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REVIEW

Tissue culture and associated biotechnological interventions for the improvement of coconut (*Cocos nucifera* L.): a review

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Abstract

Main conclusion **The present review discusses not only advances in coconut tissue culture and associated biotechnological interventions but also future research directions toward the resilience of this important palm crop.**

Coconut (*Cocos nucifera* L.) is commonly known as the 'tree of life'. Every component of the palm can be used to produce items of value and many can be converted into industrial products. 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 of seedlings for replanting remains an issue; however, tissue culture and other biotechnological interventions are 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; (iii) homozygote production via anther culture; (iv) germplasm conservation via cryopreservation; and (v) genetic transformation. Recently other advances have revealed possible new ways to improve these protocols. Although effective embryo culture and cryopreservation are now possible, the limited frequency of conversion of somatic embryos to ex vitro seedlings still prevents the large-scale clonal propagation of coconut. This review illustrates how our knowledge of tissue culture and associated biotechnological interventions in coconut has so far developed. Further improvement of protocols and their application to a wider range of germplasm will continue to open up new horizons for the collection, conservation, breeding and productivity of coconut.

Keywords Biotechnology · Coconut · Cryopreservation · Embryo culture · Germplasm conservation · Somatic embryogenesis

Abbreviations

BM72	Karunaratne and Periyapperuma (1989) medium
ABA	Abscisic acid
AC	Activated charcoal
BAP	6-Benzylaminopurine
GA_3	Gibberellic acid
2iP	2-Isopentyl adenine
2,4-D	2,4-Dichlorophenoxyacetic acid

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PGR(s)	Plant growth regulator(s)
TDZ	Thidiazuron
SE	Somatic embryogenesis
Y3	Eeuwens (1976) basal medium

Introduction

Coconut (*Cocos nucifera* L.) is one of the most important palm crops in the world, being primarily cultivated on about 12 million hectares of land in tropical and subtropical coastal lowlands (FAOSTAT 2013). Around 10 million farmers and their families are highly dependent upon the produce from this palm, and many others in rural and semi-urban locations own a small number of coconut palms that contribute to their livelihoods (Rethinam 2006). Popularly known as the 'tree of life', each part of the palm can produce items that have community value as well as providing a range of commercial and industrial products. These products include those with nutritional and medicinal properties (Foale 2003; Perera et al. 2009a). The mature kernel (solid endosperm) contains edible fibre, protein, lipid and inorganic minerals. Fruit-derived products include beverage, fresh kernel and milk (an emulsion extracted from the kernel) that are consumed locally (Lim 2012), while refined products, including virgin oil, shell charcoal, husk fibre and cortex (cocopeat for potting mixtures), are exported. Virgin oil (extracted at low temperature) possesses potent antioxidant (Marina et al. 2009) and antimicrobial properties (Chakraborty and Mitra 2008), and has potential anticancer actions (Koschek et al. 2007). Therapeutic components found in either fresh or processed coconut products have been reported to be effective in the prevention and treatment of cardiovascular disease, hypertension, diabetes, obesity, ulcers and hormonal imbalance in postmenopausal women (Ross 2005; Lim 2012). In addition, coconut wood recovered from the older portion of the trunk provides robust timber components that are used in the production of furniture, and handicrafts as well as building materials.

Coconut field cultivation faces many challenges, including the instability of the market for its traditional products. Productivity is affected by 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). Rapid spread of major pests and incurable diseases, such as phytoplasma-caused lethal yellowing and viroid-caused cadang-cadang, has resulted in a significant fall in the land area planted to coconut (Cordova et al. 2003; Harrison and Jones 2003; Lee 2013). Although there has been a breeding program aiming to increase oil yield in many countries, the general expectation of achieving 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 general and robust solution for increasing productivity (Thanh-Tuyen and De Guzman 1983; Batugal et al. 2009).

It has been 60 years now since the first in vitro culture study was carried out on coconut, when its own liquid endosperm was used as the culture medium to support embryo germination (Cutter and Wilson 1954). Since then the landmark research achievements in coconut tissue culture have not been attained as rapidly as they have for many other plant species (Fig. 1). Some of the reasons often cited for the slow advancement in tissue culture include the heterogeneous response of diverse coconut explanted tissues, the slow growth of these explanted tissues in vitro, and their further lack of vigour when planted ex vitro (Fernando et al. 2010). Nonetheless, tissue culture and associated biotechnological interventions, which aid the breeding and the development of coconut as a multi-use crop, have been achieved in the areas of: (i) embryo culture; (ii) clonal propagation via somatic embryogenesis (SE); (iii) homozygote production via anther culture; (iv) germplasm conservation via cryopreservation; and to a lesser extent (v) genetic transformation (Fig. 1). Significant achievements in zygotic embryo culture have now paved the way for the collection of rare germplasm and the rapid production of tissue culture-derived seedlings (Rillo 1998). This technique has been improved recently to deliver greater success across a wider range of cultivars (Samosir and Adkins 2014). Zygotic plumular tissue can now be used to achieve clonal propagation via SE (Pérez-Núñez et al. 2006). However, difficulties in this process are still preventing the establishment of an affordable and universal protocol for the production of plantlets on a large scale. Regarding production of homozygous inbred lines, Perera et al. (2008b) have reported the production of doubled haploid plants via anther-derived embryogenesis. Furthermore, it is now possible to cryopreserve, and then recover coconut embryos for in long-term conservation programs, without inducing morphological, cytological or molecular changes in the regenerated plants (Sisunandar et al. 2010a). Although genetic transformation in coconut has been attempted (Samosir et al. 1998; Andrade-Torres et al. 2011), achievements have been quite limited to date.

This review aims to provide a comprehensive summary of the advances to date in tissue culture and the associated biotechnological approaches applied to coconut, a historically recalcitrant species. Through a critical analysis of past notable achievements, we hope to assist researchers to refine approaches for improving the quality and resilience of the 'tree of life'.

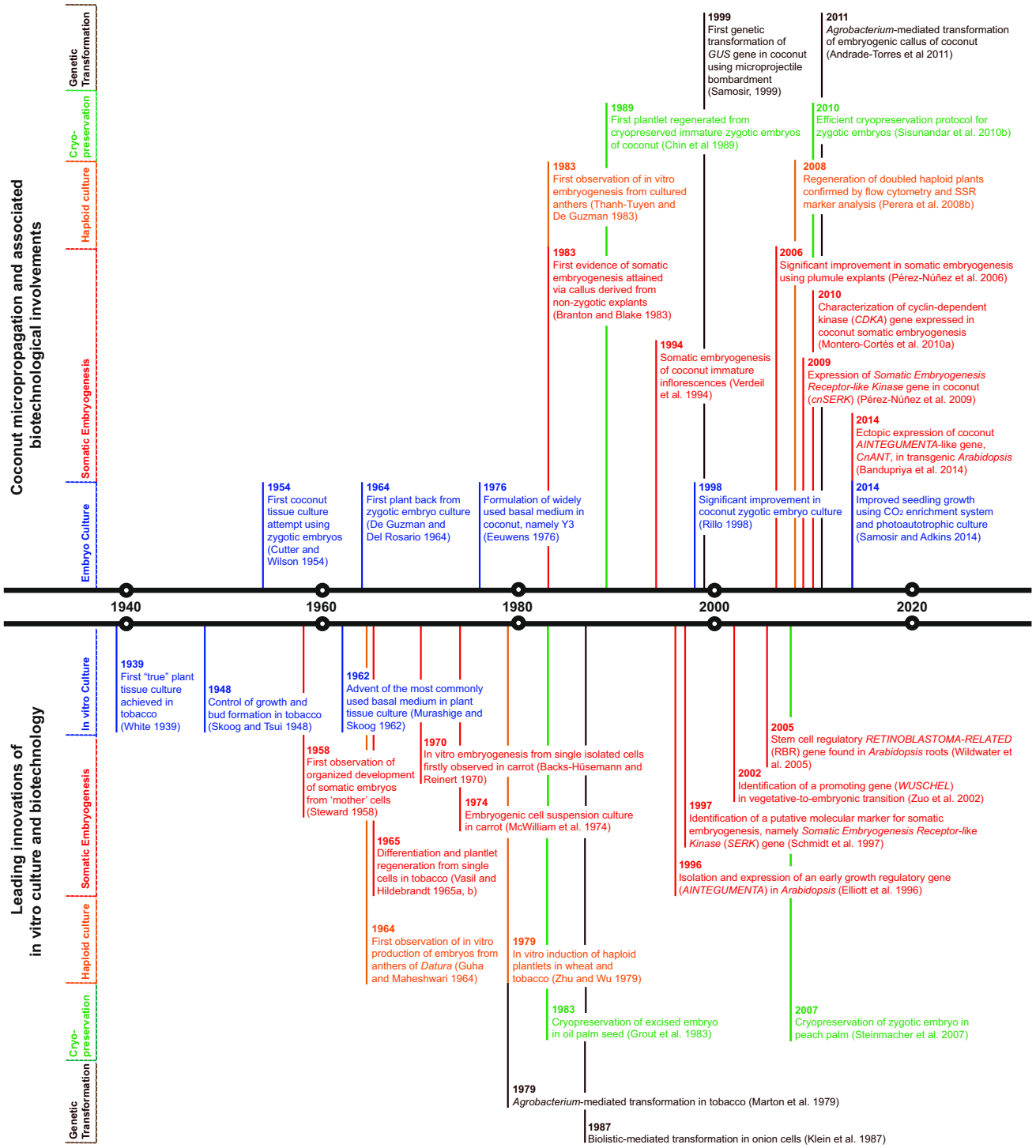

Fig. 1 Chronology of research in coconut micropropagation and biotechnological interventions in parallel with other plant examples

Embryo culture

Early attempts to isolate and culture zygotic embryos from coconut fruit date back to the 1950s (Cutter and Wilson 1954). However, it was a further decade before in vitro plantlets could be regenerated and converted into viable plants (De Guzman and Del Rosario 1964). In all studies since this time, zygotic embryos harvested 10–14 months post-pollination have been used for the establishment of cultures, with the greatest ex vitro success coming from embryos taken at 12 months (Table 1). The nutritional requirements used for embryo germination and plantlet

Table 1 In vitro culture of coconut zygotic embryos

Embryo origin/variety/cultivar[a]	Fruit maturity[b]	Culture media & PGRs (optimal combinations reported)[c]	Culture conditions[d]	Responses/results[e]	References
Unknown	Mature	Young CW (filter filtered) + Agar (1.5 %)	I: 90 µmol m^{-2} s^{-1} T: 25 °C	EG	Cutter and Wilson (1954)
Makapuno	Mature	White + CW (25 %) + Agar (1.2 %)	I: Dark (3 weeks) then light condition T: 25 °C	EG, PD	De Guzman and Del Rosario (1964)
MYD × WAT	Mature (11–12 mpp)	MS + MW Vit + Sucrose (6 %) + AC (0.2 %) + Agar (0.8 %)	I: Dark (3 weeks) then 12:12 h light:dark (55 µmol m^{-2} s^{-1}) T: 27 °C	EG, PD, PA	Assy-Bah et al. (1989)
Tonga and the Solomon Islands	Mature	MS + MW Vit + Sucrose (6 %) + AC (0.2 %) + Agar (0.8 %) for germination ↓ Y3 + NAA (200 µM) + Sucrose (4 %) + AC (0.2 %) + Agar (0.8 %) for plantlet growth	I: 16:8 h light:dark (90 µmol m^{-2} s^{-1}) T: 30–31 °C	EG, PD, AR	Ashburner et al. (1993)
MYD	Mature	Modified MS liquid + MW Vit + Sucrose (6 %) + AC (0.2 %)	I: Dark (8 weeks) then light (45 ± 5 µmol m^{-2} s^{-1}) T: 27 ± 1 °C	EG, PD, PA	Triques et al. (1997)
LT	Mature (10–11 mpp)	Y3 liquid for germination ↓ Y3 liquid + IBA or NAA (50 µM) + Sucrose (4.5 %) +AC (0.25 %) for plantlet growth	I: 9:15 light:dark (75–90 µmol $m^{-2}s^{-1}$) T: 28–30 °C	EG, PD, PA	Rillo (1998)
MGD	Mature (12–14 mpp)	Modified Y3 + AC (0.25 %) ± Gelrite (0.3 %)	I: Dark (1 week) then 16:8 h light:dark (45–60 µmol m^{-2} s^{-1}) T: 27 ± 2 °C	EG, PD, PA	Pech y Aké et al. (2004)
MGD	Mature (12 mpp)	Y3 + Sucrose (4.5 %)	I: Dark (6-8 weeks) then 16:8 h light:dark (50 µmol m^{-2} s^{-1}) T: 27 ± 2 °C	EG, PD, PA	Fuentes et al. (2005b)
MGD, MYD	Mature (12–14 mpp)	Modified Y3 + GA_3 (0.46 µM) + AC (0.25 %) ± Gelrite (0.3 %) for germination ↓ Modified Y3 + AC (0.25 %) ± Gelrite (0.3 %) for plantlet growth	I: Dark (5 weeks) then 16:8 h light:dark (45-60 µmol $m^{-2}s^{-1}$) T: 27 ± 2 °C	EG, PD, PA	Pech y Aké et al. (2007)
MYD	Mature (11–12 mpp)	MS liquid + Sucrose (4 %) + AC (0.15 %) + Lauric acid (75 µM, unbound)	–	EG	López-Villalobos et al. (2011)

Table 1 continued

Embryo origin/variety/cultivar[a]	Fruit maturity[b]	Culture media & PGRs (optimal combinations reported)[c]	Culture conditions[d]	Responses/results[e]	References
MYD	Mature (11–12 mpp)	Y3 liquid + Rillo Vit + Sucrose (6 %) + AC (0.1 %) for germination ↓ Y3 + Rillo Vit + Sucrose (6 %) + AC (0.1 %) + Bacto-agar (0.2 %) for plantlet growth	I: Dark (6–8 weeks) then 14:10 h light:dark (90 µmol $m^{-2}s^{-1}$) T: 27 ± 1 °C ↓ CO_2 enrichment system for improved seedling growth	EG, PD, PA (up to 100 %)	Samosir and Adkins (2014)

[a] *LT* Laguna Tall, *MGD* Malayan Green Dwarf, *MYD* Malayan Yellow Dwarf, *WAT* West African Tall

[b] *mpp* Months post-pollination

[c] *AC* activated charcoal, *CW* coconut water, *GA*$_3$ gibberellic acid, *IBA* indole-3-butyric acid, *MS* Murashige and Skoog (1962) medium, *MW Vit* Morel and Wetmore (1951) vitamins, *NAA* naphthalene acetic acid, *Rillo Vit* Rillo et al. (2002) vitamins, *White* White (1943) medium, *Y3* Eeuwens (1976) medium

[d] *I* illumination, *T* temperature

[e] *AR* adventitious root formation only, *EG* embryo germination, *PD* plantlet development, *PA* plantlet acclimatization

– Not mentioned

growth varied in the different studies undertaken. Even though many culture media types have been used to support zygotic embryo germination and growth, the most commonly used one is the Y3 medium developed by Eeuwens (1976). In comparison to MS (Murashige and Skoog 1962) medium, the ammonium and nitrate nitrogen contents in Y3 medium are half, while micro-elements such as iodine, copper and cobalt are tenfold greater in concentration. These alterations might better reflect the conditions of a coastal soil, a favourable habitat for coconut germination. The supplementation with a high level of sucrose (>4 %) has been reported to be essential for embryo germination and activated charcoal has been used in most studies to help prevent tissue necrosis (Table 1). Agar (1.5–0.8 %) is often used to create a solid medium for the early stages of germination; however, recent studies report the use of a two-stage system involving embryo culture in a liquid medium to obtain germination. This is followed by transfer to an agar medium (Rillo 1998) (Fig. 2a, b) 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, 2007) and the addition of plant growth regulators such as gibberellic acid (0.5 µM) have been reported to promote the rate and number of embryos germinating while certain auxin analogues such as NAA (naphthalene acetic acid) or IBA (indole-3-butyric acid) have been shown to promote root growth in the later stages of germination and early seedling growth (Ashburner et al. 1993; Rillo 1998). Also, exogenous lauric acid (75 µM), a significant endosperm fatty acid, has been shown to enhance the growth and development of plantlets (López-Villalobos et al. 2011). The environmental conditions required to optimize embryo germination and plantlet growth have been reported to be a warm temperature (25–31 °C), first in the dark (for 5–8 weeks), and then in the light (c. 45–90 µmol m^{-2} s^{-1}) once the first signs of germination have been observed (Table 1).

The acclimatization of in vitro plantlets has been achieved for a wide range of coconut cultivars using a number of potting soils and nursery conditions. For example, black polyethylene bags containing a mixture of peat moss and soil (1:1, w/w) have been shown to be ideal for raising tissue-cultured plantlets (Pech y Aké et al. 2004). The ex vitro seedling survival rate was improved by transferring plantlets through a series of different ambient conditions, firstly involving a fogging chamber, then a shaded nursery and finally a nursery under full sunlight (Talavera et al. 2005). In addition, the elevation of seedling photosynthesis has also been considered to be a key variable contributing to acclimatization success. Triques et al. (1998) highlighted the importance of the early establishment of a photosynthetic-based metabolism during in vitro plantlet development. A photoautotrophic sucrose-free

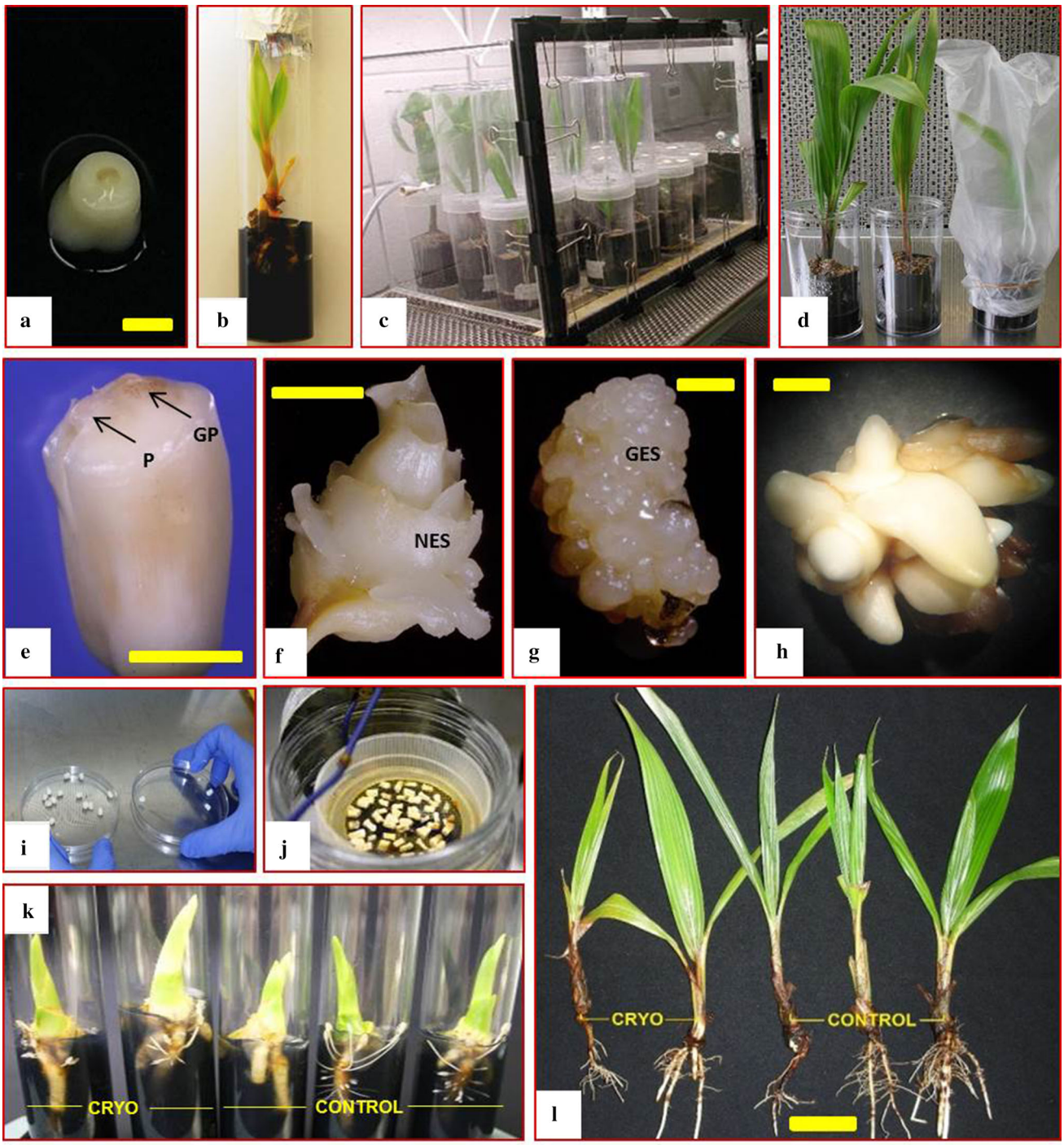

Fig. 2 Images in the steps used for of coconut embryo culture (**a**–**d**), somatic embryogenesis (**e**–**h**) and cryopreservation (**i**–**l**). **a** Initiation of a zygotic embryo culture using Y3 medium + MW Vit + 0.25 % AC + 0.8 % agar (to be kept in dark condition for 8 weeks), **b** Further development of shoot and roots on an embryo cultured plantlet. **c** Photoautotrophic system (CO_2 enrichment growth chamber) developed to improve seedling growth, **d** comparison between an acclimatized plantlet grown in a CO_2 enrichment environment and one covered by conventional plastic bag, **e** Plumule tissue emerging from a zygotic embryo and subsequently used as initial explant for callus induction, **f**–**g** different responses in callus induction media supplemented, respectively, with 200 µM and 600 µM 2,4-D, **h** Maturation of somatic embryos in a reduced 2,4-D medium, **i** aseptic isolation of zygotic embryos for cryopreservation, **j** rapid dehydration of sterilized embryos using fan-forced air apparatus, before being plunged into liquid nitrogen, **k**–**l** No significant differences in the morphology observed during the development and acclimatization of plantlets derived from cryopreserved embryos and normal embryos (these two photos are reprinted from Sisunandar et al. 2010a, with permission) (*P* plumule, *GP* germ pore, *NES* non-embryogenic structures, *GES* globular embryogenic structures). *Bar* **a**, **e**, **f**—5 mm; **g**, **h**—1 mm; **l**—5 cm

protocol using CO_2 enrichment (1600 μmol mol^{-1}) during the light phase was found to improve seedling health, growth, and the percentage of seedlings established (Samosir and Adkins 2014) (Fig. 2c, d).

The embryo culture approach has become indispensable for the collection of coconut germplasm from remote locations and their transport back to the laboratory. For many years, the traditional approach to do this was to transport the intact fruit, but this had a number of limitations, mainly due to the great size of the fruit and transmittance of pests and diseases within the fruit. An early modified form of coconut germplasm collection involved the isolation of the mature embryo in the field and placement in vials of sterile water or coconut water for transport back to the laboratory (Rillo and Paloma 1991). This technique was often inefficient due to infection of high proportion of embryos during transport. 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, then placing them in an ascorbic acid solution and holding the plugs 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 number of mature plants flourishing in soil can be low in certain cases. Therefore, the applicability of this technique to all coconut cultivars is still to be optimized. Appropriate technology transfer from the research laboratory to the smallholder is also an important step in the improvement of coconut production in some developing countries and territories.

Clonal propagation via somatic embryogenesis

Somatic embryogenesis

The concept of 'somatic embryogenesis' first came about from two independent research groups in Germany and the United States when plantlets were regenerated from cultured carrot (*Daucus carota* L.) 'mother' cells (Steward et al. 1958; Reinert 1959). Since then, the capacity to produce somatic embryogenic structures and plantlets from undifferentiated cells has become the focus of research on many species. Even though SE can be achieved in many species, it has been much more difficult to achieve in others, and this includes the coconut. The first attempts at coconut SE were undertaken over 30 years ago at Wye College, UK (Eeuwens and Blake 1977), and then by ORSTOM, France (Pannetier and Buffard-Morel 1982). These and other early studies used a number of plant somatic tissues as initial explants (i.e., young leaves, stem slices from young seedlings, sections 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 the easier to manipulate zygotic tissues (e.g., immature or mature embryos and embryo-derived plumules) to achieve SE in coconut (Table 2). While immature embryos were found to be responsive, the responsiveness of the easier to obtain mature embryos was dramatically improved by their longitudinal slicing (Adkins et al. 1998; Samosir 1999) and at a later date by the isolation and culture of the plumular tissue (Chan et al. 1998; Lopez-Villalobos 2002; Pérez-Núñez et al. 2006) (Fig. 2e). More recently, with the view that somatic tissues are the tissues that can be used to produce true-to-type clones, attention has returned to the harder-to-use somatic tissue explants such as young inflorescence tissues (Antonova 2009).

The Y3 (Eeuwens 1976) and BM72 (Karunaratne and Periyapperuma 1989) media has been the most frequently used for callus culture (Table 2) 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 (3–4 %) appears to be essential for coconut SE to take place, while activated charcoal (0.1–0.3 %) has been extensively used to prevent explanted tissues and callus from browning, a stress-related response caused by the release of secondary plant products such as phenols, or ethylene (Samosir 1999). However, the presence of activated charcoal in the culture medium interferes with the activity of the exogenously applied plant growth regulators and other media supplements, leading to uncertainty in the exact functional concentrations of these additives within the medium (Pan and van Staden 1998). Differences in particle size, and the potency of the various activated charcoal types, have been shown to influence the frequency of somatic embryogenic callus formation (Sáenz et al. 2009). Another universal toxin absorbing agent, polyvinylpyrrolidone (PVP), 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). The frequent sub-culturing of the cultured explant tissues and the developing somatic embryogenic callus is often used as another approach to reduce the exposure to the accumulation of toxic phenols (Fernando and Gamage 2000; Pérez-Núñez et al. 2006) even though the cultured tissues encounter further stress during the transfer process.

Table 2 Clonal propagation of coconut via somatic embryogenesis

Variety/ cultivar[a]	Initial explants (age)[b]	Tissue type[c]	Culture media & plant growth regulators (optimal combinations reported)[d]		Responses/ results[e]	References
			Callus induction → proliferation	Maturation → germination (modifications only)		
JMD	Seedling stem and rachillae of young inflorescences	S	Y3 + Sucrose (6.8 %) + 2,4-D (0.1 μM) + BAP (5 μM) + GA_3 (10 μM)	–	EC	Eeuwens and Blake (1977)
MDY × WAT	Young leaves	S	Y3 + MW Vit + Suc (2 %) + 2,4D (n/a) + AC (n/a)	Reducing 2,4-D (n/a) + BAP (n/a)	EC	Pannetier and Buffard-Morel (1982)
JMD	Seedling stem and rachillae of young inflorescences	S	MS macro + Y3 micro + modified Blake vit + Sucrose (5 %) + AC (0.25 %) + 2,4-D (100 μM) + BAP (5 μM) + 2iP (5 μM)	Reducing 2,4-D (0.1 μM)	EC	Branton and Blake (1983)
IWCT	MZE and stems, leaves and rachillae	Z	Modified Y3 + 2,4-D (452 μM) + NAA (27 μM) + BAP (8.88 μM) + Kin (4.65 μM) + AC (0.25 %)	Reducing 2,4-D (2.3 μM)	EC	Gupta et al. (1984)
IWCT	IZE in enclosing soft endosperm	Z	Y3 + 2,4-D (226 μM) + Kin (9.4 μM) + AC (0.1 %)	Reducing 2,4-D (4.5 μM)	Aneuploid callus cells	Kumar et al. (1985)
IWCT	MZE (8–10 mpp)	Z	B5 + IAA-asp (7 μM) + IAA-ala (7 μM)	B5 + IAA-asp (7 μM)/IAA-ala (7 μM) + Kin (9.4 μM)/BAP (8.8 μM) ↓ B5 + NAA (2.7 μM) + BAP (9.4 μM) + PVP (0.1 %)	EC, SEM, PR	Bhallasarin et al. (1986)
var. *typica*	IZE (6–8 mpp)	Z	BM72 + Sucrose (3 %) + AC (0.25 %) + 2,4-D (12-20 μM)	Reducing 2,4-D (8 μM) + BAP (10 μM)	EC, SEM, PR	Karunaratne and Periyapperuma (1989)
SLT	Young leaves	S	BM72 + Sucrose (3 %) + AC (0.25 %) + 2,4-D (12-20 μM)	Reducing 2,4-D (8 μM) + BAP (10 μM)	EC	Karunaratne et al. (1991)
MYD × WAT, WAT × MYD, and MYD	Immature inflorescences	S	Y3 + MW Vit + Sucrose (4 %) + 2,4-D (250-300 μM) + AC (0.2 %) ↓ increasing 2,4-D (450-550 μM)	Reducing 2,4-D + incorporating BAP	EC, SEM, PR	Verdeil et al. (1994)
WAT × MYD	Immature inflorescences	S	Modified MS macronutrients + Nitsch micronutrients + MW Vit + 2,4-D (450 μM)	Reducing 2,4-D + BAP (0.5 μM)	EC, SEM	Magnaval et al. (1995)
BLT	MZE slices	Z	Y3 + Sucrose (3 %) + 2,4-D (125 μM) + AC (0.25 %) + AVG (1 μM) + STS (2 μM)	Reducing 2,4-D + Putrescine (7.5 μM) + Spermine (1 μM)	EC, SEM	Adkins et al. (1998)
MMD	MZE plumules	Z	Y3 + 2,4-D (100 μM) +AC (0.25 %)	Reducing 2,4-D (1 μM) + BAP (50 μM)	EC, SEM, PR	Chan et al. (1998)

Table 2 continued

Variety/ cultivar[a]	Initial explants (age)[b]	Tissue type[c]	Culture media & plant growth regulators (optimal combinations reported)[d]		Responses/ results[e]	References
			Callus induction → proliferation	Maturation → germination (modifications only)		
MYD and BLT	MZE slices	Z	Y3 + Sucrose (3 %) + 2,4-D (125 μM) + AC (0.25 %)	Reducing 2,4-D + NAA (10 μM) + ABA (5 μM)	EC, SEM, PR	Samosir (1999)
SLT	IZE	Z	BM72 + Sucrose (4 %) + 2,4-D (24 μM) + ABA (2.5–5 μM)	Reducing 2,4-D (8-16 μM) + ABA (5 μM)	EC, SEM, PR	Fernando and Gamage (2000)
SLT	MZE plumules	Z	BM72 + Sucrose (6 %) + AC (0.25 %) + 2,4-D (0.1 μM) + BA (5 μM) ↓ increasing 2,4-D (24 μM) + Sucrose (4 %)	Reducing 2,4-D (16 μM) + ABA (5 μM) ↓ PGR-free	EC, SEM, PR	Fernando et al. (2003)
MGD	MZE plumules	Z	Y3 + Sucrose (3 %) + AC (0.25 %) + 2,4-D (600 μM)	Reducing 2,4-D (6 μM) + BA (300 μM)	EC, SEM, PR	Pérez-Núñez et al. (2006)
SLT	Unfertilized ovaries (−4, −5 and −6 stages of ovary maturity)	S	BM72 + Sucrose (4 %) + AC (0.1 %) + 2,4-D (100 μM)	Omitting 2,4-D + ABA (5 μM) + $AgNO_3$ (10 μM) ↓ Modified Y3 + 2,4-D (8-16 μM) + ABA (5 μM)	EC, SEM, PR	Perera et al. (2007a)
SLT	Unfertilized ovaries (−4 stage of ovary maturity)	S	BM72 + Sucrose (4 %) + AC (0.1 %) + 2,4-D (100 μM) + TDZ (9 μM) ↓ reducing 2,4-D (66 μM)	PGR-free ↓ BAP (5 μM) + GA3 (0.45 μM) +2iP (45 μM)	EC, SEM, PR	Perera et al. (2009b)
MYD	Immature inflorescences	S	Y3 + Sucrose (3 %) + AC (0.25 %) + 2,4-D (250 μM) + 2iP (5 μM) + BAP (5 μM) ↓ adding TDZ (10 μM)	Reducing 2,4-D ↓ PGR-free + Ancymidol (30 μM)	EC, SEM, PR (up to 56 % of PR)	Antonova (2009)

[a] *BLT* Batu Layar Tall, *JDM* Jamaican Malayan Dwarf, *IWCT* Indian West Coast Tall, *MGD* Malayan Green Dwarf; *MMD* Mexican Malayan Dwarf, *MYD* Malayan Yellow Dwarf, *SLT* Sri Lanka Tall, *WAT* West African Tall

[b] *IZE* immature zygotic embryos, *MZE* mature zygotic embryos, *mpp* months post-pollination

[c] *S* somatic tissue, *Z* zygotic tissue

[d] *ABA* abscisic acid, *AC* activated charcoal, *B5* Gamborg et al. (1968) medium, *BAP* 6-benzylaminopurine, *Blake* Blake (1972) medium, *BM72* Karunaratne and Periyapperuma (1989) medium, *2,4-D* 2,4-dichlorophenoxyacetic acid, GA_3 gibberellic acid, *IAA-ala* indole-3-acetic acid-alanine, *IAA-asp* indole-3-acetic acid-aspartate, *2iP* 2-isopentyl adenine, *Kin* kinetin, *MS* Murashige and Skoog (1962) medium, *MW Vit* Morel and Wetmore (1951) vitamins, *NAA* naphthalene acetic acid, *Nitsch* Nitsch (1969) medium, *PGR* plant growth regulators, *PVP* polyvinylpyrrolidone, *TDZ* Thidiazuron, *Y3* Eeuwens (1976) medium

[e] *EC* embryogenic callus; *SEM* somatic embryo maturation, *PR* plantlet regeneration

– Not mentioned

As seen in many 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 acclimatization of the plantlets to ex vitro conditions. Callus formation is commonly achieved with a high concentration of auxin, usually 2,4-dichlorophenoxyacetic acid (2,4-D). However, the working concentration of 2,4-D varies between different cultivars and explant types (Table 2). For instance, while a low 2,4-D (24 μM) treatment was found to be optimal to initiate callus production on zygotic embryos of Sri Lanka Tall (Fernando and Gamage 2000), a much higher dose (125 μM) was needed for Malayan Yellow Dwarf and Buta Layar Tall (Adkins et al. 1998; Samosir 1999). For callus production on immature inflorescence tissues and embryo-derived plumules, an even higher concentration of 2,4-D (450 or 600 μM) was required (Verdeil et al. 1994). Complications arise when such high concentrations of 2,4-D are used for extended periods of time as it has been shown that such treatments can induce chromosomal aberrations in the cultured tissues (Blake and Hornung 1995). In addition, it is now thought that coconut tissues can metabolize 2,4-D into fatty acid analogues, which are subsequently incorporated into triacylglycerol derivatives (López-Villalobos et al. 2004). These latter molecules represent a stable and stored form of 2,4-D that can continue to arrest somatic embryo formation even when 2,4-D has been removed from the medium. Apart from 2,4-D, other 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 gametophytic-like conditions produced by 2,4-D, are required for the 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 5–10 μM is also common (Table 2). 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 has been extended to 3 months to achieve greater callus production (Pérez-Núñez et al. 2006). Further improvement in the timely production of somatic 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). Ethylene production inhibitors, 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 for the formation of somatic embryos (Adkins et al. 1998). In several studies, the conversion of undifferentiated callus to somatic embryogenic callus was achieved by the reduction or removal of 2,4-D from the culture medium (Table 2). Furthermore, Chan et al. (1998) showed that incubating callus under a 12-h photoperiod (45–60 μmol m^{-2} s^{-1} photosynthetic photon flux density) significantly improved the rate of SE, as compared to that produced under darkness. Incorporating or increasing the amount of BAP (to between 50 and 300 μM) in the medium could also promote SE, leading to a greater number of viable plantlets at the end of the culture phase (Pérez-Núñez et al. 2006; Chan 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 also 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 few percent to 56 %.

It is worth noting that cell suspension culture systems have also been successful in raising the rate of SE for some members of the Arecaceae, including oil palm (Teixeira et al. 1995). Additionally, temporary immersion systems have been employed with date palm (Tisserat and Vandercook 1985) and peach palm (Steinmacher et al. 2011) to raise the rate of plantlet regeneration. These two techniques applied to coconut could possibly facilitate the rapid multiplication of robust plantlets, thereby creating a platform for mass clonal propagation. However, the ex vitro acclimatization of somatic embryo-derived plantlets has yet to be refined, with present rates of success of around 50 % so far (Fuentes et al. 2005a). Further improvements may come from using a photoautotrophic culture system (Samosir and Adkins 2014) and/or through the incorporation of fatty acids, notably lauric acid, into the plantlet maturation medium (López-Villalobos et al. 2001, 2011).

Biotechnological interventions for somatic embryogenesis

Somatic embryogenesis is a multi-step process which involves the transition of a single cell into a somatic proembryo structure and finally into a somatic embryo. Hence,

alterations in the physiological and biochemical characteristics of the cell must occur to create a condition in which somatic embryogenic competence can be acquired (Umehara et al. 2007; Pandey and Chaudhary 2014). To achieve such alterations, cells can be affected by a number of factors, including the presence of certain plant growth regulators, which act to change the existing pattern of gene expression, to one that promotes SE. Subsequently, these changes in competence regulate the biosynthesis of certain enzymes which drive the cell to adopt the new function (Pandey and Chaudhary 2014; Chugh and Khurana 2002; Fehér et al. 2003). This process is commonly known as cell specification and is considered to be an important genetic event in the formation of somatic embryos (Miyashima et al. 2013; Smertenko and Bozhkov 2014; Umehara et al. 2007).

Studies on specific gene expression have been used to help unravel the molecular mechanisms which regulate the process of SE in coconut (Pérez-Núñez et al. 2009). It is believed that dissecting out of the key molecular elements will help improve the efficiency of existing clonal propagation protocols. Bandupriya et al. (2013) have been able to isolate a homologous gene (i.e., *CnANT*) to the *Arabidopsis AINTEGUMENTA*-like gene in coconut, which encodes two APETALA 2 domains and a linker region. The analysis of *CnANT* transcripts demonstrated that this gene is involved in coconut SE, and is at its highest level of expression during the callus induction phase when cells are acquiring somatic embryogenic competence (Bandupriya et al. 2013, 2014). The role of *CnANT* in SE was studied in explants derived from *Arabidopsis* overexpressing lines. The upregulation of the *CnANT* gene caused increased shoot organogenesis even in culture media devoid of plant growth regulators (Bandupriya and Dunwell 2012). However, the spontaneous formation of somatic embryos as reported with other *PL/AIL* genes, was not observed with the *CnANT* gene (Bandupriya and Dunwell 2012; Boutilier et al. 2002; Tsuwamoto et al. 2010).

Similar to the *CnANT* gene, the *CnCDKA* and *CnSERK* homologs have also been isolated from coconut and shown to be associated with the induction of SE in this species (Pérez-Núñez et al. 2009). The *CnCDKA* gene encodes a cyclin-dependent kinase which regulates cell division following its activation by certain cyclins (Montero-Cortes et al. 2010a). The *CnSERK* gene encodes a protein receptor (Pérez-Núñez et al. 2009) which may be a component of a signaling cascade involved in regulating the rate of SE (Hecht et al. 2001; Schmidt et al. 1997; Santos et al. 2005; Thomas et al. 2004). In situ hybridization has shown the transcripts of both genes to be localized in the somatic embryogenic structures that form on callus, and within meristematic centres. The molecular mechanisms of *CnCDKA* and *CnSERK* genes to confer embryogenic competence to somatic cells are still unknown but experimental results indicate that these genes are reliable molecular markers for this biological process (Montero-Cortes et al. 2010a).

One further molecular strategy adopted to improve the rate of coconut SE involved the upregulation of genes that affect the formation of shoot meristem production in somatic embryos of other species. Montero-Cortes and coworkers isolated the coconut *CnKNOX1* gene, a *KNOX* class I gene, which was expressed exclusively in tissue with meristematic activity (Montero-Cortes et al. 2010b). They established that the *CnKNOX1* gene was responsive to the addition of gibberellin during coconut SE with the result of an increased rate of somatic embryo formation and germination.

Considering the limited understanding of the molecular mechanisms that underlies coconut SE, it is apparent that more research is needed in this area before a further impact upon the rate of coconut SE can be achieved. The isolation and characterization of genes which regulate the formation of the root apex, such as the *PL/AIL* genes are still in their infancy, whilst the discovery of genes which specify the shoot apex has not even commenced. The study of these embryogenic genes as well as other genes encoding regulatory factors (such as the B3 domain transcription factor family) that are involved in lipid metabolism represents an important avenue to explore in coconut research in the near future (Kim et al. 2013).

Homozygote production via anther culture

Production of doubled haploid plants is considered to be an ideal approach to overcoming the lengthy breeding cycles in certain plant species (Kasha and Maluszynski 2003). The first report of using an in vitro anther culture approach to achieve such outcomes in coconut dates back to the 1980s (Thanh-Tuyen and De Guzman 1983; Monfort 1985). In those early studies, neither ploidy level determination nor plantlet regeneration was reported. However, in a more recent series of studies it has been reported that somatic embryo structures, with root and shoot apices, have been produced through anther culture (Perera et al. 2007a, 2008a), and finally homozygotic plants (Perera et al. 2008b). The basic procedures now used employ a culture medium developed by Karunaratne and Periyapperuma (1989) and supplemented with a high concentration of sucrose (9 %) (Perera et al. 2008a, 2009c). The addition of activated charcoal (0.1 %) is also important to reduce callus necrosis. The production of microspore callus is undertaken using a moderate concentration of 2,4-D (100 μM) with the addition of TDZ (9 μM) and NAA (100 μM). In most cases, the callus cultures are produced

and maintained in the dark at 28 °C for at least 10 weeks. Subsequently, in the absence of the previously mentioned plant growth regulators, the maturation of the somatic embryos is achieved using ABA (5 μM) in combination with the ethylene action inhibitor $AgNO_3$ (10 μM) (Perera et al. 2007b). To proliferate and mature the somatic embryos, the callus is transferred to a plant growth regulator-free medium and then to a BAP-supplemented (5 μM) medium to promote their germination (Table 3). Gibberellic acid (0.35 μM) can be incorporated into the medium together with BAP (5 μM) to further improve the germination rate of the mature somatic embryos (Perera et al. 2008a, 2009c). To show the haploid nature of the callus masses and homozygotic nature of plants in soil, a flow cytometric analysis and histological study approach has been used (Perera et al. 2008b). Furthermore, through a diagnostic simple sequence repeat molecular marker (CNZ43) technique it has been shown that the production of homozygotic diploid plantlets has been achieved (Perera et al. 2008b). From this work it has been suggested that in the future it may be possible to accelerate the multiplication of plants from a single, high-value parental line, thereby avoiding generations of backcrossing. Recent reports have shed some light on sequential events during in vitro somatic embryogenesis in coconut anther culture, albeit with a low regeneration frequency (Perera et al. 2008a, 2009c). However, similar to SE in diploid tissues, the procedure in anther culture still requires further improvement to overcome the present limitations in the conversion of the induced somatic embryos to plantlets. In addition, the consistency in converting the haploid to diploid plantlets is another step in the procedure that also requires improvement.

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 plumule tissues excised from mature zygotic embryos and using a chemical dehydration method (Supplement 1). As with most species the cryopreservation protocol for coconut consists of four steps: firstly the

Table 3 Progress in haploid culture of coconut

Variety/cultivar[a]	Initial explants (age)[b]	Culture media & PGRs (optimal combinations reported)[c]		Responses/results[d]	References
		Embryogenic induction	Maturation → germination (modifications only)		
LT	Microspores (4-5 WBS)	Modified Blaydes/Keller + Sucrose (6-9 %) + CW (15 %) + AC (0.5 %) + NAA (10.8 μM)	–	ELS	Thanh-Tuyen and De Guzman (1983)
MYD × WAT and WAT × RT	Microspores	Picard and Buyser Picard and Buyser (1972) medium + Sucrose (9 %) + CW (10 %) + AC (0.3 %) + TIBA (4 μM) + Glutamine (6.8 μM)	–	ELS	Monfort (1985)
SLT	Microspores (3 WBS)	BM72 + Sucrose (9 %) + AC (0.1 %) + 2,4-D (100 μM) ↓ reducing 2,4-D (66 μM)	PGR-free ↓ BAP (5 μM) + GA3 (0.35 μM)	ELS, PR	Perera et al. (2008a)
SLT	Microspores (3 WBS)	BM72 + Sucrose (9 %) + AC (0.1 %) + 2,4-D (100 μM) + NAA (100 μM) ↓ reducing 2,4-D (66 μM) + Kin or 2iP (100 μM)	PGR-free ↓ BAP (5 μM) + GA3 (0.35 μM)	ELS, PR	Perera et al. (2009c)

[a] *LT* Laguna Tall, *MYD* Malayan Yellow Dwarf, *RT* Rennell Tall, *SLT* Sri Lanka Tall, *WAT* West African Tall

[b] *WBS* weeks before floral bud splitting

[c] *ABA* Abscisic acid, *AC* activated charcoal, *BAP* 6-benzylaminopurine, *Blaydes* Blaydes (1966) medium, *BM72* Karunaratne and Periyapperuma (1989) medium, *CW* coconut water, *2,4-D* 2,4-dichlorophenoxyacetic acid, GA_3 gibberellic acid, *2iP* 2-isopentyl adenine, *Keller* Keller et al. (1975) medium, *Kin* kinetin, *NAA* naphthalene acetic acid, *Picard and Buyser* Picard and Buyser (1972) medium, *PGR* plant growth regulators, *TIBA* 2,3,5-triiodobenzoic acid

[d] *ELS* embryo-like structure, *PR* plantlet regeneration

– Not mentioned

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 chemical dehydration sucrose, glucose and glycerol, all at high concentrations (>10 %, w/v) are the most commonly used agents, whereas dimethyl sulfoxide (DMSO) and sorbitol are less frequently used. Encapsulation using sodium alginate (3 %) following tissue dehydration using sucrose (5 %) has also been attempted using plumule tissue (N'Nan et al. 2008). For slow physical dehydration various drying durations (7–48 h) have been used across a number of coconut cultivars (Supplement 1). The outcomes can be relatively high in recovery rate but very few plantlets are produced by these methods. For rapid physical dehydration a special apparatus has been developed to dehydrate embryos using silica gel-dried, fan-forced air (Sisunandar et al. 2010b) (Fig. 2j). 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 h gave the embryos the 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).

Like many other species, a rapid freezing approach has been widely used for coconut tissues (Supplement 1). In most cases the dehydrated tissues are transferred into cryovials, and plunged directly into liquid nitrogen. Also, in most cases, a rapid thawing approach is used whereby the cryopreserved tissues are submerged into a water bath set at 40 °C for 3 min. The selection of the correct recovery and embryo germination media has been another factor critical to the success of the cryopreservation protocol. The MS (Murashige and Skoog 1962), MW (Morel and Wetmore 1951) and Y3 (Eeuwens 1976) media formulations have all been commonly used in this tissue recovery stage with the latter medium preferred in most studies (Sisunandar et al. 2010b, 2012; Sajini et al. 2011). 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 high doses of sucrose (4–6 %) has been shown to be important for the germination of the recovered embryos (N'Nan et al. 2008; Sisunandar et al. 2010b; Sajini et al. 2011). Establishment of plants in soil following cryopreservation of coconut embryos has only been reported using the chemical dehydration approach of Sajini et al. (2011) and by 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 will be the application of cryopreservation in 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.

Genetic transformation

The first attempt to undertake genetic transformation of coconut tissues was using microprojectile bombardment for insertion of the *GUS* gene into embryogenic callus and young leaf tissues (Samosir 1999). The constitutively expressed promoters Act1 and Ubi were found to produce the strongest transient expression, suggesting that these promoters could be used in future work. More recently, Andrade-Torres et al. (2011) have reported the *Agrobacterium*-mediated transformation of a number of coconut explant tissues such as immature anthers, excised zygotic embryos, plumule-derived embryogenic calli, and somatic embryogenesis-derived roots and leaves. They tested a number of reporter genes and evaluated the techniques used in antibiotic selections of transformants. Calli, which were not co-cultivated with *Agrobacterium* carrying the *gusA* gene, showed endogenous GUS-like activity. Thus, a number of alternative genes (e.g., those encoding for green or red fluorescent protein) were tested as reporter genes. It was shown that the combination of techniques (e.g., biobalistics to generate micro-wounds in explants, vacuum infiltration and co-culture with *A. tumefaciens* to introduce genes) could better facilitate gene transfer than when the techniques were applied individually (Andrade-Torres et al. 2011). Even though a genetically modified coconut plant has yet to be produced, this kind of work could be useful for the improvement of coconut SE if appropriate SE genes could be identified and isolated from other species and then introduced into coconut. Apart from this possibility, genetic transformation holds a great longer term potential for coconut by either introducing specific genes from other species for disease or stress resistance, or by modifying the expression of native genes to gain increased growth rates and oil productivity.

Conclusion and future prospects

Inefficient plantlet regeneration from in vitro culture systems remains a major bottleneck for many coconut research groups around the globe. This is the result of unresolved or partly resolved problems which relate to the variable response of explanted tissues in vitro, the slow growth of tissues in vitro, and their further lack of vigour when planted ex vitro. For these reasons, success in coconut tissue has been attained less rapidly than for many other plant species (Fig. 1). It is necessary to consider and then employ procedures that are successfully used for other species to help drive future improvements in coconut in vitro culture. The literature suggests that it may be possible to generate highly efficient embryogenic cell suspension cultures, derived from selected callus lines, to help overcome contemporary challenges, and to develop a rapid clonal propagation system for coconut. Therefore, future research should be focused on an optimization of in vitro conditions to increase the production of somatic embryos using media additives and a cell suspension culture system. Subsequent development and acclimatization could be further improved using temporary immersion and photoautotrophic systems.

It is also worth considering that, 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 of coconut water in promoting somatic embryogenesis in this otherwise recalcitrant species. Other possible improvements in the rate of somatic embryogenesis may come from the application of molecular techniques that can identify the genes involved in the regulation of somatic embryogenesis. Indeed, novel molecular tools might become available to further examine the regulation of the relevant genes, which can be precisely induced during the acquisition of embryogenic competence.

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