Addition of ionophore A23187 increases the efficiency of *Cocos nucifera* somatic embryogenesis

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Abstract

The present study reports the effect of treatment of coconut embryogenic structure explants (derived from embryogenic callus) with the calcium ionophore A23187 (0, 1, 5, 10 μ M) to promote somatic embryogenesis under in vitro conditions. The results showed no significant increase in the percentage of explants forming embryogenic callus, but with 1 μ M ionophore there were significant increases in the formation of embryogenic structures per callus (2.8-fold), of somatic embryos per callus (1.5-fold) and also a greater absolute number (1.5-fold) of developing plantlets per callus. The ionophore treatment also promoted a change of pattern of the expression of the *CnSERK* gene during embryogenic callus formation. It is proposed that if the use of ionophore A23187 treatment is coupled with an embryogenic callus multiplication process there could be a potentially greater increase in the efficiency of the formation of somatic embryos and plantlets of coconut.

Keywords Coconut · Somatic embryogenesis · Calcium ionophore A23187 · *Cn SERK*

Introduction

The coconut (*Cocos nucifera* L.) is a palm species that is cultivated in approximately 11 million hectares in nearly 90 countries around the world (FAO 2016). The markets of coconut products obtained from the fruit have been growing exponentially worldwide in the past 15 years, particularly in the case of packaged coconut water, virgin coconut oil and coconut sugar (Roolant 2014; Prades et al. 2016). Unfortunately, this success is threatened mainly by high incidence of devastating phytoplasma diseases affecting coconut and other palm species (Ntushelo and Harrison 2013; Gurr et al. 2016) and the lack of renovation of most coconut plantations, which are significantly reducing the coconut production in several countries around the world (Batugal et al. 2005). Therefore, there is an urgent need for replanting with coconut palms selected for disease resistance and productivity to sustain the increasing demand of fruit. Traditional replanting based on seed propagation is not going to be fast enough to accomplish the huge task required, a different

approach has to be used and micropropagation through somatic embryogenesis seems to be the most reliable alternative (Oropeza et al. 2005).

There are several studies reporting in vitro somatic embryogenesis protocols for different plant species and also reporting that their performance can be influenced by different factors, including genotype (Moon et al. 2013; Jiao et al. 2018), type and maturity of explant tissue source (Moon et al. 2013), choice of growth regulators (Filippov et al. 2006; Verma et al. 2018), carbon source (Gaj 2004; Moon et al. 2013), nitrogen source (Hita et al. 2003; Leljak-Levanic et al. 2004), polyamines (Jiao et al. 2018), basal medium strength (Prakash and Gurumurthi 2010), gelling agent, density of the culture (Moon et al. 2013), subculture frequency (George et al. 2008) and culture room lighting (Vidican and Cachita-Cosma 2010).

In the case of coconut, different laboratories have also worked on the development of somatic embryogenesis protocols (Nguyen et al. 2015; Sáenz et al. 2016). Different media formulations have been tested using different sources of explant and the best somatic embryogenesis responses have been obtained with inflorescence (Hornung and Verdeil 1999) and plumule (Sáenz et al. 1999) explants. A very important requirement in the media formulation to promote these responses, is the presence of an auxin (Sáenz et al. 1999), commonly

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2,4-dichlorophenoxyacetic acid (2,4-D). Twenty years ago our laboratory reported a direct somatic embryogenesis and plant regeneration protocol, using plumule explants, that was reproducible and relatively efficient (Chan et al. 1998). Over the following years, improvement of the performance of this protocol was achieved using two direct approaches. One is the modification of the media formulation, such as the addition of different growth regulators as brassinosteroids (Azpeitia et al. 2003), gibberellic acid (Montero-Cortés et al. 2010b), cytokinins (Montero-Cortés et al. 2011b) and the choice of the type of activated charcoal (Sáenz et al. 2010). The second approach is the modification of the conditions and configuration of the process, with changes such as the incorporation of the embryogenic callus multiplication and secondary somatic embryogenesis (Pérez-Núñez et al. 2006). This second approach in particular, allows the potential to produce tens of thousands of somatic embryos with high rates of conversion into plantlets, and just starting from a few explants. A process derived from these two approaches is currently under scaling-up in Mexico (Oropeza et al. 2016). However, regardless of the progress achieved so far, there is a constant need to further improve coconut micropropagation, to deal with the massive production of palms required for the urgent renewal of coconut plantations.

In addition to these direct approaches for improving coconut micropropagation, efforts have been made to gain basic knowledge to understand processes underlying the control of coconut somatic embryogenesis. As mentioned above, it is acknowledged that the auxin 2,4-D plays an essential role for the induction of somatic embryogenesis of coconut, whether from plumule explants (Rajesh et al. 2016) or rachilla explants (Sandoval-Cancino et al. 2016), and that this could be mediated by the regulation of gene expression (Nguyen et al. 2015; Rajesh et al. 2016; Sáenz et al. 2016). A particular gene of interest is *CnSERK*, a *SERK*-like gene that was reported in coconut as showing a differential pattern of expression during coconut embryogenic callus formation, and it was proposed as a marker for cells competent for the formation of somatic embryos in coconut tissue cultures (Pérez-Núñez et al. 2009), similarly as it has been considered for other plant species (Somleva et al. 2000; Hecht et al. 2001). Related reports of interest show that auxin induction of somatic embryogenesis appears to be mediated by calcium–calmodulin regulation of gene expression in wheat cultures (Mahalakshmi et al. 2007); and that treatment with A23187 ionophore, that facilitates the transport of calcium across the plasma membrane (Dedkova et al. 2000), promotes somatic embryogenesis in wheat (Mahalakshmi et al. 2007) and coffee cultures (Ramakrishna et al. 2011). Therefore, here we report a study to evaluate the effect of ionophore A23187 on somatic embryogenesis (formation of embryogenic callus, embryogenic structures and

somatic embryos) in coconut cultures and on the expression of *CnSERK* during the formation of embryogenic callus.

Methods

Plant material

Explants used were embryogenic structures that were excised from coconut embryogenic calli obtained from our coconut in vitro collection at the Clonal Micropropagation Laboratory, Centro de Investigación Científica de Yucatán (CICY), Mexico. These calli were originally produced from plumule explants of the Mexican Pacific Tall (MXPT) ecotype according to the protocol previously reported (Pérez-Núñez et al. 2006). The embryogenic structures (as defined by Sáenz et al. 2006) are pearly globular structures present on the surface of embryogenic calli, from which somatic embryos eventually form.

Culture media and culture conditions

All media and culture conditions were according to Pérez-Núñez et al. (2006). A basal medium was prepared with Y3 formulation, supplemented with gel-rite (3 g/l), sucrose (5%) and activated charcoal (2.5 g/l). The medium for the induction of embryogenic callus (medium 1) was prepared with basal medium supplemented with 0.6 mM 2,4-D. The medium for the induction of somatic embryo formation (medium 2) was prepared with basal medium supplemented with 0.3 mM 2,4-D. The medium for the somatic embryo germination (medium 3) was prepared with basal medium supplemented with 0.006 mM 2,4-D and 0.3 mM 6-benzylaminopurine (BAP) and 0.0028 mM gibberellic acid (GA_3). The medium for plantlet formation (medium 4) was prepared with basal medium supplemented with 0.006 mM 2, 4-D and 0.3 mM BAP. Media pH was adjusted to 5.75 before autoclaving (120 °C for 20 min). All chemical used are from Sigma-Aldrich, USA.

Embryogenic callus formation was induced from embryogenic structure explants when cultured on medium 1 (10 ml in a 45 ml flask) for 3 months under dark conditions. At the end of this period the process was repeated. Somatic embryo formation was obtained on embryogenic calli cultured on medium 2 (25 ml in a 150 ml flask) for 1 month under dark conditions. Germination of the somatic embryos formed on embryogenic callus was obtained by culturing these callus in medium 3 (25 ml in a 150 ml flask) for 3 months under 16-h photoperiod (45–60 $\mu\text{mol}/\text{m}^2/\text{s}$ photosynthetic photon flux density, PPFD) provided by TriPhosphor daylight tubes (F32T8, 6500 K, 32 W, MAGGMR, Mexico). Shoot development was obtained by culturing calli with germinated somatic embryos on medium 4 (25 ml in a 150 ml flask) for

2 months under 16-h photoperiod. Then cultures were subcultured 3 times into fresh medium 4. During this 8 months period, as shoots grew, they were separated from the callus and cultured as isolated shoots for further development. All cultures were kept at 27 ± 2 °C.

Ionophore A23187 trials

Embryogenic structure explants were treated with ionophore A23187 (Sigma–Aldrich, USA) and calcium ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Fermont, Mexico) to evaluate their effect on the formation of embryogenic callus in three trials.

In the first trial 21 treatments were tested. Each treatment consisted of placing three explants on medium 1 with calcium at different concentrations (0, 0.5, 1, 3 or 4 mM) and combined with an application of 10 μL of a solution of ionophore A23187 at different concentrations (50, 100, 200 or 400 μM) deposited with a micropipette on each explant evaluated. The different combinations tested are shown in Table 1.

For the second trial five treatments were tested in which calcium was not added to the medium, because of results obtained in the first trial (see “Results” section). Each treatment consisted of placing three explants on medium and applying 10 μL of a solution of ionophore A23187 (0, 1, 5 or 10 μM) deposited with a micropipette on each explant evaluated. For the third trial the embryogenic structures used as explants were obtained from the calli formed during the second trial with the treatment producing the best results, hence three explants were placed on medium 1 applying 10 μL of ionophore A23187 (1 μM) deposited with a micropipette on each explant evaluated. For each of the three trials, explants were cultured during 3 months in dark conditions at 27 ± 2 °C.

The embryogenic calli obtained from the third trial were used to evaluate the formation of somatic embryos, their germination and postgerminative development. Media and culture conditions used were as described above in “Culture media and culture conditions”.

Histology

The histological analysis was carried out according to Bufard-Morel et al. (1992) as modified by Sáenz et al. (2006) and Montero-Cortés et al. (2010a). Tissue samples were immersed in a solution of phosphate buffer (pH 7.2) with 4% (w/v) paraformaldehyde during 24 h. The samples were then dehydrated by placing them in a water solution of ethanol with stepwise increases in ethanol from 30 to 100% during 7-h period. Then samples were imbibed with the polymeric resin JB-4R (Polyscience, Warrington, PA) and then 5- μm thick sections were produced using a HM 325 microtome (Thermo Fisher Scientific, Waltham, MA). Finally the

Table 1 Effect of different treatments combining calcium with ionophore A23187 on the formation of embryogenic callus from embryogenic structure explants cultured for three month in medium 1

Ca^{2+} (mM)	A23187 (μM)	Embryogenic callus (%)	Necrosis (%)		
0	0	60.0	a	0.0	d
0.5	0	6.7	c	53.3	b, c
	50	13.3	c	40.0	c
	100	40	a,b	26.7	c, d
	200	13.3	c	40.0	c
	400	0.0	c	73.3	a, b
1	0	20.0	b,c	26.7	c, d
	50	0.0	c	53.3	b, c
	100	0.0	c	40.0	c
	200	0.0	c	86.7	a
	400	6.7	c	93.3	a
3	0	20.0	b, c	0.0	d
	50	6.7	c	6.7	d
	100	20.0	b, c	26.7	c, d
	200	0.0	c	0.0	d
	400	0.0	c	86.7	a
5	0	6.7	c	80.0	a, b
	50	6.7	c	0.0	d
	100	0.0	c	0.0	d
	200	0.0	c	0.0	d
	400	6.7	c	0.0	d
		C.V. = 257.196	C.V. = 100.806		
		$R^2 = 0.226$	$R^2 = 0.473$		

Different letters denote significant differences (ANOVA, $P \leq 0.05$)

sections were dyed with periodic acid-Schiff (PAS) reagent and protein-specific Naphthol Blue-Black (Fisher 1968).

Photographic documentation

Embryogenic calli and histological sections were observed under a stereoscope SMZ-168 TLED and photographs were captured with a Moticam 580 (Motic, British Columbia, Canada). Since embryogenic calli or histological sections did not fit into the vision field provided of Moticam 580, a series of photographs (about 60) were captured to obtain composite images with Image Composite Editor 2.0 (Microsoft Research 2017).

Statistical analysis

A completely randomized design was employed for this experiment. Data presented corresponds to averages and each explant was treated as an experimental unit and as a repetition. Each experimental group has a $n = 63$ as it is indicated in each corresponding figure or table. Data obtained were tested in a variance analysis (ANOVA) and

the procedure for average comparisons (Tukey test, $P < 0.05$) was obtained with the SAS University Edition statistical software.

TaqMan/real-time PCR assay

A TaqMan probe was designed considering the less conserved region (Fig. 1) of the sequence gen *CnSERK* (acc. Number AY791293.2) reported by (Pérez-Núñez et al. 2009). This TaqMan probe was used in the expression analysis of *CnSERK* during embryogenic callus formation from embryogenic structure explants for 90 days initiating with or without the application of ionophore A23187 (1 μ M) on the explants.

The samples were collected in triplicate for analysis every 15 days within the 90 days culture period. RNA extraction from a total of 100 mg of culture tissue of each developmental stage was carried out using TRIZOL™ (Invitrogen, Cat. No. 15596026). The Turbo DNA-free™ Kit (Thermo Fisher Scientific, Cat. No. AM1907) was used to remove possible DNA contamination from the resulting RNA samples. Quantification of RNA samples was carried out using Nanodrop 1000 (Thermo Scientific, USA). cDNA was amplified by reverse transcriptase-PCR from 1 μ g of total RNA using random primers (Thermo Fisher Scientific, Cat. No. 48190011) and the SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, Cat. No. 18080093) according to the instructions of the supplier.

The expression levels of the *CnSERK* transcripts were determined by qRT-PCR. The mixture used for this purpose had a final volume of 20 μ L and was composed by 1 μ L of cDNA, 10 μ L of Maxima Probe qPCR Master Mix (2X) (Thermo Fisher Scientific, Cat. No. K0261) and 1 μ L of the TaqMan *CnSERK* probe. *Euk 18S rRNA* (Applied Biosystems, Cat. No. 4333760T) was used as internal control to normalize the expression data obtained. Each reaction was performed in triplicate in a Rotor-Gene Q (QIAGEN, Hilde, Germany) and conditions were set as follows: 2 min at 50 °C, 10 min at 95 °C, 20 min at 72 °C, 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 1 min at 62 °C. The resulting data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

In the three trials carried out to study the effect of ionophore A23187 on coconut somatic embryogenesis, depending on the treatment, a certain percentage of explants developed embryogenic callus (as defined by Sandoval-Cancino et al. 2016). This developmental process started with embryogenic structures as explants (Fig. 2a) that after 45 days formed a callus that began developing ear-like structures, refer to as

Fig. 2 Developmental stages during embryogenic callus formation from embryogenic structures explants (a) after 30, 60 days; (b) fully formed embryogenic calli formed at day 90 of culture without (control) and with 1 μ M ionophore A23187; and (c) corresponding globular somatic embryos. TS translucent structures, ES embryogenic structures, SE somatic embryos

Fig. 1 Alignment of sequences reported at GenBank with the highest identity percentage to *CnSERK*. The region between the nucleotides on position 230 and 241 was used to design the corresponding TaqMan probe for *CnSERK*

EgSERK1	552	TTACTTGTATAATGACATAGTGTTCATTAGAG-----	584	TTGCTCTTGGA
PdPredSERK2X1	556	TTACTTGTATAATGACATAGTGTTCATTAGAG-----	588	TTGCTCTTGGA
PdPartSERK2	118	ATTTCTCTTTATAA-----	129	TTAAA-----TCTTCTCTCTAGGAAG
PdSERK1	224	ATGACATAGTGTTCATTAGAG-----	244	TTGATCTTGGAATTCCTCAGTT
EgPredSERK2	606	TTACTTGTATAATGACATAGTGTTCATTAGAG-----	638	TTGCTCTTGGA
CnSERK	206	ATGACATAGTGTTCATTAGAG-----	226	TTGATCTTGGAATTCCTCAGTT
PdPredSERK2X2	116	CATAGTGTTCATTAGAGTATGT	139	GCATTGATCTTGGAATGCACAGTT
PdPseudoSERK	206	ATGACATAGTGTTCATTAGAG-----	226	TTGATCTTGGAATTCCTCAGTT
EgPredSERK1	574	TTACTTGTATAATGACATAGTGTTCATTAGAG-----	606	TTGATCTTGGA
PdPredSERK2	604	TTACTTGTATAATGACATAGTGTTCATTAGAG-----	636	TTGATCTTGGA

translucent structures (Fig. 2a). By day 60 the callus had grown and had more translucent structures with smaller pearly white structures (embryogenic structures) growing on them (Fig. 2a).

Then after 90 days of culture the embryogenic structures became more abundant (Fig. 2b) and at this stage the callus is referred to as embryogenic callus. This developmental process for the formation of embryogenic callus occurred on explants cultured with or without ionophore A23187. In the first trial, 21 different combinations (Table 1) of concentrations of calcium (0, 0.5, 1, 3 and 5 mM) and ionophore A23187 (0, 50, 100, 200 and 400 μ M) were tested on embryogenic structure explants cultured for 90 days, after this period embryogenic callus formed on some of the explants. With the control treatment without calcium and without ionophore A23187, the percentage of explants forming callus was 60% (Table 1). With the other 20 treatments combining calcium and ionophore A23187, the percentage of explants forming embryogenic callus was lower than that of the control, ranging from 0 to 20% in most of the 20 treatments, only in one it was greater, 40% (Table 1). When considering solely the treatments with calcium without ionophore A23187, the percentage ranged from 6.5 to 20% (Table 1). Several of the explants that did not produce callus showed necrosis. In some of the treatments the percentage of explants with necrosis was as high as 93%.

Considering the negative effect of addition of calcium on embryogenic callus formation described above for the first trial, no calcium was included for the second trial and application of ionophore A23187 was tested at lower concentrations (0, 1, 5 and 10 μ M). In the control treatment with no ionophore A23187 58% of the explants formed embryogenic callus (Fig. 3). When ionophore A23187 was added, at 5 and 10 μ M there was no improvement in relation to the control treatment, but at 1 μ M the percentage of callus formation was 65%, however, the difference was not significant (Fig. 3).

A third trial was carried out, this time only testing 1 μ M ionophore A23187 with no calcium, the explants were the embryogenic structures from the calli formed in second trial. In the control treatment 47.9% of explants formed embryogenic callus, whereas with the treatment with 1 μ M ionophore A23187 the figure was 61.8% (Table 2), the difference was significant ($P \leq 0.05$).

Embryogenic structure formation

Evaluation of the amount of embryogenic structures formed per callus, showed that in the second trial it was 5.3 for control treatment and 18.1 for 1 μ M ionophore A23187 treatment (a 3.5 times increase), and in the third trial the figures were 5.3 for control treatment and 11.4 for 1 μ M ionophore A23187 treatment (a 2.1 times increase) (Table 2). The

Fig. 3 Effect of treatment of embryogenic structures explants with ionophore A23187 (0, 1, 5 and 10 μ M) on the rate of embryogenic callus formation. Different letters represent statistical differences among groups

Table 2 Effect of treatment of embryogenic structure explants with ionophore A23187 on the frequency of the formation of embryogenic callus and the number of embryogenic structures formed per callus during 3 months culture in medium 1

Trial	Treatment	Embryogenic callus (%)		Embryogenic structures per callus	
1	Control	58.3	a	5.3	b'
	A23187 (1 μ M)	65.7	a	18.1	a'
		C.V. = 6.620 R ² = 0.500		C.V. = 13.023 R ² = 0.963	
2	Control	47.9	b	5.3	b'
	A23187 (1 μ M)	61.8	a	11.4	a'
		C.V. = 9.279 R ² = 0.841		C.V. = 38.805 R ² = 0.488	

Different letters denote significant differences (ANOVA, $P \leq 0.05$)

differences were significant ($P \leq 0.05$). Histological analysis of the calli with both treatments showed that in the periphery of the embryogenic structures there was accumulation of densely stained cells (Fig. 4a, b). These cells were below a layer of unstained cells forming the protoderm (Fig. 4c, d).

Formation of somatic embryos and plantlet regeneration

Formation of somatic embryos was evaluated only in calli formed in the third trial. This was because in for the first and second trials the embryogenic calli formed were used as source of embryogenic structure explants to generate new embryogenic callus. Observations showed that calli actually

Fig. 4 Histological sections of embryogenic calli obtained after 90 days of culture without (a) and with 1 μ M ionophore A23187 treatment (b); and corresponding magnified areas ($\times 40$) within squares shown in (c) and (d). ES embryogenic structures

Table 3 Effect of treatment of embryogenic callus with ionophore A23187 on the number of somatic embryos per callus and the number of shoots per callus. Ionophore A23187 was included in medium 2 (for embryo formation), but not in medium 3 and 4 (for embryo germination and plantlet development, respectively)

Treatment	Embryos per callus		Number of calli cultured	Developing plantlet per callus
Control	8.8	b	21	4.2
A23187 (1 μ M)	12.3	a	33	6.6
	C.V. = 29.932			
	$R^2 = 0.218$			

Different letters denote significant differences (ANOVA, $P \leq 0.05$)

started forming some globular embryos even before the end of the 90 day culture in medium 1 (Fig. 2b, c), but the formation of embryos was promoted when embryogenic calli were subcultured on medium 2, and after 1 month culture there were 8.8 somatic embryos per callus in control treatment and 12.5 in the case of treatment with 1 μ M ionophore A23187, the difference was significant ($P \leq 0.05$, Table 3). Calli with somatic embryos were then subcultured on medium 3 for embryo germination. Within 3 months embryos in the two treatments started germinating and forming shoots (Fig. 5a, b). Then when shoots were subcultured on medium 4, within 2 months there were 4 developing plantlets per callus in control treatment, and 6 in treatment with 1 μ M ionophore A23187 (Table 3).

Fig. 5 Postgerminative development within 2 months of culture in ionophore-free medium 4. **a** Tissues were previously cultured in ionophore-free media. **b** Tissues were previously cultured in 1 μ M ionophore A23187-containing medium 1 (calli induction) and medium 2 (embryo formation), and ionophore-free medium 3 (embryo germination)

CnSERK expression

The expression of *CnSERK* gen was evaluated during the formation of embryogenic callus. The relative expression (Fig. 6) in the control treatment increased from 0.34 units at 15 days to 2.18 units at 45 days and 6.93 units at 60 days. Then it decreased afterwards, remaining below 1 unit for the rest of the 90-day culture period. In the case of the treatment with 1 μ M ionophore A23187 there was an increase of relative expression from 0.74 units at 15 days to 2.26 at 45 days, decreasing afterwards to 0.41 at day 60, increasing slightly to 1.04 at day 75. Then at the end of the 90-day culture period there was a large increase to 8.28 units.

Discussion

The increasing success of the coconut industry worldwide is unfortunately threaten by the decreasing coconut production due to old age of plants and diseases, therefore, there is a pressing need for replanting that accounts for at least one billion plants, 50% of those currently cultivated in the world. This cannot be accomplished by traditional seed propagation. An alternative to achieved this is by in vitro somatic embryogenesis, in a process that involves multiplication of embryogenic callus as proposed by Pérez-Núñez et al. (2006). Any changes that increase the production of callus or the embryogenic structures in the callus (that are used as explants in this process) will have an important impact on the outcome.

The induction of in vitro coconut somatic embryogenesis requires the presence of the synthetic auxin 2,4-D in the culture medium (Chan et al. 1998; Pérez-Núñez et al. 2006), as

Fig. 6 Relative expression profiles of *CnSERK* during embryogenic callus development from of *Cocos nucifera* embryogenic structure explants with and without 1 μ M ionophore A23187 treatment

it is the case for several other plant species (Altamura et al. 2016). There is evidence supporting that auxin action in different processes is mediated by intracellular calcium ions and that calcium acts as a second messenger (Du and Poo-vaiah 2005; Quint and Gray 2006). In the case of the induction of in vitro somatic embryogenesis, it has been reported that it is highly dependent on Ca^{2+} availability (Anil and Rao 2000; Kiselev et al. 2008). This could be related to different levels of cellular organization. For instance, polar auxin transport, a fundamental condition for somatic embryogenesis induction, has been reported to be highly dependent on Ca^{2+} availability (Vanneste and Friml 2013), and (as mentioned in the introduction) that the induction of somatic embryogenesis is mediated by the regulation of gene expression by the complex calcium–calmodulin (Mahalakshmi et al. 2007). Interestingly, it has been shown that a calcium ionophore (A23187) promotes somatic embryogenesis in wheat and coffee (Mahalakshmi et al. 2007; Ramakrishna et al. 2011), these studies stress the relevance of calcium and display ionophore A23187 as a tool to promote somatic embryogenesis in other plant species. Therefore, in the present study we tested the effect of ionophore A23187 and calcium whether on the induction of somatic embryogenesis in embryogenic structure explants (cultured in 2,4-D containing media).

In a first trial, explants were exposed to calcium (0, 0.5, 1, 3 or 4 mM) combined with an application of ionophore A23187 at a concentration range (0, 50, 100, 200 or 400 μ M) reported as successful in increasing the frequency of somatic embryogenesis in *Coffea canephora* (Ramakrishna et al. 2011). In the control treatment (without calcium and ionophore A23187) the percentage of explants forming embryogenic callus was 60%, but in the other treatments it was three times lower, including those with calcium but no ionophore. In those explants forming embryogenic callus, with or without treatment with calcium and ionophore A23187, the developmental process was the same. By day 30 a callus had developed that was forming earlike white structures (referred to as translucent structures by Sandoval-Cancino et al. 2016), increasing in number by day 60 when pearly white round structures started forming on the translucent structures. These pearly white structures (referred to as embryogenic structures by Sandoval-Cancino et al. 2016) were more abundant by day 90 and most of them became elongated. The callus developed at this stage was defined as embryogenic callus according to Sandoval-Cancino et al. (2016). The embryogenic calli formed in the different treatments were identical to those in the control treatment.

According to results discussed above, for the second trial calcium was not included the concentrations of ionophore A23187 tested were lower (1, 5 and 10 μ M) after Mahalakshmi et al. (2007). In this trial, an increased percentage of explants forming embryogenic callus was obtained

with μM ionophore A23187, but the difference was not significant. Therefore, in the third trial, only 1 μM ionophore A23187 was tested. This time results showed a 1.3 fold increased proportion of explants forming embryogenic calli, a 2.8-fold increased number of embryogenic structures per embryogenic callus and a 1.5-fold increased number of somatic embryos per embryogenic callus. All differences were significant. Furthermore, when calli with somatic embryos obtained with 1 μM ionophore A23187 were subsequently transferred to media to induce germination and further development, the number of developing plantlets per callus was 6.6 or 1.5 times larger than that obtained with the control treatment. Comparative results in cultures (suspension) of other plant species have shown that treatment with ionophore A23187 increases somatic embryo formation 1.7 times in wheat (10 μM , Mahalakshmi et al. 2007), 3.2 in carrot (2.5 μM , Takeda et al. 2003) and 17 in coffee (100 μM , Ramakrishna et al. 2011).

An important observation is that the embryogenic structures formed in the calli in the first trial (control treatment) were used as explants for the second trial and formed embryogenic callus. Similarly, the embryogenic structures formed in the second trial (with control and 1 μM ionophore A23187 treatments) were used as explants for the third trial (correspondingly for control and 1 μM ionophore A23187 treatments), again they formed embryogenic callus. Therefore, the use of ionophore A23187 does not preclude the capacity of embryogenic structures to form embryogenic callus. The increased formation of embryogenic structures per callus can have a cumulative effect when used as explants in several cycles of callus multiplication as proposed by Pérez-Núñez et al. (2006). For instance if we start with 100 embryogenic structures as explants and produce embryogenic calli from them, isolate the resulting embryogenic structures and repeat the operation twice, when the procedure does not include treatment with ionophore A23187, we would potentially obtain over 540 embryogenic calli, and from them, over 2,000 plantlets. When this is carried out with ionophore A23187 treatment, the yield would potentially be over 4000 embryogenic calli and over 25,000 plantlets, ten times greater than without ionophore. It will be very important to test this to determine if this potential could be achieved.

The *SERK* gene expression has been associated in different species with the establishment of somatic embryogenesis in tissues in culture (Schmidt et al. 1997; Somleva et al. 2000; Hecht et al. 2001). In the case of coconut, a previous study on the *CnSERK* expression during embryogenic callus formation showed that it increases at day 30 of culture and again at day 90 and expression is located in the periphery of the embryogenic structures, a region that histological analysis showed the occurrence of meristematic

centers located under the protoderm (Pérez-Núñez et al. 2009). In the present study, histological analysis of embryogenic calli also showed the occurrence of meristematic cells accumulating under the protoderm. Similarly a comparative analysis of the expression of *CnSERK* was carried out during the development of embryogenic callus from explants cultured with or without treatment with ionophore A23187. In both cases there was an increase of the level of expression from the beginning to the middle of the culture period and then it decreased. But there were differences; expression with treatment was twofold greater than the control during the first 30 days, then by day 60 it was the opposite about 16-fold smaller. At the end of the 90 days culture period the level of expression became over 30-fold greater with the treatment. These results may suggest that in the case of the treatment the larger *CnSERK* expression within the first 30 days may be associated with a larger number of embryogenic structures forming on the calli and the greater expression at the end could be associated with the increased formation of somatic embryos. The pattern of the expression without treatment is different to that reported by Pérez-Núñez et al. (2006), peaking at 30 days and then again at 90 days, but this might be related to the use of different types of explants, plumule explants in that case and embryogenic structure explants in the present case. Important differences between plumule explants and embryogenic structure explants have been observed in their response to cytokinins, being inhibitory for the induction of embryogenic callus formation with plumule explants (CICY, unpublished) and no effect with embryogenic structure explants (Montero-Cortés et al. 2011a, b).

In conclusion, the present study presents evidence that treating embryogenic structure explants with ionophore A23187 promotes somatic embryogenesis, and although no increase in the percentage of explants forming embryogenic callus was obtained, there were increases in the formation of embryogenic structures per callus, of somatic embryos per callus and of developing plantlets per callus, and also that this effect might be associated with changes on the gene expression, as in the case of *CnSERK*. The quantitative changes produced by the ionophore A23187 treatment could be of great practical importance. If this treatment is coupled with a process with several steps of embryogenic callus multiplication, it could potentially result in a potentially greater increase in the efficiency for somatic embryo and plantlet formation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in publication of this paper.

Research involving human and animal participant This article does not contain any studies with human subjects or animal performed by any of the authors.

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