure to 2M glycerol + 0.2, 0.6 or 0.8M sucrose for up to 60 min (Fig 12c). Other treatments such as 2M glycerol + 0.4M sucrose were fairly consistent with a decline in viability at time point 40 min.

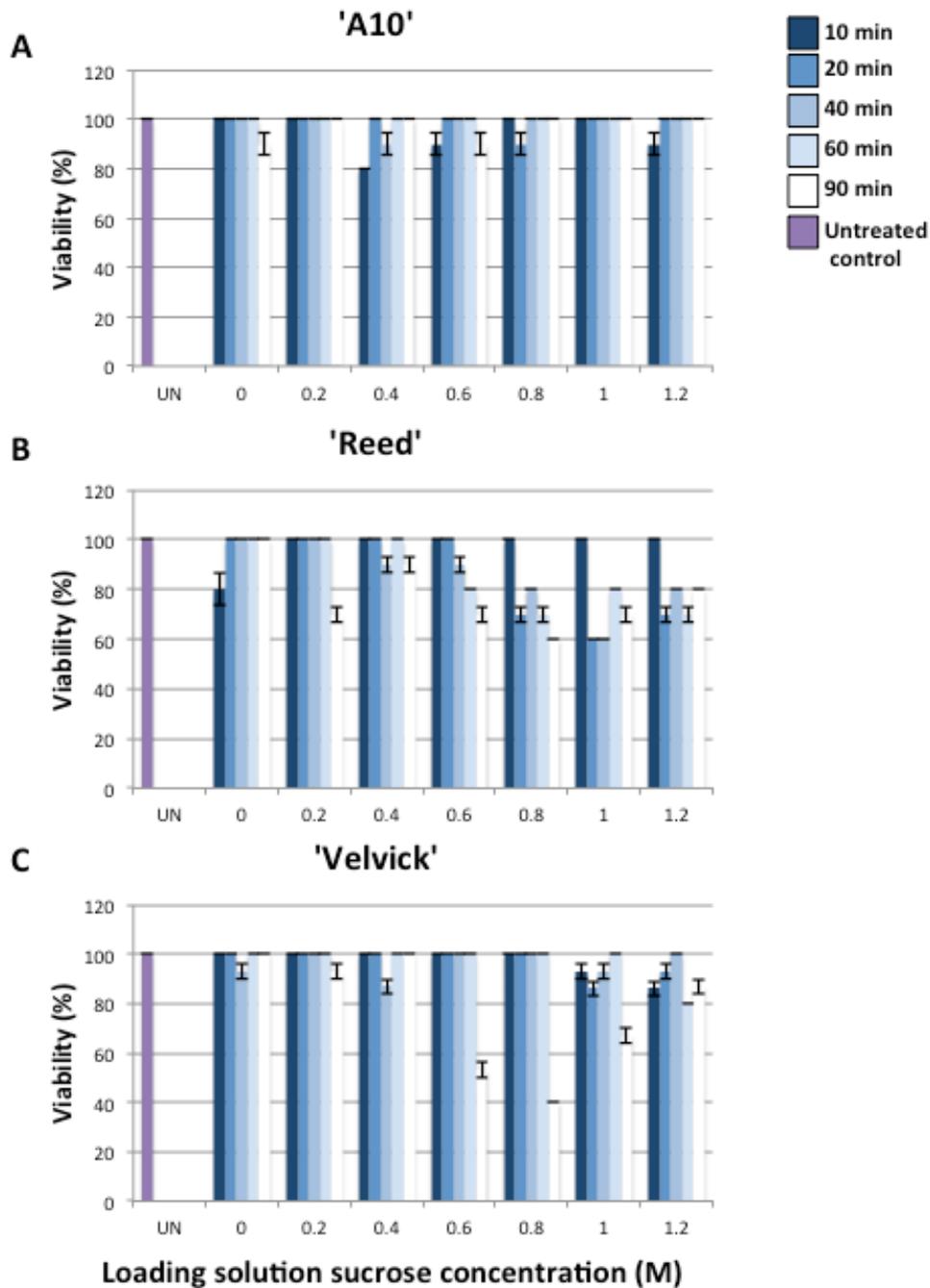


Figure 12. The effect of loading solutions with different sucrose concentrations and time on viability (%) of avocado SE, 'A10' (A), 'Reed' (B) and 'Velvick' (C). Data were recorded 20 days after treatment. Values are means of at least 10 biological replicas, 'A10' (n=10), 'Reed' (n=10) and 'Velvick' (n=15). Vertical bars represent SEM and (UN) purple bar indicated no loading solution treatment.

Effect of PVS2 exposure time and temperature on SE

Independent of the loading treatment, the toxicity of PVS2 with respect to exposure time and temperature were tested with all cultivars (Fig 13).

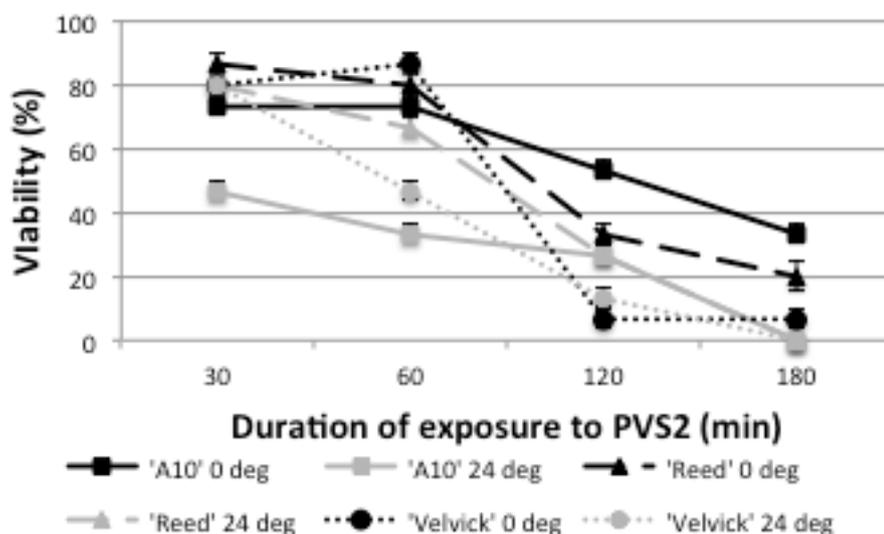


Figure 13. Viability (%) of 'A10,' 'Reed,' and 'Velvick' avocado SE after exposure to PVS2 solution for different time periods, at 0°C and 24°C. Data were recorded 20 days after treatment. Values are means of 15 biological replicas. Vertical bars represent SEM.

When 'A10' SE were exposed to PVS2 at 0°C, the percentage of viable SE remained at 73% for up to 60 min and declined drastically thereafter. At 24°C PVS2 exposure for just 30 min caused significant decrease in viability of SE (47%).

When 'Reed' SE was exposed to PVS2 at 0°C, the percentage of viable SE remained at 80% for up to 60 min and declined thereafter. When incubated at 24°C, PVS2 exposure for 60 min had least influence on viability (67%) and further exposure caused significant reduction in viability.

Cultivar 'Velvick' SE when treated with PVS2 at 0°C remained at 86% for up to 60 min and declined drastically thereafter. At 24°C PVS2 exposure for just 30 min caused significant decrease in viability of SE (47%).

SE appeared to have benefited from PVS2 treatment at low temperature, as the viabilities following the incubation at 0°C were notably higher than that at room temperature, at all the exposure times tested. Therefore all further experiments were performed at 0°C for either 30 or 60 min.

Effect of PVS3 exposure time and temperature on ‘Reed’ SE

When ‘Reed’ SE was exposed to PVS3 at 0°C for 30 min, the percentage of viable SE was only 52% (Fig 14) and declined drastically thereafter. When incubated at 24°C, PVS3 exposure for 15 and 30 min had a slightly higher viability (55%) than the 0°C but with further exposure the same trend of reduced viability was observed.

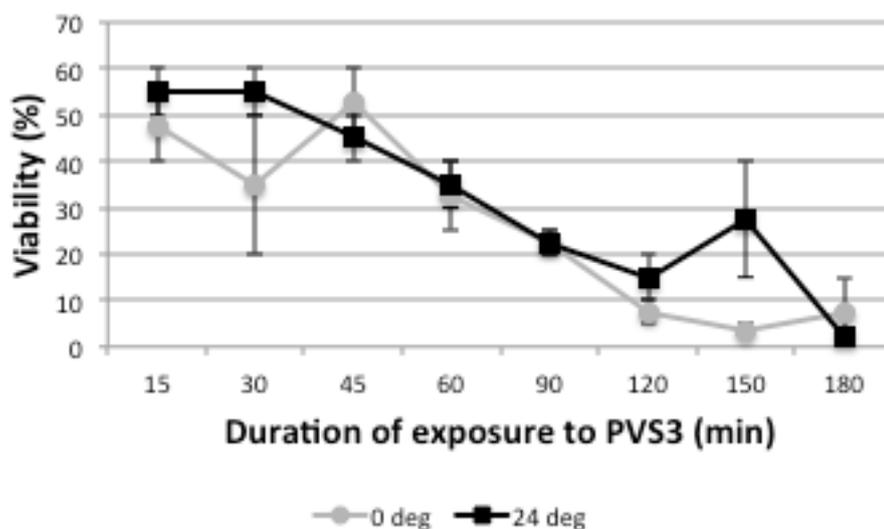


Figure 14. Viability (%) of ‘Reed’ avocado SE after exposure to PVS3 solution for varying time periods, at 0°C and 24°C. Data were recorded 20 days after treatment. Values are means of 10 biological replicas. Vertical bars represent SEM.

Due to the low recovery rate of viable avocado SE with PVS3 as compared to PVS2 treatments it was decided not to continue with PVS3 dehydration for SE.

Cryopreservation techniques for SE

1. Encapsulation-dehydration of SE

The effect of osmotic desiccation and laminar flow drying duration were tested with three cultivars, ‘A10’, ‘Reed’ and ‘Duke-7’ and, as presented in figure 15. In terms of dehydrated controls that were not subjected to LN, ‘Reed’ SE showed the highest percentage of viability ranging from 62 – 100%. In contrast, the recovery of ‘A10’ remained low (10 – 43%) after 9 weeks of post-culture and ‘Duke-7’ had 11 – 100% viability after 4 weeks of post culture depending on the treatment. ‘Reed’ SE showed some tolerance to -80°C (4 – 9%) and LN (2 – 4%), whereas ‘A10’ SE slow cooled to -80°C showed 7% viability with 2 h of osmotic desiccation and 2 h of laminar flow drying but none after LN exposure. None of the treatments with ‘Duke-7’ SE recorded any viability after slow cooling to -80°C and LN exposure.

With reference to the untreated control, it was apparent that the proliferation rate of SE varied greatly among cultivars with untreated ‘A10’ showing only 43% viability after 9 weeks of culture, while untreated ‘Duke-7’ and ‘Reed’ showed 100% viability after only 4 and 10 weeks respectively.

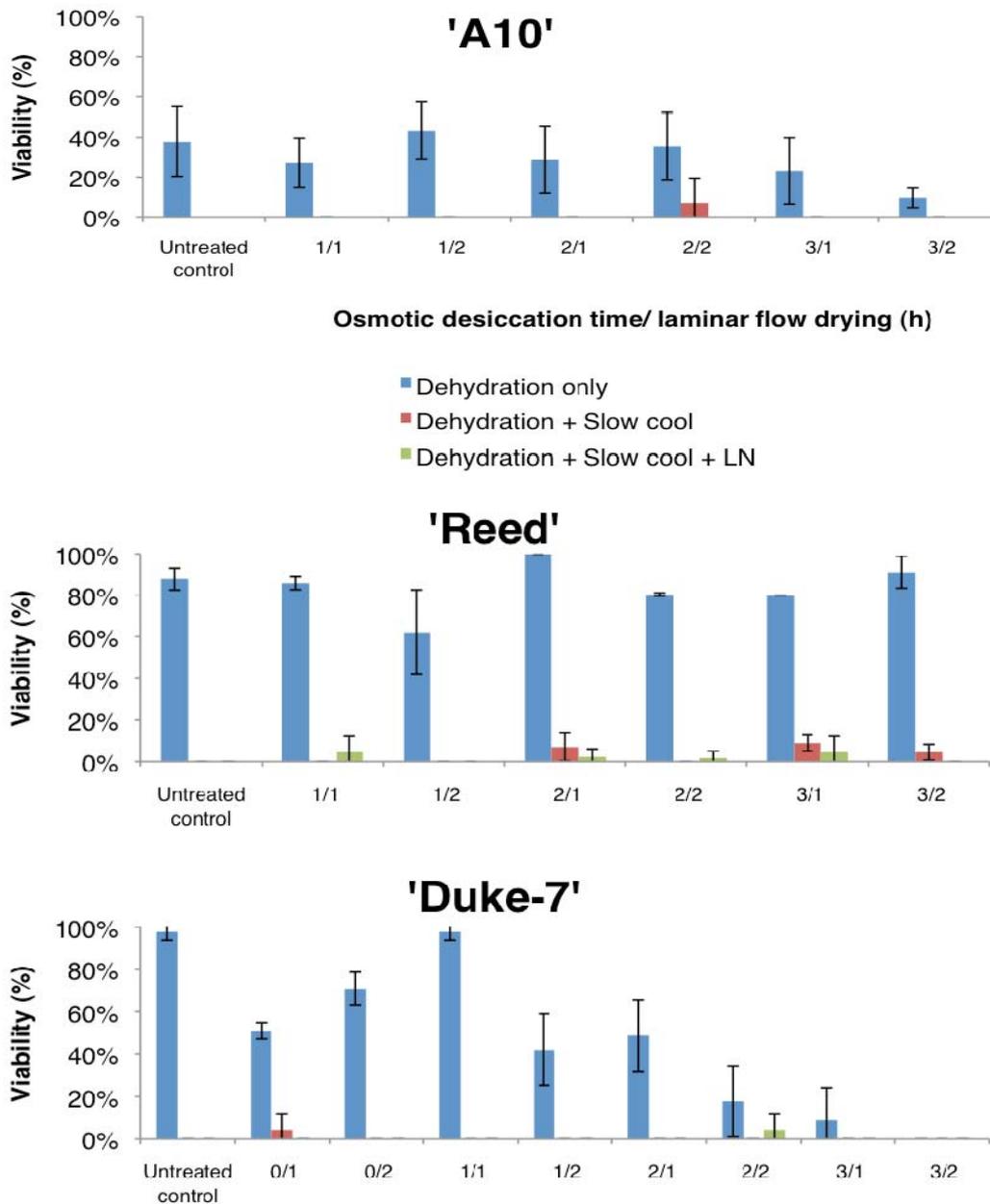


Figure 15. Percentage viability of avocado SE after encapsulation-dehydration and LN exposure. Dehydration = Osmotic desiccation followed by laminar flow drying. Dehydration + Slow cool = Dehydration followed slow freezing to -80°C . Dehydration + Slow cool + LN = Dehydration followed by slow cooling to -80°C and subsequently plunged into LN. Data are recorded 9 weeks, 10 weeks and 4 weeks of culture for 'A10' 'Reed' and 'Duke-7' respectively. Bars indicate standard error.

Based on the results obtained, encapsulation-dehydration was not found to be conducive for the three cultivars experimented as all of them recorded close to 0% viability after plunging in LN.

2. Encapsulation-vitrification of 'A10' SE

i) The effect of sucrose on encapsulated 'A10' SE

'A10' SE that were treated with loading solution only with 0.2M sucrose gave the highest viability (48%, Fig 16). For all other sucrose concentrations viability decreased with increased sucrose. Subsequent dehydration in PVS2 at 0°C for 60 min further reduced the viability. However, it was noted that SE that were in loading solutions which contained 0.8M and 1.2M sucrose concentration offered some protection towards the toxicity of PVS2. None of treatments tested here offered any protection against LN freezing with 0% viability recorded in all treatments.

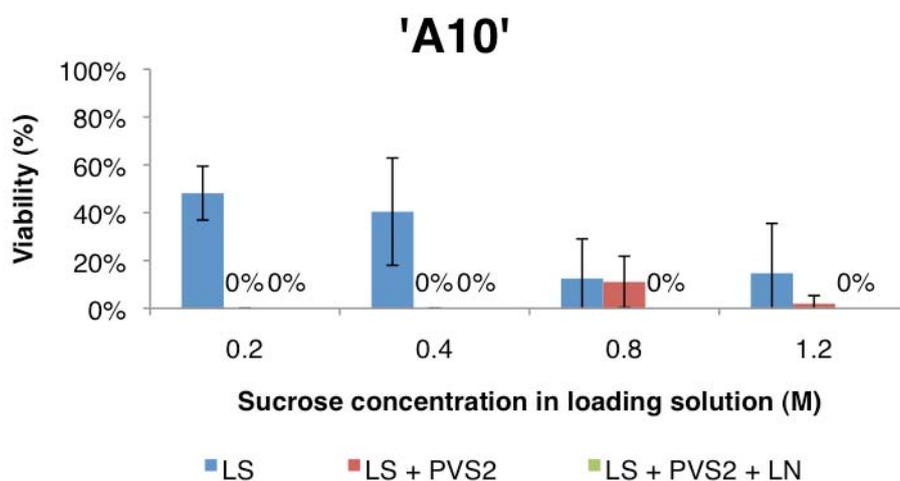


Figure 16. Effect of sucrose concentration in loading solution on the viability of encapsulated 'A10' SE by encapsulation-vitrification. LS = Loading in 2M glycerol + 0.2 – 1.2M sucrose for 20 min. LS + PVS2 = Loading followed by PVS2 dehydration at 0°C for 60 min. LS + PVS2 + LN = Direct immersion in LN after loading and PVS2 dehydration. Data was recorded 5 weeks after treatment. *Bars* indicate standard error.

ii) the effect of loading time on encapsulated SE

The effect of loading time on the viability of 'A10' was tested with 2M glycerol + 0.4M sucrose as the loading solution, as shown in figure 17.

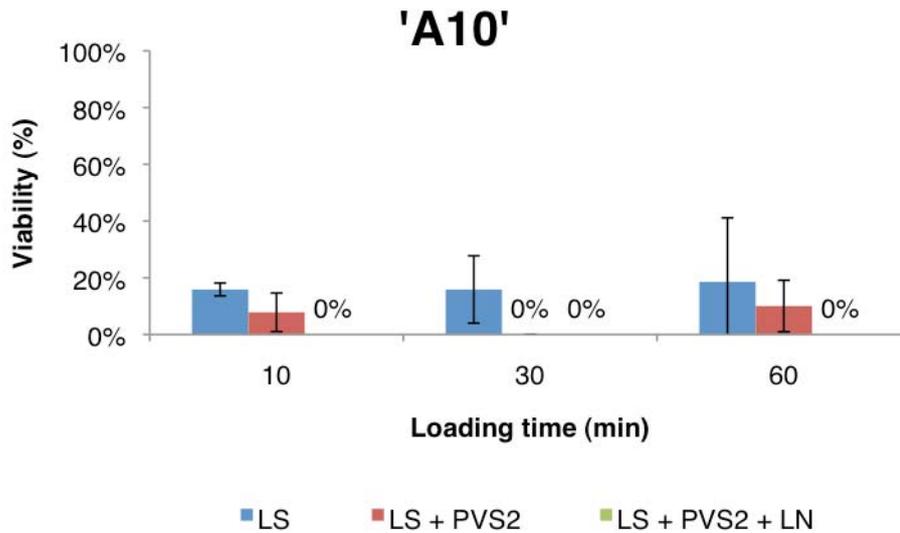


Figure 17. Effect of loading treatment duration on the viability of encapsulated ‘A10’ SE by encapsulation-vitrification. LS = Loading in 2M glycerol + 0.4M sucrose for 10, 30 and 60 min. LS + PVS2 = Loading followed by dehydration in PVS2 at 0°C for 60 min and then immersed in LN. LS + PVS2 + LN = Direct immersion in LN after loading and PVS2 treatment. Data was recorded 5 weeks after treatment. *Bars* indicate standard error.

There was no significant difference in viability between the three loading periods tested and none of them gave rise to viable SE after LN treatment.

iii) the effect of PVS2 exposure time on encapsulated SE

Independent of the loading treatment, the toxicity of PVS2 with respect to exposure time was tested using encapsulated ‘A10’ SE, as presented in figure 18.

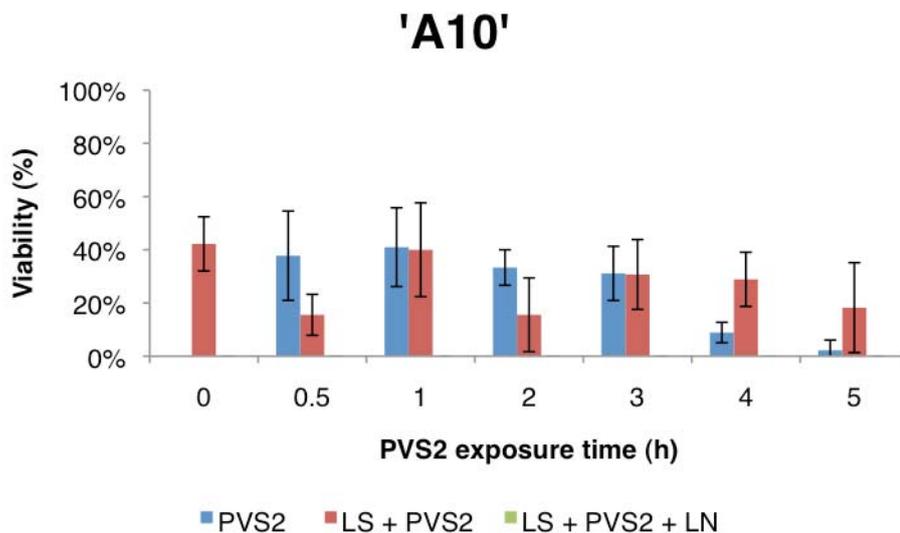


Figure 18. Effect of PVS2 exposure time on the viability of encapsulated ‘A10’ SE by encapsulation-vitrification. PVS2 = Dehydration in PVS2 at 0°C for 0 – 5 h. Dehydrated control = Loading in 2M glycerol + 0.4M sucrose for 20 min followed by PVS2 dehydration at 0°C for 0 – 5 h. LS + PVS2 + LN = Direct immersion in LN after loading and PVS2 treatment. Data was recorded 5 weeks after treatment. *Bars* indicate standard error.

'A10' SE that were treated with PVS2 only showed 31 – 41% (Fig 18) viability for the initial 3 h and declined significantly after that. When PVS2 was combined with loading solution SE were able to survive PVS2 exposure up to 5 h at all time points. However, with the addition of LN there was no viability SE observed after LN storage for encapsulated 'A10' SE.

No recovery was obtained after LN treatment using the encapsulation-vitrification method of cryopreservation so this method was abandoned.

3. Cryovial-vitrification of SE using PVS2

i) In the first series of experiments SE of each cultivar were treated with loading solution 2M glycerol + 0.4M sucrose followed by exposure to PVS2 for 30 or 60 min, and then immersion into liquid nitrogen for 60 min. SE of 'Duke-7' were subjected to loading in 2 M glycerol + 0.4 sucrose for 20 min followed by dehydration in PVS2 for 60 min at 0°C + LN plunge resulted in 87% (Fig 19) viability. For 'A10' SE, this treatment resulted in no viable embryos, even without liquid nitrogen exposure (Fig. 20a), whereas 100% viability was observed for the non-treated control. 'Reed' SE subjected to the same treatment resulted in no viable SE after liquid nitrogen immersion, and the optimal time for PVS2 exposure was 30 min without liquid nitrogen (80% viability; Fig. 20b). Viability of untreated 'Reed' SE was recorded at 100%. 'Velvick' SE incubated in PVS2 for 30 min with and without liquid nitrogen, resulted in 20 and 80 % viability, and incubation in PVS2 for 60 min with and without liquid nitrogen, resulted in 48 and 32 % viability (Fig 20c), respectively. Viable 'Velvick' embryos were obtained for PVS2 untreated controls (100% viability).

ii) In the second series of experiments optimal loading times were used for each cultivar (Table 3) in combination with PVS2 at 0°C for 60 min except in the case of 'Reed' which was 30 min. 'A10' SE which were subjected to loading in 2 M glycerol + 0.2 sucrose for 20 min followed by dehydration in PVS2 for 60 min at 0°C + LN plunge resulted in 82% (Fig 21a) viability. As expected the untreated and loading control SE yielded 100% viability. Additionally, another loading treatment of 2M glycerol + 0.8M sucrose for 20 min followed by 60 min in PVS2 for 60 min at 0°C + LN for 'A10' SE which resulted in 91% (Fig 21b) viability.

For 'Reed' SE, 2M glycerol + 0.6M sucrose for 20 min followed by dehydration in PVS2 for 30 min at 0°C + LN plunge resulted in 73% (Fig 22) viability. The untreated and loading control SE yielded 100% viability. 'Velvick' SE were subjected to loading in 2M glycerol + 0.2 or 0.8M sucrose for 20 min, followed by dehydration in PVS2 for 60 min at 0°C + LN plunge, resulted in 86 and 80% (Fig 23 a and b) viability, respectively. All three avocado SE cultivars demonstrated viability above the 40% minimum (Fig 24) required for long-term storage of germplasm (Uchendu and Reed, 2008).

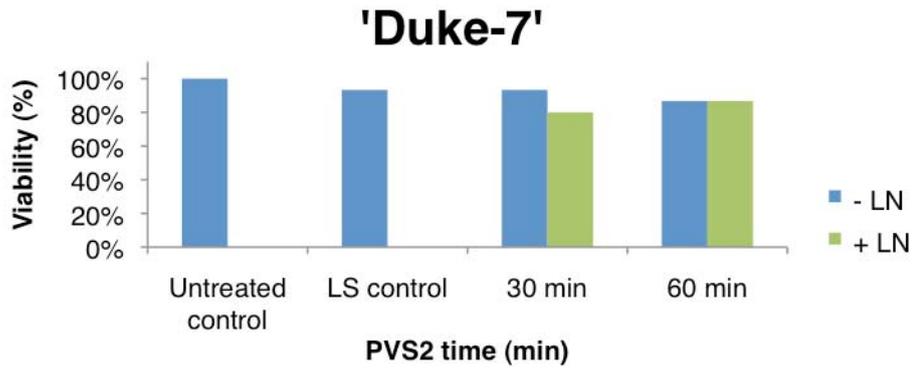


Figure 19. Viability (%) of ‘Duke 7’ avocado SE after application of the cryo-vial vitrification method, in 2M glycerol loading solution + 0.4M sucrose for 20 min, followed by exposure to PVS2 at 0°C for different time periods, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Data were recorded 10 weeks after treatment. Values are means of 15 biological replicas.

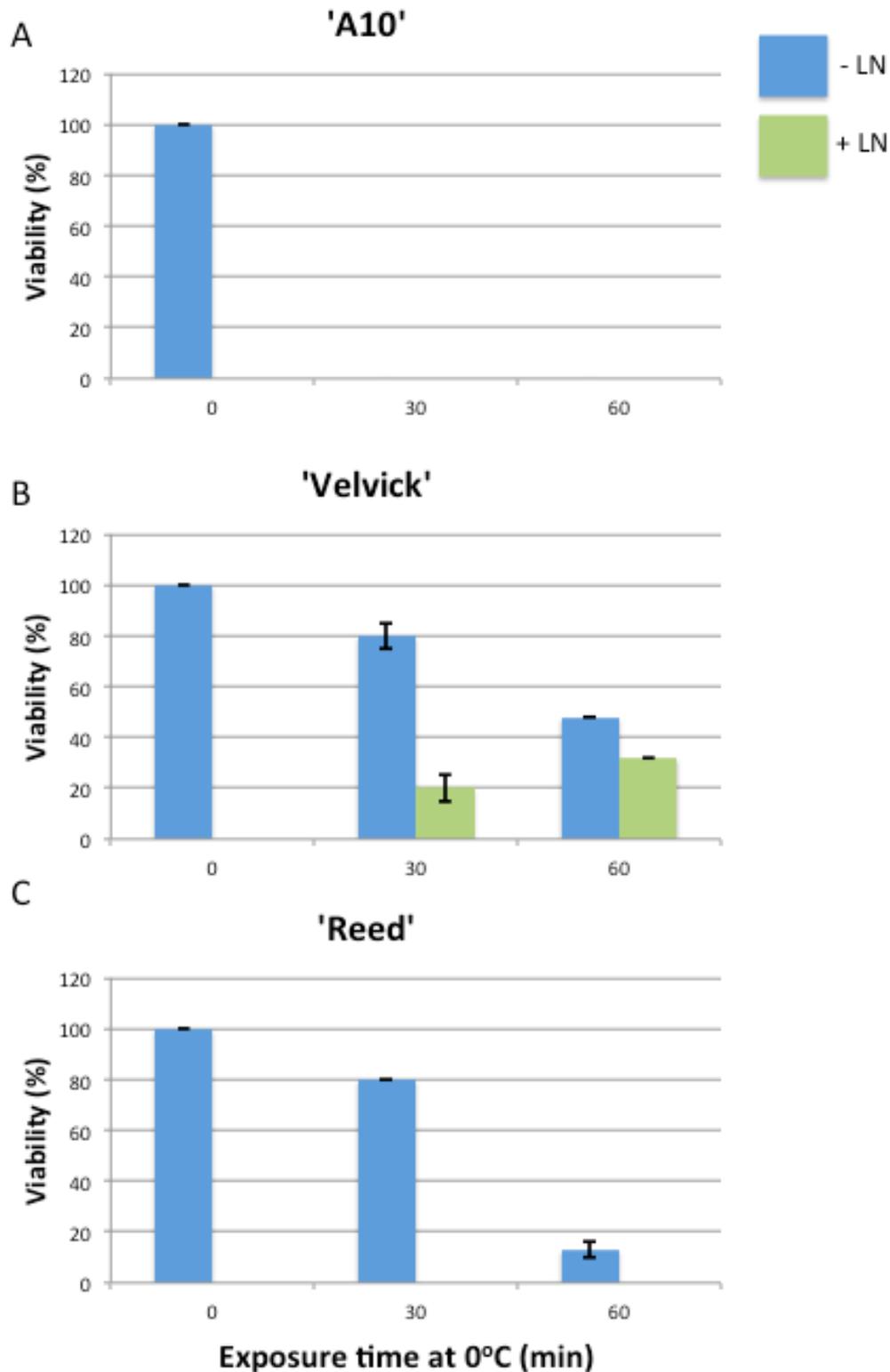


Figure 20. Viability (%) of 'A10' (a) 'Reed' (b) and 'Velvick' (c) avocado SE after application of the cryo-vial vitrification method, in 2M glycerol loading solution + 0.4M sucrose for 20 min, followed by exposure to PVS2 at 0°C for different time periods, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Data were recorded 7 weeks after treatment. Values are means of 15 biological replicas. Vertical bars represent SEM.

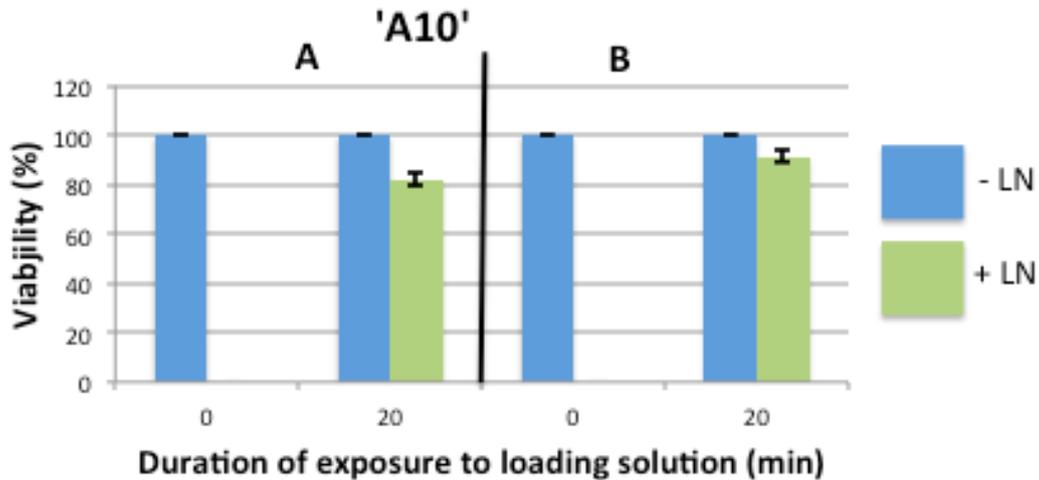


Figure 21. Viability (%) of 'A10' SE, after application of the cryovial vitrification method, in (A) 2M glycerol loading solution with 0.2M sucrose for 20 min and (B) 2M glycerol loading solution with 0.8M sucrose for 20 min. Both (A and B) were exposed to PVS2 0°C 60 min, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Vertical bars represent SEM.

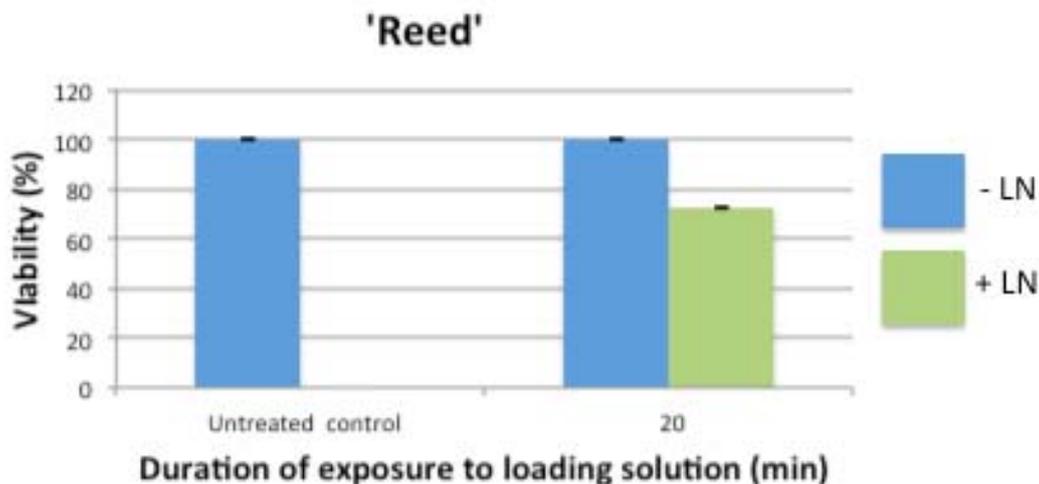


Figure 22. Viability (%) of 'Reed' avocado SE after application of the cryo-vial vitrification method, in 2M glycerol loading solution with 0.6M sucrose for 20 min, followed by exposure to PVS2 at 0°C for 30 min, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Data were recorded 5 weeks after treatment. Values are means of 15 biological replicas. Vertical bars represent SEM.

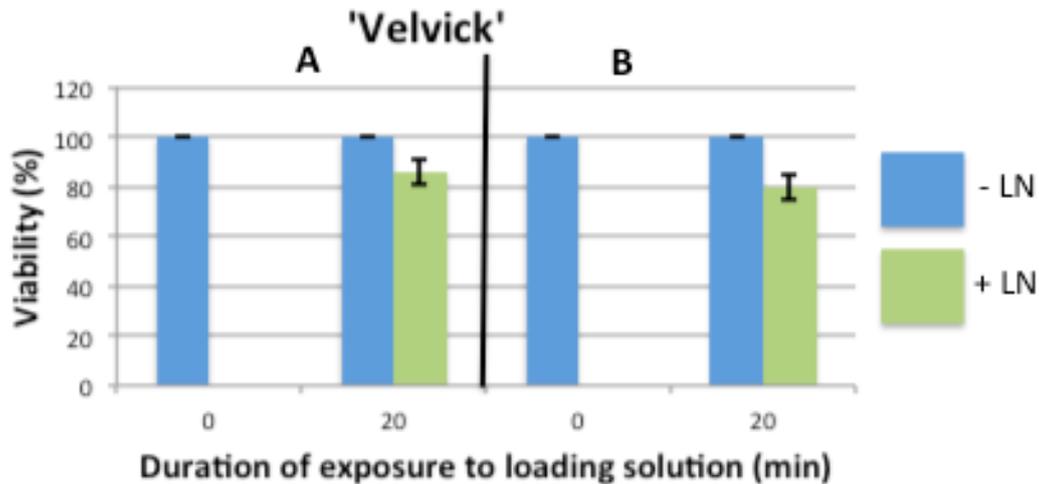


Figure 23. Viability (%) of 'Velvick' SE, after application of the cryovial vitrification method, in (A) 2M glycerol loading solution with 0.2M sucrose for 20 min and (B) 2M glycerol loading solution with 0.8M sucrose for 20 min Both (A and B) were exposed to PVS2 0°C 60 min, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen.

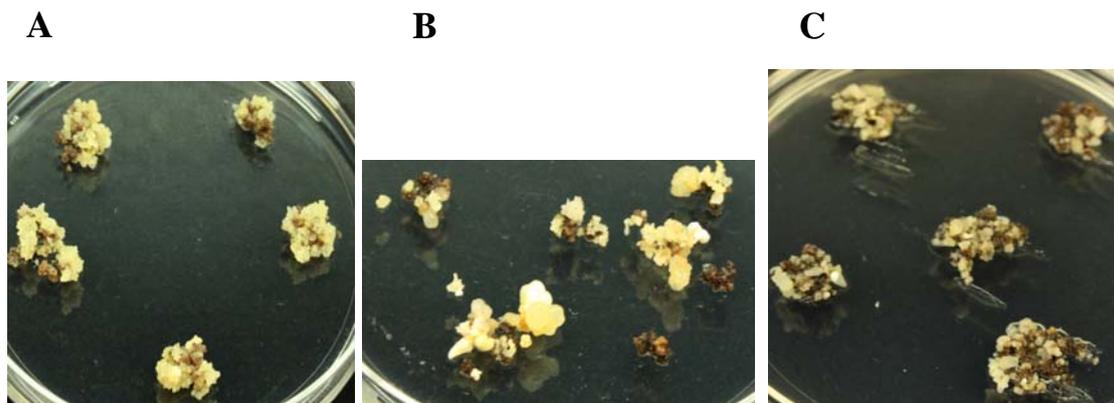


Figure 24. SE of 'A10' (A), 'Reed' (B) and 'Velvick' (C) growing on MMSE media after the cryovial method.

We now have successful method of cryovial vitrification for all cultivars tested with viability of >60%

4. Droplet Vitrification

i) In the first series of experiments for the droplet-vitrification of all cultivars were treated to loading solution 2M glycerol + 0.4M sucrose for 20 min in combination with PVS2 at 0°C for 60 min. For all cultivars +LN 0% viability was observed (data not shown).

ii) In the second series of experiments optimal loading times were used for each cultivar (Table 4) in combination with PVS2 at 0°C for 60 min. For 'A10' SE, exposure in loading solution of 2M glycerol + 0.2M sucrose for 40 min, followed by dehydration in PVS2 for 60 min at 0°C + LN plunge, resulted in 100% viability (Fig

25). The best treatment for ‘Reed’ SE, resulting in 85% viability (Fig 26), was obtained with exposure to loading solution of 2M glycerol + 0.2M sucrose for 20 min, followed by dehydration in PVS2 for 60 min at 0°C + LN plunge. For ‘Velvick’ SE, (48%) viability (Fig 27) was obtained with exposure of SE in loading solution of 2M glycerol + 0.2M sucrose for 20 min, followed by dehydration in PVS2 for 60 min at 0°C + to direct LN plunge. All three avocado SE cultivars demonstrated viability above the 40% minimum (Fig 28) required for long-term storage of germplasm (Uchendu and Reed, 2008).

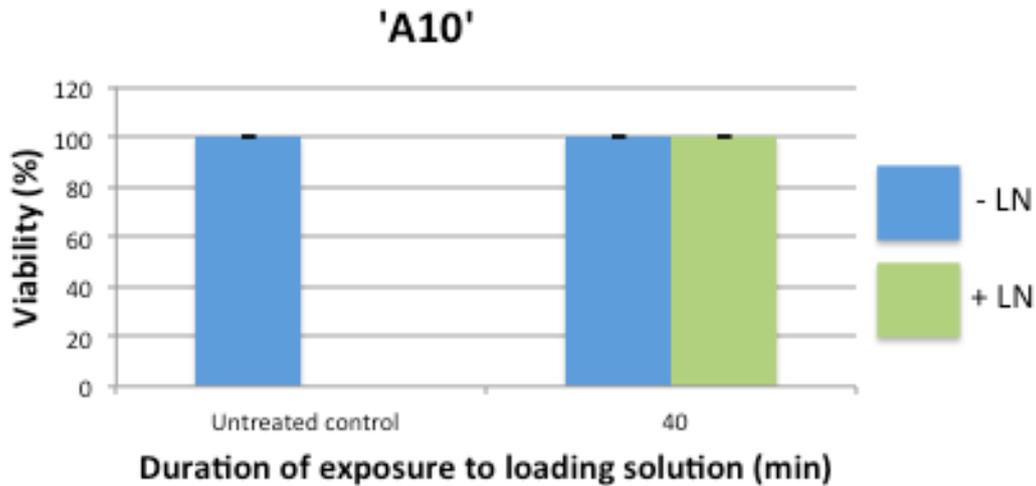


Figure 25. Viability (%) of ‘A10’ SE, after application of the droplet vitrification method, in 2M glycerol loading solution with 0.2M sucrose for 40 min + PVS2 0°C 60 min, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Vertical bars represent SEM.

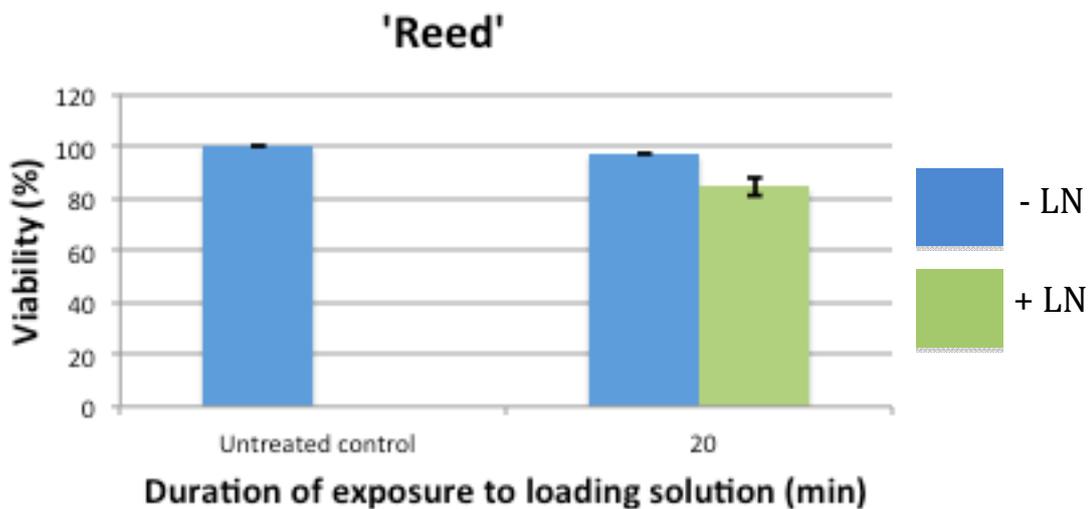


Figure 26. Viability (%) of ‘Reed’ SE, after application of the droplet vitrification method, in 2M glycerol loading solution with 0.2M sucrose for 20 min + PVS2 0°C 60 min, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Vertical bars represent SEM.

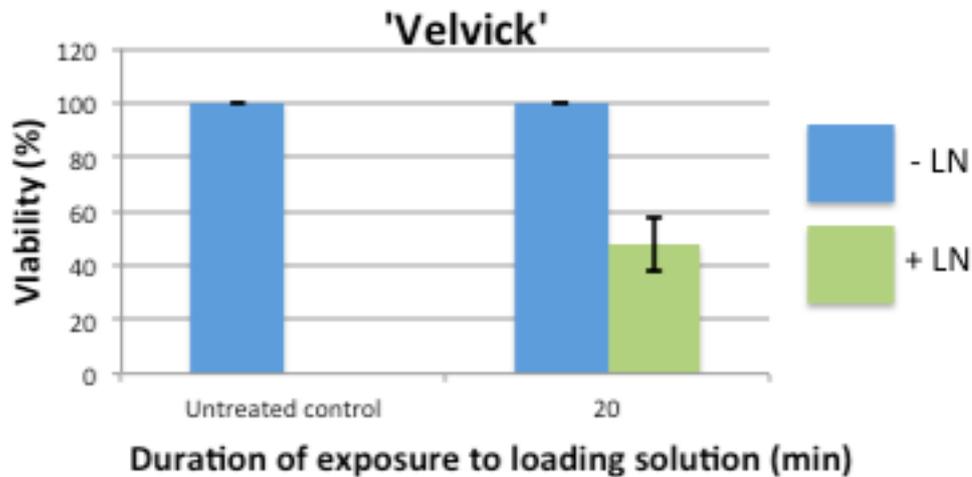


Figure 27. Viability (%) of 'Velvick' SE, after application of the droplet vitrification method, in 2M glycerol loading solution with 0.2M sucrose for 20 min + PVS2 0°C 60 min, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen.

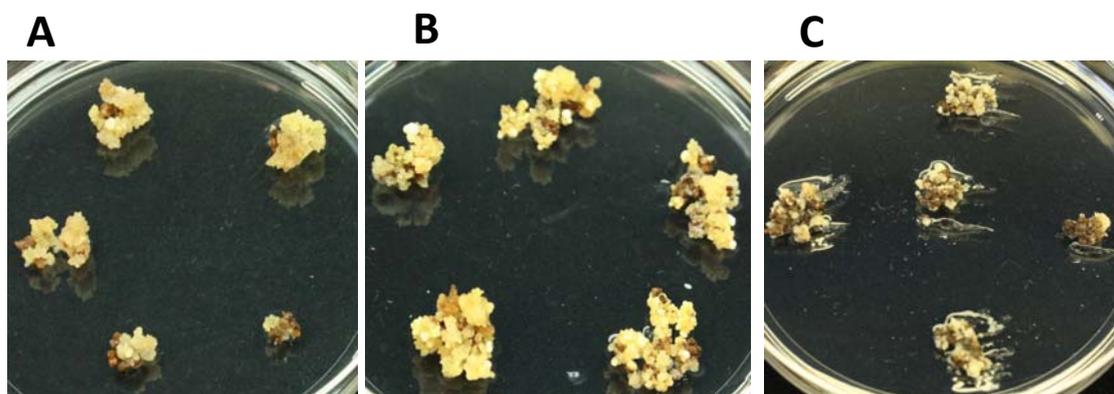


Figure 28. SE of 'A10' (A), 'Reed' (B) and 'Velvick' (C) growing on MMSE media after the droplet vitrification method.

FDA staining

SE of all cultivars were successfully stained with FDA after 5 weeks growth for both LS + PVS2 -LN and LS + PVS2 + LN by either cryovial or droplet method (Fig 29). Only viable living cells are able to be stained. The SE from the dead negative control was mostly unstained with only a slight expression which would be expected from dying tissue.

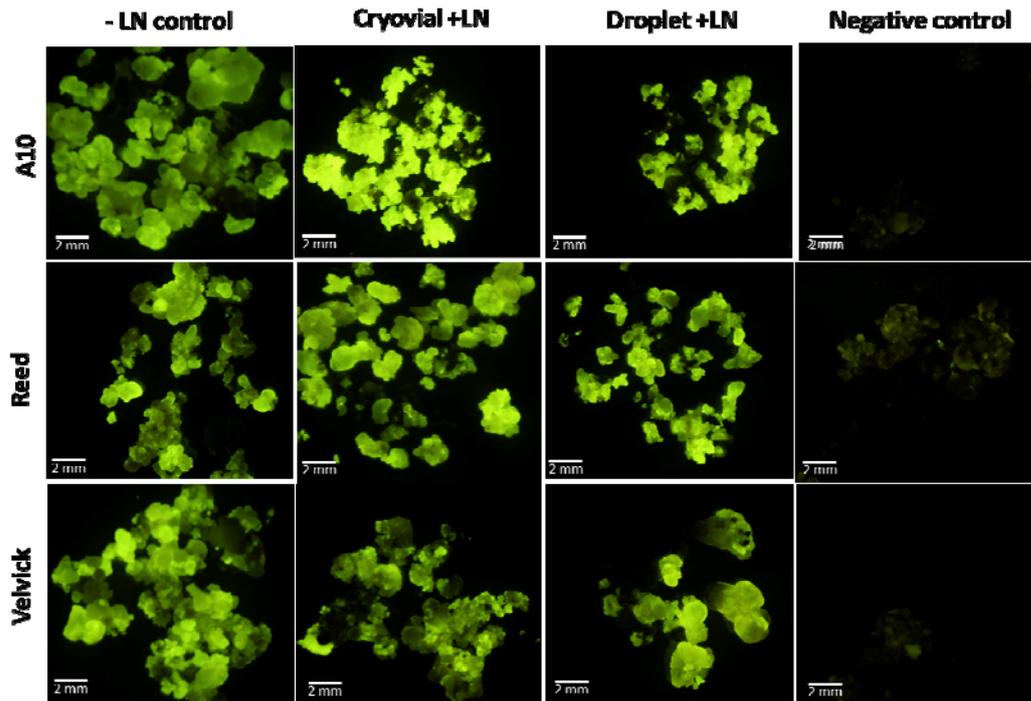


Figure 29. FDA vital staining of avocado SE 4-5 weeks post liquid nitrogen. Cryovial ‘A10’ SE, 0.8M loading solution 20 min, PVS2 at 0°C for 60 min, droplet vitrification of ‘A10’ SE, 0.2M loading solution 40 min, PVS2 at 0°C for 60 min, droplet vitrification of ‘Reed’ SE, 0.2M loading solution 20 min, PVS2 at 0°C for 60 min, cryovial of ‘Velvick’ SE, 0.8M loading solution 20 min, PVS2 at 0°C for 60 min, droplet vitrification ‘Velvick’ SE, 0.8M loading solution 20 min, PVS2 at 0°C for 60 min.

Long-term storage of ‘Reed’ and ‘Velvick’ SE

SE of cultivar ‘Reed’ and ‘Velvick’ were setup for long-term storage using the cryovial technique. Due to the time in optimizing these techniques only these two cultivars could be tested for long term storage. The average number of SE from cultivar ‘Reed’ that were still viable after 12 months of storage was 67% (Fig 30). In our experiment SE viability is recorded 5-weeks after cryopreservation i.e. LN exposure. These SE are currently at the 2-week stage, and it could be likely that at the 5 week stage the SE viability will increase slightly. The experiment is in progress.

For ‘Velvick’ SE the average that was recovered after 12 months storage was 59% (Fig 31). Also, these SE are currently at the 2-week stage, and it is highly likely that at the 5-week stage the SE viability will increase. The experiment is in progress.

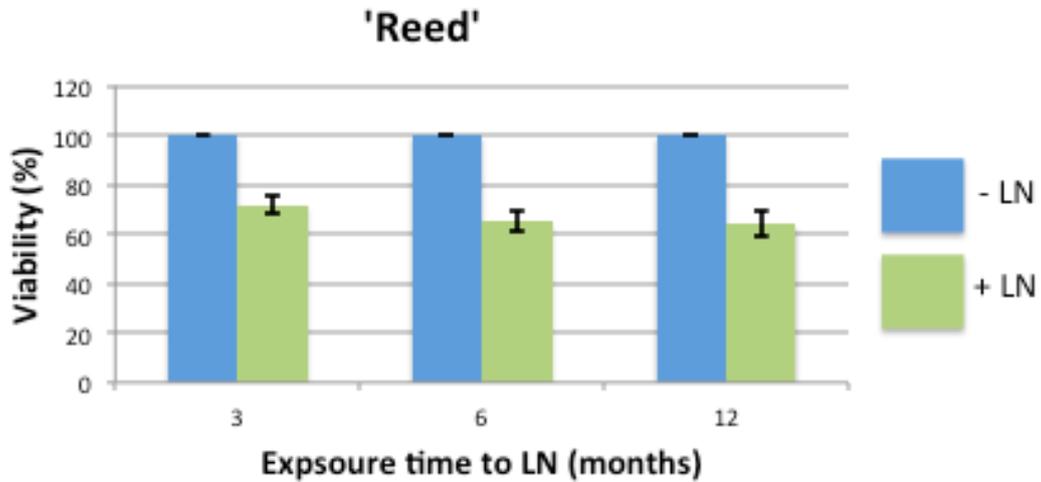


Figure 30. Viability (%) of 'Reed' SE after long term storage using the cryovial technique with a loading solution of 2M glycerol + 0.6M sucrose 20 min and PVS2 at 0°C for 30 min

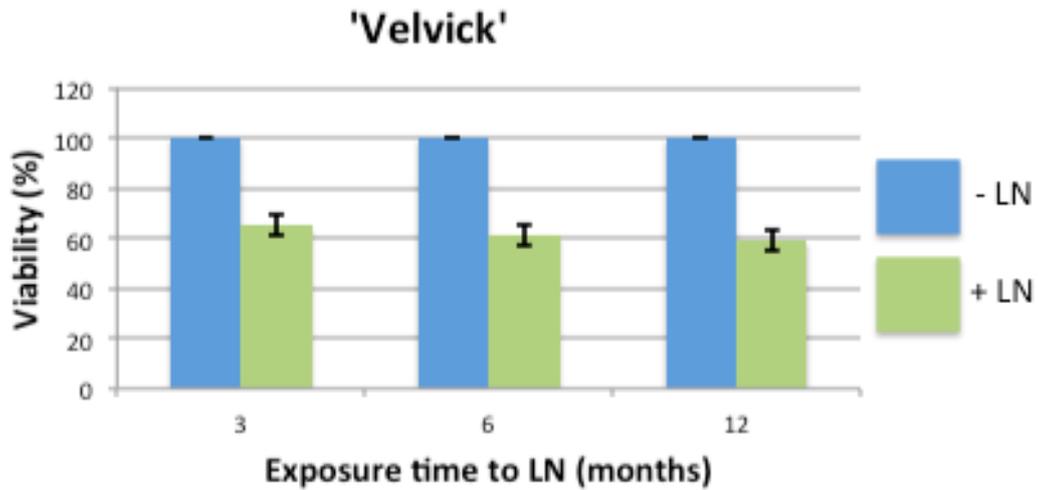


Figure 31. Viability (%) of 'Velvick' SE after long term storage using the cryovial technique with a loading solution of 2M glycerol + 0.4M sucrose 20 min and PVS2 at 0°C for 60 min.

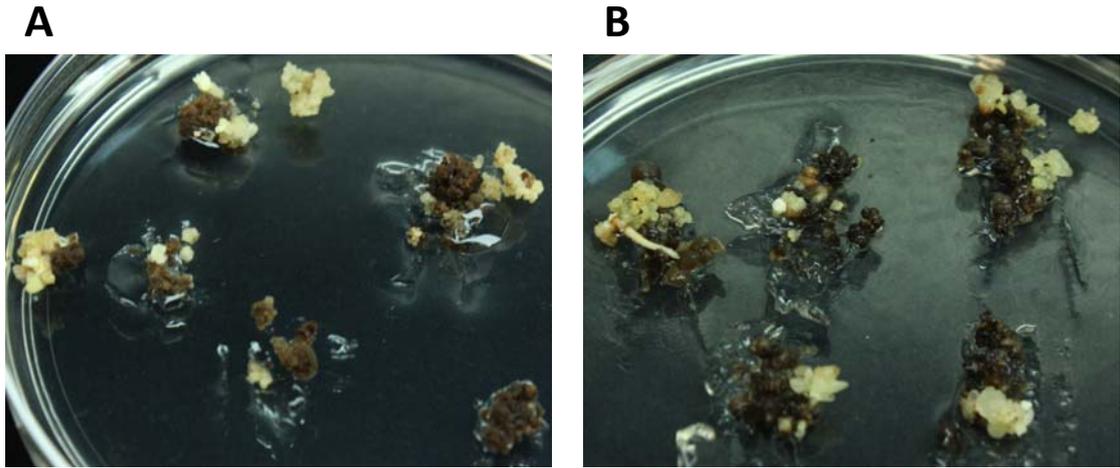


Figure 32. SE of 'Reed' (A) and 'Velvick' (B) growing on MMSE media after the 12 months liquid nitrogen storage using the cryovial method.

Discussion

Avocado genetic resources are currently maintained only in field repositories at great cost and constantly under threat of natural calamities, pests and diseases. The safest method for long-term conservation of plant genetic resources is cryopreservation. Cryopreservation of SE is valuable as it is readily retrievable for further biotechnology manipulations as well as storage of biotechnology products such as genetically transformed lines (Engelmann, 2011a).

Here, the cryopreservation of SE using different techniques was investigated. They include encapsulation-dehydration, encapsulation-vitrification, cryovial-vitrification and droplet vitrification. Among these techniques, cryovial-vitrification and droplet-vitrification protocol successfully cryopreserved avocado SE. For cryovial-vitrification, viability for 4 cultivars ranged from 73-91% (Fig 33), and for droplet-vitrification, viability for 3 cultivars ranged from 48-100% (Fig 34).

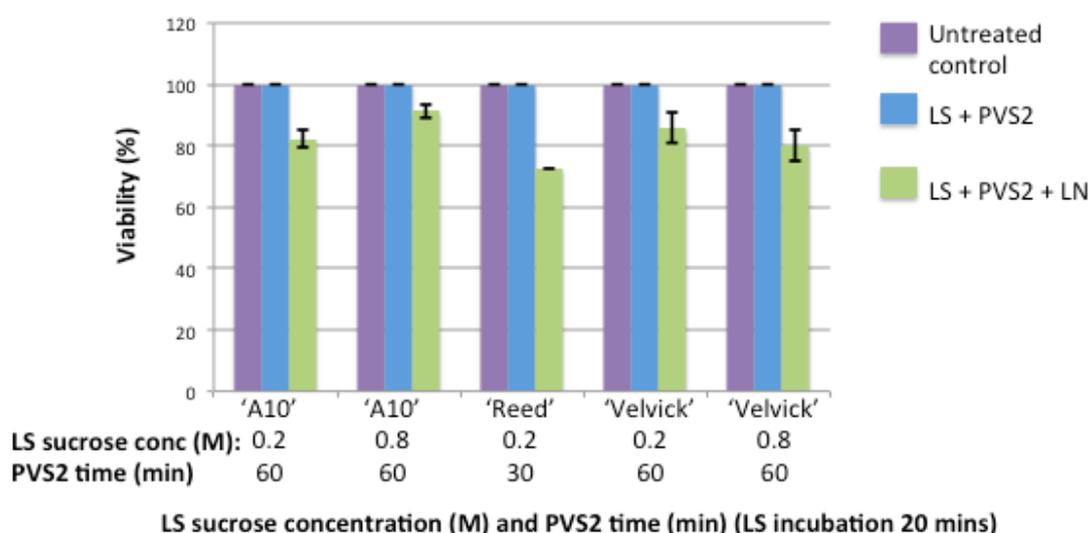


Figure 33. Viability (%) of 'A10,' 'Reed,' and 'Velvick' avocado SE after application of the cryovial vitrification method, in 2M glycerol loading solution with different concentrations of sucrose for 20 min, followed by exposure to PVS2, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. 'A10' SE exposed to 0.2M and 0.8M sucrose, respectively, and PVS2 0°C for 60 min, 'Reed' SE were exposed to 0.6M sucrose, PVS2 0°C for 30 min, 'Velvick' SE exposed to 0.2M and 0.8M sucrose, respectively, PVS2 0°C for 60 min. Data were recorded 5 weeks after treatment. Values are means of 15 biological replicas. Vertical bars represent SEM.

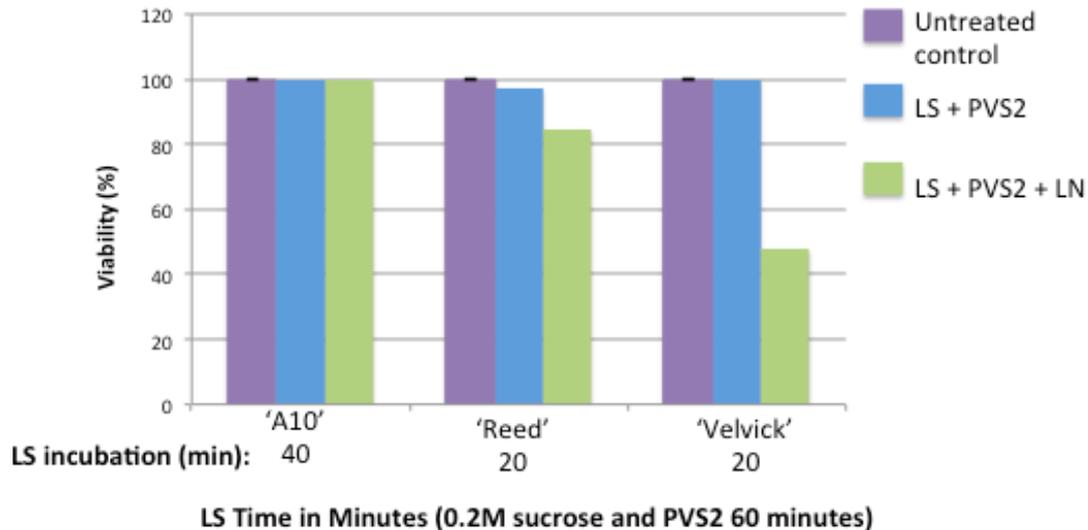


Figure 34. Viability (%) of 'A10,' 'Reed,' and 'Velvick' avocado SE after application of the droplet vitrification method, in 2M glycerol loading solution with 0.2M sucrose for different time min, followed by exposure to PVS2 0°C for 60 min, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. 'A10' SE were exposed for 40 min in LS, 'Reed' and 'Velvick' SE were exposed for 20 min in LS. Data were recorded 5 weeks after treatment. Values are means of 15 biological replicas. Vertical bars represent SEM.

1. Encapsulation-dehydration of SE

Encapsulation-dehydration protocol was designed to minimise the use of complex and toxic solutions with high osmotic potentials (e.g. PVS2) (Reinhoud et al., 2000). Encapsulation of SE in alginate beads not only enables easy manipulation and prevents mechanical stress during handling; encapsulation also reduces osmotic shock of direct exposure to highly concentrated osmotica (e.g. sucrose and glycerol) (Kaczmarczyk et al., 2012). Moreover, the presence of a nutritive bead may also enhance regrowth of the SE after cryopreservation (Panis and Lambardi, 2005). The viscosity of alginate solution is critical to maintain the integrity of beads upon freezing.

In the current study, beads previously made using Na-alginate with low viscosity of 4 – 12 cps became “mushy” after freezing, probably due to the high moisture content. None of the frozen tissues remained viable with this batch of Na-alginate. Attempted modifications made to the protocols including increased desiccation time, rapid freezing and thawing rate, failed to address this problem. Once replaced with 5.6% (w/v) alginate of 66.5 cps viscosity (PhytoTechnology Laboratories®), equivalent to 3% (w/v) Na-alginate with 125 cps viscosity described in most literature, beads recovered from LN were intact and recovery was observed.

In the current study, cryopreservation by encapsulation-dehydration only recovered < 4% viability of 'Reed' only within the cultivars and treatments tested. *Oleo europea* (Shibli and Al-Juboory, 2000), *Quercus suber* (Fernandes et al., 2008) and *Theobroma cacao* (Fang et al., 2004) SE have been successfully cryopreserved with this technique. A major distinction noted from these published protocols was that rapid plunge in LN was done, instead of the two-step freezing (slow cool to -80°C followed by LN plunge) employed in our study. This additional slow cooling may be

the reason for the low viability obtained in cryopreserved avocado SE. Moreover, Engelmann (1997) previously reported that non-organised tissue (e.g. SE and shoot tips) may not be suitable for slow cooling as sufficient dehydration is less achievable in tissue with complex structures due to different rates of water movement between and within different cell types (Engelmann, 1997).

2. Encapsulation-vitrification of SE

Encapsulation-vitrification has been applied in the cryopreservation of *Sacharum Officinarum* (Martinez-Montero et al., 2008) and *Olea europaea* (Shibli and Al-Juboory, 2000) SE. Encapsulation is thought to promote a vitrified state in the tissue regardless of the freezing and thawing rates, thus reducing damage due to ice crystal formation (Scottez et al., 1992).

Cryopreservation by vitrification requires the use of highly concentrated vitrification solution that is toxic. Loading with cryoprotectants of milder concentration prepares them for exposure to the vitrification solution as the accumulation of these cryoprotectants in the sample tissue increases the stability of membranes under conditions of severe dehydration (McBride and Zusman, 1989, Jitsuyama et al., 1997). Optimising the loading concentration and time are key to effectively inducing tolerance to dehydration by the vitrification solution (Sakai et al., 1991). In this study, some protection toward PVS2 toxicity can be observed with higher concentration of loading solutions (0.8 and 1.2 M sucrose) as shown in figure 16.

Optimisation of PVS2 exposure time and temperature is a vital step to find the balance between adequate dehydration and chemical toxicity (Panis et al., 2005; Panis et al. 1990). Many crops especially those of tropical origin require low temperature to reduce the injurious effects of PVS2 (Nishizawa et al., 1993, Tinh, 1997). This was also the case for ‘Reed’ avocado SE as substantial difference in viability was observed with 0°C (89 – 91%) and RT (7 – 76%) with PVS2 exposure up to 2 h. For *Sacharum Officinarum* (Martinez-Montero et al., 2008) and *Olea europaea* (Shibli and Al-Juboory, 2000), 10 – 15 min and 3 h in PVS2 ensured highest post-thaw viability respectively.

Combination of loading and PVS2 treatments attempted using ‘A10’ did not recover any viability after LN. This may be due to the low proliferation rate of this cultivar. The low proliferation rate of ‘A10’ is also reflected in the untreated control of encapsulation-dehydration in which ‘A10’ exhibit only half the growth as compared ‘Reed’ and ‘Duke-7’.

At this stage, the method has been tested only with ‘A10’ and no recovery was obtained faster cryopreservation. It needs to be tested and optimised with other cultivars that have a higher multiplication rate than ‘A10’.

3. Cryovial-vitrification of SE

Vitrification with or without encapsulation has been more widely applied in cryopreservation of explants with complex tissue structures (e.g. SE and shoot tips) than slow cooling (Kaczmarczyk et al., 2012). In our study, cryovial-vitrification resulted in successful cryopreservation of avocado SE in 4 out of 4 cultivars tested. These results are also in accordance to previous report in ‘Duke-7’ avocado embryogenic cultures by Guzmán-García et al. (2012) which showed that vitrification

gives a higher viability (78 – 100%) than slow cooling techniques (33 – 80%). In contrast to these findings, Efendi (2003) indicates that slow cooling techniques (53 – 80%) in three embryogenic lines provided better results than vitrification procedures where only one line showed 62% viability for avocado embryogenic cultures. Similar to encapsulation-vitrification, the viability in response to various loading treatment was tested. Theoretically, a gradual decrease of viability is expected as explants were subjected to higher concentration of loading solution for longer period. This was evident for ‘Reed’ SE and lower concentrations of sucrose in loading solutions were used for experiments, 0.2 and 0.6M (Fig 12B). With ‘A10’ and ‘Velvick’ SE the trend was not as obvious as ‘Reed’, and a sucrose concentration of >0.8M was not used in any experiments (Fig 12A and C). This may be explained by the variation of SE sizes even within the same cultivar (Fig 11).

In terms of PVS2 treatment optimisation, the results obtained support our previous findings of encapsulation-vitrification, which indicated 0°C as the preferred temperature.

The best combinations of treatments for ‘A10’, ‘Reed’ and ‘Velvick’ by cryovial-vitrification were tabulated in Table 3. ‘A10’ and ‘Velvick’ SE share the same treatments (0.2 and 0.8M sucrose), and are coincident with previous investigation in ‘Duke-7’ embryogenic culture by Guzmán-García et al. (2012) recovering a minimum of 82% viability. ‘Reed’ SE were treated at a different loading solution sucrose (0.6M) concentration and recovered with 72% viability.

Throughout the study, there is a cultivar-related difference in terms of desiccation tolerance (loading and PVS2 treatment) and subsequent freezing resistance. Similar findings have been reported by Guzmán-García et al. (2012) and (Efendi, 2003) on avocado embryogenic cultures, and also in other crops like *Pyrus* (Reed et al., 1998) and *Morus alba* (Niino et al., 1992). These differences could be attributed to the age and size of the SE.

The age of SE is defined as the number of subcultures. The proliferation potential and viability of SE has been reported to be lost over time (Witjaksono and Litz, 1999). ‘Velvick’ and ‘Duke-7’ were induced recently and therefore had been through few cycle of subcultures. Thus, sufficient cryotolerance can be induced in these cultivars with loading solution of lower concentration (2M glycerol + 0.4M sucrose). Conversely, ‘Reed’ and ‘A10’ had been maintained on plate for more than a year and hence ‘Reed’ required 0.6M sucrose for it to effectively be cryopreserved. Tessereau et al. (1994) studied the age-related difference in carrot SE and showed a larger variation in survival rate with greater number of subcultures. It is therefore important to use freshly induced SE for the reproducibility of the cryoprotocols.

As indicated in figure 11, there is a clear difference between the SE size of different cultivars. Increased cellular differentiation of larger SE, hence varying degree of vacuolation, may lead to different degree of cryotolerance (Tessereau et al., 1994). The efficient heat exchange during cooling and thawing is also more likely in smaller SE (Tessereau et al., 1994). Loading and unloading of cryoprotectants may also be difficult for larger embryos.

Some plant species such as garlic and chrysanthemum are sensitive to chemical toxicity of permeating cryoprotectants such as DMSO in PVS2. PVS3, on the other hand, serves as a milder form and are used widely when samples are heterogeneous, of large size or very sensitive to chemical toxicity and tolerant to osmotic stress (Kim et al., 2009). Inversely, in the cryovial vitrification using PVS3, viability was lost drastically upon exposure to PVS3 independent of the exposure temperature (Fig 14).

4. Droplet-vitrification of SE

Droplet-vitrification has proved to be promising with a increasing number of plant species cryopreserved with this technique, more than 300 and 540 accessions of potato and Musa, respectively, are successfully cryopreserved (Sakai and Engelmann, 2007). Work by Guzman-Garcia, (2012) employed droplet-vitrification on two 'Duke-7' embryogenic cell lines (D1 and D31) that gave promising results ranging from 77.78 – 100% recovery for both lines. D1, representative of the PEM-type, proliferate as pro embryogenic masses with a few SE. D31, representative of the SE-type, comprise mainly of SE at early developmental stages. The author's claim that the avocado cell lines, D1 and D31 used in their experiments, were representative of the embryonic cultures that can be obtained in this species, and their vitrification-based protocol may be used to cryopreserve multiple avocado cultivars.

In this study, using the droplet-vitrification method, we were able to successfully cryopreserve all three avocado cultivars, 'A10', 'Reed' and 'Velvick' with viability of 100%, 84% and 48%, respectively. A major contribution to successful of droplet-vitrification was optimisation of loading solution sucrose concentrations and exposure time (Figs 12 and 13). In comparison to cryovial-vitrification which first showed proliferation between the 2-4 weeks, droplet-vitrification treated SE demonstrated proliferation at a later time point, between 4-5 weeks. Furthermore, droplet-vitrification treated SE morphology was globular and clear, and was of better quality than cryovial-vitrification treated SE (data not shown). 'Velvick' SE treated using the droplet-vitrification method displayed the least percentage of viability (48%). Spreading SE of 'Velvick' across the plate instead of plating them in clumps resulted in better growth and higher percentages. This could be due to SE being close proximity to another and a leaching of phenolics which are detrimental to SE growth. Further research is warranted to investigate these details and improve of viability for cultivar 'Velvick'.

Chapter 2

Shoot tip cryopreservation

Introduction

Shoot tip cryopreservation is the ability to regenerate plants from cryopreserved, meristematic shoot tissue (Benson et al., 2007). This technique is especially useful for conserving genetic resources of recalcitrant seeds, vegetatively propagated species as well as rare and endangered plant species (Engelmann, 2011b). For avocado, shoot tip cryopreservation could pose as an ideal substitute for field repositories of genetically important avocado genotypes as shoot tips are true-to-type and represent the accession without any heterogeneity (Engelmann, 2011b). This is in contrast to SE of avocado derived from zygotic embryos that are heterozygous in origin. Shoot tips also have the advantage of being able to be collected anytime, unlike SE that are dependent on fruit being available at a particular time of a year and at a certain developmental stage. In addition, shoot tips permit rapid multiplication *in vitro* and have lower occurrence of somaclonal variation (Panis and Lambardi, 2005). The majority of shoot tip cryopreservation protocols deal with classical vitrification and encapsulation-dehydration techniques (Panis and Lambardi, 2005). Recently, encapsulation-vitrification techniques have been successfully applied to shoot tip cryopreservation of *Malus Domestica* Borkh. (Paul et al., 2000), *Prunus domestica* L. (De Carlo et al., 2000) and *Rubus idaeus* L. (Fig 35) (Wang et al., 2005a).



Figure 35. Plant regeneration from cryopreserved shoot tips of raspberry by encapsulation-vitrification. (A) Excision of shoot tip. (B) Encapsulation of shoot tips. (C) Recovery of shoot tip. (D) Elongation of shoot. (E) Micropropagation of shoots regenerated from cryopreserved shoot. (F) Establishment of plantlets under greenhouse conditions (Wang et al., 2005a).

Ashmore et al. (2007) reported that optimisation of PVS2 exposure is critical for successful cryopreservation of shoot tips of *Carica papaya*. Shoot tip cryopreservation has also been conducted for other species including *Malus domestica*

Borkh. (Wu et al., 1999), *Allium sativum* (Baek et al., 2003), *Deospyros kaki* Thunb. (Matsumoto et al., 2001), *Mentha piperita* L. (Volk et al., 2006) and *Ipomoea batatas* L. (Pennycooke and Towill, 2000). Baek et al. (2003) also demonstrated that higher regeneration was observed with smaller apices (1.5 or 3.0 mm in diameter) in *Allium sativum*.

To date, there is no reported literature for cryopreservation of avocado shoot tips or a standardised method that is applicable to a variety of plant species. Thus, the current proposal will be the pioneering work in this area.

Materials and methods

Plant Materials and culture conditions

Establishment and multiplication of in vitro 'Reed' avocado plantlets

In vitro plantlets were established from mature embryonic axes. Mature seeds were surface-sterilised by immersion in 2% sodium hypochlorite and Tween® 20 for 20 min (Fig 36). The seed coats were removed and the embryonic axes were dissected from the seed halves and placed on modified Murashige and Skoog (MMS) medium (Ahmed, 2002).

After two months, nodal stem cuttings dissected from the established plantlets were cultured on mango medium for plant regeneration (MMPR) (Pateña et al., 2002) amended with 3 mg/L 6-Benzylaminopurine (BAP) and 0.1mg/L 1-naphthaleneacetic (NAA), and maintained as mentioned earlier. Subsequently, shoots developed from these nodal sections were transferred onto orchid maintenance medium (*PhytoTechnology Laboratories*®, Kansas, USA) supplemented with 8 g/L agar and 10 mg/L indole-3-butyric acid (IBA) for rooting. A schematic diagram of these processes is shown in figure 36.

The pH of culture media used was adjusted to 5.65 prior to autoclaving at 121°C for 15 min and all cultures were kept in a culture room at 25±1°C under a 16 h light/8 h dark photoperiod. Axillary shoot tips of these plantlets were used as experimental materials.

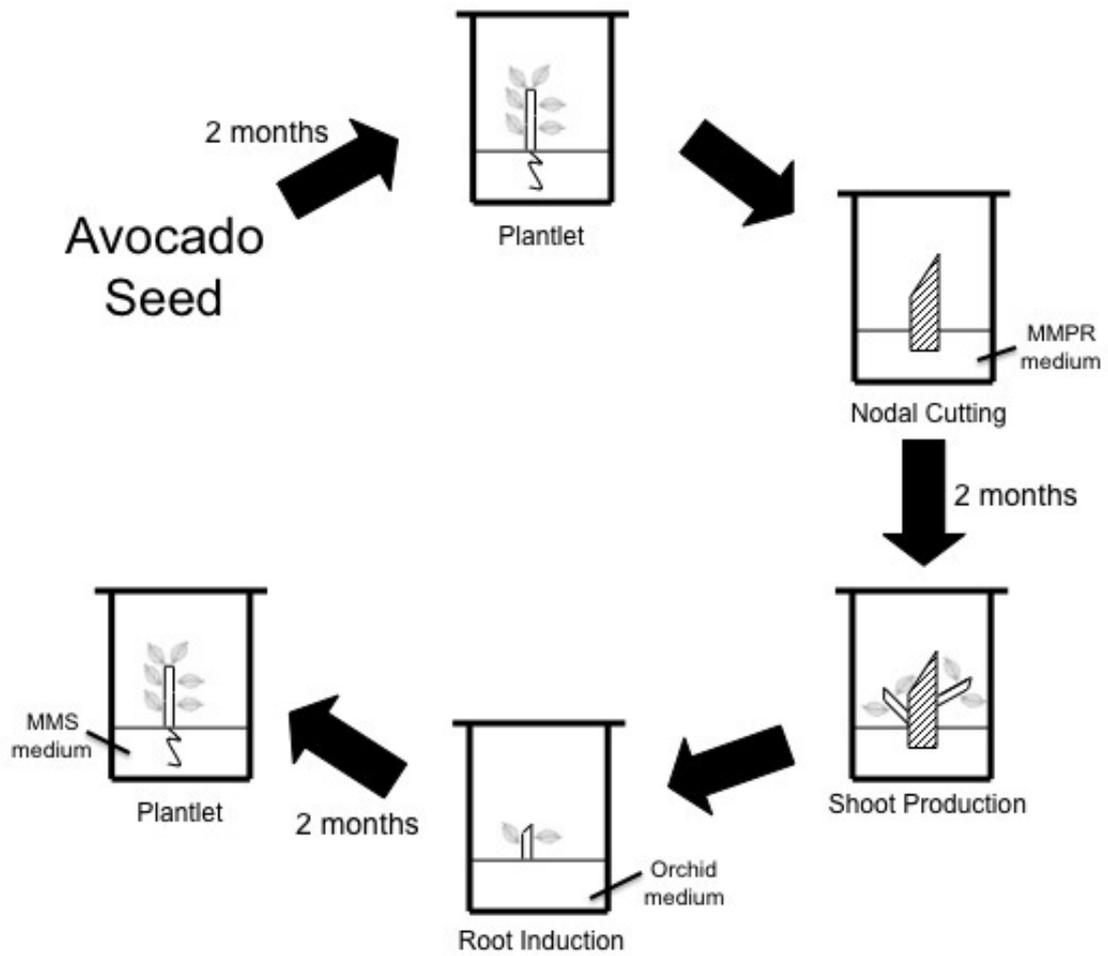


Figure 36. Schematic diagram of the processes followed for the multiplication of in vitro avocado plantlets to generate plant materials for cryopreservation.

Dissection of shoot tips

Nodal segments of *in vitro* plantlets were excised in two forms: (a) an entire nodal section (Fig 37a) and (b) v-cut wedge which ranged in size from 1-3mm (Fig 37b), each containing a single axillary bud. The excision was performed using a surgical blade (blade no. 11), with the aid of a binocular microscope (magnification x22). Explants were placed immediately on regeneration medium to retain moisture until sufficient explants were collected.

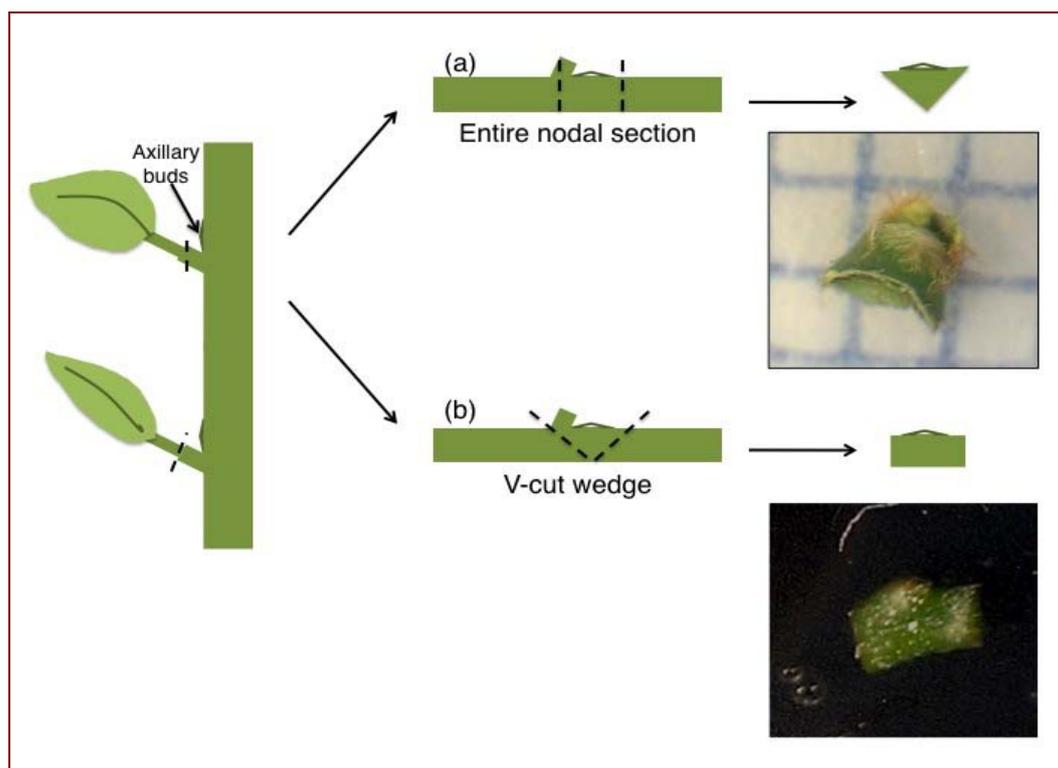


Figure 37. Schematic diagram of dissection of shoot tips as (a) an entire nodal section or (b) a v-cut wedge.

Optimisation of regeneration medium compositions

Excised explants were placed on culture media supplemented with various additives known to counteract the browning of explants and effectively promote shoot growth from the shoot tips (Table 6).

Table 6. Additives frequently used to overcome browning (Wang et al., 2005a).

Additives
(a) 2.5g/L activated charcoal
(b) 2% (w/v) polyvinylpyrrolidone (PVP)
(c) 3 mg/L BAP + 0.1 mg/L NAA
(d) 2 mg/L IAA
(e) 1 mg/L BAP + 0.1 mg/L IBA + 0.1 mg/L GA ₃

Optimization of cryoprotectant for shoot tips

Shoot tips of V-cut ‘Reed’ were either dehydrated with 30 mLs of one of the following mVSL (Table 7a), VSL (Table 7b) or PVS2 vitrification solution (Table 7c) at $0 \pm 1^\circ\text{C}$ for 0, 5, 10, 15 or 20 min. Shoot tips were then transferred to MMS medium (Table 7e) 5 buds per plate and grown as previously described and replicated twice. Data recorded after 4 weeks of growth.

Table 7. Composition of cryoprotective solutions for encapsulation-vitrification of ‘Reed’.

Solutions for optimizing cryoprotectant
(a) mVSL
20% (w/v) glycerol
10% (w/v) DMSO
25% (w/v) ethylene glycol
5% sucrose
MMS medium
(b) VSL
20% (w/v) glycerol
10% (w/v) DMSO
30% (w/v) ethylene glycol
5% sucrose
MMS medium
(c) PVS2
MMS medium
30% (w/v) glycerol
15% (w/v) DMSO
15% (w/v) ethylene glycol
0.4M sucrose
(d) Unloading solution
MMS medium
1.2M sucrose
(e) Regeneration medium
MMS medium
1 mg/L BAP
0.1 mg/L IBA
0.1 mg/L GA ₃

Cryopreservation techniques for shoot tips

1. Cryovial-vitrification of shoot tips

i) The following vitrification cryoprotocol was modified from Kaity et al. (2008). Shoot tips (5 and 10 mm in size) were excised from *in vitro* avocado plantlets. Shoot tips were then incubated in cryovial which contained loading solution (Table 8a) for 30 min at room temperature. Loading solution was then replaced by pre-chilled PVS2 (Table 8b) and explants were dehydrated for 60 min at 0°C. After cryopreservation, cryovials were thawed in water bath at 37°C for 80 s. PVS2 was replaced by unloading solution (Table 8c) and incubated for 30 min. Subsequently, 5 shoot tips/Petri dish were transferred to regeneration medium (Table 8d) and placed grown as previously described. Samples were taken at each stage in order to determine viability.

Table 8. Compositions of cryoprotective solutions used in cryovial-vitrification for shoot tip cryopreservation.

Solutions for cryovial-vitrification using PVS2

(a) Loading Solution

Liquid basal MMS medium
2M glycerol
0.4M sucrose

(b) PVS2

Liquid basal MMS medium
30% (w/v) glycerol
15% (w/v) ethylene glycol
15% (w/v) DMSO
0.4M sucrose

(c) Unloading Solution

Liquid basal MMS medium
1.2M sucrose

(d) Regeneration medium

MMS medium
1 mg/L BAP
0.1 mg/L IBA
0.1 mg/L GA₃

ii) V-cut shoot tips (2-3mm in size) were precultured overnight on MMS media + 0.75M sucrose followed by incubation in 20% PVS2 (Table 9b) at 0°C for 60 min combined with 100% PVS2 (Table 9c) at 0°C for 20 or 40 min before LN incubation. In a separate treatment V-cut 'Reed' tips were also precultured overnight but incubated in cryovial which contained PVS3 (Table 9d) at 0°C for 60 or 150 min + LN. After LN, cryovials were thawed in water bath at 37°C for 80 s. PVS2 and PVS3 was replaced by unloading solution and incubated for 30 min. Subsequently, 5 shoot tips/ Petri dish were transferred to regeneration medium, and grown as previously described.

iii) Excised V-cut shoot tips (2-3mm in size) were treated in cryovials with loading solution as previously described. Loading solution was then replaced with pre-chilled optimal cryoprotectant solutions (from Table 7); these consisted of mVSL, VSL and PVS2 at 0°C for 5 and 10 min. Cryovials were then plunged into LN for 60 min. After cryopreservation, cryovials were thawed in water bath at 37°C for 80 s. Cryoprotectant solution was then replaced with unloading solution as previously described. Subsequently, 5 shoot tips/ Petri dish were transferred regeneration medium (Table 9f) and grown as previously described. Samples were taken at each stage in order to determine viability and replicated twice.

Table 9. Compositions of cryoprotective solutions used in vitrification-based experiments for shoot tips cryopreservation.

Solutions for PVS2 and PVS3 vitrification-based experiments
(a) Pre-culture medium
Liquid basal MMS medium
0.75M sucrose
(b) 20% PVS2
Liquid basal MMS medium
6% (w/v) glycerol
3% (w/v) ethylene glycol
3% (w/v) DMSO
0.08M sucrose
(c) PVS2
Liquid basal MMS medium
30% (w/v) glycerol
15% (w/v) ethylene glycol
15% (w/v) DMSO
0.4M Sucrose
(d) PVS3 only
Liquid basal MMS medium
50% (w/v) glycerol
50% (w/v) sucrose
(e) Unloading Solution
Liquid basal MMS medium
1.2M sucrose
(f) Regeneration medium
MMS medium
1 mg/L BAP
0.1 mg/L IBA
0.1 mg/L GA ₃

2. Encapsulation-vitrification of shoot tips

i) In encapsulation-based experiments, v-cut shoot tips were first encapsulated within alginate beads before they were subjected to any treatment. In the first instance, the ability and time required for shoot tips to regrow out of the beads were evaluated. The effect of 2 weeks pre-culture on regeneration medium (MMS + 1 mg/L BAP + 0.1 mg/L IBA + 0.1 mg/L GA₃) prior to encapsulation was also tested.

ii) Beads were progressively cultured on pre-culture media (Table 10a) with increasing sucrose concentration (0.25, 0.5, 0.75M sucrose) daily. Pre-cultured beads were kept in loading solution (Table 10b) with either 0.8 or 1M sucrose at room temperature for 90 min. Subsequently beads were dehydrated in PVS2 for 5 h at 0°C and 3 h at room temperature. Dehydrated beads were placed in cryovials and plunged directly into LN. After cryopreservation, beads were thawed in water bath at 37°C for 3 min followed by incubation in unloading solution for 30 min. Rehydrated beads were rapidly surface dried and plated on regeneration medium (Table 10d).

iii) Taking the results obtained in Table 14 (results section) into account, loading sucrose concentration and PVS2 exposure time was lowered in the next experiment using cold-hardened plantlets. Shoot tips excised from plantlets hardened for 3 weeks at 4°C were encapsulated in alginate beads as previously described. Beads were then pretreated with a stepwise procedure as previously described. Beads were then treated with a loading solution of 2M glycerol + 0.4M sucrose for 60 min, exposed to PVS2 at 0°C for 30 – 150 min. Beads were not immersed into LN. All other steps were the same as previously described in section ii) of encapsulation-vitrification section.

Table 10. Compositions of pre-culture and encapsulation media used in for shoot tip cryopreservation.

Media for encapsulation and pre-culture of shoot tips
(a) Pre-culture medium Liquid basal MMS medium 0.25, 0.5, 0.75M sucrose
(b) Loading Solution Liquid basal MMS medium 2M glycerol 0.8 or 1M sucrose
(c) Calcium solution Calcium-free liquid basal MMSE medium 0.1M calcium chloride
(d) Regeneration medium MMS medium 1 mg/L BAP 0.1 mg/L IBA 0.1 mg/L GA ₃

3. Encapsulation-dehydration of shoot tips

i) V-cut shoot tips dissected from *in vitro* avocado plantlets were encapsulated in beads as previously described. Beads were pre-cultured overnight in 0.75M sucrose solution (Table 11a). Subsequently, beads were air-dried in the laminar flow hood at room temperature for 120 - 210 min after plating. Dehydrated beads were transferred into cryovials and immersed directly into LN. For rewarming, frozen cryovials were allowed to sit at room temperature for 10 min before the beads were rehydrated in liquid MMS + 1.2M sucrose for 30 min. Rehydrated beads were plated onto solid regeneration medium (Table 11b) as previously described.

Table 11. Compositions of pre-culture and encapsulation media used in for shoot tip cryopreservation.

Media for encapsulation and pre-culture of shoot tips
(a) Pre-culture medium
Liquid basal MMS medium
0.75M sucrose
(b) Regeneration medium
MMS medium
1 mg/L BAP
0.1 mg/L IBA
0.1 mg/L GA ₃

ii) V-cut shoot tips dissected from *in vitro* avocado plantlets were encapsulated in beads as previously described. The MMS medium had the following modification:

- sucrose being substituted for fructose at a lower level of 0.4M instead of 0.75M. Beads were pre-cultured on this medium for 2 weeks (Table 11a).

After pre-treatment beads were then air-dried in the laminar flow hood for 120 or 180 min at room temperature. Beads were rehydrated in liquid MMS + 1.2M sucrose for 30 min and plated onto solid regeneration medium (Table 11b). There were 5 shoot tips per Petri dish plated on regeneration media.

Assessment of Regrowth

The effect of each treatment was assessed by the regrowth of shoot tips, expressed as percentages. Regrowth is defined as shoot tips that remained green and sprouted new shoots. Callus formation was also noted. Each treatment was performed with a minimum of 5 shoot tips per treatment.

Results

Shoot multiplication to generate materials for cryopreservation

The establishment of *in vitro* 'Reed' plantlets from mature seeds took 2 months. These plantlets were multiplied by micropropagation as outlined in figure 36. Production of shoots from dissected nodal sections took another 2 months. Shoot tips used for cryopreservation in this study were sourced from these plantlets (Fig 38).



Figure 38. Shoots developed from axillary buds of stem cuttings.

Explant size, structure and plant regeneration media

In the current study, explants with size ranging from 1 – 3 mm were excised to determine the minimum size required for survival. No regeneration was observed with shoot tips of < 1 mm in size. Explants size of > 2 mm displayed higher regeneration than smaller ones and explants larger than 3 mm were found to be too large for encapsulation. Therefore, shoot tips with size of 2 – 3 mm were used as explants for cryopreservation experiments (Fig 39).



Figure 39. V-cut axillary bud of 2.5 mm in size was optimal for cryopreservation.

In a preliminary study, shoot tips showed browning and callus formation after excision and only 21% regrew after 4 weeks of culture on hormone-free MMS media (data not shown). To address these problems, shoot tips (2 – 3 mm in size) excised in two forms (v-cut wedge and entire nodal section) were grown on culture media supplemented with various anti-browning agents and hormones. Their effect of percentage regrowth and callus formation was compared in figure 40 and 42.

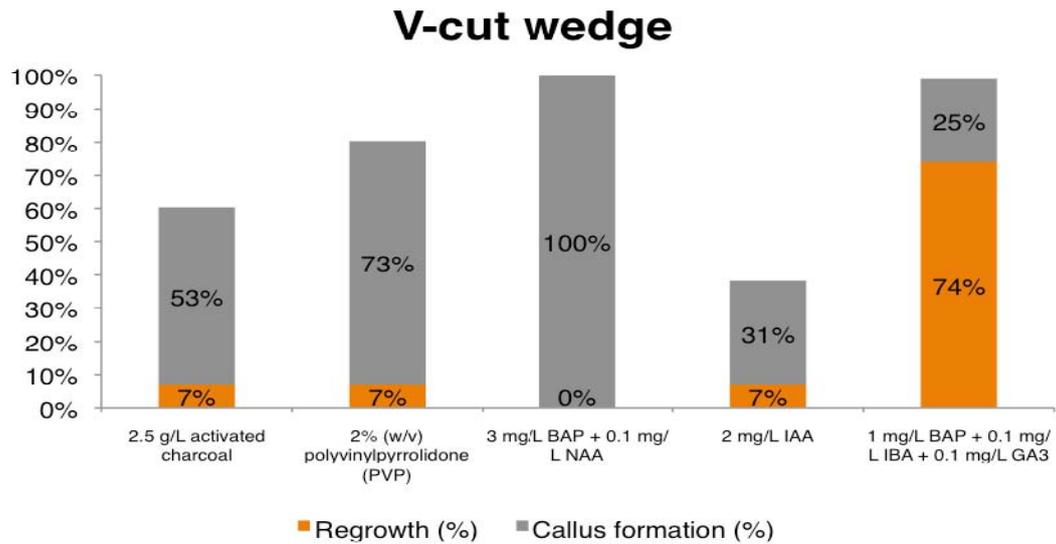


Figure 40. Percentage regrowth and callus formation of shoot tips as a v-cut wedge (2 – 3 mm) on culture media supplemented with various additives (n = 10 – 15). Data was recorded 2 weeks after treatment.

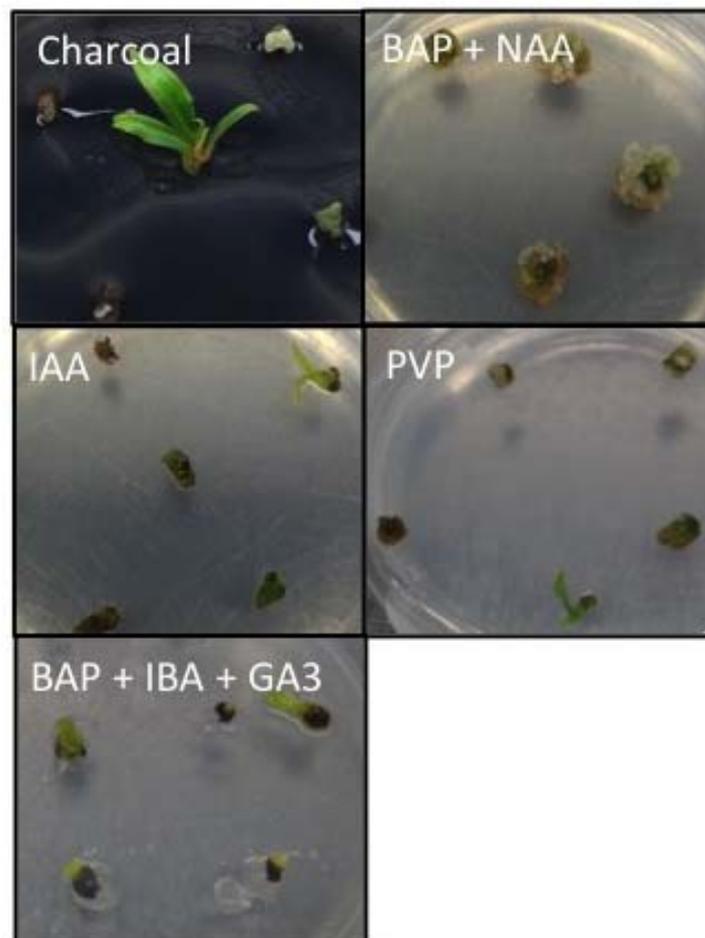


Figure 41. Growth of v-cut wedges (2 – 3 mm) containing shoot tips on media supplemented with various additives.

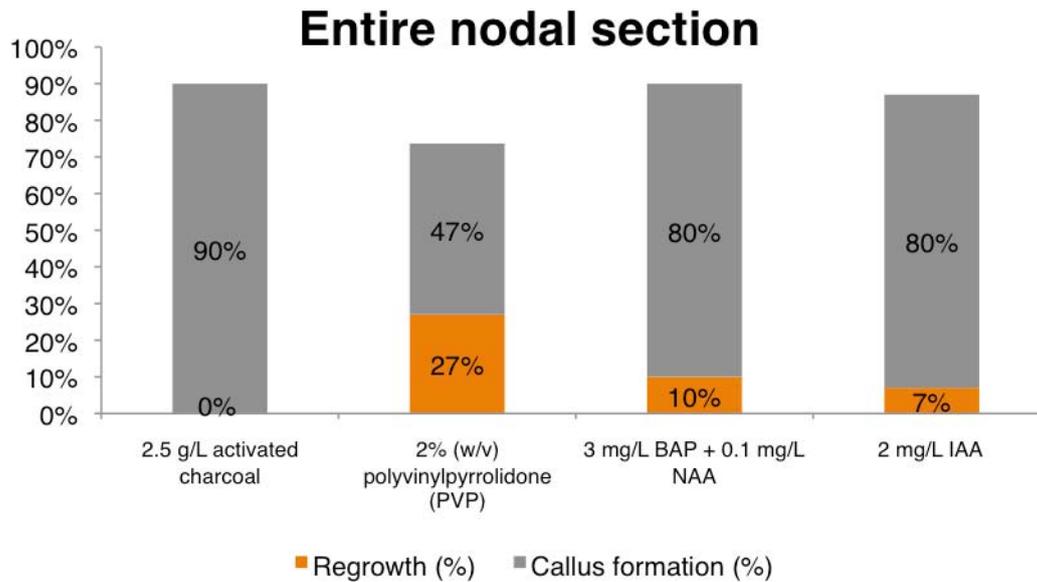


Figure 42. Percentage regrowth and callus formation of shoot tips as an entire nodal section (2 – 3 mm) on culture media supplemented with various additives (n = 10 – 15). Data was recorded 2 weeks after treatment.

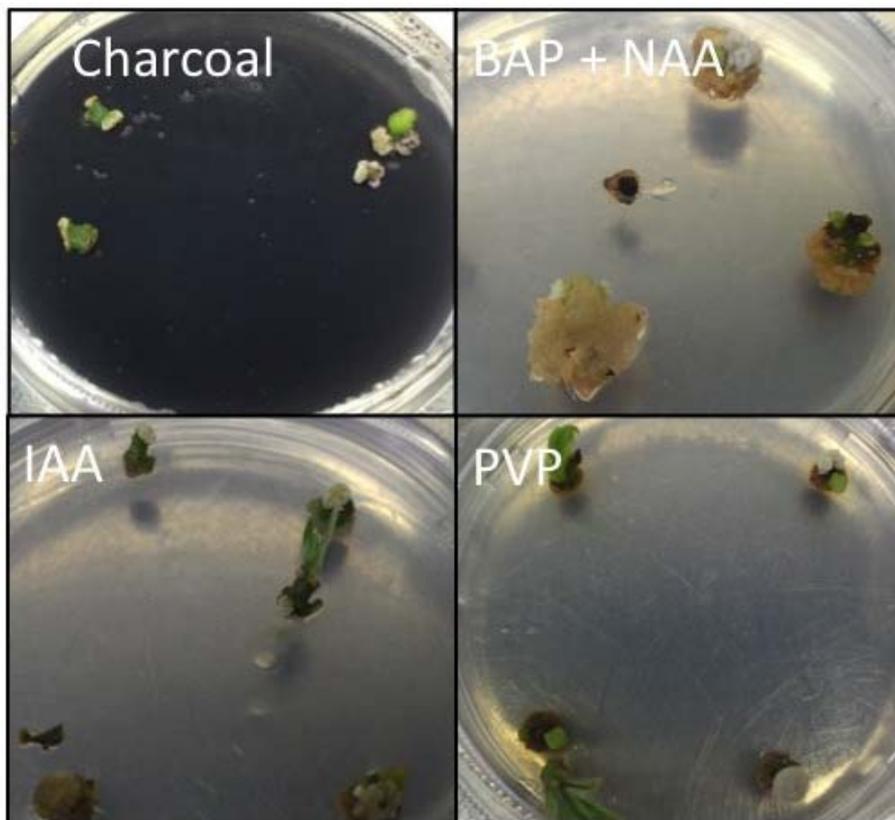


Figure 43. Growth of nodal sections (2 – 3 mm) containing shoot tips on media supplemented with various additives.

High percentage of browning and callus formation was still observed in various treatments (Fig 41 and 43). Based on Figure 40 and 41, v-cut wedges cultured on MMS supplemented with 1mg/L BA + 0.1mg/L NAA + 0.1mg/L GA₃ was found to be the best for regeneration with the highest percentage of shoot regrowth (74%) and least callus formation (25%). Media supplemented with the other additives had no positive effect on the recovery (7 – 27%) or reduction of tissue browning for both explant structures (Fig 40 and 42). Also, extensive callus formation (31 – 100%) was observed in these treatments.

For the same treatments, explants in the form of an entire nodal section recorded up to 27% regrowth. Although the length of the nodal section was kept within 2 – 3 mm, the diameter of the stem varied in different plantlets. As a consequence, some of the nodal sections may be too large for encapsulation.

For regeneration V-cut shoot tips of ‘Reed’ which were 2-3mm in size were used for all further experimentation in combination with MMS medium supplemented with 1mg/L BA + 0.1mg/L NAA + 0.1mg/L GA₃.

Optimization of cryoprotectant

When ‘Reed’ shoot tips were exposed to VSL, mVSL or PVS2 at 0°C, the percentage of viable shoot tips varied from 0-30% (Fig 44). The highest percentage was recorded at 10 mins for all three treatments. When treated for longer time periods there was a drastic decline in shoot tip viability. While the untreated control recorded 80% viability of shoot tips.

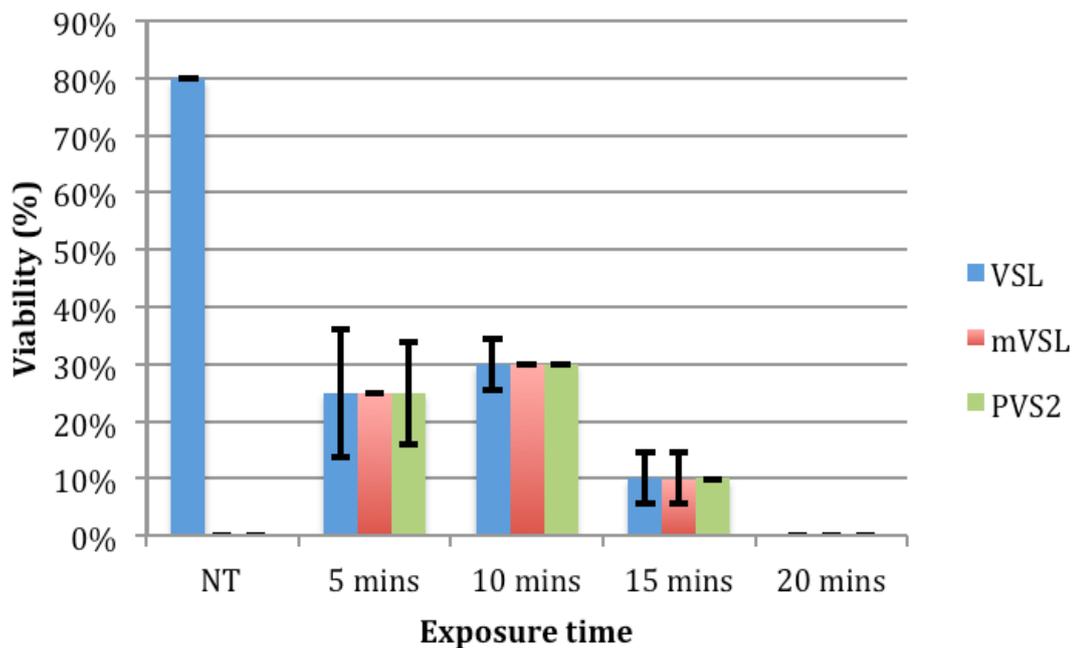


Figure 44. Percentage regrowth of shoot tips exposed to various cryoprotectant treatments (VSL, mVSL and PVS2) and times (0, 5, 10, 15 and 20 min). Shoot tips were plated on MMS medium (n = 10). Data was recorded 4 weeks after treatment.

From the optimal cryoprotectant experiment VSL, mVSL and PVS2 at 0°C for 5 and 10 min were selected to trial with LN.

1. Cryovial-vitrification of shoot tips

The first cryovial-vitrification was conducted before results of the size and structure optimisation were obtained. None of the treated explants survived freezing in LN (Table 11). Only 10% explants of 10 mm in length survived the toxicity of the loading and PVS2 treatment (Fig 45b). All dissected controls (dissected shoot tips without any treatment or LN exposure) survived on regeneration medium (Fig 45a).

Table 11. Percentage regrowth of nodal sections following cryovial-vitrification and LN exposure. Data was recorded 4 weeks after treatment.

Length of nodal sections	Treatment	Regrowth (%)	
		- LN	+ LN
10 mm (10 explants/ treatment)	Dissection control	100%	-
	LS + PVS2 60 min	10%	0%
5 mm (5 explants/ treatment)	Dissection control	100%	-
	LS + PVS2 60 min	0%	0%

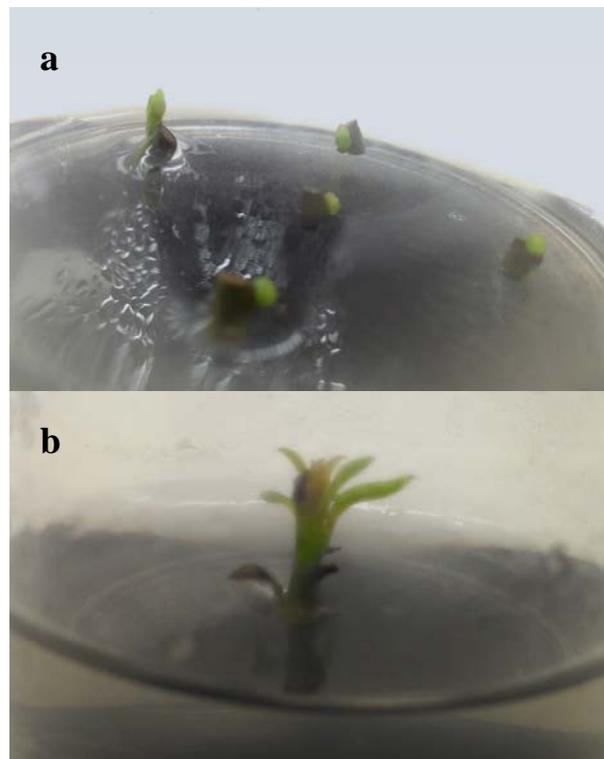


Figure 45. (a) Untreated nodal sections (10 mm in length) exhibit shoot growth 2 weeks after treatment. (b) Nodal sections (10 mm in length) that exhibit regrowth 4 weeks after treatment without LN immersion.

The use of 20% PVS2 + 100% PVS2 or PVS3 instead of PVS2 was reported to be successful in cryopreservation of shoot tips of other crops. In our study avocado shoot tips all displayed 0% viability after these treatments (Table 12).

Table 12. Percentage regrowth of v-cut shoot tips (2 – 3 mm) following cryovial-vitrification protocol modified from Kaity et al. (2008) (n = 10). Data was recorded 1 week after treatment.

Treatment	Regrowth (%)
Overnight pre-culture (0.75M sucrose)	0%
Overnight pre-culture + 20% PVS2 60 min + 100% PVS2 20 min + LN	0%
Overnight pre-culture + 20% PVS2 60 min + 100% PVS2 40 min + LN	0%
Overnight pre-culture + 100% PVS3 60 min + LN	0%
Overnight pre-culture + 100% PVS3 150 min + LN	0%

Overnight pre-culture in 0.75M sucrose appeared to be detrimental as the pre-culture alone resulted in 0% regrowth. Further experiments were designed either without a pre-culture step or with an alternative pre-culture treatment.

The use of mVSL, VSL and PVS2 with optimal times of 5 and 10 min +LN also resulted in 0% viability (data not shown).

2. Encapsulation-vitrification of shoot tips

In encapsulation-based experiments, v-cut shoot tips (2-3mm in size) which were first encapsulated in alginate beads with no treatment showed 75% regrowth (Table 13). Encapsulation in Na-alginate beads did not compromise the regrowth of shoot tips, although it delayed the sprouting to 2 weeks. Almost all (90%) of shoot tips sprouted out of the beads (Fig 46) with explants pre-cultured for 2 weeks prior to encapsulation.

Table 13. Evaluation of v-cut shoot tips (2 – 3 mm) regrowth of freshly dissected and 2-week pre-cultured shoot tips after encapsulation (n = 20). Data was recorded 6 weeks after treatment.

	Regrowth (%)
Dissection control	74%
Freshly dissected + encapsulation	75%
2-week pre-culture + encapsulation	90%



Figure 46. Sprout of green shoots from encapsulated v-cut shoot tips after 8 weeks.

Encapsulated shoot tips that were pre-cultured via the stepwise procedure, treated in loading solution of either 0.8 or 1M of sucrose and dehydrated in PVS2 minus LN showed 0% viability (Table 14). Loss of viability was also observed in the step-wise pre-culture control with only 27% of shoot tips remaining viable, in comparison to 67% viability with the untreated control (Table 14).

Table 14. Percentage regrowth of v-cut shoot tips (2 – 3 mm) following best encapsulation-vitrification treatments identified in Wang et al. (2005a). Data was recorded 10 weeks after treatment.

Stepwise Pre-culture	Loading Treatment 2 M glycerol + sucrose sucrose, 90 min	PVS2 Treatment	- LN	+ LN
Untreated control			67%	-
0.25M sucrose, 1 day, 0.5M sucrose, 1 day, 0.75M sucrose, 1 day.	-	-	27%	-
	0.8M sucrose	5 h, 0°C	0%	0%
	0.8M sucrose	3 h, RT	0%	0%
	1M sucrose	5 h, 0°C	0%	0%
	1M sucrose	3 h, RT	0%	0%

The survival of shoot tips was not markedly affected by the cold treatment with 60% of regrowth observed. The viability of the explants was halved (30%) following a stepwise pre-culture, (Fig 47). Loading in 2M glycerol + 0.4M sucrose further reduced the viability to 10%. Shoot tips dehydrated for 30 and 120 min had 30% and 10% regrowth, respectively. No regrowth was observed with other PVS2 exposure durations.

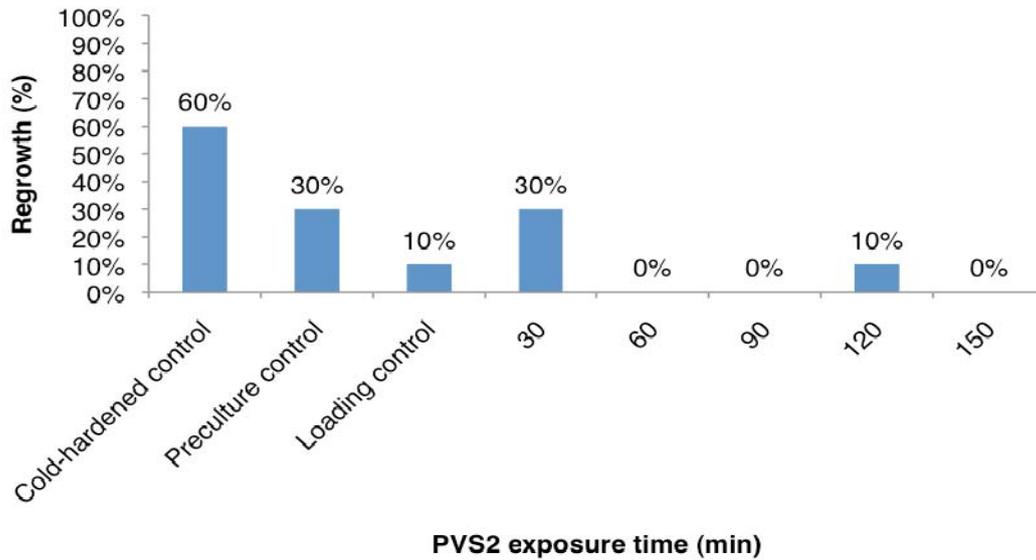


Figure 47. Percentage regrowth of v-cut shoot tips (2 – 3 mm) following encapsulation-vitrification treatments modified from Wang et al. (2005a). Shoot tips excised from plantlets hardened for 3 weeks at 4°C were pre-cultured progressively with daily increase in sucrose concentration, loaded with 2M glycerol + 0.4M sucrose for 60 min, exposed to PVS2 at 0°C for 30 – 150 min. Data was recorded 4 weeks after treatment.

3. Encapsulation-dehydration of shoot tips

In the encapsulation-dehydration protocol, encapsulated shoot tips were air-dried in laminar flow for 120 – 210 min (Fig 48).

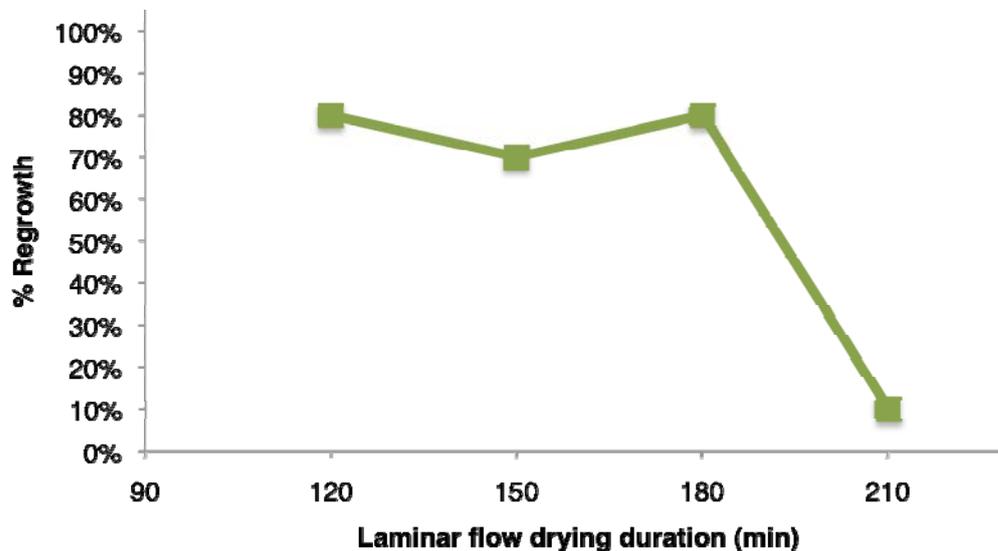


Figure 48. Effect of laminar flow drying duration on the regrowth of non-cryopreserved v-cut shoot tips (n = 10). Encapsulated shoot tips were air-dried using laminar flow for various durations, but not frozen. Data was recorded 3 weeks after treatment.

Beads desiccated for up to 180 min exhibited 80% regrowth and declined sharply with further desiccation. However, no viability after LN exposure was achieved (data not shown).

Beads which were pre-cultured on fructose media for 2 weeks and dehydrated for 120 min showed no viability. However beads which were dehydrated for 180 min with the same pre-culture treatment showed 40% viability (data not shown).

Discussion for cryopreservation of shoot tips

Natural forests of some countries still shelter diverse wild avocado germplasm that confer genes of potential significance. Due to the heterozygosity of avocado seeds, these accessions have to be maintained by clonal propagation, which is time-consuming, and labour intensive. Cryopreservation of shoot tips, on the other hand, is an ideal way to conserve this elite germplasm. Shoot tips produce true-to-type plantlets that have the identical genetic makeup to the donor plant.

Here, different techniques were explored for the cryopreservation of shoot tips. They are cryovial vitrification, encapsulation-vitrification and encapsulation-dehydration. Different factors governing the regeneration of shoot tips were also investigated prior to cryopreservation experiments.

In the current study, limitation of plant material was a major constraint. Generation of plantlets from seeds or micropropagated nodal sections took two months each. For the same reason, shoot tips were cryopreserved in this study instead of apices that were used in most studies. There are approximately 5 shoot tips per shoot as opposed to one apical bud. Successful cryopreservation of shoot tips has been reported in other species such as *Vitis vinifera* (Zhao et al., 2001), *Malus domestica* (Condello et al., 2011) and *Asparagus officinalis* (Uragami et al., 1990). Contamination was another limitation encountered in this study and occurred mainly when the explants were dissected under the binocular microscope. This can be minimised by the culture of explants on solid media for 3 days so any contamination to be noted. Only contamination free explants would be used for the subsequent procedures. A similar procedure has been done in Wang et al. (2005a).

Optimisation of regeneration conditions

Apart from the cryopreservation techniques, optimisation of the regeneration conditions of excised shoot tips is essential. In this study, the optimal explant size, structure and regeneration medium compositions have been established for cultivar 'Reed'.

Explant size

Shoot tip size influences its survival after cryopreservation and 1 – 5 mm is the range of size commonly used (Benson et al., 2007). Baek et al. (2003) clearly demonstrated the importance of explant size for cryopreservation of *Allium sativum* shoot tips and smaller apices (1.5 mm or 3.0 mm in size) displayed higher regeneration than larger ones (4.5 mm).

In this study, the explant size that gave maximum regrowth without cryopreservation ranged from 2 – 3 mm. The same shoot tip size had been used for cryopreservation of *Allium sativum* (1.5 or 3 mm) (Baek et al., 2003), *Solanum tuberosum* (Halmagyi et al., 2005) and *Capparis spinosa* shoot tips (Shatnawi, 2011).

Regeneration media

Tissue browning is commonly detected in woody plant species. It may be caused by stress of excision or cold, leading to loss of cellular compartmentalisation, leaking of phenolics and formation of insoluble brown complexes upon oxidation (Chalker-Scott et al., 1989). A callus phase prior to shoot formation, as notably observed in excised shoot tips, is undesirable because callusing lengthen the time period for regeneration and potentially increases the possibility of the occurrence of somaclonal variability (Sakai and Engelmann, 2007).

In the present study, the best regeneration media for avocado shoot tips was MMS media amended with 1 mg/L BAP + 0.1 mg/L IBA + 0.1 mg/L GA₃. The same plant growth regulators combinations were employed in the regeneration media for cryopreserved *Rubus fruticosus* and *Prunus cerasifera* apices (Vujović et al., 2011). In study with *Lavandula dentate*, shoot elongation occurred only in the presence of IBA and BAP (Marília Pereira et al., 2011). GA₃ breaks the dormancy of buds and induces the *in vitro* growth of the meristems or buds.

Sucrose pre-culture of shoot tips

In addition to reducing cellular water prior to cryopreservation, sucrose increases the stability of membranes during desiccation and cooling stresses (Crowe JH, 1989). The freezing tolerance of *Malus* (Paul et al., 2000) and *Vitis* (Wang et al., 2000) was significantly improved with direct pre-culture with sucrose concentrations of 0.75 – 1.0M. However, plant species such as *Eucalyptus gunni* and *Poncirus trifoliata* (Gonzalez-Arno et al. 1998) are intolerant to the high sucrose level and required stepwise pre-culture with increasing sucrose concentration.

In this study, avocado shoot tips were highly sensitive to the high level of sucrose. Both a direct pre-culture in 0.75M sucrose and stepwise pre-culture to 0.75M resulted in substantial loss of viability even before cryopreservation. This appears to be major constraint in most of experiments conducted here. Similar observations were recorded in the case of sweet potato where sucrose level > 0.5M proved to be toxic (Sharma, 2005). Pre-culture with lower concentrations of sucrose (e.g. 0.5M) or a different sugar osmotica such as D-mannoheptulose (avocado extract) (personal communication) or sorbitol (Yamada et al., 1991) may overcome this.

Pre-culture of parent plant on ABA enriched medium may also solve the low desiccation tolerance problem as Suzuki et al. (2006) has shown that ABA enhanced desiccation and freezing tolerance of *Gentiana scabra* shoot tips.

Cold-acclimation of avocado plantlets

Cold acclimation adapted for *in vitro* plantlets was designed based on the appraisal of plant behaviour under natural freezing conditions. In response to low temperature, physical and chemical changes take places within plant cells to improve desiccation and freezing tolerance, such as recruitment of heat-shock proteins and up regulation of “cold regulate” genes (Levitt, 1972). Niino and Sakai (1992) demonstrated that

cold acclimation is essential for survival of *Malus* shoot tips following cryopreservation.

In this study, avocado plants were cold hardened at 4°C for 3 weeks. Although its effect on the survival after LN is unknown, however the cold treatment alone did not caused any loss of viability. Tropical and warm temperature species are more sensitive to cold temperature (Sakai and Engelmann, 2007); this is not the case for avocado, despite being a subtropical fruit crop. Up to 3 weeks of cold acclimation are known to improve shoot regrowth of cryopreserved *Rubus parvifolius* from 25 to 75% (Chang and Reed, 1999).

1. Cryovial vitrification of shoot tips

In the first cryovial-vitrification experiment conducted with 5 and 10 mm nodal sections, loading and vitrification solution appeared to be highly toxic to the plant materials. Additional steps to protect the explants are required.

In the second cryovial-vitrification experiment, overnight pre-culture in 0.75M sucrose is detrimental. Owing to that, the effect of subsequent loading and PVS treatments cannot be determined as all the treatments tested involved the pre-culture step. An alternative pre-culture step is needed.

2. Encapsulation-vitrification of shoot tips

For encapsulation-based protocol, the regrowth of encapsulated shoot tips were assessed as the first step. The alginate beads protect the shoot tips against osmotic shock when exposed to high sucrose concentration (Plessis et al., 1992). Regrowth of shoot tips was not affected by the encapsulation. Pre-culture on regeneration medium for 2 weeks prior to encapsulation was found to be beneficial with 90% regrowth, as compared to freshly dissected explants with (75%) or without encapsulation (74%). The two-week pre-culture may have allowed the explants to recover from the injury caused during excision, and hormones in the media may have initiated shooting of shoot tips.

In the encapsulation-vitrification experiment, stepwise pre-culture was used and appeared to be less harmful compared to direct pre-culture, reducing the percentage regrowth by half as opposed to 0% regrowth in direct pre-culture. The incremental sucrose level reduces the osmotic shock of direct exposure. Also, encapsulation could have offered some protection.

We have optimised shoot tip regrowth form excised 2-3 mm shoot tips; however, further investigation is required to develop a protocol for shoot tip cryopreservation.

Technology Transfer

- Communication to Talking avocado.
- Industry annual reports.
- Macadamia Industry Board meeting, Ecosciences precinct, Brisbane, Australia, 2012.
- Gene Technology Workshop, Horticulture Australia Ltd, across Industry Funded Initiative, Canberra, Australia, 2012.
- Team Avocado Conference, New Zealand, 2012.
- QAAFI Annual Research Meeting, Gold Coast, Australia, 2012.
- Presentation on crop protection research and innovation to the Minister of Agriculture, Queensland Government, Queensland, Australia, 2012.
- Presentation at Annual Industry conference, 2013 and 2014.
- Presentation at Australia New Zealand Avocado Conference 2013.
- Invitation accepted to present at Avocado growers Conference New Zealand, July 2014.
- Oral presentation to be made at IHC conference Brisbane, August 2014.
- Discussion initiated with Nut Industry and Potato Industry for application of cryopreservation technology.
- Communication in progress with international partners in California, New Zealand and Ghana.

Summary and conclusions:

We report for the first time the successful application of the cryovial and droplet-vitrification methods of cryopreservation to avocado SE cultivars 'A10', 'Reed', 'Velvick' and 'Duke 7'. Both vitrification-based methods could be proposed as feasible cryopreservation protocols for avocado SE, applicable to multiple avocado cultivars, with each treatment showing greater than 60% viability after short term as well as long term storage. This work contributes towards the establishment of a standard cryopreservation protocol for avocado species. It brings the avocado research community closer to establishing a Cryo-Bank of Avocado Germplasm (CBAG) for national and international collaborations.

Outputs:

- Cryo- repository of avocado germplasm in Australia, a reality.
- Australian Avocado Industry as leaders for World's First Cryo-Bank for Avocado Germplasm, a global initiative.
- Proof of concept generated to make way for International collaboration.
- Cost effective long term conservation of commercial and wild avocado cultivars with desirable traits.
- Facilitation of world wide exchange of germplasm.
- Saving on land, labour and other resources required to maintain a field germplasm bank.
- Possibility of a 'Fee for service' arrangement with international collaborators.
- Provision of disease free plants for exchange.
- Conservation of true 'Clonal plants' if shoot tips cryopreservation is successful.

- Somatic embryos of various wild and commercial cultivars available for genetic improvement.
- Capacity to store large number of accessions at low cost of maintenance (space of just 10m² to store 1000 accessions).

Outcomes:

- Methodology established for cryopreservation of somatic embryos of avocado cultivars.
- Methodology established for cryopreservation of shoot tips of avocado cultivars (requires further work).

Recommendations

The field collections are constantly exposed to abiotic and biotic stress. Moreover, the size of gene pool, number of replications and quality of maintenance are also largely restrained by the local environmental conditions, space and funding. Consequently, many countries including Australia lack the capacity to maintain their collections. Coupled with the advancement of tissue culture techniques and marker-assisted technology, cryopreservation offers an ideal option for safe, cost-effective and long-term conservation of genetic resources. Most importantly, it offers growers a source of disease-free genetic material that can be supplied indefinitely for the establishment of orchards. Thus, the technologies developed will facilitate the establishment of Cryo-Bank for horticultural crops in Australia to conserve the germplasm/accessions available in the country. It may just require a 10m² space to store 1000 or more accessions.

Our specific recommendations include:

- Shoot tip cryopreservation work should be progressed through a HAL funded PhD scholarship.
- SE of valuable avocado germplasm in Australia should be cryopreserved (this may require funding for one 50% position).
- Discussions should be initiated to fund the cryopreservation of nuts, potato and avocado as a cross industry platform thus saving the cost to each industry
- Discussion to be initiated with international partners to facilitate further work on avocado germplasm conservation.
- Avocado SE stored in the current project should be tested for viability after 18 months and 2 years of storage.
- The outcomes and benefits of the project should be communicated to Queensland Government (Agri-Science Queensland to discuss about the possibility of setting up a Cryobank for horticultural crops in Queensland.

This will be discussed with Queensland Government and horticultural community to get the required financial support. The cryo-bank will not only serve as a repository for conservation of genetic diversity it will provide the advantage of SE being available for genetic improvement, disease free plants, easy exchange and transport and protection for pests and diseases and natural calamities.

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