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REVIEW ARTICLE

Advances in coconut micropropagation: prospects, constraints and way forward

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Abstract

Coconut is widely referred to as the "Tree of Life," holds immense value due to its versatility and significant role in numerous sectors including industry, agriculture, food and health. In recent years, a troubling decline in coconut production has been observed due to factors such as palm senility, pests, diseases and natural calamities which poses serious threats to industries and communities that rely highly on this crop thereby creating a gap between demand and supply. Addressing this gap requires innovative solutions and plant tissue culture techniques offer a promising path forward. Tissue culture techniques such as zygotic embryogenesis (mature embryo culture and sliced embryo culture), organogenesis (axillary bud culture and embryo derived shoot tip culture) and somatic embryogenesis have shown great potential for regenerating coconut plants. Axillary bud culture, offers a reliable alternative for producing elite plants with the added benefit of being free from the risk of somaclonal variation, while somatic embryogenesis, in particular has proven to be highly effective for producing large numbers of high-quality planting materials. However, each technique has its own share of shortcomings. Overcoming these challenges and closing the gap between demand and supply is critical for the mass production of elite coconut seedlings. This review explores the different micropropagation techniques, the hurdles facing coconut tissue culture and the potential for future breakthroughs.

Keywords

Cocos nucifera; micropropagation; somatic embryogenesis; organogenesis

Introduction

Coconut (*Cocos nucifera* L) often known as the elixir tree is a principal plantation crop cultivated worldwide primarily in tropical and humid subtropical regions (1).The global production of coconuts exceeds 59 million tons annually, with Indonesia, the Philippines and India identified as the leading producers (2).In India alone, coconuts contributed about $3.72 billion to the country's GDP in 2023, reflecting their essential role in agriculture and economic development.The coconut sector produces a diverse range of products, broadly categorized into oils (virgin coconut oil), edible goods (coconut cream and milk powder), beverages (tender coconut water) and industrial materials (shell charcoal and activated carbon).Both fresh and processed coconut products contain compounds with curative properties that have been found to be effective in the prevention and treatment of ailments like obesity, diabetes, ulcers, cardiovascular disease and

hormone imbalances in postmenopausal women (3).The coconut industry faces significant threats due to factors such as industrialization, urbanization, infrastructure development, changing land use pattern and natural disasters like cyclones, tsunamis, droughts as well as diseases and pests. The demand for replanting is increasing for numerous coconut plantations across the world, either due to senility or the prevalence of diseases (4). For obtaining superior planting materials in large scale, conventional propagation methods alone cannot fulfill the demand. *In vitro* propagation stands out as the only method capable of mass producing true-to-type plants, enabling the production of high quality planting materials with superior traits (5).

2. History of coconut tissue culture

Coconut tissue culture has made remarkable steps since its beginning in 1954, when Cutter and Wilson first worked with zygotic embryos with liquid endosperm of coconut. In 1964, De Guzman and Del Rosario made significant progress by successfully recovering plantlets through zygotic embryo culture (6). A major turning point came in 1976 with Eeuwens' formulation of Y3 media, which laid a strong foundation for further research in coconut tissue culture. The 1980s brought important breakthroughs in *in vitro* embryogenesis and cryopreservation techniques while the development of CRI 72 media in 1989 improved tissue culture practices (7). In the 1990s, cryopreservation methods advanced further, making it possible to preserve mature zygotic embryos and pollen, while researchers simultaneously achieved somatic embryogenesis from immature coconut inflorescences (8).With the onset of 21st century innovations like encapsulation-dehydration, molecular discoveries such as the cnSERK gene and transcriptome analysis enhanced our understanding of coconut regeneration (9). More recent milestones such as axillary shoot multiplication in 2021 and embryo-derived shoot tip culture in 2023 highlight the ongoing progress in coconut tissue culture which is vital for both conservation and commercial production (10). The history of coconut tissue culture is depicted in (Fig 1).

3. Media Composition

In 1954, Cutter and Wilson conducted the first *in vitro* culture of coconut achieving optimum growth by using a medium supplemented with sterile-filtered liquid endosperm from young green coconuts to support embryo germination. The composition of minerals present in media significantly influenced the *in vitro* growth of coconut tissues (11). Coconut tissue culture requires a relatively concentrated mineral solution with high levels of potassium and iodine to mimic the natural coastal habitat of coconut palms. To serve the goal of initiating successful tissue culture practices in coconut, Eeuwens developed Y3 medium (1976), which had contained higher quantities of potassium and iodine but lower levels of ammonium and nitrate than MS medium (12). CRI 72 which was specifically formulated for coconut micropropagation has been successful in inducing callus and somatic embryogenesis through immature embryo and plumule explants (7). Nutrient composition of several media used in coconut tissue culture has been compared in (Table 1).

Of the several media used, Y3 media was extensively used as basal and/or supplemented with phytohormones and was regarded as most suitable for coconut plant tissue culture.

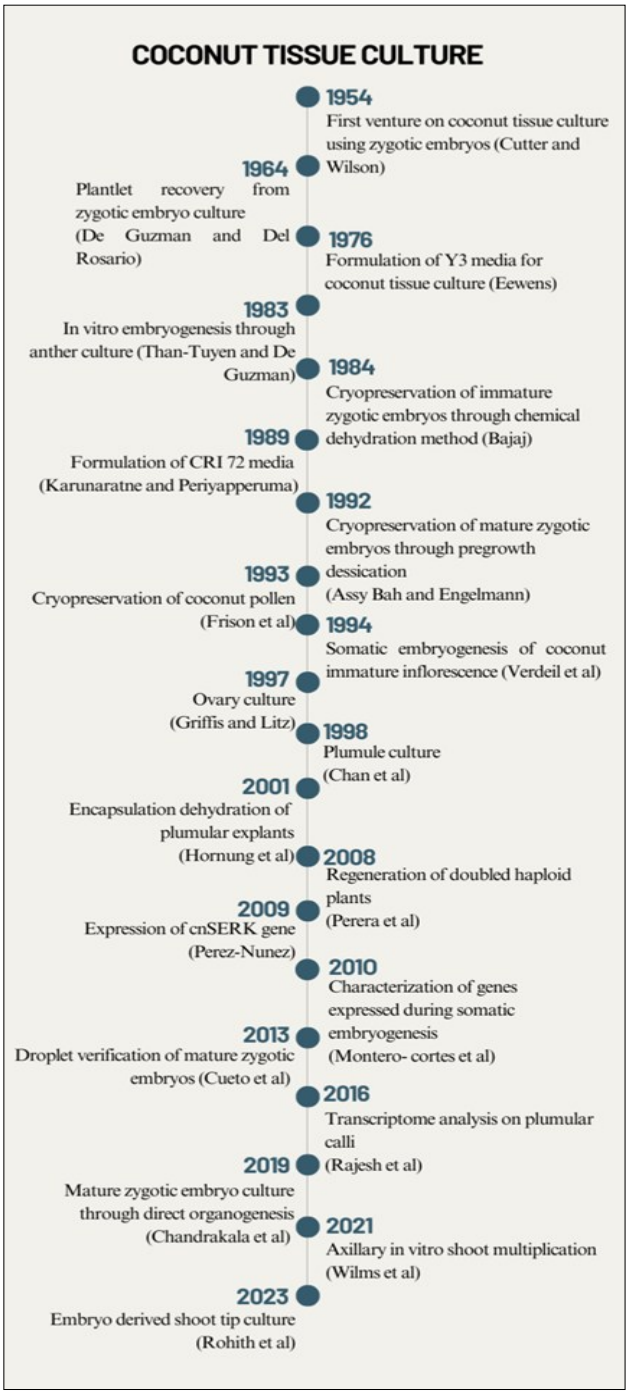

Fig. 1. History of coconut tissue culture.

Growth of coconut explants on Y3 media was considered superior when compared to White or MS media (11). Coconut embryos germinated in liquid media displayed rapid growth when compared to solid media (13). The presence of charcoal in the media makes standardization difficult as the quality and grade of activated charcoal can vary widely. Activated charcoal is commonly incorporated into tissue culture media to mitigate the browning of tissues and media by adsorbing toxic compounds, such as polyphenols, released by cultured tissues. However, its usage poses challenges for standardization due to significant variability in the quality and grade of activated charcoal. These challenges can be addressed by developing charcoal-free media, standardizing charcoal usage protocols, selecting experimentally validated grade specifications and subjecting the charcoal to pre-treatment processes such as rinsing with distilled water or mild acid solutions and autoclaving. Activated Coconut Shell Charcoal - B, produced by

Table 1. Nutrient composition of minerals and organics used in different media of coconut tissue culture

Nutrient composition (mg/l)	MS	Eeuwen's Y3	Modified Y3	CRI 72
NH_4Cl	-	535	535	-
NH_4NO_3	1650	-	-	
KNO_3	1900	2020	2020	2022
KCl	-	1492	1492	-
$MgSo_4.7H_2O$	370	247	247	361.11
$NaH_2PO_4.H_2O$	-	312	312	-
$CaCl_2.2H_2O$	440	294	294	332.94
H_3BO_3	6.2	3.1	3.1	9.27
$MnSO_4.4H_2O$	22.3	11.2	11.2	15.10
$ZnSO_4.7H_2O$	8.6	7.2	7.2	6.46
$Na_2MO_4.2H_2O$	0.25	0.24	0.24	0.25
$CuSO_4.6H_2O$	0.025	0.16	0.16	0.08
$CoCl_2.6H_2O$	0.025	0.24	0.24	0.13
$NiCl_2.6H_2O$	-	0.024	0.024	-
Na_2SO_4	-	-	-	92.32
FeEDTA	-	32.5	-	-
Na_2EDTA	37.3	-	37.3	37.22
$FeSO_4.7H_2O$	27.8	-	27.8	15.10
KI	0.83	8.3	8.3	0.83
Nicotinic acid	0.5	0.05	1	-
Pyridoxine HCl	0.5	0.05	0.05	1.23
Thiamine HCl	0.1	0.5	0.5	1.34
Riboflavin	-	-	-	3.76
Glycine	2	2	2	3.75
Biotin	-	0.05	-	0.24
Calcium Pantothenate	-	0.05	-	3.02
Myo-inositol	100	100	100	108.11
L-Glutamine	-	-	100	-
L-Asparagine	-	-	100	-
L-Arginine	-	-	100	-
L-Cysteine	-	-	-	16.22

Haycarb PLC in Sri Lanka, facilitated 63% and 65% callus induction in immature zygotic embryos and plumular explants, respectively, when combined with 175.0 µM 2,4-D (13). Additionally, using smaller particle fractions (<38 µM) achieved a 70% frequency of embryogenic callus formation in immature zygotic embryo culture, outperforming larger and whole charcoal fractions. Among eight tested activated charcoal types, PCCT (acid-washed for plant cell and tissue culture), DARCO and USP (United States Pharmacopeia testing specifications) promoted approximately 60% embryogenic callus formation under varying 2,4-D concentrations (14).The most commonly supplemented phytohormones in Y3 medium were auxins, cytokinins and gibberellic acid which were important for the regulation of developmental processes. Several plant growth regulators have been added to supplement the explants for different needs such as callusing, shoot induction and embryo germination. Modified Y3 media supplemented with equal concentrations of 2,4-D and kinetin, which was used to induce callus (15). Modified Y3 media supplemented with kinetin and/or Thidiazuron (TDZ) aided shoot induction in whole embryo culture (16). Modified Y3 media supplemented with TDZ was used to induce shoots in sliced shoot tip culture (17).

4. Embryogenesis

Embryogenesis is primarily concerned with establishing the basic shoot-root body pattern of the plant (18). Embryogenesis can be classified into two types: zygotic and somatic embryogenesis. Zygotic embryogenesis is the process of embryo development where the fertilized egg undergoes a series of molecular events in order to develop into a mature embryo (19). Somatic embryogenesis is a developmental process during which a totipotent embryonic stem cell with the ability to give rise to an embryo under specific circumstances dedifferentiates from somatic cells of a plant (20).This provides an efficient way in propagating large number of elite coconut genotypes and serves as a valuable method for genetic transformation.

4.1 Embryo Culture: First experiment regarding coconut embryo culture was conducted to find out the significance of the 'coconut milk factor' in the development and growth of excised coconut embryos by Cutter and Wilson in 1954. Later, embryo culture for the Macapuno type of coconut which possesses semisolid endosperm was reported in the 1960s by De Guzman and Del Rosario in 1964.It was reported that 62% of embryos excised from *Cocos nucifera* L. var. nana progressed to the seedling stage when inoculated into a modified Y3 media and the study revealed the capacity for *in vitro* selection of particular traits such as drought resistance in coconut (21). Experiments revealed that liquid media are more effective than solid media for the culture of zygotic embryos (22). Germination rate of 80% was observed in embryos of the Malayan Green Dwarf variety when cultured in Y3 medium supplemented with 0.46 µM GA_3 (23).

In embryo culture, elongation of primary root was observed at the concentrations of 100-300 µM NAA (24). Adventitious root formation was stimulated when primary roots developed from zygotic embryo culture were trimmed (25). *In vitro*-cultured coconut plantlets produced through zygotic embryo culture exhibited an early initiation of photosynthetic metabolism (26). Plantlets derived through embryo culture of zygotic embryos exhibited higher photosynthetic rate and low transpiration rate when compared to plants raised in nursery under field conditions (27). An

efficient protocol consisting of a miniature version of growing chamber for maintaining relative humidity and facilitating *ex vitro* rooting has been used to produce true-to-type seedlings of Kopyor coconut (*Cocos nucifera* L.) through embryo culture in large quantities (28). It was reported that 78% of shoot initiation was achieved when whole embryos were inoculated in Y3 basal medium supplemented with 200 µM TDZ (17). Embryo culture techniques have become essential for collecting coconut germplasm from faraway regions and transporting them to the laboratory (29). (Fig 2).

***4.2 Sliced Embryo Culture*:** Sliced embryo culture involves the *in vitro* cultivation of embryo sections which were sliced transversly. Coconut embryo tissues which were transversely cut and cultured in media enriched with 100 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25% of activated carbon resulted in three different kinds of proliferative responses such as whitish nodular callus, yellowish rounded callus and whitish dome like mass (30). Embryo splitting procedure was employed to double Macapuno seedling production (31). Embryo incision was carried out at the meristem region and after four weeks of culture, the embryos were then split into two and placed in MS medium supplemented with 15 µM kinetin and 2 µM IBA for recovery purposes. By following this protocol, 56 shoots were produced from 30 zygotic embryos (32).Sliced embryos resulted in maximum shoot initiation (75%) and regeneration (66.6%) when inoculated in Y3 media supplemented with 150 µM TDZ in East Coast Tall variety (ECT) of coconut (Fig 3) (16). Longitudinally sliced embryo explants of Chowghat Orange Dwarf (COD) coconut variety cultured in Y3 media enriched with 150 µM concentration of TDZ recorded high shoot induction (90.9%) and regeneration (33). Shoot induction and regeneration were observed higher when the embryo was sliced into four and cultured in Y3 media supplemented with 200 µM TDZ (17). Compared to whole embryo culture, sliced embryo culture facilitates the production of a higher number of plantlets from a single embryo, providing an efficient alternative for coconut, where side shooting is a rare occurrence.

5. Organogenesis

Organogenesis implies the development of organs like shoots and roots, under *in vitro* conditions. It relies on the balance of phytohormones and tissue response to the culture. *In vitro* organogenesis involves three key phases namely competence, differentiation and morphogenesis (34). Organogenesis is classified into direct organogenesis and indirect organogenesis. Direct organogenesis is the development of direct buds or shoots from a tissue without an intermediary callus stage. The formation of organs from the intervening callus stage is known as indirect organogenesis (35). Direct organogenesis has been considered as the effective method in several plant species. In coconut, regeneration through direct shoot organogenesis is considered to be a more secure and dependable pathway than somatic embryogenesis since the risk of somaclonal variation is less significant. While coconut is considered non-branching, the rare occurrence of shoot branching under specific natural conditions indicates the potential for producing multiple adventitious shoots *in vitro* (36).

Fig. 2. Whole embryo culture.

(A) Mature embryo (B) 30 DAI (C) 60 DAI (D) 90 DAI (E) 120 DAI (F) 180 DAI DAI - Days After Inoculation (95)

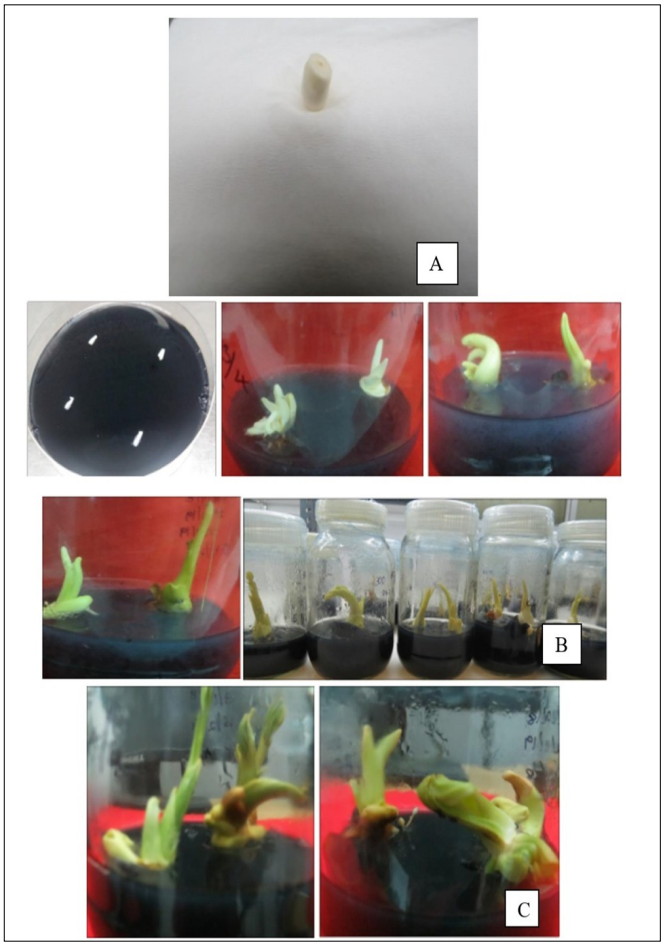

Fig. 3. Sliced embryo culture.

(A) Isolated embryo (B) Shoot initiation and shoot regeneration at various stages (C) Shoot growth and development at 150 DAI (16)

5.1 Shoot tip culture: Shoot tip culture is broadly defined as the isolation and culture of explants bearing an intact shoot meristem, that assists in shoot multiplication through the repetitive development of axillary branches under *in vitro* conditions (36). Axillary shoot formation from shoot tips, characterized by axillary origin, has been recognized as a preferred method due to its low occurrence of spontaneous mutations (37). This approach is considered a reliable and true-to-type system for *in vitro* clonal propagation. Shoot meristems, located in the shoot region responsible for cell division and elongation, drive primary plant growth and are hence classified as primary meristems. Consequently, shoot tips represent a suitable explant choice for direct shoot organogenesis. The shoot apical meristem, positioned at the apex of the dome, comprises a central zone of stem cells that contribute to the formation of leaves, stems and flowers, implying their potential in micropropagation (10). Woody plant shoot release more phenolic compounds when placed on a growth medium than herbaceous species. Percentage of shoot induction (90%, 180% and 350%) and regeneration (84%, 164% and 325%) was observed when Y3 media enriched with 200 µM TDZ was used for culturing whole shoot tip, two and four sliced shoot tips (embryo derived shoot tips) respectively. Sliced shoot tips have the potential to overcome apical dominance and can regenerate shoots (Fig 4) (17).

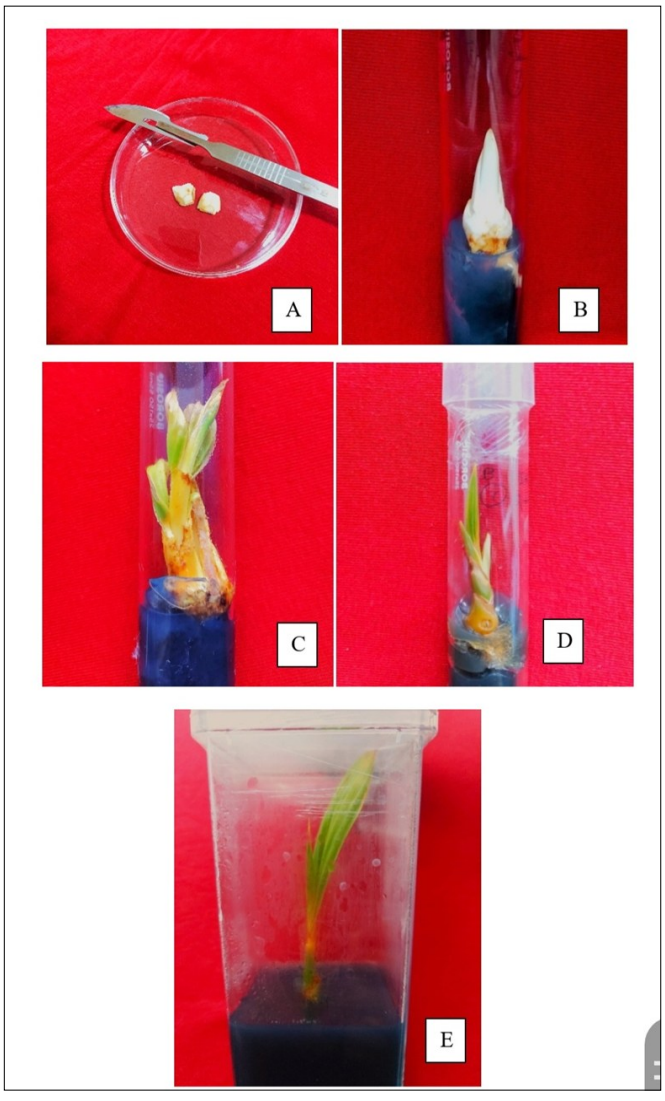

Fig. 4. Shoot tip culture.

(A) Sliced shoot tip (B) 30 DAI (C) 60 DAI (D) 120 DAI (E) 150 DAI (17)

***5.2 Axillary Bud Culture*:** Axillary meristems are often dormant due to apical dominance, but they can be activated using phytohormones like cytokinin, which directly favors their outgrowth. Phytohormones like cytokinin can directly favor axillary bud outgrowth. Two different protocols (cut and meristem) were devised to induce *in vitro* axillary shoot formation through meristem proliferation in different varieties of coconut. The induction of proliferating meristems from the initiated explants was observed at rates of 18.8% in the meristem protocol and 30.6% in the cut protocol respectively. 5 to 18 *in vitro* plantlets were produced from each regenerated meristem clump (10).

6. Somatic Embryogenesis

Many researchers consider micropropagation through somatic embryogenesis to be one of the most promising techniques for producing elite planting materials on a large scale. Several studies on coconut micropropagation indicate that somatic embryogenesis (SE) as a potential strategy for *in vitro* plant regeneration. The total calculated yield of 98 000 somatic embryos from one plumular explant was obtained through secondary somatic embryogenesis and embryogenic callus multiplication in coconut (38). The highest somatic embryogenesis frequency reported for the Al-Fayda cultivar of date palm was 89% in MS medium supplemented with 225 µM of 2,4-D (39). Similarly, in oil palm cultivar SUPPSU1, the use of OPM (Oil Palm Medium) enriched with 0.3 mg/L CPPU achieved 100% SE proliferation, producing an average of 5.16 embryos per tube after four weeks of culture (40).

Somatic embryogenesis is a process whereby a plant's somatic cell can dedifferentiate to a totipotent embryonic stem cell capable of giving rise to an embryo under appropriate circumstances (20). In somatic embryogenesis, a bipolar structure from a non-zygotic cell without vascular connection with the original tissue which resembles the zygotic embryo is developed. There are two distinct processes that induce somatic embryogenesis: direct somatic embryogenesis and indirect somatic embryogenesis (41). Direct somatic embryogenesis forms embryos directly from explants without a callus stage, whereas indirect somatic embryogenesis involves an intermediate callus formation stage (42). Somatic embryogenesis is classified into primary and secondary somatic embryogenesis. In the instance of primary somatic embryogenesis, somatic embryos develop directly from explants. Secondary somatic embryogenesis occurs when pre-existing somatic embryos produce secondary somatic embryos. Somatic embryogenesis of coconut involves the following steps: induction of embryogenic callus, formation and development of somatic embryo, maturation of somatic embryo and its maturation finally resulting in the recovery of the plantlet (43). Several factors influence somatic embryogenesis, including donor plant genotype, explants type, media composition, plant growth regulators, polyamines and DNA methylation levels (5). Nutrient deficit, wounding, cold, heat, osmotic shock, water deficit, heavy metals, medium dehydration, UV radiation and pH are all stress-related factors that induce somatic embryogenesis (44).

In coconut palm, different kinds of somatic tissues like immature embryos, stem sections from young leaves, young seedlings, plumules, rachillae, shoot tips and unfertilized ovaries were utilized to facilitate somatic embryogenesis (45). These explants and their callusing percentage are depicted in (Table 2).

Table 2. Different types of explants used in somatic embryogenesis

Explant type	Variety	Media	Observations	Time period	References
Embryo	Malayan Green Dwarf	Y3 + 0.46 µM GA_3	80% germination	32 weeks	(23)
Embryo		Y3 +125 µM 2, 4-D + 5 µM BAP	100% callusing	30 weeks	(53)
Immature zygotic embryo	Sri Lanka Tall	CRI 72 + 24µM 2,4-D + (2.5-7.5 µM) ABA	70% callusing	32 weeks	(55)
Immature inflorescence	PB 121	Y3 + 300µM 2,4-D	12.5% callusing	38 weeks	(8)
Inflorescence	*Cocos nucifera* L. var. MATAG	Y3 + 20 mg/l 2,4-D	43.75% callusing		(65)
Rachilla	West Coast Tall	Y3 + 4.54µM 2,4-D	92% callusing	30 weeks	(71)
Rachilla segments	Sri Lanka Tall	CRI 72 + 24µM 2,4-D	30% callusing	28 weeks	(73)
Plumule	Malayan Dwarf	Y3 + 0.1mM 2,4-D	40% callusing	36 weeks	(56)
Plumule	Sri Lanka Tall	CRI 72 + 24µM 2,4-D	55.2% callusing	33 weeks	(58)
Plumule	Malayan Green Dwarf	Y3 + 1µM 2,4-D	43% callusing	40 weeks	(59)
Plumule	West Coast Tall	Y3 + 16.5mg/l 2,4-D + 200mg/l glutamine	2.42% of callusing	64 weeks	(60)
Anther	Sri Lanka Tall	Y3 + 100µM 2,4-D	21% callusing		(63)
Anther	Sri Lanka Tall	Y3 + 100µM 2,4-D	22.2% callusing	44 weeks	(94)
Anther	Sri Lanka Tall	Y3 + 100µM 2,4-D + 100µM NAA	123 embryo per 100 anthers		(64)
Ovary	Sri Lanka Tall	CRI 72 +100µM 2,4-D	41% callusing	31 weeks	(68)
Ovary	Sri Lanka Tall	CRI 72 + 9µM TDZ	76.4% callusing	24 weeks	(69)
Ovary	Sri Lanka Tall	CRI 72 + 9µM TDZ + 100µM 2,4-D	74% callusing	38 weeks	(70)
Ovary	Dwarf x Tall	CRI 72 + 9µM TDZ + 100µM 2,4-D	65% callusing	38 weeks	(70)
Ovary	*Cocos nucifera* L. var. MATAG	Y3 + 20 mg/l 2,4-D	31.2% callusing	72 weeks	(66)

***6.1 Callus culture*:** Callus culture involves growing dedifferentiated plant cells on media rich in auxins or auxin-cytokinin combinations under controlled conditions. Several explants like leaf tissues, plumule, anther, immature inflorescence and embryos can be used for the induction of callus culture. Of all the explants evaluated, immature zygotic embryo tissues found to be most effective for establishing callus with the potential to initiate somatic embryogenesis (46). Explants from plant tissues when cultivated on hormone-supplemented media, these can gradually develop into a cell mass/callus which are amorphous and colorless to pale-brown. Callus cultures are homogenous enough to generate identical replicas of plants with specified characteristics through micropropagation (47). Homogeneity in coconut plant tissue culture ensures the true-to-type nature of propagated plants, which is crucial for producing genetically identical plants of elite genotypes with high performance and desirable traits. The success of micropropagation relies on maintaining this genetic uniformity; any deviation compromises the technique's purpose. Non-homogeneous cultures can result in plants with genetic variations, leading to inconsistencies in yield, disease resistance and quality. Furthermore, failing to meet genetic fidelity standards presents ethical concerns, as tissue-cultured plants must reliably exhibit the traits of elite varieties. Ensuring genetic homogeneity is essential for consistent growth, predictable outcomes in commercial cultivation and efficient agronomic practices, such as nutrient management and harvesting. It also enhances yield stability, addressing issues like variable fruit size and oil content.

Callus can be of two types: embryogenic and non-embryogenic calli. Embryogenic calluses have a smooth, translucent and well-defined somatic structure, whereas non-embryogenic calluses were rough, yellow and sponge-like texture. Histological analysis revealed distinct anatomical differences between compact embryogenic and non-embryogenic calli. Compact embryogenic calli were characterized by actively proliferating embryogenic cells with large nuclei, visible nucleoli and abundant starch granules in the cytoplasm. In contrast, non-embryogenic calli lacked meristematic cells; their central regions consisted of parenchymatous cells with faintly blue-stained nuclei, devoid of apparent nucleoli or starch granules (48). Callus can be classified as compact or friable, based on texture and structure.Compact calli are solid and rigid in texture, whereas friable calli are flexible and easily separated (49). Generally, embryogenic calli are friable in nature.

Several parameters associated in the friable callus production of coconut tissue culture include medium, nutrient reduction in the medium, frequency of subculturing, concentration of 2,4-D, level, type of nitrogen and amino acid addition (48). Significantly multiplying callus tissues were produced from more than fifty percent of cultured embryos at 12 to 20 µM concentration of 2,4-D. At higher concentrations (above 30 µM) of 2,4-D callusing was inhibited and browning of explants was observed (7). Highest callusing percentage of 2.9% was observed when young leaf explants of coconut cv MATAG were inoculated in medium containing 2,4-D (40 mg/mL) with NAA (1 mg/mL) (50). Increasing 2,4-D concentrations might improve the percentage of embryogenic callus, as well as its diameter and weight. The addition of TDZ concentration of 0.5 µM in the medium resulted in a decrease in the proportion of embryogenic callus, callus diameter on embryonic callus of kopyor coconut (51). In coconut plumular explants of West Coast Tall (WCT), callus induced with 2 µA current consistently for a month resulted in the highest weight gain, followed by callus induced with 1 µA current continuously for the same period in Y3 media supplemented with 16.5 mg/l of 2,4-D (52).

***6.2 Mature embryo culture*:** In mature embryo culture technique, fully developed embryos, typically obtained from seed nuts that are 10 to 12 months old, are cultured under *in vitro* conditions to initiate germination and plant regeneration.Highest rate of embryogenic callus formation (100%) was observed in mature zygotic embryos cultured on Y3 medium supplemented with 125 µM 2,4-D and 5 µM BAP. Multiple shoots were subsequently induced from friable embryogenic calli in Y3 media containing 10 µM kinetin, 10 µM BAP, 200 µM NAA and 0.5 µM GA_3 Cytokinins such as kinetin and BAP are key regulators of cell division and differentiation, promoting shoot induction and somatic embryo

formation. NAA, an auxin, is critical for initiating somatic embryos and facilitating proper tissue organization during embryo development. GA_3 (gibberellic acid) supports the germination of somatic embryos and enhances shoot elongation by stimulating cell elongation and growth processes. Together, these hormones create an optimal environment for shoot initiation and subsequent development (53). The highest embryogenic callus formation (55%) and translucent structure development (44%) were achieved in mature embryos of *Cocos nucifera* L. var. COD cultured on modified Y3 medium supplemented with equal concentrations of 2,4-D and kinetin (300 µM) (15). In mature zygotic embryos of COD, maximum callus and embryogenic structure formation (33%) was observed in Y3 medium supplemented with 450 µM 2,4-D and 200 µM BAP (Fig 5) (54).

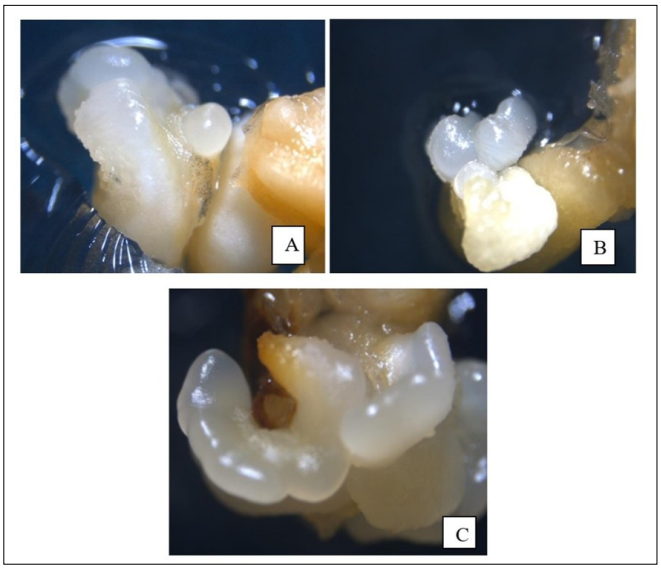

Fig. 5. Somatic embryogenesis.

(A) Callus initiation after 45 DAI (B) Translucent structures after 60 DAI (C) Embryogenic structures (54)

***6.3 Immature embryo culture*:** The process of cultivating coconut seed nut embryos that are 6 to 7 month old *in vitro* for plant germination and regeneration is known as immature embryo culture. Embryo-producing calli were successfully induced in 56% of immature coconut embryos (6 months old) of *Cocos nucifera* L var. typica using CRI 72 medium supplemented with 16 µM 2,4-D. Shoot development was observed in 22% of the cultured calli (7). Calli derived from immature zygotic embryos (7-9 months old) were maintained on CRI 72 medium supplemented with 16 µM 2,4-D for 5 weeks followed by treatment with 2.5 µM ABA resulted in 67.4% somatic embryogenesis and 9.4% shoot regeneration (55).

***6.4 Plumule Culture*:** The plumule explant, composed of the shoot meristem and bound by leaf primordia derived from mature zygotic embryos, has been found to be most suitable for embryogenic callus and somatic embryo development. Coconut micropropagation *via* plumules is potentially useful when obtained from fruit harvested from selected parents of exceptional performance, which include disease resistance. When plumular explants inoculated in Y3 media enriched with 0.1 mM 2,4-D, 40% of the plumules developed callus (56). The plumular explants reacted significantly to the brassinosteroid, increasing their ability to generate initial callus, embryogenic callus and somatic embryos. Explants treated with 0.1 µM brassinosteroid for three days yielded 10.8 somatic embryos (57). Callusing frequencies were higher when plumular explants were inoculated in different basal media which were commonly used for coconut tissue culture namely CRI 72,Y3 and MS enriched with 24 µM of 2,4-D. Callusing was reduced when the 2,4-D concentration was increased upto 100 µM (58). About 43% of coconut plumular explants cultured in media containing 1 µM 2,4 -D without activated charcoal formed embryogenic calli (59). Y3 medium supplemented with 200 mg/L glutamine and 16.5 mg/L 2,4-D significantly enhanced calloid production resulting in 90% plumular bulging and callus clump formation from plumular explants of coconut (60). Using plumule explants resulted in the formation of both embryogenic callus and secondary somatic embryos. Total calculated yield of 98,000 somatic embryos were obtained from a single plumular explant (38). The Yucatan Scientific Research Center (CICY, Mexico) developed a significantly effective protocol for coconut micropropagation which has the potential to yield over a hundred thousand somatic embryos from a single plumule explant and is presently scaled up to a semi-commercial level (61).

***6.5 Anther culture*:** On account of the high heterozygous nature of tall varieties of coconut, homozygous lines cannot be obtained through conventional methods (4). Through tissue culture techniques, homozygous lines could be produced by means of anther culture in tall varieties (62). Androgenesis was observed when the anthers were subjected to heat pretreatment for six days at 38 °C upon culturing in 100 µM 2,4-D added Y3 medium (63). When anthers were inoculated on Y3 medium supplemented with 100 µM 2,4-D and 100 µM NAA, 123 embryos per 100 anthers were obtained, although plant regeneration was observed at low frequencies (64). Callus induction (43.75%) from *Cocos nucifera* var. MATAG anthers was achieved using Y3 medium supplemented with 20 mg/L 2,4-D (65). In a similar study, callus induction percentage of 24.15% was observed when anthers obtained from *Cocos nucifera* var. MATAG were inoculated on Y3 medium supplemented with 20 mg/L 2,4-D and 1.5 mg/L activated charcoal (66). Anther-derived calli were an excellent source of explant for the development of doubled-haploid coconut plants (62).

***6.6 Ovary culture*:** Ovary culture, also referred to as gynogenesis, is the process of cultivating unfertilized ovaries to develop haploid plants from egg cells or other haploid cells in the embryo sac. The juvenilization action of surrounding meiotic tissues makes the ovary a potentially useful tissue for somatic embryogenesis. Unfertilized ovaries derived from immature female flowers were considered to be optimal explants for clonal propagation due to their somatic origin and plant regeneration efficacy (67). The *in vitro* culture of unfertilized coconut ovaries enabled the formation of callus and adventitious roots. Culturing unfertilized ovaries in CRI 72 medium with 100 µM 2,4-D and 0.1% activated charcoal resulted in consistent callogenesis. Callusing (31.67%) of cultured ovaries was detected when they were inoculated in Y3 media enriched with 20 mg/L 2,4-D (66). Culture of ovary-derived calli in media with 9 µM TDZ and 160 µM 2, 4 D resulted in a considerably increased percentage of embryogenic callus (70.4%) compared to medium without TDZ (42.6%). Slicing of ovaries for callogenesis ensured a positive effect on ovary culture, when thinly sliced ovaries exhibited a callusing percentage of 65.6% (67). Somatic embryogenesis was induced by subculturing ovary-derived calli of coconut onto CRI 72 medium containing ABA with low plant regeneration efficiency (68). Increased regeneration frequency of shoots in callus produced through ovary culture was observed when the conversion media was incorporated with GA_3. From 32 cultured

ovaries 83 plantlets were produced (69). Crushed ovaries cultured in CRI72 medium supplemented with 9 µM TDZ and 100 µM 2,4-D exhibited a 65% callus induction rate while shoot regeneration per inflorescence ranged from 1 to 200 (70).

6.7 Immature Inflorescence Culture: Immature inflorescences can be considered an ideal source of explant since the performance of parental palm is already known (71). When rachilla tissues obtained from coconut palm were used as an explant source, a greater frequency of callus induction (92%) was observed in media integrated with activated carbon and 2,4-D at 10 ppm concentration. Shoot like structures were developed under subsequent subcultures (72). About 30% of the immature inflorescence explants inoculated on medium CRI 72 enriched with 24 µM concentration of 2,4-D and 0.25% activated charcoal produced compact and highly embryogenic calli. Low shoot regeneration frequency is observed (73). Callus and embryogenic structures were formed when rachillae explants are inoculated in media containing 0.65 mM 2,4-D (74). When rachillae bits obtained from immature inflorescence were cultured in Y3 medium fortified with 4.54 µM 2,4-D maximum response in terms of white translucent outgrowth with minimum browning was observed. After 4 months of subculturing, 42% of cultures produced shoot like outgrowths (71). These findings emphasize the potential of immature inflorescences, specifically rachilla tissues, as potential explants for coconut tissue culture, as they exhibit significant callus induction and embryogenic capacity under ideal conditions. In contrast, the low frequency of shoot regeneration indicates the need for further enhancing of culture techniques in order to improve regeneration efficiency and optimize the utility of these explants

***6.8 Leaf Culture*:** Leaf tissue culture technique involves the *in vitro* cultivation of leaf segments to explore their regenerative capabilities. Experiments revealed that 2,4-D was crucial in inducing morphogenetic characters in leaf tissues of coconut and an effective range of 8-20 µM of 2,4-D was found to be significant. The embryogenic potential of leaf explants was highly correlated with their physiological maturity in young coconut palms (75). When young leaf segments of coconut cultivar MATAG inoculated in Y3 media enriched with 40 mg/l concentration of 2, 4-D, 2.9% of explants produced callus (50). Along with contamination, phenolization was a significant challenge in leaf tissue culture of coconut. Ethanol was regarded as the greatest sterilant for controlling contamination and browning rate in coconut leaf tissue culture (76).

7. Molecular basis of somatic embryogenesis

Somatic embryogenesis involves precise reprogramming of gene regulation in addition to the action of an advanced signaling system. Identification of genes and analysis of their regulation is important to gain appreciable understanding of the somatic embryogenesis process (77). Somatic Embryogenesis Receptor-Like Kinase (SERK) is a stage-specific gene that serves as a critical marker for embryogenesis, particularly in the early stages of somatic embryogenesis. An ortholog of SERK, identified in coconut as **CnSERK**, exhibits conserved domains found in SERK proteins across various species. SERK expression is restricted to embryogenic cultures and is absent in non-embryogenic tissues, making it a reliable indicator of embryogenic potential. During somatic embryogenesis, SERK is expressed in cells predicted to be embryogenic, particularly in tissue explants placed under embryogenic culture conditions. This expression is stage-specific, occurring up to the early globular stage, after which it ceases. The use of a SERK promoter-luciferase reporter gene further demonstrates that cells expressing SERK are capable of forming somatic embryos. By identifying SERK expression in the early stages, researchers can effectively select embryogenic tissues, streamlining the tissue culture process and enhancing the efficiency of somatic embryo production.Expression analysis showed that CnSERK was detected in germ cells before visible embryonic development, whereas its occurrence was low or absent in non embryogenic tissues. This represents the association of CnSERK with the occurrence and its potential as a marker to identify cells suitable for somatic embryogenesis process (78). The expression of the CDKA (Cyclin – dependent kinase) gene, which is crucial for cell cycle regulation, steadily increased during embryogenic callus formation, peaking at 90 days. As somatic embryos form, its expression progressively decreased and in germinated somatic embryos the expression reached the lowest level (79).

RNA sequencing provided quantitative information regarding gene expression and differentially expressed genes by high throughput RNA transcript analysis *via* cDNA sequencing. Transcriptomics is used to analyze gene expression variations and gene pathways in activating or repressing multiple genes during somatic embryogenesis. Analysis of the transcriptome of embryogenic calli obtained from West Coast Tall cultivar plumular explants revealed fourteen genes associated with somatic embryogenesis. Quantitative real-time PCR analyses of these fourteen genes resulted in the following findings. In the initial stage of callogenesis *CLV* (CLAVATA) was upregulated. In somatic embryo stage transcripts like *GLP* (Germin Like Protein), *GST* (Glutathione S Transferase), *PKL* (PICKLE), *WUS* (WUSHEL) and *WRKY* were expressed higher than others. The expression of *SERK* (Somatic embryogenesis receptor-like kinase), *MAPK* (Mitogen-activated protein kinase), *AP2* (APETALA2), *SAUR* (Small auxin up-regulated RNA), *ECP* (Embryogenic cell protein), *AGP* (Arabinogalactan protein), *LEA* (Late embryogenesis abundant protein) and *ANT* (AINTEGUMENTA) were higher in the embryogenic callus stage compared to initial culture and somatic embryo stages (9). Gene expression patterns studies during different stages of *in vitro* regeneration in two coconut cultivars WCT and COD revealed that expression of PKL, SERK and WUS was enhanced in embryogenic calli when compared to non embryogeniccalli in both cultivars. In West Coast Tall high expression of somatic embryogenesis was observed along with the higher expression of GLP and GST. When compared to abnormal somatic embryos, normal somatic embryos exhibited higher expression of GLP, ECP and GST genes (80).

Epigenetic mechanisms have been examined under the circumstances of coconut somatic embryogenesis by assessing morphogenetic alterations, global DNA methylation, DNA methyltransferases and expression profiles of genes linked with somatic embryogenesis. Formation of early somatic embryos is significantly improved on pretreatment with 5-Azacytidine (AzaC). Global DNA methylation peaked at the initiation of the culture, subsequently decreased after AzaC pretreatment and then reestablished at a reduced level. Increased expression of DNA methyltransferase genes observed at the initial and later stages of somatic embryogenesis indicated the importance of DNA methylation in somatic embryogenesis process (81). In the genome-wide profiling of small RNAs obtained from embryogenic

and non-embryogenic calli 110 conserved and 97 novel miRNAs were identified. These conserved and species-specific miRNAs were found to act together in regulating somatic embryogenesis in coconut (82).

8. Tissue culture in germplasm conservation

Germplasm conservation in coconut is indispensable for maintaining the genetic diversity and for ensuring security for future yield. Many factors, including the cultivation of better varieties with a limited genetic base, natural disasters, pests and diseases, urbanization and senility, pose a threat to the diversity of coconuts (83). Coconut germplasm is conserved by employing both in situ and *ex situ* methods, including *in vitro* conservation techniques such cryopreservation of zygotic embryos, embryogenic calli, plumule and anther (84).

***8.1 Embryo culture*:** The initial endeavor in cryopreservation of coconut tissues involved the application of a conventional cryopreservation protocol, where immature zygotic embryos were subjected to chemical dehydration and slow freezing techniques.No morphological, cytological, or molecular variations were observed between seedlings recovered from cryopreserved zygotic embryos and their non-cryopreserved counterparts, highlighting cryopreservation as an efficient method for coconut germplasm conservation (85). A recovery percentage of 60% was documented when mature zygotic embryos were desiccated for 10 hours and subjected to cryoprotective treatment for 15 hours in a solution containing 15% glycerol (86).High recovery of plantlets was observed when mature embryos are subjected to dessication pretreatment with 18 hours silica gel (85). Zygotic embryos subjected to chemical dehydration with plant vitrification solutions achieved a survival rate of 70 to 80% and plant recovery rate of 20 to 25% (87). For germplasm conservation, a protocol for zygotic embryo culture has been developed by Central Plantation Crops Research Institute, Kasargod.

***8.2 Plumule culture*:** Cryopreservation of embryogenic callus which were derived from plumules could be suggested as an alternative preservation strategy even though it poses challenges as each callus represents only one genotype, this method could conserve a single tissue with the potential of generating thousands of plantlets. Twenty percent of leaf shoots were recovered when plumules are immersed directly into liquid nitrogen without any intermediate callus stage. This indicates plumules can be conserved through cryopreservation by encapsulation and dehydration method(88). High amounts of embryogenic calluses were induced when the cryopreserved plumules of Brazilian Green Dwarf variety were exposed to vitrification solutions PVS2 and PVS3 for 30 minutes (89).

9. Patents

The patents in coconut tissue culture fall under the category of A01H 4 - Plant reproduction by tissue culture techniques. In 2021 a patent filed by Rural Development Administration of Korea and UK Leuven for *in vitro* clonal propagation of shoots through meristem derived from zygotic embryos entered the national phase. The patented protocol developed by the Coconut Research Institute, China achieved 72 to 78% callus induction in mature zygotic embryo cultures inoculated in Y3 medium supplemented with 25 mg/L 2,4-D. Researchers at the Yucatan Scientific Research Center (CICY) in Mexico have developed a highly efficient protocol for coconut micropropagation through plumule culture, capable of producing over 100,000 somatic embryos from a single plumule explant. This method has been scaled up to a semi-commercial level (61). The patented protocols in coconut tissue culture have been mentioned in (Table 3).

10. Challenges in coconut tissue culture

In vitro interventions have often yielded poor responses across different types of explants, cultivars, or culture conditions (90). Despite numerous research efforts in coconut tissue culture, the inherent recalcitrant nature of coconuts poses challenges, hindering their response *in vitro* making it hard to accomplish. The success of coconut *in vitro* culture is often hindered by several bottleneck factors, including low plant regeneration rates, premature necrosis, extensive browning of cultured tissues, oxidation of phenolic compounds, the effects of activated charcoal in media, the heterogenous nature of explants and poor development of plants in both *in vitro* and early *ex vitro* conditions (13). The heterogeneous nature of explants can be examined from two perspectives: the variation in response among individual palms of the same cultivar and the distinct responses observed among individual explants within a single palm (91). Among various micropropagation methods employed in coconut tissue culture, propagation through somatic embryogenesis is widely regarded as the most promising method. Although somatic tissues were regarded as the most preferable explant for micropropagation due to the known performance of the mother palm, their response is typically less than desired. The major bottlenecks in somatic

Table 3. Patents in coconut tissue culture

Patent number	Year	Inventor	Description	Link
WO1994016551A1	1994	Jacqueline Buffard-Morel Catherine Pannetier Jean-Luc Verdeil, France	Process for regenerating the coconut palm from leaf and inflorescence explants	*https://patents.google.com/patent/WO1994016551A1/en?oq=WO1994016551A1*
CN102715088A	2012	Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, China	Somatic embryogenesis through mature zygotic embryos	*https://patents.google.com/patent/CN102715088A/en?oq=CN102715088A*
CN109479714A	2018	Liu Rui and Fan Haikuo	Tissue culture method for mature zygotic embryo culture	*https://patents.google.com/patent/CN109479714A/en*
WO2020152366A1	2020	Hanns Wilms and Bart Panis	Tissue culture method of clonal multipliation via proliferating mersitems	*https://patents.google.com/patent/WO2020152366A1/en*
CN116250482A	2023	Sanya Nanfan Research Institute of Hainan University, China	Method for tissue culture and rapid propagation of coconut	*https://patents.google.com/patent/CN116250482A/en?oq=CN116250482A*

embryogenesis include the low rate of somatic embryo formation, the occurrence of abnormal somatic embryos, the generation of diverse and compact callus structures due to variations in genotype or explants maturity and the limited development of plantlets via *in vitro* methods (13). The growth of tissue culture raised plants in both *in vitro* and early *ex vitro* stages is considerably slow when compared to plants cultured from embryos. The regenerated clones appear smaller at the time of transplantation and take a longer period to reach the stage suitable for field planting (92). Browning of both explants and the medium which leads to the deterioration of tissues is a significant constraint in tissue culture of tree crops. The cut surfaces exhibit discoloration shortly after excision and the continuous release of phenolics persists even during the culturing process (93).

Conclusion

The clonal propagation of coconuts faces challenges due to the heterogeneous nature of explants. Selection of highly responsive explants, better concentration of plant growth regulators and composition of the culture medium should be followed to overcome these barriers. The obstacles hindering coconut tissue culture could be counteracted through strategies such as devising a charcoal free media, standardizing activated charcoal usage, multiplying embryogenic cell masses and inducing stress conditions to enhance somatic embryogenesis and plant regeneration. Although somatic embryogenesis is thought to be the most successful way for coconut micropropagation, it has the risks of somaclonal variation and fidelity problems. A key research gap led us to explore the organogenesis pathway, which could help minimize the risks of somaclonal variation and provide a more reliable method for coconut propagation. Organogenesis has the potential to match somatic embryogenesis in effectiveness and has been recorded to produce results more rapidly. To meet the increasing demand for high-quality planting materials, greater attention and research could be directed towards optimizing the organogenesis pathway. However, it is essential to consider that while tissue culture gives promising solutions its adoption may face challenges due to cost, infrastructure and the need for skilled personnel. Persistent collaboration between researchers, research institutions and industry stakeholders is crucial to overcoming these barriers. Only through collective efforts can the full potential of coconut tissue culture can be realized, benefiting coconut-producing regions worldwide.

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Authors' contributions

The original draft of the writing was prepared by NA and RR, with RR also contributing to the review and editing process. The conceptualization, data curation, methodology and project administration were carried out by RR and KKK. Resources, supervision, and validation were overseen by MM, SJ and TN, ensuring the project's quality and accuracy.

Compliance with ethical standards

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