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Coconut Micropropagation and Cryopreservation

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ABSTRACT

Coconut palm (*Cocos nucifera* L.) belongs to the Arecaceae, one of the most distinctive plant families emblematic of the tropics. Commonly known as the "tree of life," the coconut palm is considered one of the most important tropical crops in the world, providing protection, food, and healing. In recent decades, with the expansion of coconut-growing areas, biotic and abiotic factors, such as phytosanitary problems, the occurrence of pests and diseases, uniformity of crops, adaptation to different ecosystems, and others, have affected the production and longevity of coconut trees. In addition, unstable markets have also affected production. Biotechnology techniques have contributed to the advances in coconut production and conservation, including *in vitro* propagation methods, and large-scale mass propagation using bioreactors. Micropropagation offers advantages, such as high multiplication rates, utilization of small explants, and the potential for the production of clean plant material, free of diseases, as well as rapid multiplication of disease-resistant varieties. Cryopreservation allows for safe and long-term germplasm conservation. This review aims to provide an overview of the current status and major advances in micropropagation and cryopreservation techniques of coconut palm.

KEYWORDS: bioreactor; Cocos nucifera ; conservation; cryopreservation; in vitro propagation;

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ACSC	activated coconut shell charcoal
BA	6-benzyladenine
BGD	'Brazilian Green Dwarf' coconut
BT	'Brazilian Tall' coconut
CDKA; CnCDKA	genes involved in somatic embryogenesis
CIRAD	Agricultural Research for Development
COD	'Chowghat Orange Dwarf' coconut
CPCRI	Central Plantation Crops Research Institute
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	FAO statistical database
GA_3	gibberellic acid
GMD	'Green Malayan Dwarf' coconut
KNOX; CnKNOX1; CnKNOX2; KNOTTED	genes involved in somatic embryogenesis
LED	light-emitting diode
MS	Murashige and Skoog culture medium
NAA	1-naphthaleneacetic acid
ORSTOM/IRD	Office of Scientific and Technical Research Overseas/Research Institute for Development
PCA	Philippine Coconut Authority
PVS1; PVS2; PVS3; PVS4	pre-vitrification solutions
SE	somatic embryo
SERK; CnSERK	genes involved in somatic embryogenesis
TIS	temporary immersion system
TREC	Tropical Research and Education Center
UPLB	University of Philippines Los Baños
WCT	'West Coast Tall' coconut
XER	Ernst culture medium

I. INTRODUCTION

Coconut palm (*Cocos nucifera* L.) is considered one of the most important tropical species in the world. Known as the "tree of life," it allows the elaboration of more than 100 products and byproducts, being found in all tropical regions of the globe.

According to Foale and Harries (2011), the coconut palm originated in the ancient supercontinent of Gondwana. Coconut fruits floated in the prehistoric Tethys Seas with natural distribution on the coasts and islands of the Indian Ocean, atolls of the mid-Pacific Ocean, and coasts of South China and East Africa (Bourdeix et al. 2005). In the sixteenth century, after the discovery of the Cape of Good Hope, Portuguese and Spanish distributed coconut seedlings in Western Africa and then in the Americas and the entire tropical region of the globe. In the Caribbean, the first introduction from Cape Verde dates back to 1549, with planting in Puerto Rico. Between 1571 and 1816, coconut fruits from the Philippines were introduced to Mexico and across the Pacific coast of America down to Peru (Bourdeix et al. 2005). This species is now widespread in almost all continents, being found between parallels 23°N and 23°S in more than 200 countries (Foale and Harries 2011).

The estimated cultivation area of coconut was 12.3 m ha in 2017, with production of 60.7 m tons of fruit in 92 countries, where Indonesia, the Philippines, and India accounted for 72.7% of the total cultivated area (FAOSTAT 2017). Brazil ranked fourth, with a planted area of 215,683 ha and production of around 2.9 m tons of fruit per year, followed by Sri Lanka with about 2.5 m tons per year.

Copra is the dried kernel of the coconut, and the oil from copra is used for soap, shampoo, cosmetics, cooking oil, and margarine (Broschat and Crane 2000). Commercial exploitation in most countries that cultivate this palm is focused on the production of fruits to obtain oil and dry coconut dehydrated from copra. Virgin coconut oil is reported to have antioxidant, antimicrobial, and anticancer properties, while phototherapeutics from fresh or processed coconut products could be effective for prevention and treatment of several diseases (Nguyen et al. 2015).

Tall group varieties of coconut may reach a height of 25 to 30 m (Broschat and Crane 2000), are more heterogeneous due to cross-pollination, and fruit are mostly used for the production of copra. In contrast, dwarf varieties are shorter, are more homogeneous due to their lower allogamy, and the fruit are mainly used for the production of coconut water (Bourdeix et al. 2005). The 34 main coconut varieties grown in the world are divided into these two groups, in addition to hybrids that have resulted from crosses between the dwarf and tall varieties. The hybrids have dual purpose, for both fresh consumption and industrial processing. Other uses of coconut include the husk fiber (comprising the exocarp and mesocarp) for ropes and mats and the shell (endocarp) for charcoal (Foale and Harries 2011).

Plant tissue culture techniques have been applied to support coconut-breeding and gene conservation programs. The history of tissue culture in coconut dates back to 1954, with the development of embryos *in vitro* (Cutter and Wilson 1954; Karunaratne and Periyapperuma 1989). Embryo culture and clonal propagation via somatic embryogenesis protocols have been the focus of studies for the past 10 years, including several different coconut genotypes and cultivars, and are considered reliable tools for germplasm collection and exchange (Nguyen et al. 2015). However, variation on the responses among genotypes and slow development of tissues in culture have limited progress and the establishment of a viable protocol for the commercial large-scale clonal propagation of coconut (Adkins et al. 2018). Studies of nutrient requirements and culture media composition have been performed, and some advances have been obtained. However, somatic embryogenesis remains the most feasible technique for the clonal propagation of coconut and is necessary for the multiplication of superior genotypes on a large scale (Pérez-Nuñez et al. 2006; Sáenz et al. 2006; Perera et al. 2007; Nguyen et al. 2015).

The conservation of coconut genetic resources is mainly accomplished through field collections due to the large size and recalcitrance of the seed, making its storage difficult (N'Nan et al. 2008). Cryopreservation offers an alternative for coconut conservation (Sisunandar et al. 2010c; Nguyen et al. 2015). The process allows the storage of live plant cells, tissues, and organs at ultra-low temperatures (−196°C in liquid nitrogen, or −150°C in the vapor phase) over long periods of time (Reed 2008). The technique is a viable procedure for the conservation of valuable genotypes and biological material under stable and safe conditions, requiring little space and maintenance, and low cost (Benson 2008). Cryopreservation has been used for the conservation of genetic resources in many species, especially recalcitrant ones and for different types of tissues and organs, including seeds, somatic and zygotic embryos, shoot tips, anthers, and buds (Vendrame 2018).

This objective of this review is to provide an overview of the current status and major advances in micropropagation and cryopreservation techniques of coconut palm.

II. *IN VITRO* PROPAGATION TECHNOLOGY

A. Concept

Biotechnology tools, including plant tissue culture techniques, have contributed significantly and been widely applied in breeding programs, for germplasm conservation and exchange, to increase the genetic variability for selection purposes, in introgression of genes of interest, and in the cloning of genotypes (Trigiano and Gray 2000; Nguyen et al. 2015).

The terms *in vitro* propagation or micropropagation refer to the use of *in vitro* culture techniques for true-to-type propagation of selected genotypes using small tissues or organs as explants, such as stem tips, nodal segments, and zygotic embryos (Kane 2000; Carvalho et al. 2011).

Advances in the application of plant tissue culture for coconut micropropagation since 2006 have employed various techniques and involved diverse genotypes (Table 6.1).

Table 6.1 *In vitro* culture techniques applied to coconut and results obtained.

Technique	Cultivar	Results	References
Manipulation of concentration of exogenous sugar, light intensity, and CO_2 enrichment	Green Malayan Dwarf	Promoted photosynthetic capacity of *in vitro* plants and established autotrophic plantlets; enhanced *ex vitro* performance	Fuentes et al. (2006)
Primary somatic embryogenesis, secondary somatic embryogenesis, and embryogenic callus multiplication	Green Malayan Dwarf	Significant improvement (50 000-fold increase) in the efficiency of somatic embryo production	Pérez-Nuñez et al. (2006)
Morphological and histological evaluations in plumule explants cultured *in vitro*, from callus to somatic embryo and shoot formation	Malayan Dwarf	Integrated view into the somatic embryogenesis process in coconut	Sáenz et al. (2006)
Effects of gibberellic acid (GA_3) on *in vitro* germination of zygotic embryos, plantlet conversion, and *ex vitro* survival	Yellow Malayan Dwarf GreenMalayanDwarf	Improved germination and conversion using GA_3 on semi-solid medium	Aké et al. (2007)
Embryogenic callus production using immature inflorescences, followed by somatic embryogenesis using different medium supplements and plant growth regulators	Yellow Malayan Dwarf	Significant increase in somatic embryo formation (over 300%); increase in embryo maturation and germination	Antonova (2009)
Microspore embryogenesis using anthers as explants, and evaluation of concentrations and combinations of different plant growth regulators	Sri Lanka Tall	Induction of embryogenesis both directly and indirectly, but low conversion and presence of vitrification	Perera et al. (2009)
Callus induction from immature zygotic embryos and plumules using activated coconut shell charcoal (ACSC) in tissue culture media	Sri Lanka Tall	Callus induction observed when using two sources of ACSC in the presence of 2,4-D, but comparable to control; no conversion reported.	Fernando et al. (2010)
Evaluations of medium replenishment on growth and contamination of *in vitro* cultures, the use of supplements on *in vitro* growth and *ex vitro* survival of seedlings, *ex vitro* acclimatization techniques, and different transfer stages of *ex vitro* plantlets	Laguna Tall	Medium replenishment promoted plant growth and development of secondary roots, and reduced contamination; polyvinylpyrrolidone and polyethylene glycol reduced secondary roots and promoted plant growth and leaf production; acclimatization promoted higher seedling survival; transfer stages did not affect seedling survival.	Magdalita et al. (2010)
Evaluation of expression of the putative *CnCDKA* gene at different somatic embryo formation stages from embryogenic cultures initiated from plumules	Malayan Dwarf	Gene expression gradually decreased during somatic embryo formation, being lowest in germinating somatic embryos, providing insight into tissue embryogenic competence.	Montero-Cortes et al. (2010a)
Evaluation of occurrence of gene members of the *KNOX* family and their characterization during formation and germination of somatic embryos under the influence of gibberellic acid (GA_3)	Malayan MexicanPacific Tall	Complete sequences of two *KNOX*-like genes obtained: *CnKNOX1* and *CnKNOX2*. Expression of *CnKNOX1* was detected throughout the embryogenic process, except in globular somatic embryos, and tissues of organs with meristem. The expression of *CnKNOX2* peaked at globular stage but decreased with GA_3 application, and it was detected in tissues with and without meristems.	Montero-Cortes et al. (2010b)

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Studies on the expression of an ortholog of the *SERK* gene during somatic embryogenesis	Malayan Dwarf	A *SERK* ortholog, named *CnSERK*, was identified and its expression detected in embryogenic tissues and associated with induction of somatic embryogenesis.	Pérez-Nuñez et al. (2009)
In vitro culture of mature zygotic embryos using different protocols	Non-specified; based on several previous studies	No differences observed in germination and development between protocols; method successful for shipping embryos.	Engelmann et al. (2011)
Evaluation of lauric acid in culture media for *in vitro* culture of zygotic embryos	Yellow Malayan Dwarf	Lauric acid inhibited germination at culture initiation, but stimulated plant growth and development when added at 60 or 75 days of culture.	Lopez-Villalobos et al. (2011)
Evaluation of benzyladenine (BA) treatments in somatic embryo formation and germination	Mexican Pacific Tall	BA induced formation and germination of somatic embryos when combined with 2,4-D.	Montero-Cortes et al. (2011)
Secondary somatic embryogenesis and multiplication of embryogenic callus, and exogenous plant growth regulators	Various	Increased yields in the formation of somatic embryos	Ramos et al. (2012)
Development of a photoautotrophic system using a CO_2 enrichment step inserted into a standard *in vitro* protocol for seedling production	Yellow Malayan Dwarf	Improved and more efficient protocol with reduced cost per plant	Samosir and Adkins (2014)
Optimization of sterilization for high rate callus induction from inflorescences	Matag	Reduced contamination by improving sterilization protocol. Callus induction was relatively low.	Dalila et al. (2015)
Callus initiation and regeneration from zygotic embryos sectioned into segments	Matag	Best callus initiation obtained with combination of 2,4-D and BA, and middle segment of zygotic embryo. No regeneration was observed.	Zuraida et al. (2017)

B. *In Vitro* Propagation of Zygotic Embryos

Research involving *in vitro* propagation of coconut began more than 60 years ago with studies by Cutter and Wilson (1954) on the effect of endosperm (coconut milk) during the germination, growth, and development of zygotic embryos. Ten years later, De Guzman and Del Rosario (1964) established the first zygotic embryo culture protocol for the coconut cultivar 'Makapuno'. The definition of a basal culture medium (Y3) for the 'Malayan Dwarf' coconut by Eeuwens and Blake (1976) was a major advance for the *in vitro* culture of coconut. The Y3 culture medium (Table 6.2) was superior to other culture media previously described in the literature, and provided improvements in macronutrients, especially nitrogen (ammonia), potassium, and phosphorus, as well as micronutrients, such as iron, iodine, and molybdenum (Eeuwens and Blake 1976). By the 1980s, the first studies with coconut cloning based on somatic embryogenesis were published. This included the development of organized structures from callus derived from explants of coconut (Branton and Blake 1983) and anther culture (Thanh-Tuyen and De Guzman 1983).

Table 6.2 Y3 medium composition.

Source: Adapted from Eeuwens and Blake (1976).

Compound	Medium concentration (mg l–1)
NH_4Cl	535
KCl	1492
KNO_3	2020
$NaH_2PO_4.2H_2O$	276
H_3BO_3	3.1
$Na_2MoO_4.2H_2O$	0.24
$CoCl_2.6H_2O$	0.24
KI	8.30
$NiCl_2.6H_2O$	0.024
$CaCl_2.2H_2O$	294
$MgSO_4.7H_2O$	247
$MnSO_4.H_2O$	11.2
$ZnSO_4.7H_2O$	7.2
$CuSO_4.5H_2O$	0.25
$NaEDTA.2H_2O$	37.2
$FeSO_4.7H_2O$	13.9
Thiamine	0.5
Niacin	0.05
Pyrotoxine	0.05
CaP	0.05
Biotin	0.05
Myo-inositol	100

Coconut germplasm exchange by seeds has been gradually replaced in recent years by the culture of zygotic embryos due to several factors, such as the high cost of transportation due to the size of seeds, the risk of germination during transportation, and the potential introduction of pests and diseases. Therefore, simple and efficient embryo culture techniques have been established by several research groups in various countries to collect, exchange, and conserve germplasm mid- and long-term (Engelmann and Batugal 2002). Several protocols for the *in vitro* cultivation of zygotic coconut embryos have been developed at research institutions in different countries, such as the Philippine Coconut Authority (PCA) and the University of the Philippines Los Baños (UPLB), the Central Plantation Crops Research Institute (CPCRI) in India, and the Office of Scientific and Technical Research Overseas/Research Institute for Development (ORSTOM/IRD) in France (Engelmann and Batugal 2002). Other protocols have been established with variations in culture media in view of different responses depending on genotype. The most commonly used culture medium for coconut is the Y3, with variations in concentrations of $Fe_2SO_4.7H_2O$, Na_2EDTA, growth regulators, carbon sources, activated carbon, vitamins, and other additives in the different stages of *in vitro* culture, as a function of protocol and genotype (Verdeil et al. 1994; Verdeil and Buffard-Morel 1995; Chan et al. 1998; Aké 2004, 2007; Nguyen et al. 2015; Adkins et al. 2018).

In Brazil, some studies have been published; however, the application of these techniques requires the establishment of efficient protocols for *in vitro* embryo germination and development, and acclimatization of regenerated plantlets for proper plant adaptation and performance in field conditions. The zygotic embryo culture protocol and acclimatization of 'Brazilian Green Dwarf' (BGD) coconut seedlings were developed by Lédo et al. (2007). Culture media were defined for the germination of embryos and the development of shoots and roots, in addition to the selection of substrate composed of washed sand–dry coconut shell powder (1:1) for the acclimatization of seedlings. However, in the study of propagation of different coconut accessions by *in vitro* zygotic embryo culture, Lédo et al. (2011) observed differences in the germination percentages and seedling development among accessions. From the protocol established for the BGD genotype in 2007, Embrapa Tabuleiros Costeiros has been focusing on optimization and adaptation studies for other coconut cultivars (Lédo et al. 2007, 2011).

Iron, considered an essential element in energy transformation, can influence seedling development. For BGD coconut, the presence of 27.8 mg L^{-1} of $Fe_2SO_4.7H_2O$ provides efficient germination of zygotic embryos and formation of normal seedlings. In studies with 'Yellow Malayan Dwarf' (YMD) and 'Brazilian Tall' (BT) coconuts, a $Fe_2SO_4.7H_2O$ concentration of 13.9 mg L^{-1} in the Y3 germination medium was sufficient to favor the formation of shoots and roots (Barin and Lédo 2011). Furthermore, the addition of gibberellic acid in the range from 0.5 to 2 μM in the culture medium induced a higher germination percentage and better shoot development for both the BT and BGD varieties (Barin and Lédo 2011; Santos et al. 2012).

Additional studies have been performed on factors affecting *in vitro* zygotic coconut embryo cultures. The induction of photoautotrophic growth in YMD coconut seedlings after 4–5 months of *in vitro* cultivation by reducing the sucrose concentration (43.8 mM or less), associated with CO_2 enrichment (1600 μmol mol^{-1} CO_2, in the light phase and 350 μmol mol^{-1} CO_2 in the dark phase) for two months before acclimatization, resulted in efficient production and cost reduction (Samosir and Adkins 2014). The addition of fatty acids, such as lauric acid, in the culture medium at 60–75 days of *in vitro* culture of zygotic YMD coconut embryos accelerated seedling growth and development (Lopez-Villalobos et al. 2011).

In addition to adjustments in the *in vitro* stages, acclimatization is essential to the success of *in vitro* techniques to reduce potential losses and to increase survival. Few studies have been conducted to improve acclimatization techniques and yields. Comparing three *ex vitro* acclimatization techniques for 'Laguna Tall' coconut seedlings, Magdalita et al. (2010) observed that *ex vitro* survival was significantly improved when seedlings with two to three leaves were acclimated in either a plastic tent (81.7%) or a wet chamber (82.2%) when compared to acclimatization under mist (62.5%).

An excellent example of the application of the zygotic embryo culture technique for the large-scale production of coconut palm seedlings is that used by the Makapuno Laboratory Network in the Philippines. From a protocol established in the 1960s by De Guzman and Del Rosario (1964) due to the low seed germination of the 'Makapuno' variety in the field, a large-scale multiplication program was established in 1992 and is currently composed of six satellite laboratories (Lédo et al. 2019). The 'Makapuno' cultivar embryo culture protocol was improved in 2003, aiming to reduce costs by reducing the sucrose and activated carbon concentrations and the non-addition of myo-inositol and the plant growth regulators naphthalene acetic acid and benzylaminopurine (Areza-Ubaldo et al. 2003). Phenotypes with high value, such as 'Makapuno' and 'Lono', have been widely propagated using tissue culture techniques (Angeles et al. 2018). These results reinforce the fact that the establishment of a standard protocol for the *in vitro* culture of zygotic embryos of different genotypes cannot be routinely applied to all coconut cultivars and selections, and constant adjustments are necessary.

The embryo culture technique has also been used for the exchange of genetic resources to minimize the risks of dissemination of pests. Introduction by tissue culture is relatively safe; however, elimination of phytopathogens and maintenance of cultures free from contamination by bacteria, fungi, and micro-arthropods have been highlighted (Cassells 2012). Recent guidelines from Biodiversity International and FAO recommend the exchange of endosperm discs containing the zygotic embryo or the use of *in vitro* zygotic embryos for the safe movement of coconut germplasm (Cueto et al. 2012).

C. *In Vitro* Propagation by Somatic Embryogenesis

As vegetative propagation of coconut seems to be accomplished only by *in vitro* techniques, the development of somatic (non-zygotic) embryos becomes an alternative method of interest. Somatic embryos can develop from a variety of plant cells and tissues at different stages of development, and somatic embryos can form directly on explant tissues, or indirectly through a callus phase.

For clonal propagation of coconut, somatic embryogenesis continues to be one of the most promising techniques. Somatic embryogenesis also allows the introduction of genes through genetic transformation, which could confer resistance to pests and diseases. However, somatic embryogenesis still needs improvements in order to become an efficient *in vitro* technique.

The first studies of coconut somatic embryogenesis were performed in the 1980s using zygotic embryos, immature leaves, and inflorescences as explants (Gupta et al. 1984; Karunaratne and Peruyaperuma 1989). Due to the high efficiency of the plumule (the primary bud comprising leaves and the epicotyl) in the *in vitro* propagation process by somatic embryogenesis, this tissue has been used to propagate superior dwarf coconut genotypes that present high self-fertilization rates. Promising results have been reported for the 'Green Malayan Dwarf' (GMD) coconut (Pérez-Nuñez et al. 2006; Sáenz et al. 2010, 2018). Through histological investigation of tissue viability, Pérez-Nuñez et al. (2006) found that the insertion of repetitive callogenesis cycles and secondary somatic embryogenesis allowed an increase in the efficiency of the technique, enabling the commercial propagation of certain genotypes from plumules. However, to ensure clonal propagation, especially in tall coconut genotypes and their hybrids, somatic embryogenesis must be able to be achieved from other tissues. Different explant tissues have been reported with successful results, including inflorescences (Verdeil et al. 1994), ovaries and anthers (Perera et al. 2009, 2010; Sandoval-Cancino et al. 2016; Bandupriya 2017), the latter producing haploid plants that have significance in breeding programs.

Despite the positive results reported by Pérez-Núñez et al. (2006), whereby secondary embryogenesis was obtained in three cycles showing a 50 000-fold increase in efficiency, most studies still present unsatisfactory results for large-scale production, and new studies are being developed in several countries to improve technique efficiency. Researchers from eight countries, at the IV Workshop on Somatic Embryogenesis for Rapid Coconut Multiplication at the International Coconut Biodiversity for Prosperity Conference held in India in 2010, identified some of the problems as being variable results from plant to plant, showing that the method is genotype dependent; the effects of seasonality on the collection of explants or the stage of development of the explant; the source of activated carbon in the culture medium; and the formation of fused embryos and premature roots, making somatic embryos more difficult to germinate (Thomas et al. 2010). In addition, tissue culture may induce genetic and/or epigenetic changes in the genome, resulting in plant material that is not true to type (Angeles et al. 2018). Several constraints have been addressed for developing a successful protocol for coconut micropropagation, with considerable progress achieved to date (Bandupriya 2017). In any plant tissue culture protocol, selection of a suitable starting plant material (explant) is a major factor that governs successful plant regeneration. This explant, together with the different culture media and plant growth regulators that are used, are important factors that determine the potential genetic stability of the regenerated plants or the induction of genetic variation (somaclonal variation) and resulting abnormalities in the regenerated plants (Angeles et al. 2018).

Some advances have been obtained with the addition of activated charcoal, gibberellic acid, lauric acid, and other compounds to the culture medium. For example, Sáenz et al. (2010) studied the effects of different brands of activated charcoal and particle size on the induction of somatic embryogenesis of mature GMD coconut plumules and found that particles smaller than 0.38 µm induced a higher percentage of embryogenic calluses (70%). The addition of 0.5 µM gibberellic acid in the culture medium increased the number of embryogenic calluses, as well as the number of somatic embryos per callus, and the germination of embryos from 'Malayan Dwarf' when it was initiated from plumule explants (Montero-Cortes et al. 2010b).

Histological studies of *in vitro* coconut culture have been developed at CIRAD (Agricultural Research for Development) in France (Verdeil and Buffard-Morel 1995). The technique has since been improved and has contributed to the elucidation of the processes associated with somatic embryogenesis. For example, morphological and histological evaluations were performed in plumule explants of 'Malayan Dwarf' cultured *in vitro*, with observations of development from callus to somatic embryo and shoot formation. This provided an integrated view into the somatic embryogenesis process in coconut, which can contribute to the further improvement of this technique (Sáenz et al. 2006). Histological and biochemical analyses of 'Kopyor' coconut plantlets regenerated from embryogenic cultures were also performed during *ex vitro* rooting and nursery stages to verify improvement in morphological characteristics that led to higher rates of survival and enhanced plant development (Sisunandar et al. 2018). The expression of diverse genes involved in the process of somatic embryogenesis has also been studied in an attempt to further improve the production of somatic embryos and the regeneration of plants. For example, studies on the expression of CnSERK, CnCDKA, and KNOTTED genes in the process of somatic embryogenesis of the 'Malayan Dwarf' variety were conducted by Pérez-Nuñez et al. (2009) and Montero-Cortes et al. (2010a,b). The expression of the CnCDKA gene was found to be higher during the embryogenic callus formation phase, coinciding with its *in situ* location at the meristematic cell centers from which the somatic embryos developed. Gene expression progressively reduced with embryo development. However, according to Nguyen et al. (2015), because of the limited knowledge of the molecular mechanisms that control the expression of somatic embryogenesis in coconut, additional research is needed to improve the yield of somatic embryos.

The slow *in vitro* development of cultures and low *ex vitro* vigor of plantlets, and the general recalcitrant characteristics of coconut, account for the low efficiency of the current *in vitro* clonal propagation systems when compared to other species. In addition, most studies previously cited use semi-solid media, such as the Y3 medium that is solidified with either agar or other gelling agents. Consequently, different approaches to *in vitro* cultures should be evaluated, including the use of liquid cultures, as in the case of oil palm (*Elaeis guineenses*) where Marbun et al. (2015) employed temporary immersion bioreactors to increase the multiplication capacity and efficiency of somatic embryogenesis in MS liquid culture medium (Murashige and Skoog 1962), a system that allows biofactories in some parts of the world to produce seedlings on a large-scale basis.

III. BIOREACTOR TECHNOLOGY

A. Concept

The concept of bioreactor technology involves the automated *in vitro* culture of cells, tissues, and organs in liquid medium. This system allows significant reduction of labor and production costs while providing faster and more efficient large-scale *in vitro* propagation of plants, compared to the conventional *in vitro* system using semi-solid agar-based media (Ziv 2000, 2005). The temporary immersion bioreactor system involves controlling the frequency (number of immersions per day) and duration of each immersion of explants in the liquid medium. These systems have been efficiently used to improve the quality and *in vitro* multiplication of several species, such as banana, carrot, coffee, potato, and rubber (Alvard et al. 1993; Akita and Takayama 1994; Archambault et al. 1995; Teisson and Alvard 1995; Etienne et al. 1997). Etienne and Berthouly (2002) described several types of temporary immersion bioreactor systems that have been successfully used in the processes of *in vitro* multiplication of buds, microcuttings, microtubers, and somatic embryos in several species, including grape (*Vitis vinifera* L.), orchids (*Potinara* spp.), date palm (*Phoenix dactylifera* L.), radiata pine (*Pinus radiata* D. Don), banana (*Musa acuminata* L.), potato (*Solanum tuberosum* L.), coffee (*Coffea arabica* L.), rubber tree (*Hevea brasiliensis* Wild. ex A. Juss.), sugarcane (*Saccharum* spp.), and pineapple (*Ananas comosus* (L.) Merr.), among others. In addition to reducing labor and production costs, other advantages of bioreactors include less space required for production, better plant quality and vigor, increased productivity, and overall efficiency (Etienne and Berthouly 2002).

B. Bioreactor Technology for Coconut Production

In order to further improve *in vitro* coconut production using somatic embryogenesis as well as other *in vitro* techniques, a new temporary immersion system (TIS) has been evaluated with some preliminary studies being performed at the Tropical Research and Education Center (TREC) at the University of Florida. The SETIS^{TM} temporary immersion bioreactor system is a novel concept within TIS technology, using the twin-bottles principle of two connected vessels: the plant material and growth media vessels (Vervit 2020).

This technology has been optimized to provide an efficient system for large-scale clonal propagation of several species. The bioreactors receive selective lighting from specific light-emitting diodes (LEDs), which can improve *in vitro* productivity through enhanced photosynthesis. The SETIS^{TM} system has been successfully used for banana, sugarcane, and yam, among others (Balogun et al. 2018; Bello-Bello et al. 2019; Silva et al. 2020). At TREC, this system has been successfully tested for the micropropagation of banana, sugarcane, bromeliads, and orchids (W.A. Vendrame, unpubl.). The SETIS^{TM} bioreactors could, therefore, be a feasible platform for large-scale *in vitro* propagation of coconut. In 2017, TREC initiated studies to improve the protocol for coconut production aimed at *in vitro* mass multiplication of the variety 'Fiji Dwarf', whose genotype has demonstrated tolerance to lethal yellowing. Preliminary results, although promising, show that the slow growth of *in vitro* cultures and the recalcitrant nature of coconut continue to be some of the limitations to the full development of a successful protocol for clonal propagation of coconut (W.A. Vendrame, unpubl.). International collaboration as a means to promote improvement in the development of such *in vitro* protocols, including the use of TIS bioreactors, should be fostered.

IV. CRYOPRESERVATION

A. Concept

Conservation of plant species can be achieved using two biotechnology approaches: *in vitro* culture and cryopreservation. *In vitro* conservation allows the maintenance of many genotypes safeguarded from diseases and pests (Uyoh et al. 2003). However, conservation of *in vitro* cultures is limited to short- and medium-term storage. It requires constant sub-culturing into fresh culture medium, which could possibly lead to the loss of genotypes due to contamination or to human error during the process (Engelmann 1991; Escobar et al. 1997; Charoensub et al. 1999). Another limitation is the potential for genetic instability and variation due to the maintenance of species under *in vitro* conditions over long periods (Rao 2004).

Cryopreservation is a method of conserving plant cells, tissues, and organs in liquid nitrogen at ultra-low temperature (−196°C in liquid nitrogen or −150°C in nitrogen's vapor phase), with low risk of genetic variations or physiological changes over long-term storage periods (Gonzalez-Benito and Perez 1997; Reed 2008; Welewanni et al. 2017). Cryopreservation can also be used for cryoselection, which involves selection through freezing of samples with special properties, and also for cryotherapy, whereby cryopreservation of a virus-infected plant's apex can assist with the elimination of the virus (Engelmann 2004). In addition, cryopreservation allows the preservation of shoot tips and meristems to conserve the germplasm of species that propagate vegetatively, to maintain clones of elite genetic stocks, for seeds of recalcitrant species, and for plants that are important for economic production and conservation through micropropagation (Benson and Harding 2012).

Although not suitable for field maintenance, cryopreservation provides a backup against the loss of valuable genotypes that may occur under field conditions. It is reliable for maintaining viability of germplasm under stable and low-cost conditions, and safe from diseases or environmental damage (Harvengt et al. 2004).

A variety of tissues and organs can be cryopreserved and stored, including shoot tips, somatic and zygotic embryos, whole seeds, pollen, anthers, and buds. For complex organs, such as embryos and shoot apices, there are new cryopreservation techniques that are more appropriate and can be used in any basic tissue culture laboratory (Rao 2004). These techniques expand the applicability of cryopreservation to a wide range of plant materials, including non-cold hardy tropical plant species (Hirai and Sakai 2003). Among the techniques reported for cryopreservation, seven vitrification-based procedures have been developed and are the most commonly utilized, including encapsulation–dehydration, vitrification, encapsulation–vitrification, dehydration, pregrowth, pregrowth–dehydration, and droplet freezing (Engelmann 2000; Panis et al. 2005). Some of these techniques have been applied to coconut cryopreservation protocols, as reported in Section IV.B.

B. Cryopreservation of Coconut

Field gene banks are the traditional way of maintaining coconut germplasm collections, since their seeds are too large and, therefore, not suitable for conventional storage (Engelmann et al. 2005). The first studies on cryopreservation of coconut date back to the 1980s using immature zygotic embryos and a technique that included chemical dehydration followed by slow freezing (Bajaj 1984). Later studies of cryopreservation focused on mature zygotic embryos (Assy-Bah and Engelmann 1992) where the vitrification technique was used with mature zygotic embryos of four varieties of coconut: the hybrid PB 121 ('Malayan Yellow Dwarf' × 'West African Tall'), 'Cameroon Red Dwarf', 'Indian Tall', and 'Rennel Tall'. More recently, new protocols have been developed and refined showing positive and promising results for the cryopreservation of coconut with a range of tissues, including zygotic embryos, plumules, and pollen, using different techniques (Karun and Sajini 2010; Sajini et al. 2011; Cueto et al. 2014; Machado et al. 2014). Given that the *in vitro* culture of zygotic embryos has become routine practice (Engelmann et al. 2005), the small size of tissues opens up the possibility of long-term storage of coconut germplasm using cryopreservation techniques. However, cryopreservation of embryos, plumules, or pollen has some limitations, as each protocol is specific to a single genotype (Bourdeix and Prades 2017). In addition, abnormalities in seedlings derived from cryopreserved material have been reported (Welewanni et al. 2017). Consequently, protocols need to be developed for each coconut variety separately.

Advances in cryopreservation of coconut over the previous 10 years are listed in Table 6.3.

Table 6.3 Cryopreservation techniques applied to coconut and results obtained.

Technique	**Cultivar**	**Results**	**Reference**
Immature and mature embryos, plumules cryopreserved after dehydration, encapsulation–dehydration, and encapsulation–vitrification	Various	Low survival and abnormalities in further development of immature embryos; high survival of mature embryos submitted to dehydration pretreatment; low survival of plumules	Engelmann at al. (2005)
Cryopreservation of pollen using desiccation	West Coast Tall Chowghat Orange Dwarf	Best germination on medium containing sucrose and boric acid under high humidity	Karun et al. (2006)
Effect of sucrose and abscisic acid (ABA) on plumule cryopreservation by encapsulation–dehydration	Sri Lanka Tall	Pretreatment with ABA in the presence of sucrose improved survival and recovery of cryopreserved encapsulated plumules.	Bandupriya et al. (2007)
Cryopreservation of plumules by encapsulation–dehydration	Malayan Yellow Dwarf	Optimal conditions of sucrose concentration combined with dehydration pretreatment duration improved regrowth, although regrowth percentage was low (29%).	N'Nan et al. (2008)
Effect of transport/store conditions on cryopreservation of encapsulated plumules using sucrose and dehydration	Sri Lanka Tall	Improved recovery (40%) in plumules pretreated with sucrose	Bandupriya et al. (2010)
Review of cryopreservation methods: air desiccation, pre-growth desiccation, dehydration–encapsulation, and vitrification; pollen cryopreservation	Various	Variable results on the effectiveness of various methods for successful recovery of plantlets. A methodology for efficient pollen cryopreservation is presented.	Karun and Sajini (2010)
The influence of zygotic embryo maturity on recovery after cryopreservation and genetic fidelity of seedlings germinated	Nias Yellow Dwarf Takome Tall	Zygotic embryos isolated from 11-month-old fruits returned the highest number of normal seedlings (28%) when compared to younger or older fruits. No morphological, cytological, or genetic abnormalities were observed.	Sisunandar et al. (2010a)

Review of cryopreservation methods: air desiccation, pre-growth desiccation, dehydration–encapsulation, and vitrification; pollen cryopreservation	Various	Variable results on the effectiveness of various methods for successful recovery of plantlets. A methodology for efficient pollen cryopreservation is presented.	Karun and Sajini (2010)
The influence of zygotic embryo maturity on recovery after cryopreservation and genetic fidelity of seedlings germinated	Nias Yellow Dwarf Takome Tall	Zygotic embryos isolated from 11-month-old fruits returned the highest number of normal seedlings (28%) when compared to younger or older fruits. No morphological, cytological, or genetic abnormalities were observed.	Sisunandar et al. (2010a)
Genetic fidelity of seedlings recovered from cryopreserved zygotic embryos using rapid dehydration, rapid freezing, rapid thawing, and *in vitro* recovery, followed by acclimatization	Nias Yellow DwarfNias Green Dwarf Sagerat Orange DwarfTakome Tall	Morphological, cytological, and molecular comparisons were performed, but no differences were observed.	Sisunandar et al. (2010b)
Rapid dehydration step prior to cryopreservation	Malayan Yellow Dwarf	A rapid (8 h) dehydration followed by rapid cooling was the most efficient protocol, but best recovery was achieved when a rapid warming step and an optimized *in vitro* step were used.	Sisunandar et al. (2010c)
Summary of studies performed, including plumule cryopreservation by dehydration–encapsulation	Malayan Yellow Dwarf Nain Jaune Malais Sri Lanka Tall	Encapsulation–dehydration returned a low recovery rate (20%). ABA added to pretreatment medium prior to dehydration and freezing improved recovery to 40%.	Malaurie et al. (2011)
Preculture conditions, vitrification, and unloading solutions for survival and regeneration of cryopreserved zygotic embryos	West Coast Tall	The most effective protocol (70–80% survival) for cryopreservation was preculture of embryos in medium with 0.6 M sucrose, PVS3 treatment, rapid cooling and rewarming, and unloading in 1.2 M sucrose liquid medium.	Sajini et al. (2011)
Assessment of viability of cryopreserved zygotic embryos by electrolytic conductivity and potassium leaching	Brazilian Jiqui Green Dwarf (AVeJBr)	Pre-treatment of zygotic embryos with sucrose and glycerol increased viability in electrolytic conductivity and potassium leaching tests.	Gomes-Copeland et al. (2012)
Simplified cryopreservation protocol for mature zygotic embryos using one-step dehydration combined with glucose and silica gel	Niew Leka Dwarf Malayan Yellow Dwarf Sri Lanka Green Dwarf Cameroon Red Dwarf Brazil Green Dwarf Vanuatu Tall Tagnanan Tall Sri Lanka Tall Panama Tall West African Tall	Germination of embryos recovered from cryopreservation ranged between 13.3% and 74.7% among the different varieties.	N´Nan et al. (2012)

Effect of different transportation methods on the cryopreservation of plumules	Sri Lanka Tall	Plumules excised from embryos transported or stored in solidified agar medium and pretreated with sucrose had 71.8% survival and 56% recovery. Supplementation with ABA increased survival to 77.5% and recovery to 65%.	Bandupriya et al. (2014)
Alternative cryopreservation techniques; droplet–vitrification of plumular cube, vitrification of meristematic pole disc, vacuum-assisted vitrification of intact embryos, and preculture–desiccation of intact embryos	Malayan Yellow Dwarf	Bacterial contamination during preculture was a problem that needs to be addressed, progressive sucrose preculture provides desiccation tolerance, and preculture–desiccation of intact embryos and vitrification of plumular cubes are recommended for routine cryobanking of coconut collections.	Cueto et al. (2014)
Pollen cryopreservation by direct immersion in liquid nitrogen	West Coast Tall ChowghatOrange Dwarf	Pollen germination ranged from 26 to 29% for COD and 28–32% for WCT cryopreserved pollen. Seed set was normal for both varieties for pollen cryostored for 6 months and 4 years.	Karun et al. (2014)
Pollen viability under storage at different low temperatures: refrigerator (−4°C), freezer (−20°C), ultra–freezer (−80°C), and liquid nitrogen (−196°C)	BrazilianGreen DwarfBrazilian Tall Cameroon Red Dwarf	Best viability was achieved at −4°C, −80°C, and −196°C for Brazilian Tall; −20°C and −196°C for Cameroon Red Dwarf; and −20°C, −80°C, and −196°C for Brazilian Green Dwarf.	Machado et al. (2014)
Ultrastructural changes during cryopreservation as affecting survival of plumules and embryos using the dehydration–encapsulation protocol	Malayan Yellow Dwarf	Three types of damages identified in cells of seed samples affected by dehydration and freezing, and two of them identified as irreversible and responsible for lack of regrowth of samples.	N'Nan et al. (2014a)
Dehydration–encapsulation of plumules excised from zygotic embryos	Brazilian Green Dwarf Cameroon Red DwarfMalayanYellow Dwarf Panama Tall Tagnanan Tall Vanuatu Tall	Survival varied from 6 to 66% and recovery from 0 to 24% after 2 and 7 months of culture *in vitro*, respectively, and depended on variety.	N´Nan et al. (2014b)
Effect of embryo maturity using a four-step protocol: rapid desiccation, rapid freezing, rapid thawing, and recovery and acclimatization for 4 months in greenhouse	Nias Yellow Dwarf Tebing Tinggi DwarfTakome Tall Bali Tall	Embryos isolated from 11-month-old fruits returned the highest germination (~40%) and highest percentage of normal seedlings (~ 30%) after cryopreservation as compared to younger embryos.	Sisunandar et al. (2014)
Dehydration and cryoprotection methods for mature zygotic embryos	Brazilian Green Dwarf	Pretreatment of embryos with sucrose and glycerol returned highest viability (75–80%) by tetrazolium test.	Gomes-Copeland et al. (2015)
Rewarming procedures and recovery media for zygotic embryos cryopreserved by vitrification	Brazilian Green Dwarf	No significant differences observed for rewarming procedures. Recovery medium Y3 + GA_3 + activated charcoal + gelrite returned 72.5% regeneration.	Lédo et al. (2018)
Techniques to control bacterial contamination of zygotic embryos for cryopreservation protocols	Malayan Yellow DwarfWestern African Tall	Endogenous bacteria were identified, most considered non-pathogenic. Surface sterilization of embryos combined with antibiotics was successful in reducing contamination. The preculture–desiccation procedure resulted in 90% germination and 35% regeneration.	Kim et al. (2019)
Cryopreservation of meristem tips from *in vitro* shoots using droplet–vitrification	Malayan Yellow DwarfWestern African Tall	Over 50% of the meristem tips regenerated into shoot tips after cryopreservation.	Wilms et al. (2019)
Effect of vitrification solutions and exposure time on cryopreservation of plumules by droplet vitrification	Brazilian Green Dwarf	Exposure time of 30 min, regardless of vitrification solution (PVS2 or PVS3), resulted in 30% embryogenic callus formation after cryopreservation. Survival was over 80%.	Lédo et al. (2020)

Among the coconut explants available for cryopreservation, zygotic embryos and pollen have been shown to be the most suitable for long-term conservation. However, the parent's genetic identity would not be maintained as a whole, particularly for tall genotypes, as cryopreservation of pollen or zygotic embryos only allows the preservation of the progeny of a single genotype (Bourdeix and Prades 2017). Cryopreservation of pollen can be used for long-term storage from varieties with unique characteristics for breeding programs, for the exchange of germplasm, and to allow pollination over a long period of time (Karun et al. 2006; Karun and Sajini 2010). There are some reports relating to the cryopreservation of zygotic embryos from accessions collected in Brazil. For example, Machado et al. (2014) observed the viability of cryopreserved pollen from the 'Brazilian Tall', 'Brazilian Green Dwarf', and 'Red Cameroon Dwarf' coconut varieties. Pollen from 'Brazilian Tall' showed best viability when stored in refrigerator (−4°C), freezer (−80°C), and liquid nitrogen (−196°C) conditions. Pollen from 'Red Cameroon Dwarf' had best viability when stored under freezer (−20°C) and liquid nitrogen (−196°C), while for 'Brazilian Green Dwarf', freezer (−20°C and −80°C) and liquid nitrogen (−196°C) conditions promoted the best viability. Preliminary studies have been published to evaluate the viability of cryopreserved 'Brazilian Green Dwarf' pollen by means of electrolytic conductivity and potassium leaching tests (Gomes-Copeland et al. 2012, 2015); however, no regeneration was achieved after cryopreservation.

Recent studies have shown the feasibility of the vitrification and droplet–vitrification techniques for the cryopreservation of zygotic embryos of 'Brazilian Green Dwarf' (Lédo et al. 2018, 2020). However, there is a need for further studies that address adjustments to, and the adequacy of, current protocols as they relate to a specific variety and/or to accessions, as results appear to be genotype dependent and regeneration continues to be a major limitation. For example, studies with regeneration of cryopreserved 'Malayan Yellow Dwarf' coconut plumules submitted to encapsulation–dehydration returned only 20% survival (N'Nan et al. 2008).

Regeneration techniques using somatic embryogenesis after recovery from cryopreservation are important in relation to breeding and conservation of genetic resources, because they allow a higher number of plants to be obtained (Nguyen et al. 2015). Hence Adkins et al. (2018) also claim that the application of cryopreservation to somatic embryogenic cell cultures is important, considering that successful preservation would enable the production of many more coconut plants from one initial explant. A vitrification technique that involves the cryoprotection of explants was reported for various coconut genotypes, with good performance of vitrification solution 3 (PVS3), according to Nishizawa et al. (1993). Sajini et al. (2011) subsequently reported that PVS1, PVS2, and PVS4 vitrification solutions were harmful to zygotic embryos, while PVS3 was not.

For over 30 years, protocols for cryopreservation of coconut tissues and organs have been evaluated with mixed results (Table 6.3). There is limited availability of explants from the plant materials that are currently used in cryopreservation, such as zygotic embryos or plumules. Consequently, other alternatives should be considered, such as inflorescences or somatic embryos. The successful development of cryopreservation protocols for coconut is of relevance for breeding and genetic improvement, as well as for conservation programs.

V. SUMMARY AND CONCLUSIONS

The achievements reported to date for *in vitro* propagation of coconut have faced several limitations, such as the recalcitrant nature of the species, the slow growth and consequent development of cultures *in vitro*, the diversity of responses *in vitro* observed due to the various types of explants that have been used, and the genetic variation among the different varieties that are available. The main techniques that seem feasible for *in vitro* propagation of coconut include zygotic embryo culture, plumule culture, and somatic embryogenesis. There is also some reported success in regard to the regeneration and survival of plantlets. Additional studies should focus on further refinement and standardization of protocols for embryos and plumules, and should evaluate such improved protocols with various genotypes before they can be applied to large-scale clonal propagation and application for genebanks.

Cryopreservation offers considerable potential for the long-term conservation of coconut genetic resources and represents an important additional technique for allowing conservation of genes. The focus of future studies should be on further development and refinement of an appropriate technique that could be applied to several genotypes and with higher rates of recovery and survival, as well as the regeneration of plantlets.

The implementation of coordinated and collaborative international efforts is essential to promote exchange of information and to accelerate the development and improvement of *in vitro* protocols for the clonal propagation and cryopreservation of coconut.

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