

## ■ Somatic Embryo Cryopreservation of multiple avocado (*Persea Americana* Mill.) Cultivars using two vitrification based techniques

C. O'Brien<sup>1</sup>, M. Constantin<sup>2</sup>, A.Walia<sup>2</sup>, N. Mitter<sup>2</sup>.

<sup>1</sup>. Agri-Science Queensland, Department of Agriculture, Fisheries and Forestry, Brisbane, Australia

<sup>2</sup>. Queensland Alliance for Agriculture and Food Innovation, The University of QLD St Lucia, Australia

Avocado genetic resources are currently maintained in the form of field repositories at great cost and risk of natural disasters, pest and diseases. Cryopreservation offers a necessary, complimentary method that is safe, cost-effective and long-term. Long-term maintenance and regeneration of plantlets from avocado somatic embryos has been a major barrier in the development of new avocado varieties due to low germination rates and loss of embryogenic competence.

Somatic embryo viability to different sucrose concentrations in loading solutions, the effect of Plant Vitrification Solution-2 (PVS2) incubation times and temperatures were optimised. Two protocols for vitrification-based cryopreservation (cryo-vial and droplet) of avocado somatic embryos were investigated. Globular somatic embryos of three avocado cultivars ('A10', 'Reed' and 'Velvick') were tested, and the ability to withstand cryopreservation is attributed to cultivar-dependent differences in desiccation tolerance and subsequent freezing resistance, possibly due to their size and culture age. Somatic embryos were stored in liquid nitrogen for 3 and 12 months. Somatic embryos were successfully recovered post liquid nitrogen using a rapid thawing system at 37°C. Higher frequencies of regrowth of somatic embryos ranging from 75 -100% were obtained on MMSE after 5-10 weeks. Mature somatic embryos selected from MMSE plates were regenerated using a two-step regeneration system (Encina *et al* 2014). The system developed for storing and regenerating avocado somatic embryos for multiple cultivars will add considerable value towards cryopreservation of avocado germplasm as well as the generation of new and improved avocado varieties.

Keywords: Droplet-vitrification, Loading solution, PVS2, Germplasm.

### INTRODUCTION

Avocado genetic resources across the globe are currently being maintained *ex situ* in field repositories. This allows researchers to physically evaluate and characterise the accessions for various phenotypic and genotypic parameters of importance to growers (Barrientos-Priego *et al.*, 1992; Benz, 2012). Countries including the USA, Mexico, Israel and New Zealand have dedicated land-leased repositories to maintain avocado germplasm (Lopez *et al.*, 1994; Ben Ya'acov *et al.*, 2003; Ayala-Silva *et al.*, 2004). However, these repositories are exposed to natural disasters and always at the risk of abiotic and biotic stresses (Barrientos-Priego *et al.*, 1992). The recent threat posed by Laurel Wilt to the avocado germplasm in the USA is a glaring example (FDACS, 2012). Wild avocado germplasm resources are also under threat due to deforestation; and according to some predictions we have already lost 40% of the forest cover in developing countries (Barrientos-Priego, 1999; Panis and Lambardi, 2005; Ayala-Silva *et al.* 2004). Even under the best of circumstances, field repositories require considerable inputs in the form of land, labour and management. Consequently, many countries including Australia lack the capacity to maintain their own collections. This exemplifies the urgent need for a long-term germplasm conservation method that is not exposed to the vagaries of climate change, disease or pest incursions (Barrientos-Priego, 1999; Ben-Ya'Acrov and Barrientos, 2003).

Cryopreservation, that is storage at ultra low temperature (liquid nitrogen, -196°C), offers a more cost-effective and environmentally friendly alternative to field collections; greatly reducing the space required to store thousands of accessions and providing long-term storage of clean, disease-free material. To date, cryopreservation has been applied successfully to almost 40 different species of crop, fruit and forest trees (coffee, apple, dates, citrus etc.) by the use of somatic embryos (SE) (Engelmann, 2011). However, only few studies have focused on the cryopreservation of avocado SE due to the loss of morphogenic competence of SE in as little as 3-4 months after induction, depending on cultivar (Witjaksono and Litz, 1999) and their incomplete maturation i.e. SE are unable to display bipolar regeneration (Ammirato, 1987). Work from our laboratory has described an *in vitro* regeneration protocol for SE of avocado using a two-step system supplemented with glutamine. For cultivar 'Reed', the two-step culture system resulted in the total regeneration of 58.5% and plant/shoot recovery of 36.7%; significantly higher than any other treatment investigated to date (Encina *et al.* 2014).

Efendi (2003) described the effect of cryogenic storage on five avocado cultivars ('Booth 7', 'Hass', 'Suardia', 'Fuerte', and 'T362') using two protocols, controlled-rate freezing and classical vitrification. For controlled-rate freezing, three out of five embryogenic cultivars were successfully cryopreserved, with a recovery of 53 - 80 %. Using classical vitrification, cultivar 'Suardia' showed 62 % recovery whereas 'Fuerte' had only a 5 % recovery. The 'droplet-vitrification' method is a new and highly valuable procedure with promising results, more than 300 and 540 accessions of potato and Musa, respectively, are successfully cryopreserved (Sakai and Engelmann, 2007; Kim *et al.* 2009). Guzmán-García *et al.*, (2012) had promising results using droplet-vitrification on two 'Duke-7' embryogenic cell lines, with 77.78 - 100 % recovery.

In this paper we demonstrate for the first time two cryopreservation protocols (cryovial and droplet vitrification) for the conservation of avocado SE that can be applied to multiple cultivars through the optimization of loading sucrose concentrations and PVS2 temperature and times. Furthermore, cryopreservation of SE will aid in the usage of avocado SE as research material for genetic improvement.

## MATERIALS AND METHODS

### Plant materials

Avocado (*Persea americana*) cultivars 'A10', 'Reed' and 'Velvick' were used in this study. 'A10' is a hybrid cross between Mexican and Guatemalan trees. 'Reed' was introduced in 1960 from a chance seedling that was planted in 1948. It belongs to the Guatemalan race which evolved in upland mountain cloud forests. 'Velvick' belongs to the Mexican race which is both 'tropical highland' and borderline 'cool subtropical' in adaptation.

### Induction of embryogenic cultures

Embryogenic cultures were induced from immature zygotic embryos. Immature fruits of avocado (5 – 15 mm in size) were surface-sterilised in 2 % sodium hypochlorite solution supplemented with 500 µL/L of Tween® 20 (Sigma-Aldrich, Missouri, USA) for 20 min. The sodium hypochlorite solution was then drained and fruits were rinsed three times with sterile deionised water in a laminar flow cabinet. These fruits were bisected longitudinally to extract the zygotic embryos. Zygotic embryos were 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.

### Maintenance of embryogenic culture

SE generated were transferred to Petri dishes (90 x 15 mm) containing 30 – 40 mL of mango somatic embryo medium (MMSE) which consisted of Gamborg's B5 major salts (Gamborg *et al.*, 1968), MS minor salts, MS vitamins and MS Fe-EDTA (Murashige and Skoog, 1962), 0.4 g/L glutamine, 0.5 mg/L 2, 4-dichlorophenoxyacetic acid, 100 mL/L coconut water, 60 g/L sucrose and 2.5 g/L gellan gum (Gelrite® Merck) pH 5.7 (Pateña *et al.*, 2002). Plates were sealed with Parafilm® and the cultures were maintained as previously described and subcultured every 4 – 5 weeks.

### Culture conditions

Approximately 100 mg of globular SE that were actively multiplying after 3–4 weeks of subculture were used in all cryopreservation experiments. The pH of all culture media and cryoprotective solutions was adjusted to 5.7 prior to autoclaving at 121 °C for 15 min. The only exception was in the case of plant vitrification solution 2 (PVS2) which was filter-sterilised using a 0.22 µm filter. SE were submerged in liquid nitrogen (LN) for 60 min for all experiments.

### Pre-treatment of SE

Before cryopreservation techniques could be compared, the effect of sucrose pre-treatment on SE was trialled with 'A10', 'Reed' and 'Velvick'. SE were incubated at 25 ± 1 °C for 0, 10, 20, 40, 60 or 90 min in various loading solutions (LS) containing 2 M glycerol and sucrose concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1 or 1.2 M. SE were then transferred to MMSE medium in clumps of 5 and grown as previously described and replicated twice.

### Cryovial-vitrification of SE using PVS2

- i) The first series of cryovial-vitrification experiments were based on the procedures developed by Guzmán-García *et al.* (2012). SE of each cultivar were incubated at room temperature for 20 min in LS containing 2 M glycerol + 0.4 M sucrose. LS was then replaced by PVS2 at 0 ± 1 °C and incubated for either 30 or 60 min. Before the completion of PVS2 dehydrated SE were transferred into 2 mL cryovials (100 mg SE per cryovial) containing 1 mL of fresh ice-cold PVS2 and after incubation were plunged directly in LN. After cryopreservation, cryovials were thawed in a water bath at 37 ± 1 °C for 80 sec. PVS2 was replaced by unloading solution (MMSE liquid + 1.2 M sucrose) and incubated for 30 min. Subsequently, SE were transferred to MMSE medium in clumps of 5 per plate and placed in the dark as previously described. Samples were taken at each stage i.e. 1) untreated SE, 2) LS + PVS2 – LN and 3) LS + PVS2 + LN in order to determine viability and replicated three times.
- ii) A second series of experiments was conducted as above and replicated three times using the cryovial-vitrification method but with LS containing 0.6 M sucrose for 'Reed'; 0.2 and 0.8 M sucrose for 'A10' and 'Velvick' respectively.

### Droplet-vitrification of SE using PVS2

Droplet-vitrification was also based on the protocol by Guzmán-García *et al.* (2012). SE were first incubated in LS containing 2 M glycerol + 0.2 M sucrose at room temperature for 20 min. After removal of LS, dehydrated SE in PVS2 at 0 ± 1 °C for 60 min. 10 min before the completion of PVS2 incubation, dehydrated SE were placed individually into a 15 µL 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 and the aluminium strip and SE were placed into a 2 mL cryovial before being plunged directly into LN. For thawing, 1 mL of 37 ± 1 °C unloading solution (MMSE liquid + 1.2 M sucrose) was added to the cryovial before the strip and SE were poured into Petri dishes containing 15 mL of the same unloading solution. SE were left in unloading solution for 30 min before being plated in the same manner as described for the cryovial-vitrification experiment. Samples were taken at each stage in order to determine viability. All experiments were plated on MMSE media in clumps of 5 and grown in the dark as previously described and replicated three times.

### Assessment of viability

Cryopreserved SE were plated onto MMSE medium to encourage new growth. For cryovial and droplet-vitrification experiments, cryopreserved SE were divided into 5 clumps (approximately 100 mg per clump) on each Petri dish. Embryo viability was recorded and expressed as percentage viability. Viability corresponds to any manifestation of growth of SE and percentage viability was calculated as the percent of proliferating clumps out of the total number of clumps.

### Long-term cryopreservation storage

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

## RESULTS AND DISCUSSION

### Cryovial-vitrification of SE using PVS2

SE of 'A10', 'Reed' and 'Velvick' were first cryopreserved using the method described by Guzmán-García *et al.* (2012). The untreated SE of all cultivars demonstrated 100 % viability. For the LS + PVS2 – LN controls 'Reed' and 'Velvick' SE resulted in high viability (80 - 93 %) however no viability was recorded for 'A10'. No viability was also recorded for SE of 'A10' or 'Reed' after LN treatment. However for 'Velvick' a viability of 32-48% was recorded after LN.

SE of 'A10', 'Reed', and 'Velvick' were trialled with the optimized LS sucrose concentrations and times from the pre-treatment experiment. For 'A10' and 'Velvick' a LS with a sucrose concentration of 0.2 and 0.8 M was used and for 'Reed' 0.6 M was selected. SE were treated in the varying LS + PVS2 at 0 ± 1 °C - LN and 100 % viability was observed for all cultivars. Viable SE of all three cultivars was recorded after LN treatment. 'A10' showed a viability of 82% (0.2 M) or 91% (0.8 M). The same high viability was recorded for 'Velvick' at either sucrose concentration i.e. 0.2 M (80 %) and 0.8 M (86 %). For 'Reed' SE a viability of 71% was recorded.

### Droplet-vitrification of SE using PVS2

SE of 'A10', 'Reed' and 'Velvick' were used in the first droplet-vitrification experiment following the method described by Guzmán-García *et al.* (2012). All untreated controls displayed 100 % viability. For 'Reed' and 'Velvick' SE treated with LS + PVS2 – LN 80 % of SE remained viable, whereas the same treatment for 'A10' SE resulted in 0 % viability. All three cultivars treated with LS + PVS2 + LN recorded 0 % viability. SE of 'A10', 'Reed', and 'Velvick' were trialled with the optimized LS sucrose concentrations and times from the pre-treatment experiment. For 'A10', 'Reed' and 'Velvick' a LS with a sucrose concentration of 0.2 M was selected.

SE of 'A10', 'Reed' and 'Velvick' all showed high viability (98 – 100 %) in the treatment LS + PVS2 – LN. When SE were treated with LS + PVS2 + LN high viability was recorded for 'A10' (100 %) and 'Reed' (85 %) however only (45 %) of 'Velvick' SE could be recovered after LN storage.

### Long-term cryopreservation storage

SE of 'A10', 'Reed' and 'Velvick' were successfully recovered after 3 months storage in LN using the best treatments for the cryovial and droplet-vitrification treatments. 'Reed' and 'Velvick' SE were the only cultivars tested for 12 months LN storage due to the time it took to optimize protocols. SE of 'Reed' and 'Velvick' were successfully recovered after 12 months storage using the cryovial treatment.

## CONCLUSIONS

To date, this is the first report on the application of the cryovial and droplet vitrification methods for cryopreservation of multiple avocado cultivars. These findings demonstrated the feasibility of long-term storage in LN for avocado SE. The two-step regeneration system (Encina *et al.*, 2014) involving the transfer of white opaque SE to a liquid medium as an intermediate step for inducing germination can be applied to white SE as they become available. This work contributes towards the establishment of a standard cryopreservation protocol for avocado species.

## ACKNOWLEDGEMENTS

This project has been funded by HAL using the Avocado Industry levy and matched funds from the Australian Government and supported by the Queensland Department of Agriculture, Fisheries and Forestry and Graham Anderson (Anderson Horticulture). We would like to thank Graham Anderson Junior for experimental assistance.

## REFERENCES

- Ayala-Silva, T., Schnell, R.J., Meerow, A.M., Goenaga, R., and Zee. F. 2004. Current status of the subtropical and tropical germplasm repositories of the National Plant Germplasm System. *Proc. Fla. State Hort. Soc.* 117:182-187.
- Ammirato, P.V., 1987. Organizational events during somatic embryogenesis. In: Green, C.E., Somers, D.A., Hackett, W.P., Biesboer, D.D. (Eds.), *Plant Biology, Plant Tiss and Cell Cult.* 3:57-81.
- Barrientos-Priego, A.F., Borys, M.W., Escamilla-Prado, E., Ben-Ya'acov, A., De La Cruz-Torres, E. and López- López. 1992. Study of avocado germplasm resources, 1988-1990. IV. Findings in the Mexican Gulf region. In: C.J. Lovatt (ed.) *World Avocado Congress II Proceedings.* April 21 – 26, 1991. Orange, CA pp: 551-558.
- Barrientos-Priego, A.F. 1999. The importance of the conservation and evaluation of avocado genetic resources. *Plant Breeding and Genetics,* 27-28 October 1999. Riverside, CA, 12 – 15.
- Benz, B. 2012. The Conservation of Cultivated Plants. *Nature Education Knowledge* 3(10):4.
- Ben-Ya'acov, A. and Barrientos, P. 2003. The persea germplasm resources potential as discovered during an international collection project. *Proceedings V World Avocado Congress (Actas V Congreso Mundial del Aguacate)* 2003, 21-26.

- Effendi, D. 2003. Transformation and cryopreservation of embryogenic avocado (*Persea americana* Mill.) cultures. Ph.D. 3127651, University of Florida.
- Encina, C.L., Parisi, A.P., O'Brien, C.M. and Mitter, N. M. 2014. Enhancing somatic embryogenesis in avocado (*Persea americana* Mill.) using a two-step culture system and including glutamine in the culture medium. *Scientia*. 165:44-50.
- Engelmann, F., 2011. Cryopreservation of embryos: an overview. *Methods Mol Biol*. 710:155-184.
- FDACS. 2012. Florida Department of Agriculture and Consumer Services Identifies Laurel Wilt Disease in Avocado Production Area of Miami-Dade County [Online]. Tallahassee, Florida. Available: <http://www.freshfromflorida.com/newsroom/press/2012/05012012.html> [Accessed 11 August 2014].
- Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res*. 50:151–158.
- Guzmán-García, E., Bradaï, F. and Sánchez-Romero, C. 2012. Cryopreservation of avocado embryogenic cultures using the droplet-vitrification method. *Acta Physiol Plant*. 35:183–193.
- Kim, H.H., Lee, Y.G., Park, S.U., Lee, S.C., Baek, H.J., Cho, E.G., Engelmann, F. 2009. Development of alternative loading solutions in droplet-vitrification procedures. *Cryo Letters*. 30:291-299.
- López-López, L., Rubi Arriaga, M., Ben-Ya'acov, A.D. and Barrientos-Priego, A.F. 1994. Panorama y potencial de los recursos genéticos del género *Persea*, preservados en el estado de México. Memoria de la fundación Salvador Sánchez Colin-CICTAMEX, S.C. 1994. Coatepec Harinas. México, 83-88.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant* 15:473–497.
- Panis, B. and Lambardi, M. 2005. Status of cryopreservation technologies in plants (crops and forest trees). In: Ruane, J. and Sonnino, A. (eds.) *The Role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources*. Food & Agriculture Org.
- Pateña, L.F., Carlos-Refuerzo, L.R., Barba, R.C., 2002. Somatic embryogenesis and plantlet regeneration in mango (*Mangifera indica* L.). In *Vitro Cell. Dev. Biol. Plant* 38:173–177.
- Sakai, A., and Engelmann, F., 2007. Vitrification, encapsulation-vitrification and droplet-vitrification: a review. *Cryo Letters*. 28:151-172.
- Witjaksono and Litz, R.E. 1999. Induction and growth characteristics of embryogenic avocado cultures. *Plant Cell, Tissue and Organ Culture*. 58:19-29.



# ACTAS • PROCEEDINGS

## VIII CONGRESO MUNDIAL DE LA PALTA 2015

del 13 al 18 de Septiembre. Lima, Perú 2015

[www.wacperu2015.com](http://www.wacperu2015.com)

