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RESEARCH ARTICLE

5-Azacytidine promotes the induction of embryogenic calli and somatic embryos from transverse thin cell layer (tTCL) cultures in coconut

Keezhath Thazha Kuniyil Amritha[1, 2], Kilingar Subrahmanya Muralikrishna[1], Jasmin Habeeb[1], Kunduchi Pereye Chandran[1], Santhappan Paulraj[1], and Muliyar Krishna Rajesh[1, 3*]

[1]Indian Council of Agricultural Research-Central Plantation Crops Research Institute (ICAR-CPCRI), Kasaragod 671124, Kerala, India.
[2]Mangalore University, Mangalagangotri, Mangaluru 574199, Karnataka, India.
[3]Indian Council of Agricultural Research-Central Plantation Crops Research Institute (ICAR-CPCRI), Regional Station, Vittal 574243, Karnataka, India.
*Corresponding author (rajesh.mk@icar.gov.in).
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ABSTRACT

Coconut (*Cocos nucifera* L.) is highly recalcitrant to in vitro interventions. There is a need to overcome various bottlenecks to standardize a repeatable protocol for in vitro regeneration in coconut to meet the requirements of quality planting materials. Epigenetic processes, especially DNA methylation, are known to assay crucial roles in regulating genes controlling plant growth and development, especially somatic embryogenesis. In this study, we demonstrate that the supplementation of 5-azacytidine (5-azaC), a demethylating agent, in the coconut tissue culture media can enhance the formation of embryogenic calli, somatic embryos, and plantlet regeneration from transverse thin sections of mature zygotic embryos. Transverse thin cell layer (tTCL) sections of zygotic embryos were cultured onto Y3 medium supplemented with different concentrations of 5-azaC (0, 10, 15, and 20 µM), auxins (2,4-D, picloram and atrazine; 75 and 100 µM) and thidiazuron (TDZ;4.5 µM). Explants were exposed to constant 5-azaC and reduced auxin concentration in subsequent sub-culturing. Our results indicated supplementing 15 µM 5-azaC, in combination with picloram (75 µM) and TDZ (4.54 µM), improved the percentage of callusing (95.8%) and formation of embryogenic calli (87.5%), and formation of somatic embryos (4.7) and plantlets (4.0) per explant in comparison with control having 80.8%, 75.0%, 1.6 and 0.67, respectively from tTCL sections of mature zygotic embryos. The results will form the basis for designing more efficient coconut tissue culture protocols.

Key words: Atrazine, 5-azacytidine, coconut, *Cocos nucifera*, embryogenic calli, somatic embryogenesis, picloram, thidiazuron, transverse thin cell layer cultures.

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is an oil-yielding crop belonging to Arecaceae, the palm family. Coconut is predominantly propagated through seed nuts; this can influence the variations in tree and fruit characteristics because of the highly cross-pollinated nature of the tall genotypes. Crop improvement programs in this perennial crop aim to enhance yield, resistance to diseases and pests, and climate resilience. A high degree of heterozygosity, seed propagated nature, long juvenile phase, and long-life cycle make conventional coconut breeding challenging (Rajesh et al., 2018). Somatic embryogenesis (SE) is a process whereby a whole plant is derived from somatic cells that have achieved competence to enter into the embryogenic pathway in vitro (Méndez-Hernández et al., 2019). The SE has been used for the clonal propagation of elite genotypes and the study of the molecular regulation of plant embryogenesis. Dramatic changes in cellular components like proteins, histones, polyamines, and polysaccharides have been observed during the process of SE (Méndez-

Hernández et al., 2019). The SE pathway is regulated by the expression of a repertoire of genes involved in synthesizing and transporting these cellular components (Elhiti and Stasolla, 2022).

Coconut tissue culture, which has been attempted using a repertoire of explants, is fraught with many bottlenecks (Rajesh et al., 2018; Kong et al., 2024), leading to its in vitro recalcitrance. Irrespective of the explant source, in vitro regeneration has been mainly reported to occur indirectly, i.e., involving an intervening callus phase. Production of friable, embryogenic calli is a prerequisite for successful plant regeneration in coconut. Among other factors, the presence of auxin in the medium is critical for successful callogenesis (Rajesh et al., 2014; 2018). The most commonly used auxin in coconut tissue culture media has been 2,4-D, with the concentration of 2,4-D supplemented in the medium depending on the type of explant and concentration of activated charcoal in the culture medium (Kong et al., 2023). Generally, SE in coconut calli has been induced by the gradual reduction of auxin concentration with a corresponding increase in the cytokinin level in the medium (Rajesh et al., 2014).

In contrast, SE in coconut has also been induced by an early increase in 2,4-D concentration followed by a gradual reduction or a rapid decrease in 2,4-D concentration and supplementation of high concentration of cytokinin (Rajesh et al., 2018; Kong et al., 2023; 2024). Another study suggested that an alternate treatment, i.e., removing auxin and incorporating abscisic acid (ABA) into the culture medium, could produce more consistent plant regeneration from coconut calli (Rajesh et al., 2018). In a recent study, Wilms et al. (2021) reported the induction of axillary shoots in vitro by supplementation of thidiazuron (TDZ) in the culture medium.

The role of epigenetic mechanisms, like DNA methylation, in regulating plant SE has not received due recognition until recently. The DNA methylation is the post-synthesis deoxycytosine methylation in the 5'-position of the pyrimidine ring of cytosine forming methyl deoxycytosine. In plants, DNA methylation is more common in CG/CHG repetitive motifs, referred to as 'CG islands' (Brukhin and Albertini, 2021). The DNA methylation can control the transposon activities via demethylation in their characterized 'CHG' islands, which results in the transposable elements moving across the genome; this could result in the disruption of the genes, thereby silencing them and causing phenotypic variability (Kumar and Mohapatra, 2021). The DNA methylation is a dynamic response to an external stimulus or transition to a different developmental stage (Sarpan et al., 2020).

Among the factors that regulate the expression of genes involved in various steps of SE, epigenetic changes such as chromatin remodeling and DNA methylation have also been implicated to play a significant role in regulating SE (Mozgová et al., 2017). The DNA methylation is the post-synthesis deoxycytosine methylation in the 5'- position of the pyrimidine ring of cytosine forming methyl deoxycytosine. The nucleotide analog 5-azacytidine (5-azaC), a methylation inhibitor, has been widely utilized to understand the epigenetic mechanism regulating plant SE. Studies have been undertaken to assess the effect of 5-azaC on in vitro regeneration in various plant species like *Brassica napus* and *Hordeum vulgare* (Solís et al., 2015), *Theobroma cacao* (Pila Quinga et al., 2017), *Arabidopsis thaliana* (Grzybkowska et al., 2018) and *Cocos nucifera* (Osorio-Montalvo et al., 2020). The main focus of this study was to assess the ability of different concentrations of 5-azaC to control SE in transverse thin cell layer (tTCLs) cultures, a novel explant in coconut, in a bid to expand the choices for viable in vitro regeneration pathways that would permit a repertoire of downstream biotechnological applications, such as large-scale multiplication of elite genotypes, genetic transformation, and gene editing feasible.

MATERIALS AND METHODS

Plant materials

The procedure outlined by Rajesh et al. (2014) was followed for extracting plumular explants. In brief, matured nuts (11-12 mo old) were harvested from 'West Coast Tall' *Cocos nucifera* L. palms, split open, zygotic embryos and endosperm were excised using a cork borer and placed in sterile, distilled water. The endosperm plugs enclosing the embryos were sterilized with 0.01% $HgCl_2$ (HiMedia, Thane, India) for 5 min and rinsed thrice with sterile distilled water to remove the traces of $HgCl_2$. Healthy embryos, extracted from endosperm plugs, were surface sterilized in 20% sodium hypochlorite solution (HiMedia) for 20 min and rinsed with sterile distilled water thrice. Transverse thin cell layers (tTCLs) about 0.2-0.3 mm thick, containing portions of the plumular region, were directly sliced from the surface-sterilized matured embryos in an aseptic condition using a sterile blade (surgical blade No.24, HiMedia) and scalpel holder (holder No.4, HiMedia).

Medium preparation and culture conditions

Initially, an experiment was conducted to test the effect of different concentrations of 5-azacytidine (5-azaC; 0, 10, 15, 20 µM) on primary callus induction, formation of embryogenic calli, and somatic embryos. Transverse TCLs (four sections for each embryo from the plumular end) were placed with the basal cut in contact with basal Y3 medium, which has been designed specifically for coconut (Eeuwens, 1976); Y3 medium has higher amounts of potassium chloride (KCl) and potassium iodide (KI) in comparison to MS-based media. In the present study, Y3 media were supplemented with 2,4-D (75 µM) (Sigma-Aldrich, St. Louis, Missouri, USA), thidiazuron (TDZ) (4.54 µM) (Sigma-Aldrich), based on the medium described earlier by Rajesh et al. (2014), and the concentrations mentioned above of 5-azaC. All media, which contained 3% (w/v) sucrose and 0.1% (w/v) activated charcoal, were adjusted to pH 5.8 and solidified with 0.6% agar (Sigma-Aldrich) before autoclaving at 105 kPa and 121 °C for 20 min and poured into 9 cm Petri dishes (*ca*. 25 mL medium in each) (Borosil, Mumbai, India). Based on the results obtained from this initial experiment, the best concentration of 5-azaC was selected, and three auxins (*viz.*, 2,4-D, picloram, and atrazine 75 and 100 µM) were tested.

At monthly intervals, the cultures were transferred to medium with a gradual decrease of the auxin (75 µM→45.24 µM→22.62 µM), while the concentration of 5-azaC and TDZ were maintained at a constant rate. The cultures were finally transferred to a medium devoid of any growth regulators. Once the somatic embryos were formed, they were transferred to Y3 medium supplemented with 6-benzyl amino purine (BAP) (2.2 µM) and naphthalene acetic acid (NAA) (2.6 µM) in culture jars (250 mL; 12 cm high and 6 cm in diameter) (ELS instruments, Bengaluru, India). Germinating somatic embryos were placed in Y3 medium supplemented with BAP (4.4 µM) (Sigma-Aldrich), NAA (5.3 µM) (Sigma-Aldrich), and indole-3-acetic acid (IAA) (Sigma-Aldrich) (2.8 µM).

Plant establishment and hardening

Plantlets with a minimum of 2-3 opened leaves and 3-4 primary roots with well-developed secondary roots were carefully taken out of the culture bottles. The medium was gently washed away for acclimatization. After that, the rooted plants were moved to plastic pots (10 cm diameter and 9.5 cm height) filled with sterilized soil, sand, and coir dust in equal proportions. The plantlets were covered with polythene bags for 3 wk. Plants were placed in a controlled environment room maintained at 25 ± 2 °C, with a 16:8 h photoperiod. After 1 wk, the bags were perforated to reduce humidity, and later, the bags were removed during the night. After 4 wk, the bags were removed completely. Watering was done in the greenhouse every 3 d to reduce the possibility of fungal diseases, as plants recently transitioned from tissue culture are generally more susceptible.

Histology and photographic documentation

For histological studies, the cultures were fixed in formaldehyde (Merck, Bengaluru, India) followed by serial dehydration using different concentrations of ethanol (70%, 80%, 90% and 100%) and different concentrations of ethanol and butanol (3:1, 1:1, 1:3) for 24 h in each. Dehydrated tissues were infiltered with molten wax, and the tissue specimens were embedded in paraffin wax to prepare paraffin blocks. Thin sections (10 µm) were prepared using the microtome (RM 2145, Leica, Wetzlar, Germany) with knife steel blades. Blocks were trimmed, and wax ribbons with sections were prepared. Gelatin (HiMedia) (3%) was used to affix the sections onto the microscopic glass slides. After the staining procedure, the slides were observed under a stereo zoom microscope (Leica) and binocular microscope (Leitz Diaplan, Schönwalde-Glien, Germany). Photos were captured through a camera (Leica) fitted to the microscope and connected to a desktop via Leica Application Suite (LAS) software.

Scanning electron microscopy (SEM)

Tissues, *viz*., tTCL explant, embryogenic and non-embryogenic calli, and somatic embryos of both control and 5-azaC (15 µM) were fixed with the powder sample spread on the double-sided conductive carbon tap in turn fixed on the stub and placed in the sample chamber of ESEM (Environmental Scanning Electron Microscope; Quanta 250, FEI, Brno, Czech Republic). After attaining a high vacuum, the filament was switched on. Various parameters like electron beam, intensity, spot size, voltage, and emission current were adjusted, and the images were captured in different magnifications.

Statistical analysis

A completely randomized design (CRD) was employed. The data presented correspond to means of six replicates per treatment, with each replication consisting of four slices from three zygotic embryos. The data obtained from the embryogenic calli induction, somatic embryo initiation, development, and germination experiments were recorded using MS Excel 2013. The rate of primary calli formation, somatic embryogenesis, and plantlet formation induction rates were evaluated. Statistical analysis was performed using ANOVA and expressed as the mean ± standard error (SE). The significant differences were determined at a 5% level with Duncan's multiple range tests (DMRT) using SPSS 24.0.

RESULTS

The results indicated the potential of transverse thin cell layer (tTCL) sections from mature zygotic embryos as an explant for the initiation of in vitro cultures (Figure 1a). Initial signs of morphological changes in the tTCL were evident during the second week of culturing in all the treatments, including control, marked by swelling of the entire explant. Callogenesis, characterized by compact nodular structures with unorganized growth from the portion consisting of the plumular portion, was apparent after 4 wk culture incubation. The coloration of the nodular callus ranged from white to creamy yellowish. However, none of the tTCL sections responded in vitro, as no change was observed in the anterior-most section of the embryo. In contrast, the rest of the sections resulted in calli formation, with the highest (100%) observed from the middle two sections (Figure 1b). Subsequently, the surrounding portion of the plumular region darkened by the fourth week. In absolute control, devoid of growth hormones, bulged sections did not form calli and resulted in either germination or enlargement of primordial leaves.

Initially, tTCLs were tested for callogenesis and somatic embryogenesis (SE) in different concentrations of 5-azaC along with 2,4-D and TDZ. Results revealed the positive influence of 5-azaC on callus initiation as 15 µM treatment predisposed it by 3 to 4 d. Even though percent callus initiation was highest in 5-azaC at 15 µM treatment, overall, it was non-significant among the treatments (Table 1). After 40-45 d culture incubation, embryogenic calli, characterized by translucent ear-shaped structures, emerged (Figure 1c). Among the treatments, the response was insignificant. However, the overall percent embryogenic calli were highest in treatment consisting of 15 µM 5-azaC (Table 1). Interestingly, the embryogenic callus obtained by the 12^{th} week of culturing from 5-azaC (15 µM) treatment was heavier than that of control. A significant increment of 43% in the average fresh weight of the callus was recorded per tTCL in 15 µM 5-azaC compared to its counterpart, where the average fresh weight recorded was 0.47 g $tTCL^{-1}$.

The onset of SE was evident in cultures by the fourth monthly subculture (Figures 1d-1e). Statistical analysis revealed that the number of somatic embryos per explant was significantly more in the 5-azaC treatment than in the control (Table 1). Among 5-azaC concentrations, 15 µM resulted in the highest number of somatic embryos. Microscopic images of the embryogenic calli revealed the presence of multiple meristematic regions (Figure 2q). The influence of 5-azaC was also obvious in the germination of somatic embryos. Results indicated that 97% of the somatic embryos germinated in 15 µM 5-azaC treatment compared to 85% in control (Figure 1f). Similarly, significantly improved plantlet formation from somatic embryos was observed in 15 µM 5-azaC treatment compared to its control counterpart (Figures 1g-1m). Plantlet formation was 80% of the somatic embryos in the former treatment and 61% in the latter. Well-rooted plantlets were transferred to the plastic cups containing sterile soil, sand, and farmyard manure and then successfully acclimatized in the polyhouse conditions (25 ± 2 °C, 80% relative humidity) for further growth.

Various stages of the SE process were studied using scanning electron microscopy (SEM) analysis. The non-embryogenic callus had a more uneven surface. In contrast, the embryogenic callus of both control and 5-azaC treatment (15 µM) showed clusters of well-defined smooth round protuberances, which led to the formation of somatic embryos on the surface of the callus. The study also revealed that in embryogenic calli 45 d after inoculation, 5-azaC treatment resulted in more protruding structures and advanced development compared to the control samples (Figures 2a-2n).

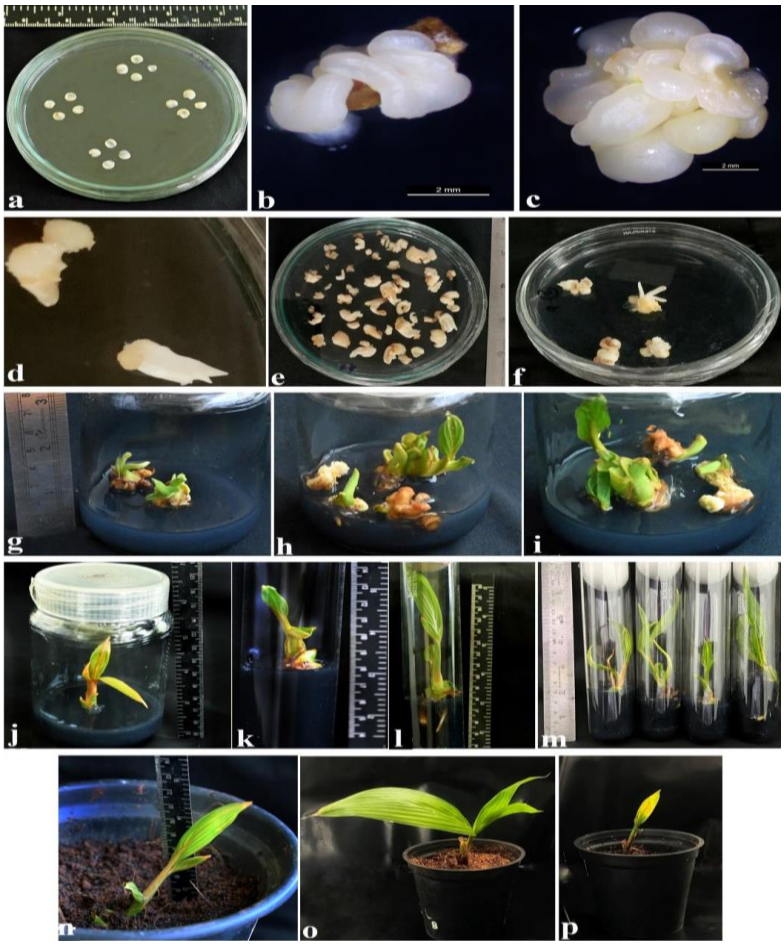

Figure 1. Callus initiation and somatic embryogenesis in transverse thin cell layer (tTCL) subjected to 5-azacytidine (15 µM) treatment along with 2,4-D. The tTCL inoculated in the callogenesis medium (a), callus initiation (b), embryogenic callus formation (c), somatic embryogenesis and germination (d-f), plantlet development (g-m), plantlets transferred to pots for acclimatization (n-p).

Table 1. Effect of 2,4-D and thidiazuron (TDZ) in combination with different concentrations of 5-azacytidine (5-azaC) on callus initiation, formation of embryogenic callus, somatic embryogenesis and plantlet regeneration in plumular explants of coconut. Data represents mean ± SE of six replicates per treatment. Four slices from three zygotic embryos each formed one replicate. Means within the same column, followed by different letters, are significantly different according to Duncan's multiple range test (DMRT) at a 5% level. Arc sine transformation values for callus induction are given in parentheses.

Treatments	Callus	Embryogenic callus	Somatic embryos per explant	Germinated somatic embryos per explant	Plantlets produced per explant
	%	%	Nr explant^{-1}	Nr explant^{-1}	Nr explant^{-1}
Control: Y3 alone	0.00 ± 0.00^{a} (0.00)	-	-	-	-
Y3 + 2,4-D (75 µM) + TDZ (4.54 µM)	86.11 ± 3.93^{a} (70.39)	73.61 ± 4.77^{a} (59.73)	1.83 ± 0.14^{a}	1.56 ± 0.07^{a}	1.11 ± 0.13^{a}
Y3 + 2,4-D (75 µM) + TDZ (4.54 µM) +5-azaC (10 µM)	84.72 ± 3.79^{a} (67.70)	76.39 ± 3.79^{a} (61.43)	2.78 ± 0.27^{b}	2.56 ± 0.24^{b}	1.56 ± 0.11^{a}
Y3 + 2,4-D (75 µM) + TDZ (4.54 µM) +5-azaC (15 µM)	88.89 ± 3.93^{a} (74.17)	80.56 ± 1.68^{a} (63.94)	3.67 ± 0.08^{c}	3.56 ± 0.07^{c}	2.94 ± 0.32^{b}
Y3 + 2,4-D (75 µM) + TDZ (4.54 µM) +5-azaC (20 µM)	87.50 ± 1.78^{a} (69.56)	73.61 ± 3.19^{a} (59.28)	2.33 ± 0.28^{ab}	2.00 ± 0.26^{ab}	1.56 ± 0.27^{a}

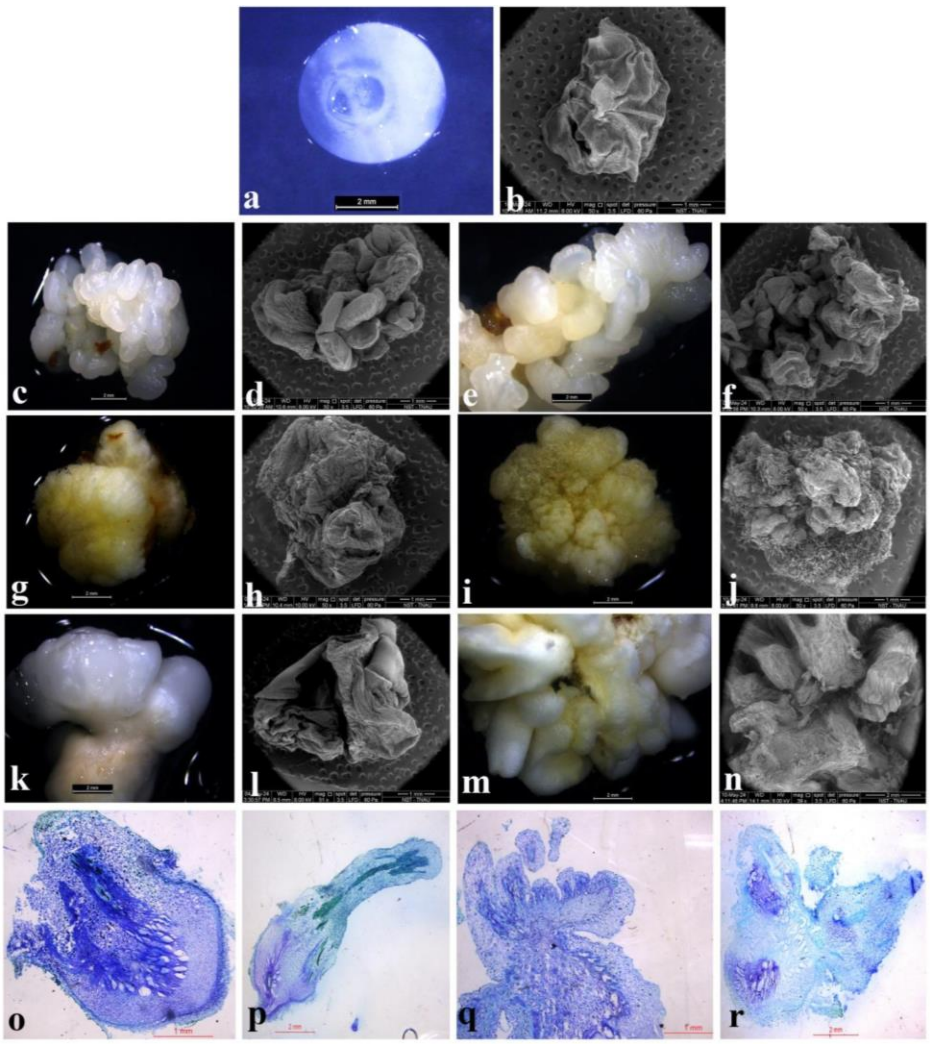

Figure 2. Morphohistology of regeneration in coconut transverse thin cell layers (tTCLs). Scanning electron microscopic and light microscopic images during somatic embryogenesis induction (a-n); plumular portion from the tTCL section during inoculation of mature zygotic embryo (bar 11.2 mm) (a-b); embryogenic callus from control sample (bar 10.6 mm) (c-d); embryogenic callus from 5-azacytidine (5-azaC) sample (bar 10.3 mm) (e-f); non-embryogenic callus from control sample (bar 10.4 mm) (g-h); non-embryogenic callus from 5-azaC sample (bar 9.8 mm) (i-j); normal mature somatic embryo formed from the control sample (bar 8.5 mm) (k-l); normal mature somatic embryo formed from the 5-azaC sample (bar 14.11 mm) (m-n); histology of the structures en-route somatic embryogenesis (o-r); control with single meristematic region (o-p); explants in medium supplemented with 15 µM 5-azaC showing multiple meristematic regions (q-r).

Different structures en route to coconut SE were studied to provide insights into the changes occurring during SE, as coconut is considered highly recalcitrant to in vitro culture. During SE, several histological and morphological changes take place. The histological observations of the transverse section of the embryo tissue fixed after 100 d of the initial culture of both control and treatment showed noticeable growth differences. This could be attributed to the formation of new multiple meristematic zones formed of parenchyma cells and vascular tissues in matured somatic embryos by dividing provascular cells in the treatment with 5-azaC compared to the control (Figures 2o-2r).

Encouraged by the positive results obtained with 15 µM 5-azaC, tTCL explants were tested in a medium with additional auxins such as picloram and atrazine apart from 2,4-D, at two concentrations, *viz*., 75 and 150 µM. Similar results were obtained in the case of 2,4-D, as observed in the preliminary experiment. However, increasing the basal concentration of 2,4-D to 150 µM did not improve somatic embryogenesis with or without 5-azaC. On the other hand, callus initiation and embryogenic calli formation were poorly influenced by atrazine at both the tested concentrations, even in the presence of 5-azaC (Table 2). We observed a significant interaction of 5-azaC and picloram on callus initiation. This treatment resulted in 96% callogenesis compared to 87% in picloram at 75 µM alone.

Similarly, embryogenic calli production increased significantly by 10% in cultures, and the number of somatic embryos increased by 55% in the presence of 5-azaC and picloram. Germination of somatic embryos and plantlet formation were also influenced similarly (Table 2). Percent callus formation declined when the concentration of picloram increased to 150 µM.

Table 2. Effect of different auxins in combination with 5-azacytidine (5-azaC) on callus initiation, formation of embryogenic callus, somatic embryogenesis and plantlet regeneration in plumular explants of coconut. Data represents mean ± SE of six replicates per treatment. Four slices from three zygotic embryos each formed one replicate. Means within the same column, followed by different letters, are significantly different according to Duncan's multiple range test (DMRT) at a 5% level. Arc sine transformation values for callus induction are given in parentheses. TDZ: Thidiazuron.

Treatments	Callus	Embryogenic callus	Somatic embryos per explant	Germinated somatic embryos per explant	Plantlets produced per explant
	%	%	Nr explant^{-1}	Nr explant^{-1}	Nr explant^{-1}
Control: Y3 alone	0.00 ± 0.00^{a} (0.00)	-	-	-	-
Y3 + 5-azaC (15 µM)	0.00 ± 0.00^{a} (0.00)	-	-	-	-
Y3+ 2,4-D (75 µM) + TDZ (4.54 µM)	80.83 ± 3.25^{de} (64.24)	75.00 ± 1.29^{cd} (60.02)	1.67 ± 0.30^{a}	1.00 ± 0.00^{a}	0.67 ± 0.30^{a}
Y3+ 2,4-D (75 µM) + TDZ (4.54 µM) + 5-azaC (15 µM)	90.83 ± 1.49^{f} (72.50)	82.50 ± 8.06^{d} (69.36)	4.00 ± 0.52^{c}	3.67 ± 0.30^{e}	3.67 ± 0.30^{b}
Y3+ 2,4-D (150 µM) + TDZ (4.54 µM)	69.17 ± 1.97^{c} (56.30)	60.00 ± 2.24^{b} (50.78)	1.67 ± 0.30^{a}	1.33 ± 0.30^{ab}	1.00 ± 0.00^{a}
Y3+ 2,4-D (150 µM) + TDZ (4.54 µM) + 5-azaC (15 µM)	72.50 ± 1.29^{c} (58.39)	68.33 ± 1.97^{bc} (55.78)	2.00 ± 0.00^{ab}	1.67 ± 0.30^{abc}	1.00 ± 0.52^{a}
Y3+picloram (75 µM) + TDZ (4.54 µM)	86.67 ± 0.75^{f} (68.60)	77.50 ± 1.29^{d} (61.71)	3.00 ± 0.52^{bc}	2.67 ± 0.30^{d}	2.00 ± 0.52^{a}
Y3+ picloram (75 µM) + TDZ (4.54 µM) + 5-azaC (15 µM)	95.83 ± 1.97^{g} (80.39)	87.50 ± 1.29^{e} (66.70)	4.67 ± 0.30^{d}	4.33 ± 0.30^{e}	4.00 ± 0.00^{b}
Y3+ picloram (150 µM) + TDZ (4.54 µM)	78.33 ± 1.97^{d} (62.32)	68.33 ± 2.69^{d} (55.80)	2.33 ± 0.30^{bc}	2.00 ± 0.00^{bcd}	1.33 ± 0.30^{a}
Y3+ picloram (150 µM) + TDZ (4.54 µM) + 5-azaC (15 µM)	85.83 ± 0.75^{ef} (67.91)	78.33 ± 1.97^{d} (62.32)	3.00 ± 0.00^{bc}	2.33 ± 0.30^{cd}	1.67 ± 0.60^{a}
Y3+ atrazine (75 µM) + TDZ (4.54 µM)	26.67 ± 1.49^{b} (31.07)	0.00 ± 0.00^{a} (0.00)	-	-	-
Y3+ atrazine (75 µM) + TDZ (4.54 µM) + 5-azaC (15 µM)	22.50 ± 1.29^{b} (28.29)	0.00 ± 0.00^{a} (0.00)	-	-	-
Y3+ atrazine (150 µM) + TDZ (4.54 µM)	27.50 ± 1.29^{b} (31.61)	0.00 ± 0.00^{a} (0.00)	-	-	-
Y3+ atrazine (150 µM) + TDZ (4.54 µM) + 5-azaC (15 µM)	27.50 ± 1.29^{b} (31.61)	0.00 ± 0.00^{a} (0.00)	-	-	-

DISCUSSION

The availability of an efficient and large-scale clonal propagation technique can cater to the high demand for quality planting materials in coconut, mainly to replace existing senile palms or to establish new coconut gardens. Unfortunately, somatic embryogenesis (SE) in coconut has several bottlenecks (Rajesh et al., 2018; Kong et al., 2024), which must be overcome to achieve a commercially viable protocol for in vitro regeneration.

Identifying ideal explant(s) and growth regulator(s) is crucial for the successful induction of SE and subsequent plantlet regeneration in any plant species. With respect to coconut, this exercise assumes high importance given the need to overcome in vitro recalcitrance.

Many recent studies have extolled the importance of extensive genetic reprogramming, specifically epigenetic regulation, which occurs during SE induced by auxin treatment and is often mediated by microRNAs (miRNAs) and modifications of chromatin (Wójcik et al., 2020; Wójcikowska et al., 2020). Determination of the epigenetic landscape underlying SE could shed light on the regulatory factors shaping the embryogenic response of various explants, which is deeply influenced by the delicate balance between exogenous and endogenous regulators (Wójcikowska et al., 2020). The identification of spatio-temporal gene regulatory networks involved in DNA and histone methylation, subsequently leading to epigenetic reprogramming during early zygotic embryogenesis (ZE), has provided vital clues and resulted in drawing parallels between ZE and SE (De-la-Peña et al., 2015; Méndez-Hernández et al., 2019; Sarpan et al., 2020). In addition, a lower rate of DNA methylation in embryogenic calli (EC), in comparison to non-embryogenic calli (NEC), results in more genes being active transcriptionally in EC; this results in an enhanced rate of SE from EC (De-la-Peña et al., 2015).

5-Azacytidine (5-azaC), a nucleotide analog to the methyl derivative of cytosine, has been used to study the role of DNA methylation in plant and animal developmental processes. Incorporating 5-azaC into DNA stretches leads to their unmethylation, which has been attributed to the inhibition of the 5-DNA-methyltransferase enzymes by 5-azaC. Many studies have highlighted the role of 5-azaC in improving SE and subsequent regeneration in plant species like *Brassica napus* and *Hordeum vulgare* (Solís et al., 2015) and *Theobroma cacao* (Pila Quinga et al., 2017).

The response of coconut explants to in vitro interventions, especially the formation of embryogenic calli and induction of SE, has been worked out by many researchers (Rajesh et al., 2014; Jayaraj et al., 2015; Sáenz et al., 2018; Osorio-Montalvo et al., 2020). These responses were influenced by the composition of the culture medium and the type/concentration/ratio of growth regulators, both endogenous and exogenously supplemented, especially the auxins and the cytokinins. The average response recorded from various explants has been 40%-70%, with respect to the formation of embryogenic calli with a mean of 3 to 7 somatic embryos per embryogenic calli (Osorio-Montalvo et al., 2020).

The dynamics of the role of DNA methylation during in vitro culture of coconut remains to be deciphered. Osorio-Montalvo et al. (2020) reported a 4 to 10-fold enhancement of the formation of somatic embryos from plumular explants on their pre-treatment with two concentrations of 5-azaC. In the present study, transverse thin cell layer (tTCL) cultures derived from mature zygotic embryos of coconut were used. Enhanced induction of SE has been reported during in vitro culture of many plants from tTCLs, which are explants of 0.5-1.0 mm thickness and comprising only a few layers of cells (Stefenon et al., 2020). According to da Silva and Dobránszki (2019), the better response of tTCLs to in vitro interventions, compared to bulkier explants, could be ascribed to a larger surface area being in contact with the culture conditions in vitro.

A combination of auxin and 5-azaC, at specific concentrations, was observed to enhance the formation of somatic embryos in coconut in the current study compared to the control. Auxins, especially 2,4-D, have proven to be crucial for inducing embryogenic calli and somatic embryos in coconut, with their absence resulting in the failure of occurrence of both these processes. The exact role of changes in DNA methylation levels induced by the auxin-5-azaC interaction in controlling plant developmental stages remains unclear. Whether these changes either directly govern the developmental processes or reflect the silencing of transposable elements and other repetitive sequences is uncertain. Exposure to explants to auxins alone could increase overall methylation levels, triggering stimulation of cell division and dedifferentiation. This process may potentially induce various mechanisms, including induction of embryogenic calli and SE, as discussed by von Aderkas and Bonga (2000). The metabolic conditions induced by 2,4-D in the explants could lead to elevated DNA methylation levels. Consequently, the use of hypomethylating drugs may disrupt this pattern, suggesting that the observed response in the current study, specifically in terms of embryo conversion rates in response to 5-azaC, may be attributed to this disruption. Therefore, there is a potential for inducing embryogenesis in recalcitrant species by experimentally altering their methylation patterns using auxins and hypomethylating chemicals, as proposed by von Aderkas and Bonga (2000).

There are contrasting reports on the effect of supplementation of 5-azaC in the induction of SE in plants. In the case of *Medicago truncatula*, embryogenic and non-embryogenic cultures induced a medium devoid of 5-

azaC exhibited normal development, characterized solely by cell proliferation. Nevertheless, the inoculation of embryogenic cultures to a medium containing 5-azaC resulted in the formation of somatic embryos. In contrast, the non-embryogenic line, subjected to the same medium, displayed the death of cells. Another study revealed that prolonged stress exposure to cacao led to a reduction in embryogenic potential. This condition could be reversed by adding 5-azaC (Wójcikowska et al., 2020).

In our current investigation, the presence of 5-azaC significantly altered the response pattern in the cultured coconut explants, leading to a higher number of somatic embryos than in the control. While Osorio-Montalvo et al. (2020) have pre-treated the coconut embryogenic structures for 3-7 d in 5-azaC, in the present study, tTCL from fresh zygotic embryos were exposed to 5-azaC for longer durations in a culture medium. In contrast to these observations, in *Daucus carota* and *Coffea canephora*, using 5-azaC has been associated with embryogenic competence loss (Fehér, 2015).

Fehér (2015) highlighted the importance of considering the unique requirements of different plant species when adding exogenous 5-azaC in culture media for induction of SE. Understanding these variations is crucial for optimizing concentrations and treatment durations to obtain effective outcomes. Our study enhanced SE from tTCLs in coconut by an optimal concentration of 5-azaC. Optimization is absolutely necessary as a higher concentration of 5-azaC has been reported to inhibit somatic embryo formation (Fehér, 2015). It is essential to strike a balance between the cytotoxic impact of 5-azaC and its hypomethylating capabilities, as emphasized by Fehér (2015).

One of the main bottlenecks in coconut tissue culture is the exudation of polyphenols by the explants (Rajesh et al., 2018). Various evidence has been put forth across plants indicating that phenolic compounds released by explants into the culture medium may disrupt SE (Nic-Can et al., 2015) have suggested that interactions between epigenetic mechanisms and secondary metabolites could impact SE response in coffee. Phenolic compounds have been reported to disrupt the activity of DNA methyltransferases (Leri et al., 2020). Based on their studies in grapevine, Dal Santo et al. (2022) have hypothesized that the accumulation of secondary metabolites during initial culture conditions, coupled with stress responses, could potentially inhibit DNA methylation pathways associated with SE. Activated charcoal is generally added to the medium to adsorb the polyphenols. However, the supplementation of 5-azaC in a culture medium along with activated charcoal in the case of coconut can reduce the availability of 5-azaC (Osorio-Montalvo et al., 2018).

The SEM analysis has been utilized in various plant systems to delineate structural differences arising during SE and regeneration. Recently, SEM was conducted in *Digitalis lanata* to study the different developing stages of somatic embryoids from embryogenic callus, confirming the origin of somatic embryos from the callus, which later progressed through different stages of SE (Bhusare et al., 2020). In the present study, the number of protruding structures in the embryogenic callus was increased, as indicated by the SEM images (Figures 2a-2n), suggesting an enhancement in the formation of somatic embryos.

Evidences has been put forth in multiple plant species that phenolic compounds released by explants into the culture medium can interfere with SE, and interactions between epigenetic mechanisms and the effect of secondary metabolites on the SE response have been proposed in coffee (Nic-Can et al., 2015). Phenolic compounds are known to inhibit DNA methyltransferases, and most plant-derived anti-cancer drugs that influence epigenetic mechanisms are polyphenols. The early accumulation of secondary metabolites, especially polyphenols, combined with stress responses exceeding a certain threshold, may suppress the DNA methylation pathway associated with embryogenesis (Chen et al., 2020; Nic-Can et al., 2020; Dos Santos et al., 2023). This inhibition can affect various cellular processes, including gene expression and plant development, especially in recalcitrant species; this inhibition could be overcome by supplementing the tissue culture medium with 5-azaC. The 5-azaC causes DNA demethylation, which can counterbalance the hyper-methylation that polyphenols might induce. This helps restore normal gene expression patterns and can promote the growth and regeneration of recalcitrant species in vitro. By altering the methylation landscape, 5-azaC can help reprogram the epigenetic state of cells (Osorio-Montalvo et al., 2018; Nic-Can et al., 2020). It is plausible that this reprogramming can activate genes necessary for regeneration and development that might otherwise be suppressed due to methylation changes caused by polyphenol exposure.

CONCLUSIONS

In conclusion, 5-azacytidine (5-azaC) is emerging as a powerful tool in manipulating plant somatic embryogenesis; its role in DNA demethylation and the subsequent reprogramming of somatic embryogenesis (SE) opens avenues for advancements in plant regeneration and crop improvement. In the present study, transverse thin cell layers (tTCLs) from fresh coconut zygotic embryos were treated with 5-azaC in culture medium. An optimal concentration of 5-azaC was observed to enhance SE from tTCLs. To our knowledge, this is the first report of the tTCL technique for the regeneration via SE in coconut.

Author contributions

Conceptualization: M.K.R. Methodology: A.K.T.K., K.S.M., M.K.R. Validation: J.H., P.S. Formal analysis: A.K.T.K., K.S.M., M.K.R., C.K.P. Investigation: A.K.T.K., K.S.M. Resources: M.K.R. Data curation: A.K.T.K., K.S.M., J.H., C.K.P., P.S., M.K.R. Writing-original draft: A.K.T.K., K.S.M., M.K.R. Writing-review & editing: K.S.M., J.H., C.K.P., P.S. Visualization: K.S.M., A.K.T.K. Supervision: M.K.R. Project administration, M.K.R. Funding acquisition: M.K.R. All co-authors reviewed the final version and approved the manuscript before submission.

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