Cloning Coconut via Somatic Embryogenesis

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Coconut [Cocos nucifera L.] is often called “the tree of life” because of its many uses in the food, beverage, medicinal, and cosmetic industries. Currently, more than 50% of the palms grown throughout the world are senile and need to be replanted immediately to ensure production levels meet the present and increasing demand for coconut products.

Keywords: coconut ; somatic embryogenesis ; cloning ; explants ; plant growth regulators ; medium ; acclimatization ; genetics

1. Introduction

Coconut [*Cocos nucifera* L.] is grown in most tropical and subtropical regions of the world. It offers food, shelter, and income to people via the hundreds of products it provides [1][2]. Demand for coconut products has increased up to five-fold in the last 10 years; however, production has not kept up with this growing demand [3]. Very little or no replanting of coconut palms has been undertaken in most production countries and there are now reports of regions carrying more than 70% senile palms [4]. Apart from senility, coconut production currently faces other constraints, including biotic and abiotic stresses, and a lack of quality planting materials from seednuts. Coconut is susceptible to pest and diseases such as the coconut rhinocerous beetle (*Oryctes rhinoceros* L.), the red palm weevil (*Rhynchophorus ferrugineus* Olivier), lethal yellowing diseases including Bogia coconut syndrome, and viroid diseases such as cadang-cadang, and climatic conditions such as drought intensified by recent climate change events [5]. Replanting efforts have been limited due to the lack of good quality and true-to-type planting materials. As Tall coconut types cross-pollinate, the establishment of new palms from seednuts may show a high degree of variation in fruit yield and other traits, which can be observed only at palm maturity [6]. In such Tall types, controlled pollination is required to produce true-to-type palms, and this is difficult to achieve on a large scale due to the height these palms can attain at maturity. In addition, when disease-resistant plants are identified, they are usually few in number and therefore unable to produce sufficient seednuts [7] to meet the high demand for disease-resistant seedlings. Similarly, some elite varieties cannot be grown from seed, such as the makapuno and aromatics [8][9][10]. Due to these constraints, large quantities of high-quality planting materials to meet the growing demand cannot be supplied by traditional means, and no vegetative propagation methods are available for plantlet production [9]. In vitro culture has the potential to rapidly multiply a chosen genotype to produce numerous clonal plantlets. Ideal application could result in the mass production of early-bearing, disease-free, and resistant palms that have high productivity or other valuable characteristics [5][11]. Somatic embryogenesis (SE) has been identified by many as the most feasible technique for large-scale production of high-quality coconut plantlets [12][13][14].

Coconut SE involves, firstly, the induction of embryogenic callus, the formation and development of the somatic embryo, its maturation, then germination, and finally the recovery of the plantlet formed [15]. During the process of SE, the dedifferentiated somatic cells regain their epigenetic and biochemical competence to form somatic embryos that progress through a series of developmental stages such as zygotic embryogenesis [16]. Somatic embryogenesis in the palm family was first described for oil palm (*Elaesis guineensis* Jacq.) by Rabechault et al. in 1970 [17], and later in coconut by Eeuwens and Blake, in 1977 [18]. To date, these techniques have been considerably improved to be able to produce coconut plantlets in vitro on a large scale [7][19].

At every stage in the SE approach, from somatic cells to whole plantlet formation, numerous factors have been found to play a crucial role in success, including the genotype of the donor plant, the explant type cultured, the media and plant growth regulators (PGR) used, and the acclimatization procedures applied to the germinated plantlets. Several coconut tissues have been used for explants *viz*., shoot tips, plumules, rachillae sections, young leaves, unfertilized ovaries, and immature embryos, with the response varying depending on the explant type [13]. Plant growth regulators used in the media are known to be another major influencer of the rate of success in the production of plantlets via SE. For example, a low concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) is known to induce the best quantity of embryogenic cells in

several Sri Lankan Tall varieties [20], whereas a high concentration of 2,4-D was required for Malayan Yellow Dwarf to achieve the similar results [21].

2. Importance of Suitable Explants

Cell totipotency is an important characteristic of plant cells, but not all cells are totipotent. The somatic cells that demonstrate a sensitivity to embryogenesis and are capable of undergoing SE are described as being totipotent. The capability of tissue to produce somatic embryos is mainly dependent on a restricted fraction of their cell population, rather than a distinct area within this cell population [22]. Hence, an explant with a source of totipotent cells is the key prerequisite for SE formation by external inducement. The SE response can also be influenced by the age of the explant. Various types of explants have been used for undergoing SE in other species, and include seedlings, leaves, petioles, shoot meristems, roots, seeds, cotyledons, zygotic embryos, and immature zygotic embryos [23].

Somatic tissues from the coconut, such as young leaves, stem sections from young seedlings, and tissues from immature inflorescences have all been studied as explants to generate the embryogenic callus of coconut. The culture of living tissues of coconut was first undertaken by Eeuwens and Blake, 1977 [18], who used seedling stem and immature inflorescence sections as explants. The first SE attempts on coconut explants showed a calloid-like structure to be formed, which is a more organized structure of the callus [24]. Under suitable conditions, plantlets can be produced from the somatic embryos that formed from this embryogenic callus [25]. Although this early approach was inefficient, the embryogenic callus induction rate was increased in later years [26]. In many species it is known that the selection of an explant from a healthy and vigorously growing plant is particularly important to obtain good tissue culture outcomes [27]. In coconut, the type and stage of explant development can significantly affect the responsiveness of the explant to tissue culture [28]. Somatic tissues, such as rachilla segments from immature explants, were commonly used in the early studies of coconut tissue culture because true-to-type clones could be produced from such tissues [7]. However, it is challenging to use these types of coconut tissues. For example, the correct age of the inflorescence tissue to be used as an explant can be difficult to determine [29], and harvesting this material is destructive if not fatal to the palm. Furthermore, the callus induction rate from inflorescence tissues is often lower than 30%, with poor repeatability [30][31].

Zygotic tissues such as embryos or plumules have also been used as explants in coconut SE work [13]. Adkins et al., 1999 [32] found that immature zygotic embryos were superior explant tissues for SE, with a callus induction rate of 50%, compared to just 3% from mature embryos. Similarly, Karunaratne and Periyapperuma 1989 [33] reported that embryos over 8 months of age were already undergoing germination and therefore they were not suitable for callus formation. Whole embryos, in addition to sections of embryos, have been used as explants [32][34]. For embryo sections, the middle portion was found to be the best for callus formation, ranging from 58% to 83% [32][35]. This may be because the central tissue contains much of the embryo axis. The callus induction rate was shown to be dramatically decreased when the surrounding, cotyledonary tissue was used [7][34].

More recently, plumular tissues from germinating zygotic embryos [11][36][37][38] and rachilla sections from immature inflorescences [39][40] have been successfully used for coconut SE. Of these, the plumular tissue was found to be the most suitable for SE [30][41][42][43]. Perez-Nunez et al., 2006 [11] hypothesized that 98,000 somatic embryos could be produced from a single zygotic plumule. The technique would need to use the repeated subculture of plumular callus, which could rapidly increase the total cell biomass and promote the formation of secondary somatic embryos [44]. Such an approach, however, may not produce true-to-type plantlets unless the self-pollinating Dwarf varieties of coconut are cultured, and the process may suffer from somaclonal variation if the number of callus subcultures is numerous.

Leaves from 5 year old coconut palms have also been used as explants, but the callus induction rate was very low, at less than 20%, and somatic embryos took up to 6 months to develop [45][46]. Shoot meristem explants isolated from zygotic embryos achieved a 79% callus induction rate [47]. However, the shoot meristem explant excised from the in vitro germinated embryos showed a lower percentage of SE callus formed, and this may be due to meristematic multiplication inhibition by the presence of cotyledonary tissues [47]. Although anthers have also been used as an explant source [14][48][49][50][51], the frequency of plantlet production was low, at only 0 to 7% from calli/embryos that were subcultured in SE induction and maturation medium [52]. Unfertilized ovaries have been used as another novel explant type for coconut SE, particularly as such somatic tissues would be capable of producing true-to-type plantlets from all coconut varieties. Using such explants, a callus induction rate of 41% [42] has been achieved with an efficiency of plantlet production of about 30%, resulting in 83 plantlets from 32 ovaries [52], which is still below what would be needed for a commercial application [53][54]. Thus, the results suggest that plumular tissues are the preferred explant type for large-scale production of Dwarf coconut types. However, further work is required on somatic tissues, such as ovaries and young inflorescence tissues, to enable

the production of true-to-type clones from Tall coconut types. The studies on coconut explants and varieties are summarized in **Table 1**.

Table 1. In vitro studies on explant, media composition, and plant growth regulators in order from older to recent studies in coconut.

Explant	Media Composition and Plant Growth Regulators			Variety/Hybrid	References
	Basal Media (Callus Induction)	Plant Growth Regulator (Callus Induction)	Plant Growth Regulator (Embryo Maturation)		
Rachilla and stem	Eeuwen's Y3 basal medium and sucrose (6.8%), and agar (0.39%)	2,4-D (0.1 µM), BAP (5 µM), and GA_3 (10 µM)	BAP	Jamaican Malayan Dwarf	[18]
Young foliage tissue	Eeuwens' salts, Morel's Vitamin, sucrose (30 g/L) and agar (0.8%)	2,4-D or TCPP	BAP	Malayan Yellow Dwarf (MYD) × West African Tall (WAT)	[45]
Root, stem and leave	Murashige and Skoog (MS) macro-nutrients, Y3 micro-nutrients, improved Blake vitamin, sucrose (5%), Activated Charcoal (AC; 0.25%), 300 mgL^{-1} casein hydrolysate and myo-inositol (100 mgL^{-1}), 5% agar	2,4-D (100 µM), BAP (5 µM) and 2iP (5 µM)	2,4-D (100 µM), BAP (5 µM) and 2iP (5 µM)	Jamaican Malayan Dwarf	[55]
Rachilla, stem and foliage	Eeuwen's Y3, sucrose (5%), and AC (0.25%), and agar (0.6%)	2,4-D (452 µM), NAA (2.69 µM), BAP (8.88 µM), Kinetin (4.65 µM)	2,4-D (2.3 µM)	West Coast Tall	[56]
Young embryo	Gamborg's B5 medium, and agar (0.7%)	IAA, NAA, 2,4-D, BAP or Kinetin (0.5 mg/L to 5 mg/L)	IAA (2 mg/L)	West Coast Tall	[57]
Embryo	Broad spectrum tissue culture medium, sucrose (30 g/L), AC (0.25%) and agar (0.8%)	2,4-D (12–20 µM)	2,4-D (8 µM and 2 µM), BAP (10 µM) and Kinetin (10 µM)	Typica	[33]
Immature inflorescence	Eeuwen's Y3, Morel and Wetmore (MW) Vitamins, sucrose (116.8 mM) and AC (2 g/L)		2,4-D and BAP (10^{-5} M)	MYD × WAT, WAT × MYD and MYD	[58]
Immature inflorescence	Modified MS macro nutrients, Nitsch micronutrients, MW Vitamins, EDTA (26 mg), iron (24.9 mg), sucrose (20 g/L), ascorbic acid (100 mg/L), malic acid (100 mg/L), adenine sulfate (30 mg/L), agar (7.5 g/L) and AC (3 g/L)	2,4-D (100 mg/L) and BAP (1 mg/ L)	2,4-D (130 mg/L) and BAP (140 mg/L)	PB 121 (MYDxWAT)	[59]
Embryo slice	Eeuwen's Y3, sucrose (90 mM), AC (2.5 g/L) and Agar (0.7%)	2,4-D (125 µM), AVG (1 µM) and	2,4-D (50 µM)	Batu Layar Tall	[60]
Plumule	Eeuwen's Y3, AC (2.5 g/L) and gelrite (3 g/L)	2,4-D (0.1 mM)	2,4-D (1 µM) and BAP (50 µM)	Malayan Dwarf	[41]
Mature embryo slice	M2, sucrose (0–100 g/L), and AC (2.5 g/L), and agar (7.5 g/L)	2,4-D (125 µM) and ABA (0–90 µM)	2,4-D	Batu Layar Tall	[34]
Immature embryo	BM72, sucrose (40 g/L) and AC (0.25%), and agar (0.8%)	2,4-D (24 µM), and ABA (2.5–7.5 µM)	2,4-D and cytokinin (2–10 µM)	Sri Lanka Tall	[20]
Plumule	BM72, sucrose (4% w/v), and agar (0.8%)	2,4-D (24 µM)	N/A	Sri Lanka Tall	[61]

Explant	Media Composition and Plant Growth Regulators			Variety/Hybrid	References
	Basal Media (Callus Induction)	Plant Growth Regulator (Callus Induction)	Plant Growth Regulator (Embryo Maturation)		
Plumule	Eeuwen's Y3, sucrose (30 g/L), AC (2.5 g/L), gelrite (3 g/L)	2,4-D (600 µM)	2,4-D (6 µM), and BAP (300 µM)	Green Malayan Dwarf	[11]
Plumule	Eeuwen's Y3, gelrite (3 g/L) and AC (2.5 g/L)	2,4-D (0.65 mM)	2,4-D (6 µM), and BAP (300 µM)	Green Malayan Dwarf	[62]
Unfertilized ovary	CRI 72 and agar (2%)	2,4-D (100 µM)	2,4-D (66 µM) and ABA (5 µM)	Sri Lanka Tall	[29]
Immature inflorescence	Eeuwen's Y3, sucrose (30 g/L) and AC (2.5 g/L)	Spermine (0.01 µM), Smoke-saturated-water (10%) and auxin (500 µ M).	2,4-D and PGR free no PGR and	Malayan Yellow Dwarf	[63]
Inflorescence	CRI 72, sucrose (40 g/L) and AC (0.1%)	2,4-D (100 µM) and TDZ (9 µM)	No growth regulators	Sri Lanka Tall	[52]
Rachilla	Eeuwen's Y3, AC (2.5 g/L) and gelrite (3 g/L))	2,4-D (0.65 mM)	2,4-D (0.325 and 0.006 mM), BAP (0.3 mM) and GA3 (0.0046 mM)	MYD × MXPT Malayan Red Dwarf) MRD × (Tagnanan) TAGT	[40]
Plumule	Eeuwen's Y3, MW vitamins, agar (2.5 g/L)	2,4-D (600 µM)	2,4-D (6 µM) and BAP (300 µM)	MYD, Makapuno, XXD and PB121	[10]
Plumule	Eeuwen's Y3, sucrose (50 g/L), AC (2.5 g/L) and gelrite (3 g/L)	2,4-D (600 µM)	2,4-D (6 µM) and (300 µM BAP)	Green Malayan Dwarf	[38]

2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), thidiazuron (TDZ), abscisic acid (ABA), gibberellic acid (GA3), indole acetic acid (IAA), naphthaleneacetic acid (NAA), aminoethoxyvinylglycine (AVG).

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