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ORIGINAL ARTICLE

A peak in global DNA methylation is a key step to initiate the somatic embryogenesis of coconut palm (*Cocos nucifera* L)

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Abstract

Key message **DNA methylation, morphogenesis and gene expression during the somatic embryogenesis of Coconut are affected by 5-Azacytidine pretreatments, indicating that DNA methylation is an important factor throughout this process.**

Abstract Somatic embryogenesis (SE) is a process that can aid in the production of elite *Cocos nucifera* palms. It has been well established that epigenetic mechanisms are regulators of cell differentiation programs; however, their role in the coconut somatic embryogenesis has not yet been addressed. To this end, the morphogenetic changes, the global DNA methylation and the expression profiles of the SE-related genes and DNA methyltransferases genes were evaluated during the SE process, with and without the presence of 5-Azacytidine (AzaC). The results show that three days of pretreatments with 15 µM and 20 µM of AzaC significantly increased early somatic embryo formation (four- and tenfold, respectively). A clear peak of the global percentage of DNA methylation (approximately 13%) was determined at the beginning of the culture, followed by a re-establishing stage and a steady increase thereafter; in all cases, the levels of DNA methylation were lower after the pretreatments with AzaC. Additionally, the expression profiles of the *SERK*, *WUS*, *BBM* and *LEC* genes are modulated during the SE process and the pretreatments with AzaC affect the expression profiles of these genes, even at early stages. Furthermore, increased levels of expression were observed for the genes encoding for DNA methyltransferases (*MET, CMT* and *DRM*) at early and late stages of SE, indicating that DNA methylation is an important factor throughout the SE.

Keywords 5-Azacytidine · In vitro tissue culture · Gene expression · Epigenetics

Abbreviations

AzaC	5-Azacytidine
BBM	BABY BOOM
CMT	Chromomethylase
DNMTs	DNA methyltransferases
DRM	Domains Rearranged Methyltransferase
LEC1	*LEAFY COTYLEDON 1*
MET	Methyltransferase
PKL	*PICKLE*
SE	Somatic embryogenesis
SERK	*SOMATIC EMBRYOGENESIS RECEPTOR KINASE*
WUS	*WUSCHEL*
2,4-D	2,4-Dichlorophenoxyacetic acid

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Introduction

The coconut palm (*Cocos nucifera* L.) is a perennial and multi-purpose tree that offers a vast variety of products with domestic and industrial applications (Arunachalam and Rajesh 2017). *C. nucifera* is cultivated in most tropical and subtropical countries, having a great influence on the rural economy and on the livelihood of millions of people around the world. It is projected that the demand for coconut products will increase exponentially in the coming years, mainly

for products such as coconut water, milk and oil (Rodrigues et al. 2018). During a recent meeting of the International Coconut Genetic Resources Network (COGENT) it was concluded that replanting of coconut trees at a massive scale is required if the coconut producing countries are to meet the world's rapidly growing demand for coconut products (Bourdeix and Prades 2018).

A viable alternative for the production of large quantities of seedlings, with potential to satisfy the rising demand of coconut palms, is the massive micropropagation of plants through somatic embryogenesis (SE). In plants, SE is a process by which somatic cells cultured under in vitro conditions can develop structures very similar to zygotic embryos but without the fusion of gametes (Elhiti and Stasolla 2016). SE is considered a very powerful biotechnological tool, especially to propagate species with very long reproductive cycles or with low seed production (Smertenko and Bozhkov 2014). Although SE is a powerful tool to multiply plants, the embryogenic response might vary, mainly due to the differences in the totipotent capacity of diverse plant species.

In this regard, great efforts have been carried out over the last two decades to develop and optimize propagation methods of coconut plantlets through SE (Sandoval-Cancino et al. 2016). In 1998, an efficient SE protocol was reported using plumule explants (Chan et al. 1998) and since then, numerous studies have been carried out to increase the efficiency of this protocol (Azpeitia Morales et al. 2009; Montero-Cortés et al. 2011a, b; Montero-Córtes et al. 2010; Pérez-Núñez et al. 2006; Rivera-Solís et al. 2018; Sandoval-Cancino et al. 2016; Sáenz et al. 2010). As a result, a protocol for the multiplication of embryogenic calluses has been developed, which is being used as the basis for a massive propagation process of coconut plants (Sáenz et al. 2018).

The study of numerous internal factors (physiological, metabolic, genetic, epigenetic, morphogenetic, etc.) have helped to further understand the SE process of coconut and to harvest its potential (Sáenz-Carbonell et al. 2016). However, the epigenetic mechanisms occurring during coconut SE have not been addressed so far and could be a key element for a better control of the process. It has previously been reported that epigenetic mechanisms are active during the SE of several plant species (De-la-Peña et al. 2015; Feher 2015; Ikeuchi et al. 2015) and that DNA methylation is especially involved in the initiation and development of SE in plants (Kumar and Van Staden 2017). It was already reported as early as in the 1980's that the embryogenic capacity could be conditioned by DNA methylation levels (Bhojwani and Razdan 1986). It is also known that DNA methylation during the SE plays an important role in the regulation of SE-related gene expression (Karim et al. 2016; Mahdavi-Darvari et al. 2015). A group of genes has been specifically studied due to their essential role during this process in plants: *BABY BOOM* (*BBM*), *LEAFY COTYLEDON* 1 (*LEC1*), *PICKLE* (*PKL*), *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*), and *WUSCHEL* (*WUS*), (Karim et al. 2016; Kumar and Van Staden 2017; Milutinovic et al. 2003).

On the other hand, DNA methylation is catalyzed by enzymes known as DNA methyltransferases (DNMTs) such as: Methyltransferase (MET), Domains Rearranged Methyltransferase (DRM), and Chromomethylase (CMT) playing an important role during SE of plants (De-la-Peña et al. 2015). For instance, a correlation between DNA methylation, expression of DNMTs and embryogenic competence has been reported for *Boesenbergia rotunda* (Rezaul Karim et al. 2018).

Finally, a pharmacological approach such as the use of 5-Azacytidine (AzaC), an inhibitor of DNA methylation, could assist to gain a better understanding of the epigenetic mechanisms that occur during the SE process (Nic-Can et al. 2013). It has been shown in several plant species (*Arabidopsis thaliana*, *Brassica napus*, *Coffea canephora*, *Daucus carota*, *Elaeis guineensis*, etc.) that the use of AzaC can alter the epigenetic mechanisms in many in vitro culture systems (reviewed by Osorio-Montalvo et al. 2018).

Therefore, this work aims at obtaining better insights into the SE process of coconut through the effect of the DNA methylation inhibitor 5-Azacytidine by focusing on the morphogenetic responses, the levels of global DNA methylation and the expression of genes that regulate the SE process and that encode for DNA methyltransferases.

Materials and methods

Plant materials

Embryogenic structures from embryogenic calluses (Fig. 1a, c) were used as explants as described by Pérez-Nuñez et al. (2006). The embryogenic structures were excised from embryogenic calluses, previously cultured for three months in medium I, as described below. The embryogenic calluses come from the Mexican Pacific Tall line MI-192-17 obtained in the Clonal Micropropagation Laboratory, Centro de Investigación Científica de Yucatán (CICY).

Medium preparation and culture conditions

The treatments media were prepared based on the Y3 medium formulation (Eeuwens 1976) supplemented with 3 g L^{-1} Gelrite™ + 2.5 g L^{-1} activated charcoal (Sigma-Aldrich®, C6289) + 650 µM of 2,4-dichlorophenoxyacetic acid (2,4-D) (Medium I). Explants were cultured for 90 days in 35 mL glass vessels (Vitro, Tlalnepantla, Mexico) fitted with a polypropylene cover (Möller, Nezahuatcoyotl, Mexico) with 10 mL of medium, under complete darkness at 27 ± 2 °C. For the induction of somatic embryo formation,

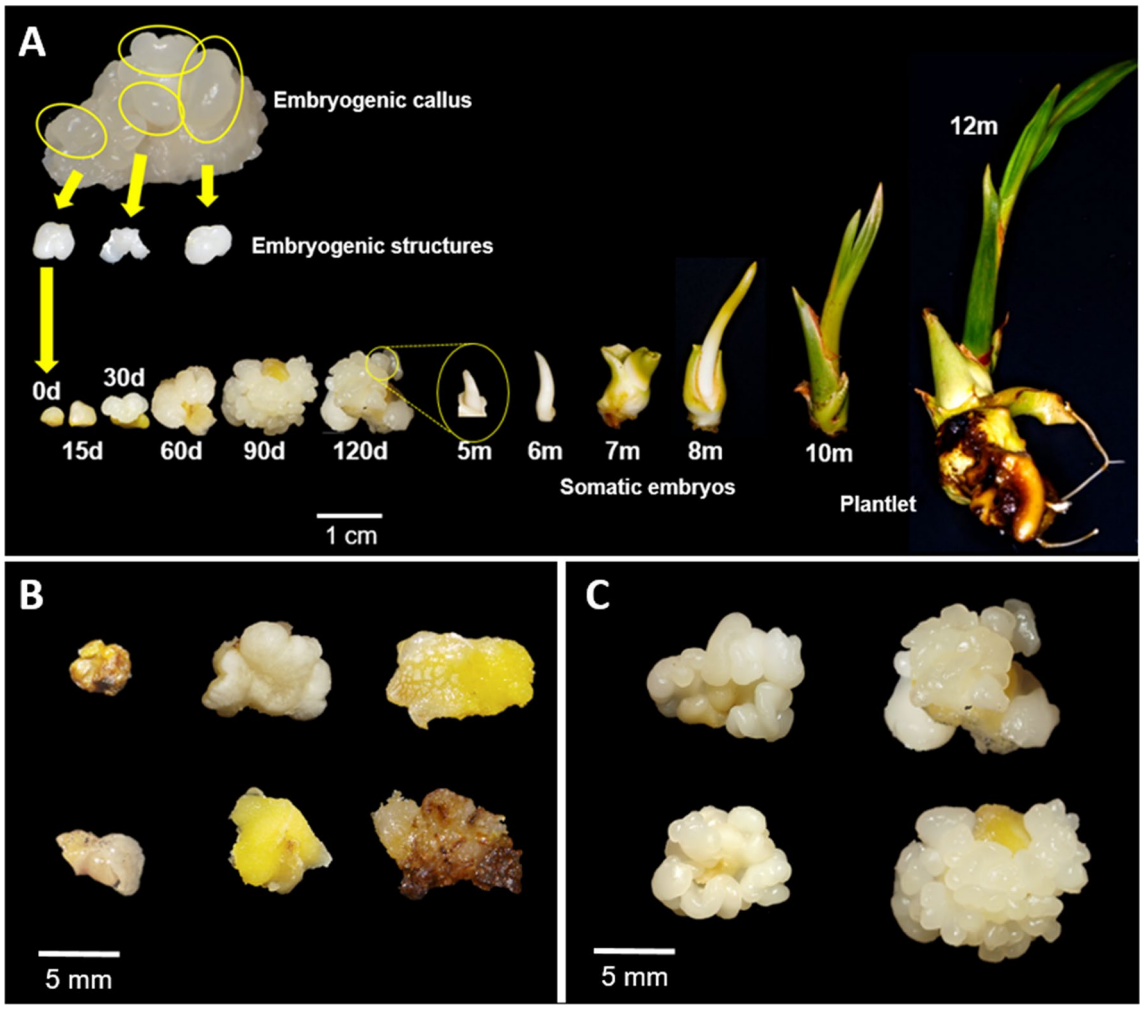

Fig. 1 **a** Somatic embryogenesis process using embryogenic structures from embryogenic calluses as explants. The SE process lasts approximately 12 months, from embryogenic structures to plantlet. d = days, m = months. **b** Non-embryogenic calluses at 120 days of in vitro culture. **c** Embryogenic calluses at 120 days of in vitro culture

embryogenic calluses were transferred to Medium II: Y3 medium + 2.5 g L^{-1} activated charcoal + 3 g L^{-1} Gelrite™ + 325 µM of 2,4-D. Cultures were established in 100 mL glass vessels (Vitro, Tlalnepantla, Mexico) equipped with a polypropylene cover (Möller, Nezahuatcoyotl, Mexico) and in complete darkness at 27 ± 2 °C for 30 d. In all cases, media pH was adjusted to 5.75 before autoclaving (120 °C for 20 min). All chemicals used were acquired from Sigma-Aldrich, USA.

5-Azacytidine treatments

Each pretreatment consisted of a medium Y3 with 3 g L^{-1} Gelrite™, without activated charcoal + 6.5 µM of 2,4-D added with 0, 5, 10, 15 and 20 µM of 5-Azacytidine (Sigma-Aldrich®, A2385). AzaC was filter-sterilized as an AzaC stock solution and it was added to the autoclaved medium at final concentrations based on the pretreatment. After either, 3 or 7 days, the explants were transferred to Medium I for 90 days and then they were transferred to Medium II during 30 days with the same conditions mentioned above. Control explants were not submitted to any pretreatment. The effect of each pretreatment was evaluated by measuring the formation of embryogenic calluses, somatic embryos, percentage of global DNA methylation and gene expression. The experimental design is shown in Fig. 2.

DNA methylation analysis

Genomic DNA from in vitro explants of *C. nucifera* was extracted following the protocol reported by Doyle and Doyle (1987) with modifications made by Harrison et al. (1994). Briefly, 100 mg of embryogenic structures/calluses were collected at the stages of -3 days (in preconditioning

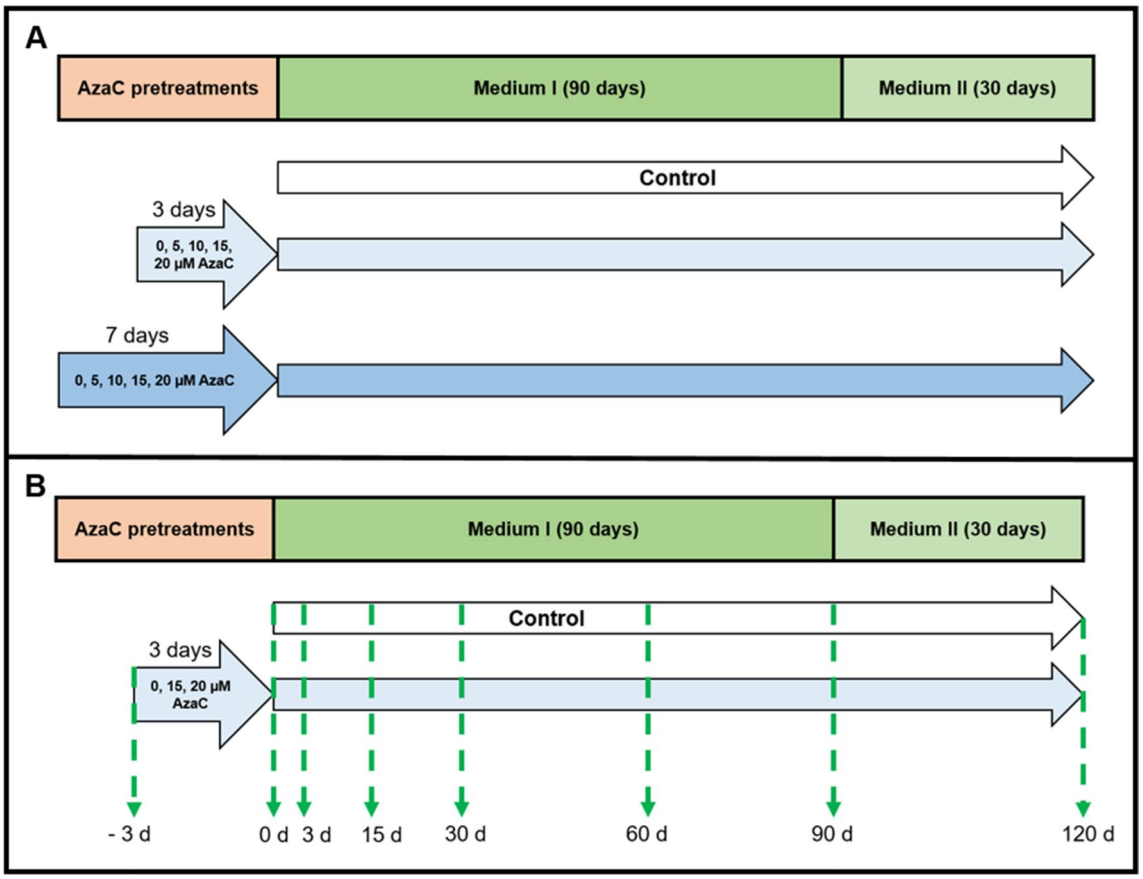

Fig. 2 **a** Experimental scheme clarifying the timing and concentrations of applied 5-Azacytidine (AzaC), and the posterior transfers of explants to Medium I and Medium II. **b** Experimental scheme (once the three-day pretreatments with AzaC is chosen) clarifying the sampling points of plant material for DNA and RNA extraction during the SE process. Green arrows indicate the sampling points

treatments), 0, 3, 15, 30, 60, 90, and 120 days, for control, 0 µM of AzaC, 15 µM of AzaC and 20 µM of AzaC pretreatment groups (Fig. 2b). Each sample was pulverized in liquid nitrogen with a mortar and pestle, mixed with 500 µL of hot (65 °C) 2% CTAB buffer. Samples were then incubated at 65 °C for 30 min and cooled to room temperature. The resulting extracts were then emulsified with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) and centrifuged at 14,000×*g* for 5 min. Total nucleic acids were precipitated from the upper aqueous phase by the addition of cold isopropanol and pelleted by centrifugation as mentioned before. Nucleic acid pellets were dried briefly, resuspended in 100 mL of TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8) and incubated with RNase for 1 h at 37 °C. DNA samples were quantified using a Thermo Scientific™ NanoDrop™ One UV–visible spectrophotometer. Afterwards, the obtained nucleic acid was digested and the nucleosides were separated as described by De-la-Peña et al. (2012). Briefly, DNA from each sample (5 µg) were hydrolysed and mixed with 5 µL of 10X DNA digestion buffer (200 mM acetic acid, 200 mM glycine, 50 mM magnesium chloride, 5 mM zinc acetate, 2 mM calcium chloride adjusted with sodium hydroxide to pH 5.3), 2 µL of DNase I (D2821-Sigma, 10 U/mL) and 1 µL of Nuclease P1 (N8630-Sigma, 1.25 U/µL). The samples were incubated overnight at 37 °C, after which were mixed with 5 µL of 100 mM NaOH and 2 µL calf intestine alkaline phosphatase (P4879-Sigma, 1 U/mL). Samples were then incubated for 3.5 h at 37 °C and afterwards mixed with the mobile phase D (50 mM dibasic ammonium phosphate, 15 mM ammonium acetate adjusted with phosphoric acid to pH 4.1) and centrifuged at 18,000×*g*. Chromatographic analyses were carried out using an Agilent 1200 series system for high performance liquid chromatography (HPLC). Global DNA Methylation percentages (GDM%) were obtained from the reversed-phase chromatograms, using the area under the curve of the corresponding peaks to establish the concentration of 2′-deoxycytosine (dC) and 5-methyl-2′-deoxycytosine (5mdC) in the sample (% 5mdC = C 5mdC / [C 5mdC + C dC] × 100), where C is concentration. All the analyses were performed with three independent biological samples.

Molecular analysis

Plant material was collected and immediately frozen in liquid nitrogen at the stages of -3, 0, 3, 15, 30, 60, 90 and 120 days (in preconditioning treatments), for the control group and for pretreatments with 0, 15 and 20 µM of AzaC (Fig. 2b). Total RNA was extracted as reported by Chomczynski and Sacchi (1987), with some modifications. Briefly, total RNA was extracted with 0.1 M Tris–HCl, pH 8.0; 0.2 mM EDTA, pH 8.0; 2% SDS and 0.15% α-monothioglycerol, followed by an extraction with phenol–chloroform (1:1). Subsequently, the mixture was incubated at 70 °C for 5 min, centrifuged and the supernatant was collected. RNA was precipitated with cold isopropanol incubated at − 20 °C for 10 min and centrifuged again. The pellet was washed with 75% ethanol. DNA of the samples was removed with a DNAse I treatment (Ambion, USA). The amount of RNA was determined by spectrophotometry (OD260). The first strand cDNA was prepared using random hexamers (Invitrogen, USA), 1 µg of RNA and a Superscript II reverse transcriptase (Invitrogen, USA), according to the protocol recommended by the manufacturer.

Real-time quantitative PCR reactions (qRT-PCR) were performed using SYBR™ Green PCR Master Mix-UDG (Applied Biosystems by Thermo Fisher Scientific, UK) and analyzed in a Rotor-Gene thermal cycler (QIAGEN, Germany), according to the manufacturer's instructions. Primer sequences used can be consulted in the electronic supplementary material (Table S1). PCR conditions were: a hold cycle at 95 °C for 2 min followed by 40 cycles at 95 °C for 30 s and 61 °C for 30 s; and finally a melting curve for analysis. Expression of the *18S* ribosomal subunit gene was used as internal control. Three independent biological samples by duplicate were carried out. Real time PCR results were analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen 2001). An analysis of variance (ANOVA) and a Tukey test was performed to test the differences between the means.

Photographic documentation

Embryogenic structures and embryogenic calluses were observed under a SMZ-168 TLED stereoscope and the photographs were captured with a Moticam 580 camera (Motic, British Columbia, Canada). Images of bigger structures were composed from a series of photographs to obtain composite images with the aid of the software Image Composite Editor 2.0 (Microsoft Research 2017).

Statistical analysis

A completely randomized design was employed for this trial. The data presented corresponds to means of three repeats with ten replicates each. In this trial, one explant was considered an experimental unit and a replicate. The number of explants used per treatment were 30. Percentage values obtained in the in vitro tissue culture experiments were normalized by transformation using the arcsine function $\left(\arcsen\sqrt{\left(\frac{X}{100}\right)}\right)$. Significant differences between groups ($P<0.05$) were assessed by a one-way analysis of variance (ANOVA) using the Tukey post-hoc test to identify differences with the aid of SigmaPlot® software, version 11.0.

Results

Morphogenetic response during somatic embryogenesis

Using the conventional protocol of coconut micropropagation through somatic embryogenesis (herein referred to as Control) 55% of explants formed embryogenic calluses after 90 days (Figs. 1c, 3a), with 12% of explants becoming necrotic. Early embryo formation is not usually observed during in vitro culturing of initial explants in medium I (Fig. 4). In this case, 6.6% of calluses produced an average of two early somatic embryos (Fig. 3b). The formation of somatic embryos in coconut occurs after 30 days of in vitro culture in medium II, totaling 120 days of in vitro culture. The results herein show that the embryogenic callus produces an average of 4.5 embryos.

Effect of 5-Azacytidine on the morphogenetic response

Explants that were pretreated with AzaC with 10 and 15 µM for 3 days showed a significant increase in embryogenic calluses formation compared with the control. 75% of embryogenic calluses formation was obtained with 15 µM of AzaC with 6% of explants becoming necrotic, while a pretreatment with 20 µM of AzaC resulted in the lowest yield in calluses formation (62%) and the highest percentage of necrotic explants (12%) (Fig. 3a). However, it should be noted that the pretreatment without AzaC also resulted in a significant increase in the percentage of embryogenic calluses (73%) compared to the control (55%) (Fig. 3a).

The explants exposed to AzaC for seven days showed lower percentages of embryogenic calluses formation when compared to the three-day pretreatment. When pretreated for seven days, a significant increase in the percentage of embryogenic calluses formation (73%) was obtained with the pretreatment without AzaC, similarly to what was observed when applying a three day pretreatment, while the lowest percentage of embryogenic calluses formation (51%) was obtained with 10 µM of AzaC (Fig. 3a).

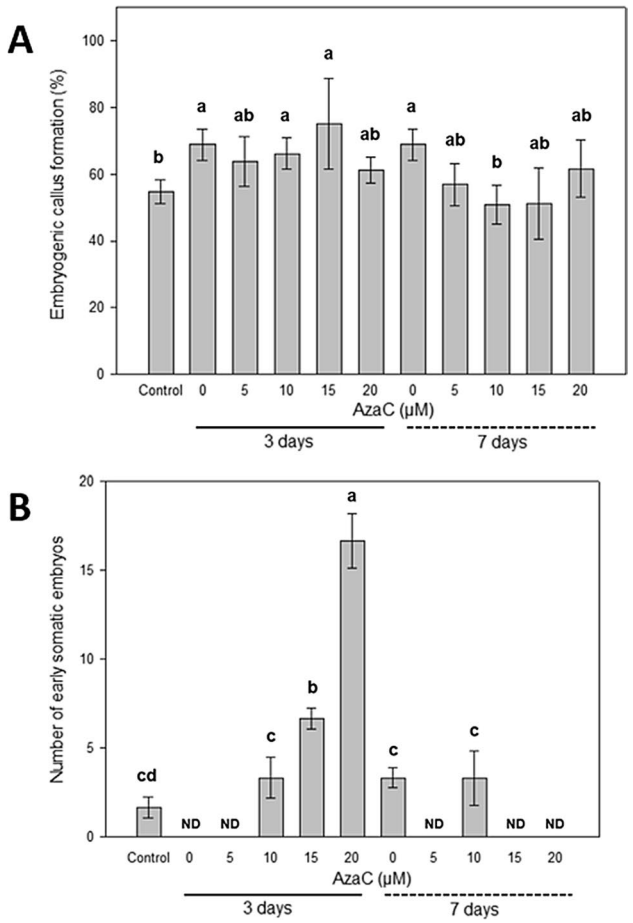

Fig. 3 Effect of different pretreatments with 5-Azacytidine (AzaC) in the formation of embryogenic calluses (**a**) and total number of early somatic embryos at 90 days in medium I (**b**). Bars represent means ± SD of three repeats with 10 replicates each. Values followed by the same letter are not significantly different, according to the Tukey test ($P \leq 0.05$)

Additionally, necrosis was observed in explants from the 15 and 20 µM treatments (6.14%).

When pretreated with 15 and 20 µM of AzaC in medium I, 13 and 26% of calluses responded and produced a total of 7 and 17 early somatic embryos per experimental repeat, respectively. A significant four- and tenfold increase, respectively, when compared to the control. As expected, the 3-day pretreatment without AzaC did not result in the formation of early somatic embryos (Fig. 3b).

With regards to the formation of somatic embryos in medium II, three-day pretreatments (0, 5, 10, 15 µM of AzaC) yielded 3.6, 3.9, 3.5 and 4 embryos per callus, respectively. Explants pretreated with 5, 10, 15 and 20 µM of AzaC for seven days generated, in average, lower amounts of somatic embryos per callus as those obtained with the three-day pretreatment (2.6, 2.5, 3.2 and 3.1, respectively).

Global DNA methylation levels during somatic embryogenesis

The percentage of global DNA methylation (GDM%) was analyzed during a 120-day period (see sampling points in Fig. 2b). The results show a large variation of GDM% during the somatic embryogenesis, characterized by a rapid increase of DNA methylation at day 3 (from 10.84% to 22.99%), a subsequent decrease at day 15 (11.69%), followed by a gradual increase of the GDM% between days 30 and 90 (from 14.39% to 18.3%) and a notable increase of the GDM% at day 120 (39.63%) (Fig. 5), when somatic embryos were already formed (Fig. 1a).

Effect of 5-Azacytidine on global DNA methylation levels

The effect of AzaC on the GDM% was evaluated on the explants with three-day pretreatments, using 0, 15 and 20 µM of AzaC. Only these pretreatments were selected for further analyzes, since they showed the highest yields of embryogenic calluses formation and the highest percentage of early somatic embryo occurrence. Both AzaC pretreatments showed similar variations of the GDM% throughout the in vitro culture (Fig. 5). Furthermore, the observed trend is also similar to that of the control without AzaC. Namely, the appearance of a peak in GDM% three days after the beginning of the pretreatment (from 10.9 to 24.4 and 10.4 to 19.5%, respectively), a resettle to basal levels (6.2 and 5.7% at day 15, respectively), followed by a gradual increase until reaching a new maximum at day 120 of the culture (28.5 and 29%, respectively). The values observed applying the pretreatment without AzaC were similar to those of the control, except for days 3 and 15 of the culture, where values were 3.7 and 2.6% lower, respectively.

SE-related gene expression during somatic embryogenesis

The expression levels of the genes regulating the SE process (*SERK*, *WUS*, *BBM*, *LEC* and *PKL*) was evaluated during the SE process of coconut. The expression profiles of the aforementioned genes were compared to those at day 0 (0 d) (Fig. 6a–e, respectively). The *SERK* gene expression profile presented two peaks of expression at 30 days (3.1-fold) and 90 days (6.2-fold) and then decreased to the initial levels (Fig. 6a). Alternatively, *WUS*, *BBM* and *LEC* share a similar expression pattern (Fig. 6b–d): a basal expression at 0 days followed by a steady increase before reaching a maximum level at 90 days (1.2-fold) in the case of *BBM* and at 120 days in the cases of *WUS* (1.9-fold increment) and *LEC* (2.6-fold). The

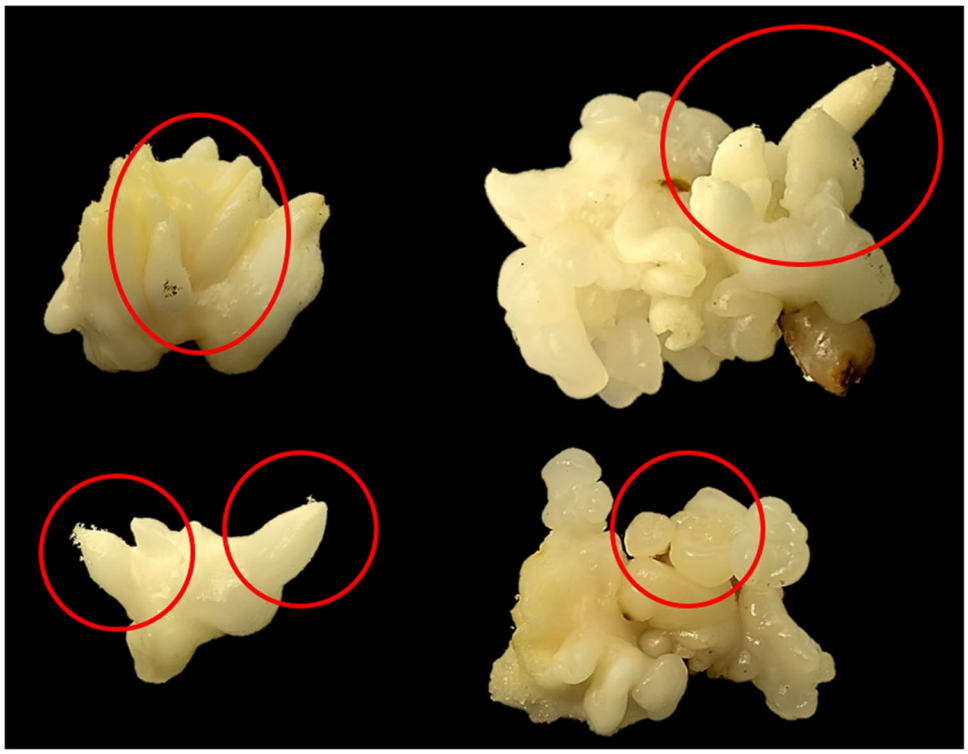

Fig. 4 Embryogenic calluses with early somatic embryos at 90 days of culture pretreated with 20 µM of AzaC. Red circles highlight the somatic embryos (color figure online)

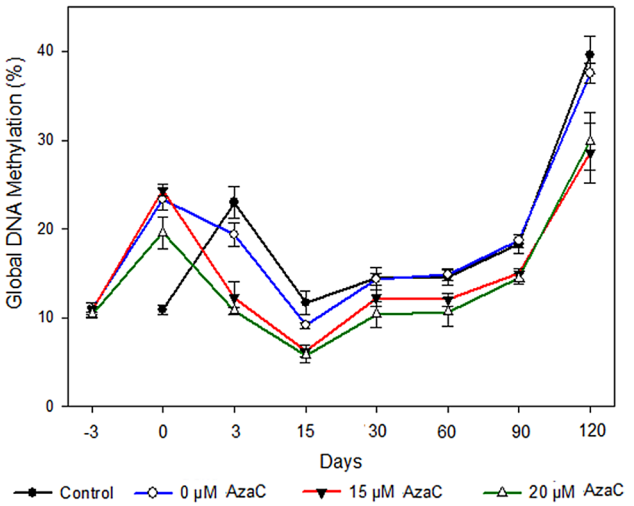

Fig. 5 Percentage of global DNA methylation from explants during somatic embryogenesis of *Cocos nucifera* with and without 5-Azacytidine. Symbols represents means of three independent biological samples ± SD

expression of the gene *PKL* presented a peak at 15 days (1.80-fold) and gradually decreased afterwards (Fig. 6e).

Effects of 5-Azacytidine on SE-related gene expression

To identify the effects of AzaC on the expression level of the SE-related genes described above, explants submitted to three-day pretreatments with 15 and 20 µM of AzaC were analyzed and compared to the expression levels of explants pretreated without AzaC. The results show a difference in gene expression when samples are pretreated with AzaC. The expression of *SERK* varies significantly at 30 days, with a fold increase of 2.2 for the 15 µM and 2.7 for the 20 µM treatments, and at 90 days with a 1.9 and 1.3 fold decreases in expression, respectively (Fig. 6a). *WUS* showed a 4.9 and 15.3-fold significant decrease in expression for each treatment at 3 days, and a 2.1 and a 2.4-fold significant increase at 90 days for each respective treatment (Fig. 6B). Similarly, *BBM* first showed a significant decrease in expression at 3 days (16.7 and 18.2-fold) with a significant increase with 20 µM treatment (1.0 fold) at 120 days (Fig. 6c). The expression of *LEC* decreased 2.5 and threefold at 3 days and increased 1.5-fold for both treatments at 60 days (Fig. 6d). Finally, the expression of *PKL* decreased significantly 1.4 and 0.9 fold at 60 days and 1.5-fold for both treatments at 90 days (Fig. 6e).

DNA methyltransferases (DNMTs) gene expression during somatic embryogenesis

Different expression patterns can be observed for the genes that encode for DNMTs (*MET*, *CMT* and *DRM*) during the SE process (Fig. 7a–c). Firstly, *MET* expression presented a small peak at 3 days (1.5-fold increase) that eventually increased further until reaching its highest level at 120 days (2.05-fold) (Fig. 7a). *CMT* and *DRM* showed a

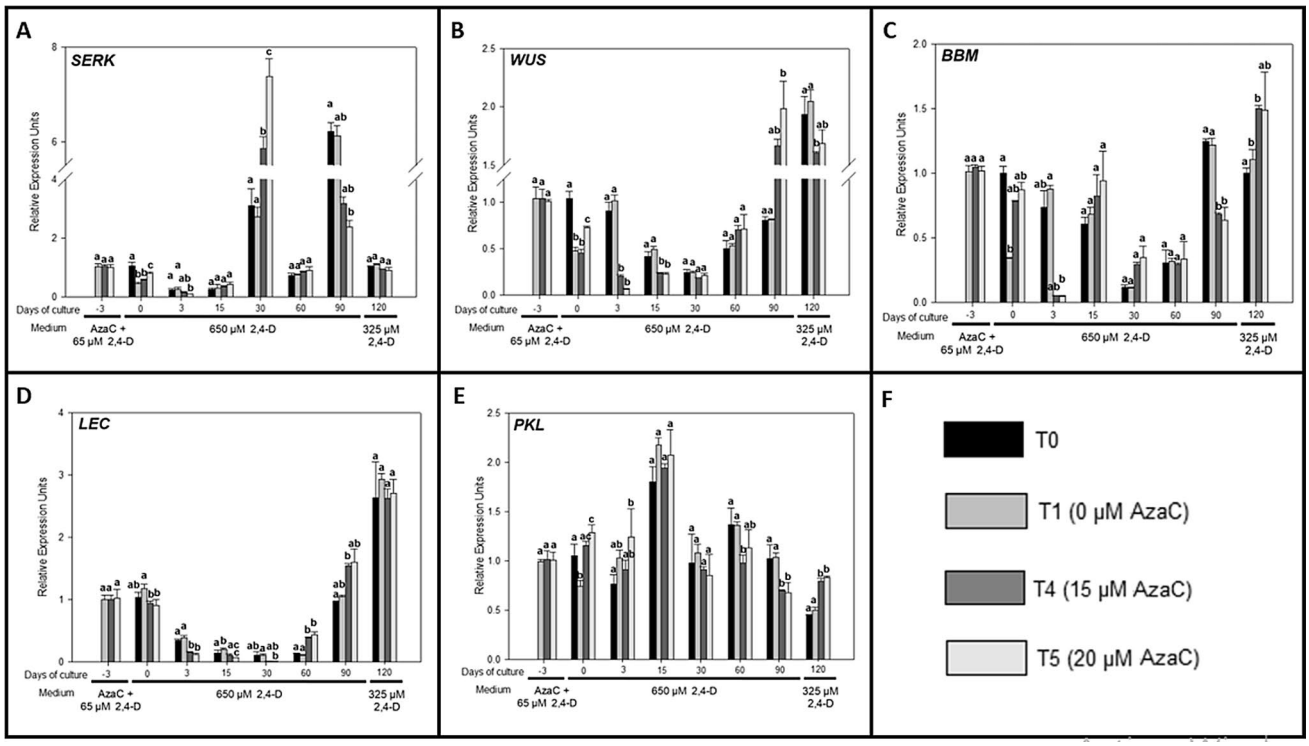

Fig. 6 **a**–**e** Relative gene expression levels of five genes that regulate somatic embryogenesis (*SERK*, *WUS*, *BBM*, *LEC* and *PKL*) during somatic embryogenesis of *Cocos nucifera*. **f** Analyzed pretreatments. Gene expression was quantified at − 3 days (**d**), 0 d, 3 d, 15 d, 30 d, 60 d, 90 d and 120 d time points. Bars represent means ± SD of three independent biological samples by duplicate. Differences in values followed by the same letter are not statistically significant, according to the Tukey test ($P \leq 0.05$)

steady and gradual increase until reaching their highest level of expression at 120 days (11.5 and 1.9-fold, respectively) (Fig. 7b, c).

Effects of 5-Azacytidine on DNA methyltransferases (DNMTs) gene expression

Pretreatments with 15 and 20 µM of AzaC had the following effects on the expression of DNMTs genes. The expression of *MET* increased significantly 1.6 and 1.5-fold at 0 days, respectively; at 3 days it increased 1.5 and 1.6-fold and at 90 days it decreased significantly 1.6 and 1.7-fold, respectively (Fig. 7a). *CMT*'s expression decreased 2.8 and 3.5-fold at 60 days and it decreased further (2.0 and 2.2-fold, respectively) at 90 days. A 3.4 and 3.5-fold decrease in the expression of *DRM* was observed at 3 days, with an additional 1.5-fold decrease in expression at 15 days, for both treatments (Fig. 7c). The pretreatment without AzaC presented a significant higher expression levels of the analyzed DNMT encoding genes after the subculture (2.4, 7.5 and 2.1 fold, respectively) and after three days of culture (1.3, 4.4 and 3.2 fold) when compared to the control.

Discussion

Traditional in vitro cultures of coconut aimed at the induction of SE require the use of activated charcoal (AC) to avoid browning and necrosis of the explants (Sáenz et al. 2010). In this case, the adsorptive properties of AC would reduce the availability of AzaC, thus affecting the response of the explants to the DNA-methylation inhibitor. For this reason a strategy was designed to avoid AzaC adsorption but also inhibit the browning and necrosis of the explants. In a first step, lasting three or seven days and referred to as pretreatment, AC was not included in the culture medium, after which the explants were transferred to Medium I (containing AC). A similar strategy had previously been reported for *Acca sellowiana* (Fraga et al. 2012), *C. nucifera* (Azpeitia et al. 2003) and *Picea omorica* (Leljak-Levanic et al. 2009).

The SE process consists of two steps: induction and development. Our study was focused on the induction stage that encompasses the phases of dedifferentiation, totipotency, and acquisition of the embryogenic competence (Elhiti et al. 2013). In coconut SE the induction step

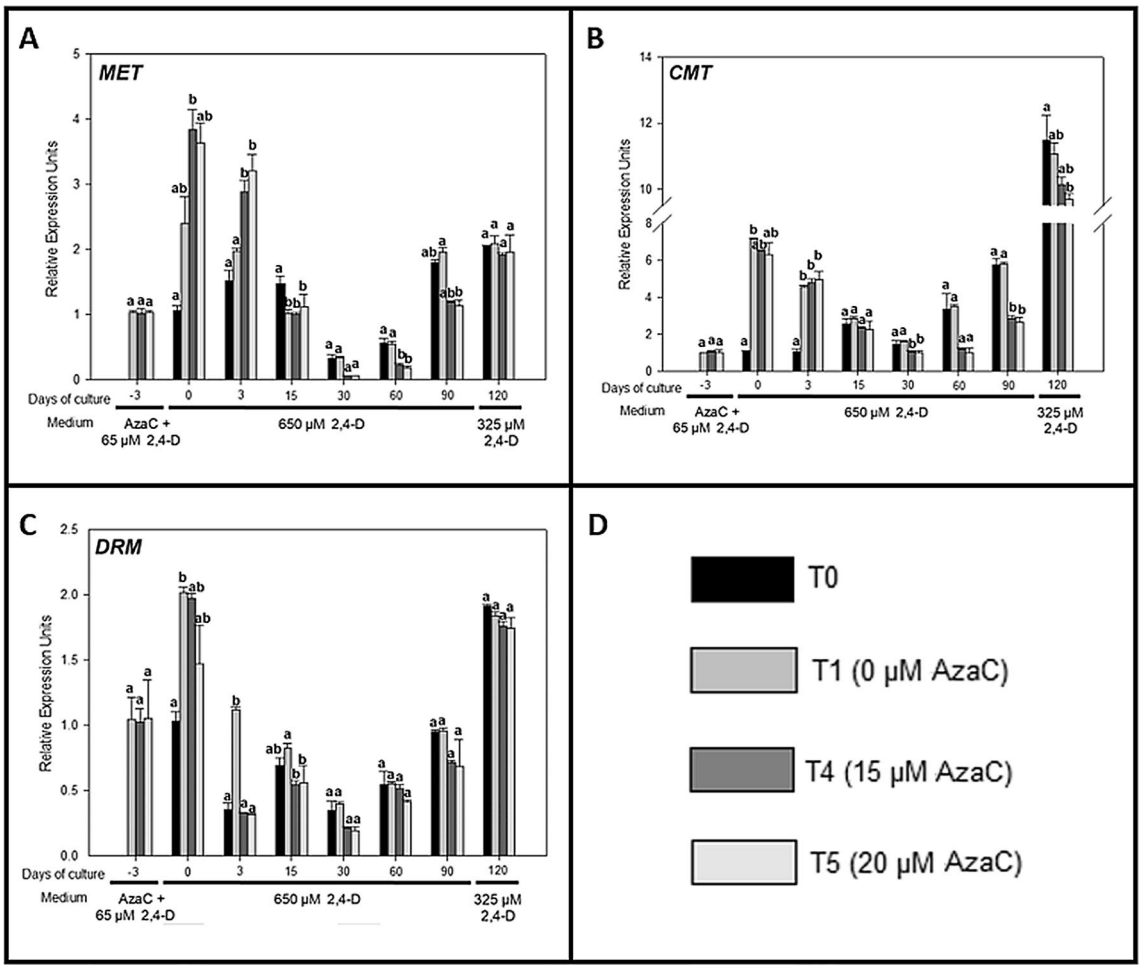

Fig. 7 **a**–**c** Relative gene expression levels of three genes that encode for DNA methyltransferases (*MET, CMT* and *DRM*) during somatic embryogenesis of *Cocos nucifera*. **d** Analyzed pretreatments. Gene expression was quantified at − 3 days (d), 0 d, 3 d, 15 d, 30 d, 60 d, 90 d and 120 d time points. Bars represent means ± SD of three biological samples by duplicate. Differences in values followed by the same letter are not statistically significant, according to the Tukey test ($P \leq 0.05$)

initiates at day 15 with the beginning of the appearance of calluses. At day 30 a well-developed callus is obtained and by day 90 an embryogenic callus, with pearly embryogenic structures on its surface, is formed (Pérez-Nuñez et al. 2006). The formation of globular embryos occurs after 120 days. In our experience these initials step are of crucial importance to obtain somatic embryos and eventually plantlets.

The morphogenetic response during the SE process of coconut palms has already been reported in previous studies (Pérez-Nuñez et al. 2006; Sáenz-Carbonell et al. 2018). The percentage of embryogenic calluses formation reported so far has oscillated between 40 and 70% and the number of somatic embryos was of 3–7 embryos per callus. In the present study the mean percentage of embryogenic calluses formation was of 55%, with five somatic embryos per callus. The three-day pretreatments with 15 µM of AzaC achieved the highest percentages of embryogenic calluses formation (75%) after 90 days of culture. However, the same pretreatment but without AzaC showed a similar response (73%). These results seem to indicate that the subculture of the explant to a fresh medium with 2,4-D, after a short pretreatment period, could increase the embryogenic response. This increase in embryogenic response is most likely due to the fact that 2,4-D induces an increase in the accumulation of endogenous indoleacetic acid, as has already been reported for carrot (Michalczuk et al. 1992) and a higher endogenous concentration of indoleacetic acid has been associated to an increased embryogenic response in different plant species (Ivanova et al. 1994; Michalczuk and Druart 1999).

The addition of AzaC (20 µM) to the culture medium had a significant positive effect on the formation of early globular somatic embryos reducing it to 90 days. This type of response has been observed sporadically in Medium I but the addition of AzaC seems to reduce the embryogenic response to less than 90 days while also increasing the amount of

early somatic embryos. The pretreatment without AzaC did not present any embryogenic calluses with early somatic embryos, which could indicate that the presence of AzaC induces the premature formation of embryos from coconut calluses by generating molecular or epigenetic changes. AzaC has previously shown to have short and long term effects on the initiation and progression of embryogenesis by microspores in the cases of *B. napus* and *Hordeum vulgare*. The treatment of *B. napus* and *H. vulgare* with 2.5 µM of AzaC for four days increased the percentage of somatic proembryos, whereas a longer treatment produced the opposite effect (Solís et al. 2015). Furthermore, the exposure of *C. canephora* explants to a concentration of 20 µM of AzaC not only improved the yields of globular somatic embryo formation but also seemed to have synchronized the process (Nic-Can et al. 2013). A similar effect was observed in *A. sellowiana* where the use of 10 µM of AzaC increased the formation of somatic embryos by more than 240% (Fraga et al. 2012).

The global DNA methylation was monitored to determine whether changes in DNA methylation occur during the SE process. The results for the control treatment show a peak in GDM% at the beginning of the culture (approx. 13%), a re-settlement of the GDM% to lower levels and finally a steady increase throughout the rest of the culture. The methylation dynamics of the AzaC pretreated cultures show similar trends to that of the control but with lower GDM% levels. GDM% resulting from the pretreatments with 15 and 20 µM of AzaC were clearly lower when compared to those observed for the pretreatment without AzaC, mainly at day three of the culture when the highest differences were observed (7.1 and 8.6%, respectively). These differences were maintained across the in vitro culture but to a lesser extent. This dynamic of DNA methylation during SE has also been observed for *D. carota* (LoSchiavo et al. 1989), for which the GDM% was of 16% during the induction process with 2,4-D, followed by 14% for the pre-globular embryos, 19% in the hearted embryo stage and 20% in seedlings. GDM% in *C. canephora* have also shown small increase from 23% at the beginning of the induction to 25% at day 14, and a significant increase at the end of the SE process (day 56), at which point the somatic embryos have already been formed (Nic-Can et al. 2013). Our results in coconut suggest that the de-differentiation of explants is accompanied by an increase in DNA methylation, which may be caused by the combined effects of the endogenous auxins and of the 2,4-D present in the medium. Auxins have previously been reported to be capable of increasing the levels of DNA methylation (Ayil-Gutiérrez et al. 2013; LoSchiavo et al. 1989; Osorio-Montalvo et al. 2018).

The levels of expression of *SERK, WUS, BBM, LEC* and *PKL* in specific stages of the SE process were also analyzed, to further understand the development of the SE and the effects of AzaC on it. This analysis revealed that *SERK* exhibits high levels of expression at days 30 and 90 of the in vitro culture, similarly to what had already been reported by Pérez-Núñez et al. (2009). *SERK* is a gene that has been widely studied and pointed out as a marker gene for SE induction (Mahdavi-Darvari et al. 2015; Schmidt et al. 1997). Furthermore, the peaks observed at days 30 and 90 coincided with the formation of initial calluses and embryogenic calluses, respectively. Additionally, the pretreatments with AzaC increase the expression of this gene at day 30. The expression of *WUS*, a homeobox gene that regulates the establishment and maintenance of the shoot apical meristem (Mayer et al. 1998), showed its highest level of expression at day 120, when somatic embryos had already been formed. When pretreated with AzaC, the levels of expression of *WUS* increased twofold at day 90, compared to the explants exposed to the pretreatment without AzaC. *BBM* is a member of the AP2/ERF transcription factor family. It promotes the activation of genes involved in cell wall modifications and it has been related to cell division and growth during SE (Boutilier et al. 2002; Solís-Ramos et al. 2012). Our analysis of the expression of *BBM* during the SE process of coconut explants shows a gradual decrease up to day 60 and followed by an increase. The levels of expression of *BBM* in explants pretreated with AzaC decreased during the first days of culture but reached twice the level of expression at day 30 than those explants pretreated without AzaC. *LEC* is a *B-HAP3*-like gene with a fundamental role as a transcription factor, it regulates the expression of other genes involved mainly in the induction and later in the embryonic differentiation during SE (Gaj et al. 2005; Rocha and Dornelas 2013). In the present work, the expression of *LEC* during SE begins to increase after day 60 and reaches its peak at day120, when the somatic embryos were already formed. The expression levels of *LEC* in the pretreatments with AzaC were higher at day 60 and 90, when compared to the pretreatment without AzaC. It had already been reported that the expression of *LEC1* and *BBM1* was strongly inhibited by AzaC during the SE process (Nic-Can et al. 2013). *PKL* is a B-HAP3 type chromatin remodeling factor and it plays a fundamental role in the maturation of embryos since it represses embryonic characteristics (Ogas et al. 1999; Radoeva and Weijers 2014). In addition, *PKL* has a repressive activity towards *LEC* (Dean Rider Jr et al. 2003). In the analyzed coconut explants, the expression of *PKL* in the SE process reached a maximum level at day15, when the explants begin to differentiate and form calluses and it reached a minimum at day 120 when somatic embryos are already formed. It should be noted that the expression pattern of *PKL* is inversely proportional to that of *LEC*. Our results show that the profile of expression of the genes *SERK*, *WUS*, *BBM*, and *LEC* are modulated during the somatic embryogenesis process of coconut. They also show that AzaC affects the expression

profiles of these genes by generally inducing an earlier appearance of their peaks of maximum expression. Furthermore, the minimum level of GDM% reached at day 15, coincides with the beginning of calluses formation, indicating that it could be a trigger to the embryogenic response of the explant. The significant increase in the expression of *SERK* at day 30 and the decrease of the expression of *PKL* seems supports this hypothesis. These results show that the hypomethylating effect of AzaC on DNA could be a key step to positively modulate the activity of SE-related genes in coconut, which is consistent with the observed earlier formation of somatic embryos.

DNMTs are the enzymes responsible of transferring methyl groups to the cytosines of DNA resulting in the methylation of DNA, which generally has a repressive effect on gene expression (De-la-Peña et al. 2015). In 2014, various elements involved in RNA-directed DNA methylation (RdDM) were identified and analyzed in leaves and seeds of coconut (Huang et al. 2014), among them *MET*, *CMT* and *DRM* genes. These findings suggested that all the above elements are important for the development of embryos and the endosperm of coconut seeds. Our results show that the genes encoding for DNMTs analyzed during the SE process of coconut present a peak of expression at day 0 of culture, followed by a reduction (from day 0 to 30) and an increase from day 30 until day 120, a pattern that seems to coincide with that of the GDM%. It is worth noting that maintenance and de novo DNA methylation is important throughout coconut SE, since a higher expression of *MET*, *CRM* and *DRM* was observed at early and late stages of the embryogenic process. In our study, the genes encoding for DNMTs from explants pretreated with and without AzaC presented a relatively high expression levels when compared to those of the control, during the first days of culture, contrary to the observed reduction of GDM%. This phenomenon might be explained as a response of the cells in an attempt to restore the normal levels of GDM%, pharmacologically reduced by AzaC. Also, it cannot be ruled out that the observed levels of expression might be induced by the higher 2,4-D concentrations, while the DNA methylation is being inhibited at a protein level, either by a cellular accumulation of AzaC or by an unknown mechanism. This opposing effect of AzaC on GDM% has previously been reported during the SE of *Boesenbergia rotunda* in which the reduction of DNA methylation coincided with increased expression levels of the analyzed DNMTs encoding genes (Rezaul Karim et al. 2018).

We hypothesize that the induction of explants in a new medium with a fresh supply of 2,4-D induces a peak increment of GDM% during the first days of in vitro culture during the de-differentiation process, perhaps as a mechanism to suppress the expression of SE-related genes. Subsequently, the observed minimum levels of methylation reached at the beginning of calluses formation (day 15) would allow for a cellular reprogramming, turning on the transcriptional machinery typical of embryogenic competence. The gradual

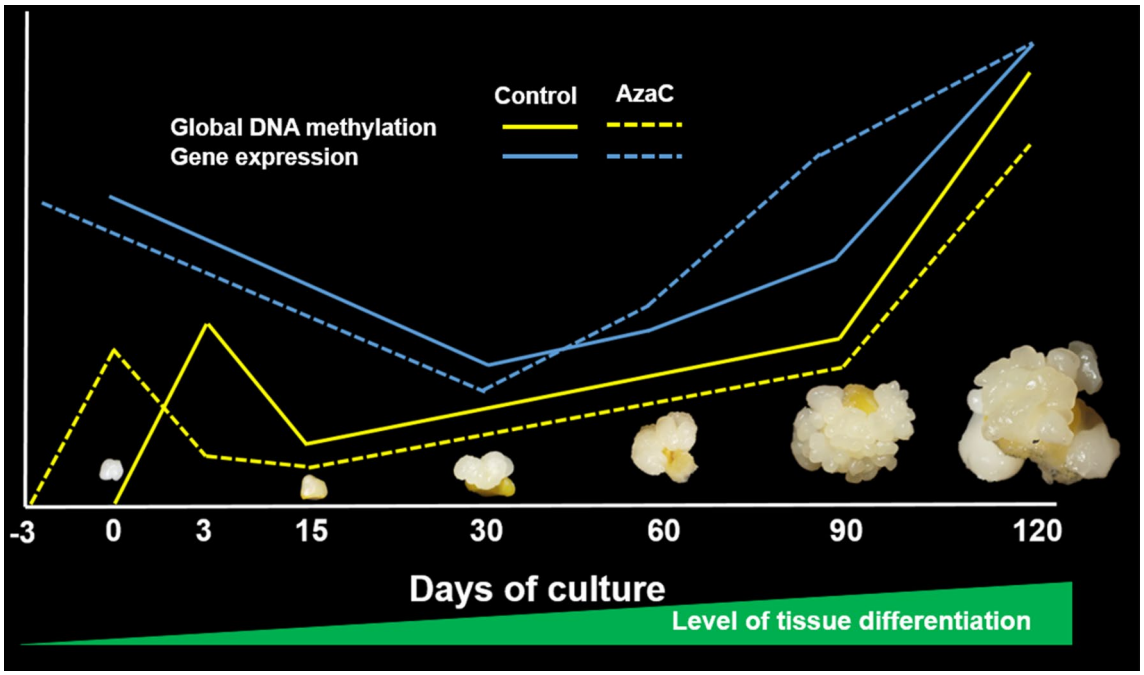

Fig. 8 Proposed scheme of the events that occur during the somatic embryogenesis process in *Cocos nucifera,* including the global DNA methylation and gene expression dynamics

increase of DNA methylation that follows probably favors the expression of genes that positively regulate the SE. In that sense, AzaC is likely to modulate the peak of GDM% and to induce epigenetic changes that result in higher expression levels of key genes regulating SE, such as *SERK* and *LEC* (Fig. 8). The described scenario could explain the stimulation of the earlier appearance of somatic embryos.

In conclusion, the evidence presented by our study reveals that the SE process of coconut is characterized by significant changes in DNA methylation, morphogenesis and gene expression. Furthermore, our results clearly indicate that the changes induced by the pretreatments with AzaC lead to an earlier generation of somatic embryos in *C. nucifera* explants, becoming an interesting alternative for the improvement of micropropagation protocols of coconut palms.

Author contribution statement POM performed the experiments and wrote the manuscript; LSC, CDS and COS directed the project, discussed the results and reviewed the manuscript; ICC and ECC provided technical assistance for the experiments; GNC reviewed the manuscript and discussed the results.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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