Cord 2016, **32** (2)

Improving the Availability of Valuable Coconut Germplasm using Tissue Culture Techniques

Steve Adkins*, Quang Thien Nguyen and Mike Foale

Abstract

Coconut cultivation faces a number of acute problems that reduce its productivity and competitiveness. These problems include various biotic and abiotic challenges as well as an unstable market for its traditional oil-based products. Around 10 million small-holder farmers cultivate coconut palms worldwide on c. 12 million hectares of land, and many more people own a few coconut palms that contribute to their livelihoods. Inefficiency in the production and supply of appropriate seedlings for replanting a generally ageing resource remains an issue, especially where there is uncertainty about the ability of local populations to resist potential disease threats. However, tissue culture in such cases is expected to provide pragmatic solutions. Over the past 60 years much research has been directed towards developing and improving protocols for (i) embryo culture; (ii) clonal propagation via somatic embryogenesis and (iii) germplasm conservation via cryopreservation. Recent advances have provided new ways to improve these protocols, especially cryopreservation. Although effective embryo culture and cryopreservation are now possible, the low efficiency of conversion of somatic embryos to *ex vitro* seedlings still restrains the large-scale clonal propagation of coconut. Although tissue culture in coconut has developed over the recent decades, further improvement of protocols and their application to a wider range of germplasm will boost their adoption for the breeding, conservation and propagation of coconut.

Keywords: Coconut, Cryopreservation, Embryo culture, Germplasm conservation, Somatic embryogenesis

Introduction

Together with the instability of the market for its traditional products, coconut field cultivation faces a multitude of challenges. In addition, productivity is affected by palm age, declining steadily after 35 years due to a decline in leaf area, by the rundown of soil nutrients, and through damage caused by cyclones, storms and tsunamis (Sisunandar *et al*. 2010a; Samosir and Adkins 2014). Speedy invasion of major pests, such as leaf beetles, and incurable diseases, such as phytoplasma-related lethal yellowing and viroid-caused cadang-cadang, have all resulted recently in a significant decline in the land area planted to coconut (Cordova *et al*. 2003; Harrison and Jones 2003; Lee 2013). Despite major efforts, through breeding programs aiming to increase oil yield in many countries, the general expectation for a higher, stable yield has not been realized (Samosir and Adkins 2004). A 'conventional' breeding approach to coconut improvement alone, involving multiple generations of inbreeding and finally hybridization, is unlikely to be a robust solution for increasing productivity (Thanh-Tuyen and De Guzman 1983; Batugal *et al*. 2009). The expectation that a plant adapted to the

*Professor in Plant Physiology, School of Agriculture and Food Sciences (SAFS), Centre for Plant Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St. Lucia 4072 Brisbane, Australia. E-mail: s.adkins@uq.edu.au

strand could be highly productive in a plantation structure, matching the oil palm for yield, for example, is perhaps misplaced.

It has been more than 60 years now since the first *in vitro* culture study was carried out on coconut (Cutter and Wilson 1954). Since then the landmark research achievements in coconut tissue culture have not been attained as much as they have for many other plant species. Some of the reasons often refer to the slow development of tissues in culture, and the varied response of the diverse range of coconut explant tissues used Dominant features are the slow growth of these explant tissues *in vitro*, and their further lack of vigor when planted *ex vitro* (Fernando *et al.* 2010). Nonetheless, tissue culture and associated biotechnological interventions for coconut have been achieved in these three major aspects: (i) embryo culture; (ii) clonal propagation via somatic embryogenesis (SE); and (iii) germplasm conservation via cryopreservation. Significant achievements in zygotic embryo culture have now enabled the collection of rare germplasm, and the rapid production of tissue culture-derived seedlings (Rillo 1998). The technique has been improved recently to meet a greater expectation across a wider range of cultivars (Samosir and Adkins 2014), while zygotic plumular tissue can now be used to achieve clonal propagation via SE (Pérez-Núñez *et al*. 2006). However, difficulties in this process still hinder the establishment of a feasible protocol for the production of plantlets on a commercial scale.

This paper reviews the advances to date in tissue culture and the associated biotechnological approaches applied to coconut, a physiologically recalcitrant species. Through an analysis of past notable achievements, we hope the paper can assist researchers to refine and discover novel approaches for improving the quality and resilience of the 'tree of life'.

Germplasm rescue and sterile movement via embryo culture

The most commonly used culture medium for coconut embryo culture is the Y3 medium developed by Eeuwens (1976), even though different ones have been shown to support zygotic embryo germination and growth. A high level of sucrose (> 4%) has been reported as an essential supplement for *in vitro* embryo germination. Activated charcoal has been used in many cases to help prevent tissue browning (Ashburner *et al*. 1993; Triques et al. 1997). Medium solidifiers, such as agar (1.5 to 0.8%), are often used to create a gelled substrate for the early stages of germination; however, recent studies report the use of a two-stage system including embryo culture in a liquid medium to obtain germination. This is followed by transfer to a gelled medium (Rillo 1998) or to nutrient-saturated vermiculite (Samosir and Adkins 2014) for seedling growth. More recently, other gelling agents such as Gelrite (Pech y Aké *et al.* 2004; Pech y Aké *et al.* 2007) and the addition of plant growth regulators including gibberellic acid (0.5 μM) have been reported to promote the rate and number of embryos germinating. Furthermore, synthetic auxin analogues such as naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) have been shown to promote root growth in the later stages of germination and during early seedling growth (Ashburner *et al.* 1993; Rillo 1998). The acclimatization of embryo-cultured plantlets has been achieved in many coconut cultivars using a number of potting soils and nursery conditions. A photoautotrophic and sucrose-free protocol, using CO_2-enrichment (1,600 μmol mol^{-1}) during the light phase, was found to improve seedling health and growth, and also the percentage of seedlings established from culture (Samosir and Adkins 2014).

Embryo culture has become an indispensable method for the collection of coconut germplasm from remote areas, and allowing long-distance delivery to the laboratory. An early-modified form of coconut germplasm collection involved the isolation of the mature embryo in the field, and its placement in a vial filled with sterile water or coconut water before being transported back to the laboratory (Rillo and Paloma 1991). A more proficient protocol was then developed which retained the embryos in a sterile state, embedded in a plug of solid endosperm recovered using a 2.5-cm diameter cork borer. This technique was further improved by the on-site surface sterilizing of the

endosperm plugs, placement in an ascorbic acid solution and retention at a cool temperature (*ca*. 5°C) during transport back to the laboratory (Adkins and Samosir 2002).

Even though embryo culture has been successfully achieved with many coconut cultivars, and can serve as a reliable tool for germplasm collection and exchange, the success rate of mature plants flourishing in soil can be low. There is also the risk of pathogenic viruses being present in embryo-cultured materials when they are intended to be moved and replanted. Therefore, the applicability of this technique to all coconut cultivars is still to be assessed and optimised as well as developing a mode of delivery to the smallholder.

Clonal propagation via somatic embryogenesis

Coconut SE research commenced over 30 years ago at Wye College, UK (Eeuwens and Blake 1977), and was continued by ORSTOM, France (Pannetier and Buffard-Morel 1982). These studies made use of a number of plant somatic tissues as initial explants (i.e. young leaves, stem slices from young seedlings, sections taken from rachillae of young inflorescences) to form embryogenic calli (Branton and Blake 1983; Gupta *et al*. 1984). However, more recently, there has been a shift to use either somatic tissues (e.g. immature inflorescences, ovaries) or to the easier to manipulate zygotic tissues (e.g. immature or mature embryos and embryo-derived plumules) to achieve SE in coconut (Samosir *et al*. 1998; Pérez-Núñez *et al.* 2006). More recently, recognising that only true somatic tissues can be used to produce true-to-type clones, some attention has returned to tissue explants taken from young inflorescence tissues (Antonova 2009).

The Y3 (Eeuwens 1976) and BM72 (Karunaratne and Periyapperuma 1989) media have been the most frequently used for callus culture, while MS (Murashige and Skoog 1962) and B5 (Gamborg *et al*. 1968) have been found to be less effective (Branton and Blake 1983; Bhallasarin *et al*. 1986). The inclusion of sucrose (3 to 4%) has proved to be important for coconut SE to take place, with activated charcoal (0.1 to 0.3%) typically applied to prevent explanted tissues and callus from browning, which is a stress-related response caused by the release of secondary plant products such as phenols, or ethylene (Samosir 1999). Dissimilarity in particle size, and the potency of the various activated charcoal types, has been shown to influence the frequency of somatic embryogenic callus formation (Sáenz *et al*. 2009). Polyvinylpyrrolidone (PVP), another common toxin-absorbing agent, was tested in coconut leaf-derived cell suspension cultures, but without any significant effect (Basu *et al*. 1988). However, polyvinylpolypyrrolidone (PVPP), used in zygotic embryo–derived callus culture, was found to have some positive effect in promoting the rate of SE (Samosir 1999).

Similar to other species, the sequential development of clonally propagated coconut plantlets is typically divided into three stages: firstly the production of callus and its proliferation; secondly the formation, maturation and germination of somatic embryos; and thirdly the acclimatisation of the plantlets to *ex vitro* conditions. Apart from 2,4-D, other synthetic auxins such as NAA (27 µM) in combination with 2,4-D (452 µM) have been used to promote callus formation on rachillae explants (Gupta *et al.* 1984). In addition, a study of the ultrastructural changes that take place during the acquisition of SE potential suggests that the gametophyte-like conditions produced by 2,4-D are required for a successful transition from the vegetative into the embryogenic state (Verdeil *et al.* 2001).

Supplementation of the callus proliferation and maturation medium with a cytokinin such as 6-benzylaminopurine (BAP), thidiazuron (TDZ), kinetin (Kin) or 2-isopentyl adenine (2iP), at 5 to 10 µM is also common place (Perera *et al.* 2009b). Callus formation is often best achieved in the dark for at least 1 month after culture initiation, and at 28 ± 1 °C (Adkins *et al.* 1998). However, in one study, dark incubation was extended to 3 months to achieve greater callus production (Pérez-Núñez *et al.* 2006). Further improvement in the timely production of embryogenic callus has been achieved by

applying into the medium one of the multi-functional polyamines, particularly putrescine (7.5 mM) or spermine (1.0 µM), to protect the explanted tissue from ethylene damage and/or to promote the rate of SE (Adkins *et al.* 1998). Inhibitors of ethylene production, such as aminoethoxyvinylglycine (AVG) and ethylene-action inhibitors such as silver thiosulphate (STS), have also been shown to provide a beneficial environment for callus multiplication, and the formation of somatic embryos (Adkins *et al.* 1998).

Abscisic acid (ABA), when applied at a moderate concentration (*ca*. 5 µM), has been shown to enhance the formation and the maturation of somatic embryos (Samosir *et al*. 1999; Fernando and Gamage 2000; Fernando *et al.* 2003). In addition, the use of osmotically-active agents such as polyethyleneglycol (PEG 3%) in combination with ABA (45 µM) has been shown to be beneficial, not only for the production of somatic embryos but also for their subsequent maturation and germination (Samosir *et al.* 1998). In a more recent study using immature inflorescence explants, Antonova (2009) demonstrated the benefits of using a specific growth retardant ancymidol (30 µM) to elevate the somatic embryo germination frequency from a low proportion to 56%.

It is worth noting that cell suspension culture systems have been successful in raising the rate of SE for some other members of the Arecaceae, including oil palm (*Elaeis guineensis* Jacq.: Teixeira *et al.* 1995). Further improvements may come from using a photoautotrophic culture system to raise plantlets (Samosir and Adkins 2014), and through the incorporation of fatty acids, notably lauric acid, into the plantlet maturation medium (López-Villalobos *et al.* 2001, 2011).

Germplasm conservation via cryopreservation

Over the past 30 years, scientists have been trying to develop a method for the safe and long term conservation of coconut germplasm. In the 1980s, the first attempt to cryopreserve coconut tissues was undertaken with immature zygotic embryos using a chemical dehydration and slow freezing technique (Bajaj 1984). However, more recently attention has shifted towards using mature (11 months post pollination) zygotic embryos (Sisunandar *et al*. 2014) and using a physical dehydration method; or using plumular tissues excised from mature zygotic embryos and employing a chemical dehydration method. As with most species, the cryopreservation protocol for coconut consists of four steps: firstly the pre-culture of the explanted tissues in preparation for drying; secondly tissue dehydration; thirdly tissue freezing; and finally tissue recovery involving thawing and plantlet production. Three tissue dehydration methods have been attempted: chemical dehydration, slow physical dehydration (desiccation taking place in a laminar air flow hood), and fast physical dehydration (fan forced drying using silica gel). For rapid physical dehydration a special apparatus has been developed to dehydrate embryos by exposure to dry air by fan-forcing the air over silica gel (Sisunandar *et al.* 2010b). By following the water loss during the physical drying of embryos (using differential scanning calorimetry) it was found that drying to 20% moisture content in a period of 8 hours gave the embryos to best chance of surviving cryopreservation. Upon recovery of embryos, this approach gave the higher proportion of plants growing in soil (up to 40%), a level that had not been achieved using any previous method. It was also shown that this cryopreservation method did not induce any measurable genetic change in the recovered plants *(Sisunandar et al.* 2010a).

The MS (Murashige and Skoog 1962), MW (Morel and Wetmore 1951) and Y3 (Eeuwens 1976) media formulations have all been commonly used in the tissue recovery stage, after cryopreservation, with the latter medium preferred in most studies (Sisunandar *et al*. 2010b; Sajini *et al*. 2011; Sisunandar *et al.* 2012). It is noteworthy that the application of auxins (2,4-D, NAA) or kinetin, either alone or in combination, did not significantly help embryo germination or plantlet recovery (Bajaj 1984; Chin *et al.* 1989). On the other hand, the addition of a high dose of sucrose (4 to 6%) has been shown to improve the germination of the

recovered embryos (N'Nan *et al.* 2008; Sisunandar *et al.* 2010b; Sajini *et al.* 2011). Successful establishment of plant in soil following cryopreservation of the embryo has been reported twice, using the chemical dehydration approach of Sajini *et al.* (2011) and the physical dehydration approach of Sisunandar *et al.* (2010b).

Up until now the majority of coconut cryopreservation work has focused on the use of zygotic embryos or isolated plumular tissues, the availability of which can be limited. Therefore, an interesting field for future research would be the application of cryopreservation to somatic embryogenic cell cultures. The successful preservation of such cultures would enable the production of many more coconut plants from one initial explant, as well as providing a new way to transfer germplasm around the globe.

Conclusion and future prospects

Ineffective plantlet conversion from *in vitro* culture systems to field-growing palms, remains a major bottleneck for many coconut research groups around the world. This is the result of unresolved or partly resolved problems which relate to the variable response of explanted tissues and their slow growth *in vitro*, and their further lack of vigour when planted *ex vitro*. As a result, achievements in coconut tissue have been attained less rapidly than for many other plant species. It is essential to reflect on, and then trial, procedures that work for other species to identify future improvements in coconut *in vitro* culture. The assembled information suggests that it may be possible to generate highly efficient embryogenic cell suspension cultures, derived from suitable callus lines, to help overcome contemporary challenges, and to develop a rapid clonal propagation system for coconut. Therefore, future research should be focused on the optimization of *in vitro* conditions to increase the production of somatic embryos using media additives, combined with a cell suspension culture system. Subsequent development and acclimatization could be further improved by using a temporary immersion approach together with a photoautotrophic system to promote rapid plantlet growth. As the coconut seed possesses a substantial source of natural plant nutrients and growth factors within its own liquid endosperm, further investigation may identify a role for coconut water in promoting SE in this generally recalcitrant species. Other possible improvements in the rate of SE may come from the application of molecular techniques that can identify the regulatory genes involved, and then promote their activity. Indeed, novel molecular tools might become available to further examine the regulation of the relevant genes, which can be selectively switched on during the acquisition of embryogenic competence.

Acknowledgement

The authors would like to thank the Australian Agency for International Development (AusAID) for a scholarship awarded to Quang T. Nguyen. We also thank the Australian Centre for International Agricultural Research (ACIAR) for financial support. We also acknowledge the independent reviews from Professor Jeffrey Adelberg (Clemson University, USA) and Dr Yohannes M. S. Samosir (Bakrie Agriculture Research Institute, Indonesia).

References

Adkins SW, Samosir YMS (2002) Embryo culture activities at the University of Queensland. In: Engelmann F, Batugal P, Oliver L (eds) Coconut embryo in vitro culture: Part II. Merida, Mexico, pp 163-168

Adkins SW, Samosir YMS, Ernawati A, Godwin ID, Drew RA (1998) Control of ethylene and use of polyamines can optimise the conditions for somatic embryogenesis in coconut (*Cocos nucifera* L.) and papaya (*Carica papaya* L.). In: Drew RA (ed) Proceedings of the international symposium of biotechnology in tropical and subtropical species. Brisbane, Australia, pp 459-466

Andrade-Torres A, Oropeza C, Sáenz L, González-Estrada T, Ramírez-Benítez J, Becerril K, Chan J, Rodríguez-Zapata L (2011) Transient genetic transformation of embryogenic callus of Cocos nucifera.

Biologia 66:790-800. doi:10.2478/s11756-011-0104-4

Antonova ID (2009) Somatic embryogenesis for micropropagation of coconut (*Cocos nucifera* L.). PhD Thesis, The University of Queensland, Australia,

Ashburner GR, Thompson WK, Burch JM (1993) Effect of alpha-naphthaleneacetic acid and sucrose levels on the development of cultured embryos of coconut. Plant Cell Tiss Org 35:157-163

Bajaj YPS (1984) Induction of growth in frozen embryos of coconut and ovules of citrus. Curr Sci 53 (22):1215-1216

Basu A, Sethi U, Guhamukherjee S (1988) Induction of cell division in leaf cells of coconut palm by alteration of pH and its correlation with glyoxalase-I activity. J Exp Bot 39:1735-1742. doi:10.1093/jxb/39.12.1735

Batugal P, Bourdeix R, Baudouin L (2009) Coconut breeding. In: Jain SM, Priyadarshan PM (eds) Breeding plantation tree crops: tropical species. Springer, New York, pp 327-375. doi:10.1007/978-0-387-71201-7_10

Bhallasarin N, Bagga S, Sopory SK, Guhamukherjee S (1986) Induction and differentiation of callus from embryos of *Cocos nucifera* L. by IAA-conjugates. Plant cell reports 5:322-324

Blake J, Hornung R (1995) Somatic embryogenesis in coconut (*Cocos nucifera* L.). In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants. Kluwer, Dordrecht, pp 327-349

Branton RL, Blake J (1983) Development of organized structures in callus derived from explants of *Cocos nucifera* L. Ann Bot 52:673-678

Chakraborty M, Mitra A (2008) The antioxidant and antimicrobial properties of the methanolic extract from *Cocos nucifera* mesocarp. Food Chem 107:994-999. doi:10.1016/j.foodchem.2007.08.083

Chan JL, Saenz L, Talavera C, Hornung R (1998) Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. Plant cell reports 17:515-521

Chin HF, Krishnapillay B, Hor YL (1989) A note on the cryopreservation of embryos from young coconuts (*Cocos nucifera* var. Mawa). Pertanika 12 (2):183-186

Cordova I, Jones P, Harrison NA, Oropeza C (2003) In situ PCR detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease. Mol Plant Pathol 4:99-108. doi:10.1046/j.1364-3703.2003.00152.x

Cutter VM, Jr., Wilson KS (1954) Effect of coconut endosperm and other growth stimulants upon the development in vitro of embryos of *Cocos nucifera*. Bot Gaz 115:234-240. doi:10.2307/2472513

De Guzman EV, Del Rosario DA (1964) The growth and development of *Cocos nucifera* L. makapuno embryo in vitro. The Philippine Agriculturist 48:82-94

Eeuwens CJ (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured in vitro. Physiol Plant 36:23-28

Eeuwens CJ, Blake J (1977) Culture of coconut and date palm tissue with a view to vegetative propagation. Acta Hort 78:277-286

FAOSTAT (2013) Food and Agriculture Organization of the United Nations - World coconut harvested areas in 2013 http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567 - ancor. Accessed 22/11/2014

Fernando SC, Gamage CKA (2000) Abscisic acid induced somatic embryogenesis in immature embryo explants of coconut (*Cocos nucifera* L.). Plant Sci 151:193-198. doi:10.1016/S0168-9452(99)00218-6

Fernando SC, Verdeil JL, Hocher V, Weerakoon LK, Hirimburegama K (2003) Histological analysis of plant regeneration from plumule explants of *Cocos nucifera*. Plant Cell Tiss Org 72:281-283. doi:10.1023/A:1022345011002

Fernando SC, Vidhanaarachchi VRM, Weerakoon LK, Santha ES (2010) What makes clonal propagation of coconut difficult? AsPac J Mol Biol Biotechnol 18:163-165

Foale M (2003) The coconut odyssey: the bounteous possibilities of the tree of life. ACIAR Monography No. 101. Canberra

Fuentes G, Talavera C, Desjardins Y, Santamaria JM (2005) High irradiance can minimize the negative effect of exogenous sucrose on photosynthetic capacity of in vitro grown coconut plantlets. Biol Plant 49:7-15

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151-158. doi:10.1016/0014-4827(68)90403-5

Gupta PK, Kendurkar SV, Kulkarni VM, Shirgurkar MV, Mascarenhas AF (1984) Somatic embryogenesis and plants from zygotic embryos of coconut (*Cocos nucifera* L.) in vitro. Plant cell reports 3:222-225

Harrison NA, Jones P (2003) Diseases of coconut. Diseases of tropical fruit crops. CABI Publishing, 44 Brattle Street, 4th Floor, Cambridge, MA, 02138, USA. doi:10.1079/9780851993904.0197

Karunaratne S, Periyapperuma K (1989) Culture of immature embryos of coconut, *Cocos nucifera* L.: callus proliferation and somatic embryogenesis. Plant Sci 62:247-253

Koschek PR, Alviano DS, Alviano CS, Gattass CR (2007) The husk fiber of *Cocos nucifera* L. (Palmae) is a source of anti-neoplastic activity. Brazilian J Med Biol Res 40:1339-1343. doi:10.1590/s0100-879x2006005000153

Lee RF (2013) Cadang-cadang disease of palm and other diseases. Phytopathol 103:177-177

Lim TK (2012) *Cocos nucifera*. In: Lim TK (ed) Edible medicinal and non-medicinal plants. Springer-Verlag Berlin, Berlin, pp 301-334. doi:10.1007/978-90-481-8661-7_45

Lopez-Villalobos A (2002) Roles of lipids in coconut (*Cocos nucifera* L.) embryogenesis. University of London,

López-Villalobos A, Dodds PF, Hornung R (2001) Changes in fatty acid composition during development of tissues of coconut (*Cocos nucifera* L.) embryos in the intact nut and in vitro. J Exp Bot 52:933-942

López-Villalobos A, Dodds PF, Hornung R (2011) Lauric acid improves the growth of zygotic coconut (*Cocos nucifera* L.) embryos in vitro. Plant Cell Tiss Org 106:317-327. doi:10.1007/s11240-011-9924-8

López-Villalobos A, Hornung R, Dodds PF (2004) Hydrophobic metabolites of 2,4-dichlorophenoxyacetic acid (2,4-D) in cultured coconut tissue. Phytochem 65:2763-2774. doi:10.1016/j.phytochem.2004.08.034

Marina AM, Man YBC, Nazimah SAH, Amin I (2009) Antioxidant capacity and phenolic acids of virgin coconut oil. Int J Food Sci Nutr 60:114-123. doi:10.1080/09637480802549127

Morel G, Wetmore RH (1951) Fern callus tissue culture. Am J Bot 38:141-143. doi:10.2307/2437837

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497. doi:10.1111/j.1399-3054.1962.tb08052.x

N'Nan O, Hocher V, Verdeil JL, Konan JL, Ballo K, Mondeil F, Malaurie B (2008)

Cryopreservation by encapsulation-dehydration of plumules of coconut (*Cocos nucifera* L.). CryoLett 29:339-350

Pan MJ, van Staden J (1998) The use of charcoal in in vitro culture - a review. Plant Growth Regul 26:155-163. doi:10.1023/a:1006119015972

Pannetier C, Buffard-Morel J Production of somatic embryos from leaf tissues of coconut, *Cocos nucifera* L. In: Proceedings of the 5th International Plant Tissue Culture Congress, Tokyo, Japan, 1982.

Pech y Aké AE, Maust B, Orozco-Segovia A, Oropeza C (2007) The effect of gibberellic acid on the in vitro germination of coconut zygotic embryos and their conversion into plantlets. In Vitro Cell Dev Plant 43:247-253

Pech y Aké AE, Souza R, Maust B, Santamaría JM, Oropeza C (2004) Enhanced aerobic respiration improves in vitro coconut embryo germination and culture. In Vitro Cell Dev Plant 40:90-94. doi:10.1079/ivp2003480

Perera L, Perera SACN, Bandaranayake CK, Harries HC (2009a) Coconut. In: Vollmann J, Rajcan I (eds) Oil Crops, vol 4. Springer, New York, pp 369-396. doi:10.1007/978-0-387-77594-4_12

Perera PIP, Perera L, Hocher V, Verdeil JL, Yakandawala DM, Weerakoon LK (2008) Use of SSR markers to determine the anther-derived homozygous lines in coconut. Plant cell reports 27:1697-1703. doi:10.1007/s00299-008-0592-z

Perera PIP, Vidhanaarachchi VRM, Gunathilake TR, Yakandawala DMD, Hocher V, Verdeil JL, Weerakoon LK (2009b) Effect of plant growth regulators on ovary culture of coconut (*Cocos nucifera* L.). Plant Cell Tiss Org 99:73-81. doi:10.1007/s11240-009-9577-z

Pérez-Núñez MT, Chan JL, Sáenz L, González T, Verdeil JL, Oropeza C (2006) Improved somatic embryogenesis from *Cocos nucifera* (L.) plumule explants. In Vitro Cell Dev Plant 42:37-43. doi:10.1079/ivp2005722

Reinert J (1959) Über die kontrolle der morphogenese und die induktion von adventivembryonen an gewebekulturen aus karotten. Planta 53 (4):318-333. doi:10.1007/BF01881795

Rethinam P (2006) Asian and Pacific coconut community activities, achievements and future outlook. ACIAR Proceedings Series 125:15-21

Rillo EP (1998) PCA's embryo culture technique in the mass production of Makapuno coconuts. In: Batugal PA, Engelmann F (eds) Coconut embryo in vitro culture: Part I. Proceedings of the first workshop on embryo culture, Banao, Guinobatan, Albay, Philippines, 27-31 October 1997. International Plant Genetic Resources Institute (IPGRI), Rome, pp 69-78

Rillo EP, Paloma MBF (1991) Storage and transport of zygotic embryos of Cocos nucifera L. for in vitro culture. Plant Genet Resources Newslett 86:1-4

Ross IA (2005) *Cocos nucifera* L. In: Ross IA (ed) Medicinal Plants of the World, Vol 3. Humana Press, pp 117-154. doi:10.1007/978-1-59259-887-8_3

Sáenz L, Herrera-Herrera G, Uicab-Ballote F, Chan JL, Oropeza C (2009) Influence of form of activated charcoal on embryogenic callus formation in coconut (*Cocos nucifera*). Plant Cell Tiss Org 100:301-308. doi:10.1007/s11240-009-9651-6

Sajini KK, Karun A, Amarnath CH, Engelmann F (2011) Cryopreservation of coconut (*Cocos nucifer*a L.) zygotic embryos by vitrification. CryoLett 32:317-328

Samosir YMS (1999) Optimisation of somatic embryogenesis in coconut (*Cocos nucifera* L.). PhD Thesis, The University of Queensland, Australia,

Samosir YMS, Adkins SW (2004) Embryo transplantation and *ex vitro* germination

for germplasm exchange and the production of high value, endosperm mutant coconuts. In: Peiris TSG, Ranasinghe CS (eds) Proceedings of the International Conference of the Coconut Research Institute of Sri Lanka: Part II. The Coconut Research Institute Institute of Sri Lanka, Lunuwila, Sri Lanka, pp 92-102

Samosir YMS, Adkins SW (2014) Improving acclimatization through the photoautotrophic culture of coconut (*Cocos nucifera*) seedlings: an in vitro system for the efficient exchange of germplasm. In Vitro Cell Dev Plant 50:493-501. doi:10.1007/s11627-014-9599-z

Samosir YMS, Godwin ID, Adkins SW (1998) An improved protocol for somatic embryogenesis in coconut (*Cocos nucifera* L.). In: Drew RA (ed) Proceedings of the International Symposium of Biotechnology in Tropical and Subtropical Species, vol 461. Brisbane, Australia, pp 467-475

Samosir YMS, Godwin ID, Adkins SW (1999) The use of osmotically active agents and abscisic acid can optimise the maturation of coconut somatic embryos. In: Oropeza C (ed) Current Advances in Coconut Biotechnology. CAB International, UK, pp 341-354

Sisunandar, Novarianto H, Mashud N, Samosir YMS, Adkins SW (2014) Embryo maturity plays an important role for the successful cryopreservation of coconut (*Cocos nucifera*). In Vitro Cell Dev Plant 50:688-695. doi:10.1007/s11627-014-9633-1

Sisunandar, Rival A, Turquay P, Samosir Y, Adkins SW (2010a) Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos does not induce morphological, cytological or molecular changes in recovered seedlings. Planta 232:435-447. doi:10.1007/s00425-010-1186-x

Sisunandar, Sopade PA, Samosir YM, Rival A, Adkins SW (2010b) Dehydration improves cryopreservation of coconut (*Cocos nucifera* L.). Cryobiol 61:289-296. doi:10.1016/j.cryobiol.2010.09.007

Sisunandar, Sopade PA, Samosir YMS, Rival A, Adkins SW (2012) Conservation of coconut (*Cocos nucifera* L.) germplasm at sub-zero temperature. CryoLett 33:465-475

Steinmacher DA, Guerra MP, Saare-Surminski K, Lieberei R (2011) A temporary immersion system improves in vitro regeneration of peach palm through secondary somatic embryogenesis. Annals of Botany 108 (8):1463-1475. doi:10.1093/aob/mcr033

Steward FC, Marion OM, Mears K (1958) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Am J Bot 45 (10):705-708. doi:10.2307/2439728

Talavera C, Contreras F, Espadas F, Fuentes G, Santamaría JM (2005) Cultivating in vitro coconut palms (*Cocos nucifera*) under glasshouse conditions with natural light, improves in vitro photosynthesis nursery survival and growth. Plant Cell Tiss Org 83:287-292. doi:10.1007/s11240-005-7052-z

Teixeira JB, Sondahl MR, Nakamura T, Kirby EG (1995) Establishment of oil palm cell suspensions and plant regeneration. Plant Cell Tissue and Organ Culture 40 (2):105-111. doi:10.1007/bf00037662

Thanh-Tuyen NT, De Guzman EV (1983) Formation of pollen embryos in cultured anthers of coconut (*Cocos nucifera* L.). Plant Sci Lett 29:81-88

Tisserat B, Vandercook CE (1985) Development of an automated plant culture system. Plant Cell Tiss Org 5:107-117. doi:10.1007/bf00040307

Triques K, Rival A, Beule T, Dussert S, Hocher V (1997) Developmental changes in carboxylase activities in in vitro cultured coconut zygotic embryos: comparison with

corresponding activities in seedlings. Plant Cell Tiss Org 49:227-231

Triques K, Rival A, Beule T, Morcillo F, Hocher V, Verdeil JL, Hamon S (1998) Changes in photosynthetic parameters during in vitro growth and subsequent acclimatization of coconut (*Cocos nucifera* L.) zygotic embryos. In: Drew RA (ed) Proceedings of the international sympodium of biotechnology in tropical and subtropical species, vol 461. Acta Hort. (ISHS), pp 275-284

Verdeil JL, Hocher V, Huet C, Grosdemange F, Escoute J, Ferrière N, Nicole M (2001) Ultrastructural changes in coconut calli associated with the acquisition of embryogenic competence. Ann Bot 88:9-18. doi:10.1006/anbo.2001.1408

Verdeil JL, Huet C, Grosdemange F, Buffard-Morel J (1994) Plant regeneration from cultured immature inflorescences of coconut (*Cocos nucifera* L.): evidence for somatic embryogenesis. Plant cell reports 13:218-221