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ORIGINAL PAPER

Morphological and polyamine content changes in embryogenic and non-embryogenic callus of sugarcane

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Abstract Differences in competence acquisition and subsequent embryo maturation in embryogenic and non-embryogenic callus of sugarcane var. SP79-1011 were evaluated using histomorphological analysis, growth curves, numbers of somatic embryos, and polyamine contents. Embryogenic callus was formed by cells with embryogenic characteristics such as a rounded shape, prominent nuclei, a high nucleus: cytoplasm ratio, small vacuoles and organized globular structures. However, non-embryogenic callus presented dispersed, elongated and vacuolated cells with a low nucleus: cytoplasm ratio; these characteristics did not allow for the development of somatic embryos even upon exposure to a maturation stimulus. These results suggest that non-embryogenic callus does not acquire embryogenic competence during induction and that maturation treatment is not sufficient to promote somatic embryo differentiation. The use of activated charcoal (AC; 1.5 g L^{-1}) resulted in a higher somatic embryo maturation rate in embryogenic callus but did not yield success in non-embryogenic callus. Embryogenic callus incubated with control (10 μM 2,4-dichlorophenoxyacetic acid) and maturation (1.5 g L^{-1} AC) treatments for 28 days showed similar patterns of total free polyamines; these results differed from the results observed with non-embryogenic callus, suggesting that embryogenic callus already exhibits a characteristic pattern of endogenous polyamine levels. At 28 days of culture with maturation treatment, embryogenic callus exhibited significantly higher levels of free Spm than embryogenic callus incubated with control treatment and non-embryogenic callus incubated with both treatments. This result suggests that Spm could be important for the acquisition of embryogenic competence and somatic embryo maturation in sugarcane var. SP79-1011.

Keywords Embryogenic competence · Polyamines · Sugarcane · Somatic embryogenesis

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Introduction

Sugarcane is grown on more than 23.98 million hectares of land worldwide, generating 1.71 billion tons of harvested cane in 2010 (FAO 2012). As a major source of the world's sugar, sugarcane has been grown on an industrial scale in more than 90 countries worldwide for approximately 100 years (Arruda 2011). In recent decades, the ability of this crop to accumulate large quantities of biomass and sucrose has highlighted its potential role as an important biofuel feedstock that may help to alleviate the global demand for sustainable energy (Arruda 2011; Taparia et al. 2012). Most currently cultivated sugarcane varieties are

derived from the interspecific hybridization of *Saccharum officinarum* and *Saccharum spontaneum*, which was accomplished in the late 1800s (Snyman et al. 2011). Modern day hybrids have a limited gene pool and complex polyploid, aneuploid genomes (Butterfield et al. 2001). Consequently, breeding for superior traits and increased crop yield is slow and difficult, and a period of 10–14 years is generally required before a commercially viable line can be released (Butterfield and Thomas 1996). Limited breeding success has been achieved because of the complexity of the sugarcane genome (Arruda 2012).

Modern sugarcane breeding programs involve biotechnological approaches such as marker-assisted breeding, DNA mapping and genetic transformation (reviewed by Lakshmanan et al. 2005). Genetic transformation, which can introduce genes that encode desirable traits into elite sugarcane cultivars, provides an alternative method for the improvement of pest and disease resistance as well as yield. This method has previously been used to effectively increase sugar content and improve crop performance (Arruda 2012).

Improvement of sugarcane will require the development of an optimized tissue culture and plant regeneration system for the production of genetically modified sugarcane. In vitro techniques for the mass propagation of sugarcane plantlets via organogenic pathways are well established; however, the production of embryogenic callus is critical to many ongoing efforts aimed at improving sugarcane germplasms through genetic transformation (Lakshmanan et al. 2005). Somatic embryogenesis via highly nodular embryogenic callus is preferred for the regeneration of sugarcane plants that have been transformed using either particle bombardment or *Agrobacterium*-mediated transformation (Arruda 2012).

Somatic embryogenesis is analogous to zygotic embryogenesis, in which a single cell or a small number of somatic cells are the precursors to the formation of a somatic embryo (Tautorus et al. 1991). The induction of somatic embryogenesis is only possible using totipotent plant cells, which may be genetically reprogrammed to differentiate into any cell type, thereby leading to the creation of an entire embryo from a group of cells or even a single somatic cell (Verdeil et al. 2007). This reprogramming is only possible if the cells are both competent and able to receive the appropriate inductor stimuli.

During the induction of sugarcane somatic embryogenesis, both embryogenic and non-embryogenic callus can be obtained. These types of callus differ in their growth and can be visually differentiated by their morphology; however, little is known about the biochemical and molecular events that take place when somatic cells become competent to produce somatic embryos (Nieves et al. 2003).

The ability to produce embryogenic callus during sugarcane somatic embryogenesis has been described as a genotype-dependent response (Cidade et al. 2006; Lakshmanan 2006; Snyman et al. 2011), and protocols must be adapted for each variety used in a given study. Low embryogenic competence and somaclonal variation are among the main factors that limit the development of efficient genetic transformation protocols in sugarcane (Desai et al. 2004; Lakshmanan et al. 2005). Additionally, the use of growth regulators to modulate embryogenic competence and allow the progression and maturation of somatic embryos is not completely understood (Jimenez 2005).

Improved knowledge of the factors that affect the acquisition of competence in embryogenic and non-embryogenic callus would provide a useful experimental system for studying the mechanisms of morphogenesis in vitro and could improve the success of genetic transformation efforts in sugarcane. Several compounds have been implicated in the acquisition of somatic embryogenesis competence and morphology development including polyamines (PAs), which are ubiquitous compounds that mediate fundamental aspects of growth and development as well as abiotic and biotic stress responses in plants (Kuznetsov et al. 2007; Baron and Stasolla 2008; Wu et al. 2009; Alcázar et al. 2010). PAs are low molecular weight, aliphatic, polycationic compounds that carry positively charged nitrogen atoms, a property that facilitates electrostatic interactions with macromolecules such as DNA, RNA, phospholipids, cell wall components and proteins (Wallace et al. 2003; Baron and Stasolla 2008). Although their precise modes of action have yet to be understood, extensive studies support a role for PAs in the modulation of various physiological processes that include the cell growth and differentiation observed during somatic and zygotic embryogenesis, which occurs in several plants (Minocha et al. 1999, 2004; Bertoldi et al. 2004; Silveira et al. 2006; Santa-Catarina et al. 2007; Steiner et al. 2007; El Meskaoui and Trembaly 2009; Wu et al. 2009) including sugarcane (Nieves et al. 2003, 2008). Further research on somatic embryogenesis in sugarcane, especially studies comparing embryogenic and non-embryogenic callus, is fundamentally required to understand the role of these compounds in the acquisition of competence and somatic embryo development.

In this sense, morphological and biochemical analyses could be employed to understand differences in the acquisition of competence and somatic embryo development between embryogenic and non-embryogenic sugarcane callus. Such an understanding would aid in the optimization of protocols for plantlet regeneration in genetic transformation assays. The aim of this study was to determine the effects of maturation treatments on morphology, growth and

endogenous PA metabolism and to determine the relationship between maturation treatment and somatic embryogenesis competence and evolution in sugarcane var. SP79-1011.

Materials and methods

Plant material

Embryogenic and non-embryogenic callus induced from sugarcane (*Saccharum* spp.) variety SP79-1011 were used in the maturation experiment. Briefly, immature nodal cane segments with axillary buds were collected from field-grown plants and planted in plastic trays containing the commercial substrate PlantMax (DDL Agroindustria, Paulínia, Sao Paulo, Brazil). The trays were maintained under ambient conditions for approximately 3 months. During this time, new plants originated from sprouting axillary buds and grew approximately 45 cm high. After removing the mature leaves, the shoot apical meristem were used as explants for callus induction. The apical meristem were surface sterilized in 70 % ethanol for 1 min, immersed in 50 % commercial bleach (hypochlorite from 1 to 1.25 %) for 30 min and subsequently washed 3 times with sterilized water. The apical meristem were transversely sectioned into 2-mm-thick slices, which were cultured in assay tubes (150 × 25 mm) containing 20 mL of MS medium (Murashige and Skoog 1962) (Phytotechnology Lab, Overland Park, KS, USA), supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel® (Sigma-Aldrich, St. Louis, MO, USA) and 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich). The tubes were maintained in the dark at 25 ± 1 °C for approximately 45 days.

The induced callus was transferred to and maintained in Petri dishes (90 × 15 mm) containing 20 mL of the same culture medium. The callus was subcultured in maintenance medium, which was renewed every 20–25 days. These cultures were maintained in the dark at 25 ± 2 °C. During the maintenance period, the callus was isolated and separated into 2 types—embryogenic and non-embryogenic—according to Lakshmanan (2006) using a light stereomicroscope. Smooth and compact callus was classified as embryogenic, and friable or soft callus was classified as non-embryogenic.

Maturation of embryogenic and non-embryogenic callus

Three colonies containing 200 mg of fresh mass (FM) obtained from embryogenic to non-embryogenic callus were inoculated in a Petri dish (90 × 15 mm) containing 20 mL of MS culture medium supplemented with sucrose (20 g L^{-1}), Phytagel® (2 g L^{-1}) and various concentrations of activated charcoal (AC; 0, 1.5 and 3.0 g L^{-1}) (Sigma-Aldrich) in the presence or absence of 2,4-D (10 or 0 μM, respectively). The pH of the culture medium was adjusted to 5.8 before Phytagel® was added. The culture medium was sterilized by autoclaving at 121 °C for 15 min. At least 15 dishes were used for each treatment for both types of callus. After inoculation, the cultures were maintained in a growth chamber at 25 ± 1 °C and grown in the dark for the first 7 days. Thereafter, photoperiods of 16 h of light (60 μmol m^2 s^{-1}) were employed for 21 days.

Samples were collected at 0, 7, 14, 21 and 28 days of incubation. For both types of callus, 3 separate dishes per treatment were examined at each sampling time. Growth was determined based on increases in callus FM and the number of somatic embryos produced. Sections of this callus (one per colony) were removed for histomorphological analyses, and the remaining callus was stored at −20 °C for later quantitation of free PAs.

Growth analyses and the number of somatic embryos

Growth under the different treatments was measured based on increases in FM and the number of somatic embryos by examining 3 Petri dishes per treatment and selecting 2 colonies per dish (the initial FM was 200 mg per colony). Growth measurements were performed at the beginning of the experiment (day 0) and after 7, 14, 21 and 28 days of culture; for each treatment, the FM (mg) at each time point was compared with the initial FM of the colonies. Somatic embryo maturation was examined after 28 days of culture using a light stereomicroscope, and the presence or absence of somatic embryos was examined in each colony.

Histomorphological analyses

Histomorphological analyses were performed on embryogenic and non-embryogenic callus incubated in a medium that did not allow somatic embryo formation (MS medium supplemented with 10 μM 2,4-D) and medium that was optimal for somatic embryo formation (MS medium supplemented with 1.5 g L^{-1} AC), hereinafter referred to as the control and maturation treatments, respectively. To study the morphology of the embryogenic and non-embryogenic callus, samples were collected at 0, 7, 14, 21 and 28 days of incubation for histological analysis using light and scanning electron microscopy.

For both types of microscopy, samples were fixed in a solution containing 2.5 % glutaraldehyde (Merck, Darmstadt, Germany) and 4 % *p*-formaldehyde (Merck) in 100 mM sodium cacodylate (pH 7.2) (Merck) for 24 h at room temperature. The samples were subsequently washed with

100 mM sodium cacodylate solution (pH 7.2) for 45 min at room temperature.

To prepare specimens for light microscopy, the samples were dehydrated twice in an ethanol series of 30, 50, 70, 90 and 100 % for 1 h each. After dehydration, the samples were infiltrated with HistoResin (Leica, Wetzlar, Germany) and 100 % ethanol (1:1, v/v) for 12 h and subsequently with 100 % HistoResin for 24 h before embedding in HistoResin. Sections (approximately 5 μm thick) were cut and stained with a 1 % solution of toluidine blue (Sigma-Aldrich). The samples were observed under an Axioplan light microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam MRC5 digital camera (Carl Zeiss), and the images were analyzed using AxioVisionLE version 4.8 software (Carl Zeiss).

To prepare specimens for scanning electron microscopy, the samples were post-fixed in 1 % osmium tetroxide (Sigma-Aldrich), which was buffered in 100 mM sodium cacodylate solution (pH 7.2) for 1 h. The fixed tissues were dehydrated twice using an ethanol (Merck) series of 30, 50, 70, 90 and 100 % for 1 h each. Finally, tissue samples were placed into absolute ethanol at room temperature, critical point dried with CO_2 using a CPD-030 BAL-TEC (Vaduz, Liechtenstein,) and sputter coated with gold. Images were obtained using a DSM 962 scanning electronic microscope (Carl Zeiss).

Analyses of free PAs

PAs were analyzed in embryogenic and non-embryogenic callus incubated in the control and maturation treatments as described by Silveira et al. (2004). Three samples (300 mg of FM each) were pulverized in 1.6 mL of 5 % (v/v) perchloric acid (Merck). After 1 h of incubation at 4 °C, the samples were centrifuged at 20,000×*g* for 20 min at 4 °C. Free PAs were determined directly from the supernatant by derivation with dansyl chloride (Merck) and subsequent high performance liquid chromatography with a 5-μm reverse-phase column (Shimadzu Shin-pack CLC ODS). The gradient was developed by mixing increasing proportions of absolute acetonitrile (Merck) with 10 % acetonitrile in water (pH 3.5). The gradient of absolute acetonitrile was programmed as follows: 65 % over the first 10 min, from 65 to 100 % between 10 and 13 min and 100 % between 13 and 21 min. The flow rate was 1 mL min^{-1} at 40 °C. The concentrations of total PAs, putrescine (Put) (Sigma-Aldrich), spermidine (Spd) (Sigma-Aldrich) and spermine (Spm) (Sigma-Aldrich) were determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with PA standards.

Statistical analysis

The maturation experiment was performed using a completely randomized design. The data were analyzed by analysis of variance (ANOVA) ($P < 0.05$) followed by the Student–Newman–Keuls (SNK) test (Sokal and Rohlf 1995) using the SAEG® Program v. 9.1 (Fundação Arthur Bernardes, Viçosa, Brazil). ANOVA was performed to determine variation in the number of somatic embryos within embryogenic callus under all treatments at 28 days of culture. ANOVAs of the growth curve and PA data were performed considering the combination of treatment and callus type as the independent variable (control-embryogenic, control-non-embryogenic, maturation-embryogenic and maturation-non-embryogenic) at each time point.

Results

The effects of maturation treatments on somatic embryo formation and the morphology of embryogenic and non-embryogenic callus

The potential for somatic embryo development in response to the various maturation treatments was analyzed by counting the number of somatic embryos produced per colony (the initial FM was 200 mg each) during 28 days of incubation (Fig. 1). The morphology was also monitored during this time (Fig. 2).

The formation of somatic embryos was not observed in non-embryogenic callus during 28 days of incubation regardless of treatment (Fig. 2) and despite the presence of a maturation stimulus. Non-embryogenic callus morphology during the incubation was similar among the different treatments; this callus was more soft and friable than

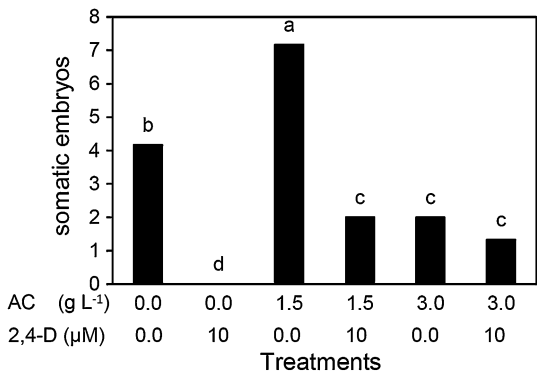

Fig. 1 The number of somatic embryos/colonies in the embryogenic callus of sugarcane var. SP79-1011 after 28 days of incubation with various maturation treatments containing AC (0, 1.5 and 3.0 g L^{-1}) with or without 2,4-D (0 or 10 μM). Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test (n = 6; coefficient of variation = 35.3 %)

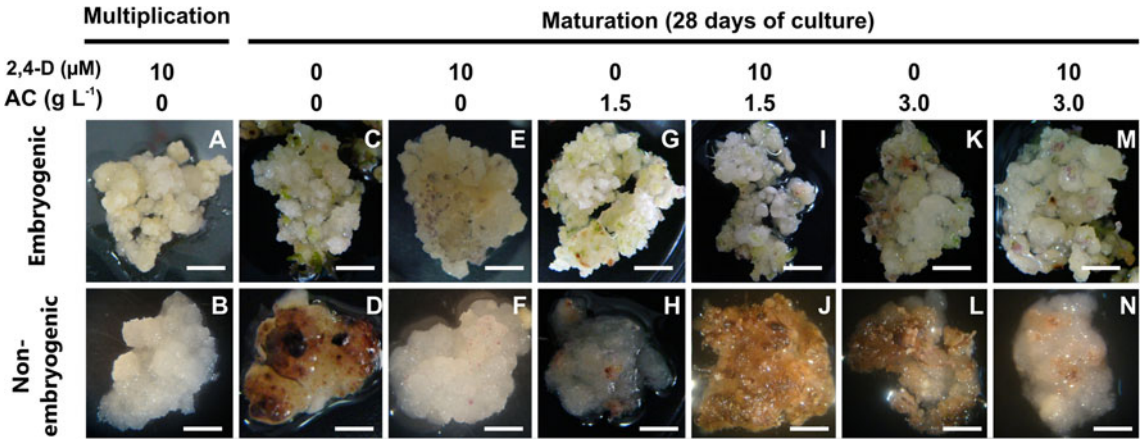

Fig. 2 Morphological aspects of embryogenic and non-embryogenic callus of sugarcane var. SP 79-1011 on multiplication medium and after 28 days of culture with different maturation treatments with and without AC (0, 1.5 or 3.0 g L^{-1}) and 2,4-D (0 or 10 μM). In **a**, **c**, **e**, **g**, **i**, **k** and **m** (embryonic), the *bars* represent 3.5 mm; in **b**, **d**, **f**, **h**, **j**, **l** and **n** (non-embryonic), the *bars* represent 2.9 mm

embryogenic callus (Fig. 2) and developed a more brownish color, most likely resulting from exposure to light (Fig. 2).

In contrast, embryogenic callus incubated with 1.5 g L^{-1} AC exhibited increased production of somatic embryos at 28 days of incubation, and this result was significantly different from the level of production observed when 3.0 g L^{-1} AC and other treatments were applied (Fig. 1). Thus, the treatment containing 1.5 g L^{-1} AC was considered to be the best treatment for embryogenic callus maturation in sugarcane var. SP79-1011 because smooth and compact callus with green points was observed at 28 days of incubation (Fig. 2). Embryogenic callus exhibited less oxidation than non-embryogenic callus (Fig. 2).

Supplementation of the culture medium with 2,4-D (with or without AC) reduced or inhibited somatic embryo formation during the maturation phase. Combining 2,4-D with 1.5 g L^{-1} AC resulted in an approximate 70 % reduction in somatic embryo formation compared with the treatment containing 1.5 g L^{-1} AC without 2,4-D (Fig. 1). The addition of 3.0 g L^{-1} AC with or without 2,4-D (10 μM) resulted in a reduced but similar level of somatic embryo development. Somatic embryo formation could be visualized at 28 days of incubation (Fig. 1) and was generally indicated by the presence of green points in the callus (Fig. 2). The somatic embryos obtained were capable of germination in culture flasks containing 20 mL of MS culture medium supplemented with sucrose (20 g L^{-1}) and Phytagel® (2 g L^{-1}) (Supplementary Figure 1).

Based on the results presented in Figs. 1 and 2, the histomorphological analysis, PA measurements and growth assays were limited to embryogenic and non-embryogenic callus incubated in the treatments MS medium supplemented with 10 μM 2,4-D (control) or MS medium supplemented with 1.5 g L^{-1} AC (maturation).

The effects of maturation treatments on the growth of embryogenic and non-embryogenic callus

The effects of the applied treatments on the growth of embryogenic and non-embryogenic callus were analyzed in terms of increased FM. During these treatments, no significant differences in FM were observed between the embryogenic and non-embryogenic callus, although a larger FM increase was exhibited by the embryogenic callus at 28 days of incubation (Fig. 3).

The effect of maturation on the histomorphological aspects of embryogenic and non-embryogenic callus

During the multiplication phase of the induced callus, both embryogenic and non-embryogenic callus was observed, and both types of callus retained their respective morphological aspects (Supplementary Figure 2). The callus was defined as embryogenic or non-embryogenic during the subculture, and each type was separated into fresh medium.

We characterized the embryogenic and non-embryogenic callus of sugarcane var. SP79-1011 before and during a maturation experiment. These callus types presented morphologies similar to those described by Lakshmanan (2006); embryogenic callus was smooth and compact (Fig. 4a), whereas non-embryogenic callus was friable or soft and translucent (Fig. 4b). Both types of callus were monitored by light and scanning electron microscopy.

Embryogenic callus (Fig. 4a) exhibited a more organized structure and morphology, even when cultured under multiplication conditions (MS medium supplemented with 10 μM 2,4-D) before beginning the maturation experiment (time 0). Structures were observed that were slightly rounded, more developed toward the outside and generally easily distinguished from other portions of the callus

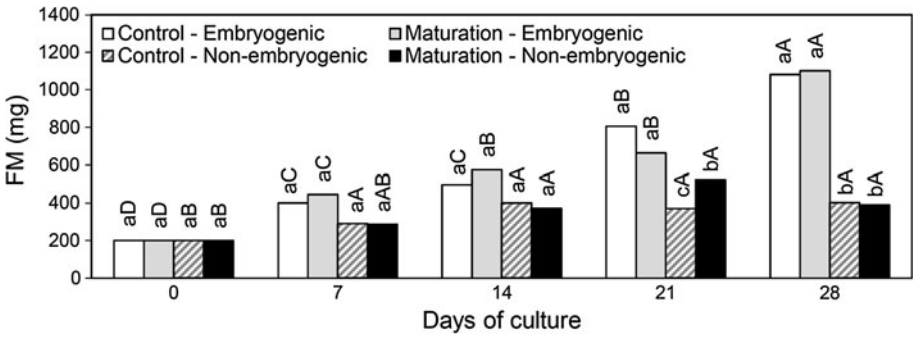

Fig. 3 Growth of embryogenic and non-embryogenic callus of sugarcane var. SP79-1011 during 28 days of incubation with control (10 μM 2,4-D) and maturation (1.5 g L^{-1} AC) treatments. *Lowercase letters* denote significant differences between treatments for each day of culture. *Capital letters* denote significant differences among days of culture with the same treatment. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test (n = 6; coefficient of variation = 10.5 %)

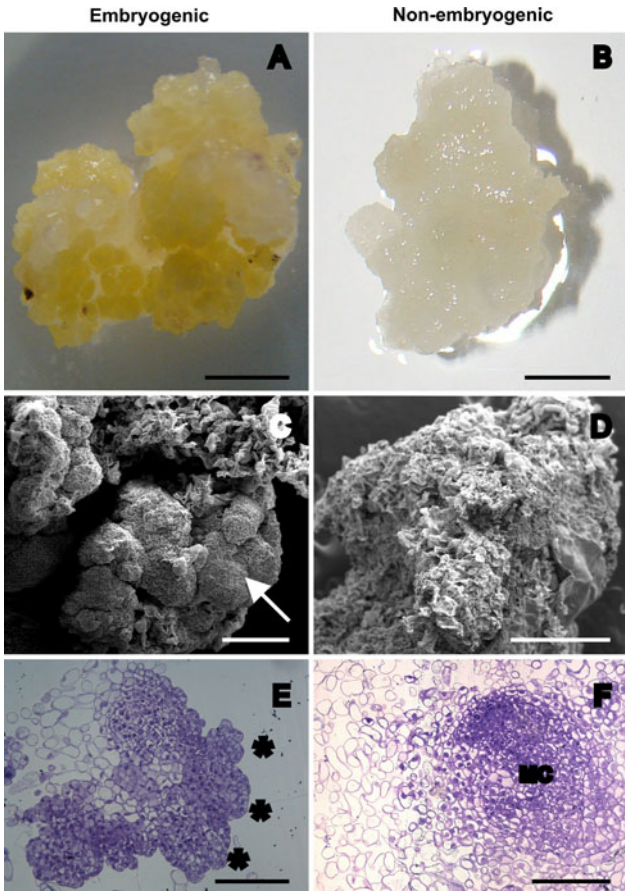

Fig. 4 Histomorphological aspects of embryogenic (**a**, **c** and **e**) and non-embryogenic (**b**, **d** and **f**) callus of sugarcane var. SP79-1011 on MS medium supplied with 10 μM of 2,4-D before culture with maturation treatments. *Asterisks* (*) indicate organized structures on the surface of embryogenic callus. MC = a meristematic center in a non-embryogenic callus, showing non-organized and dispersed cells. *Bars*: **a** 3.5 mm; **b** 3.0 mm; **c** 200 μm; **d** 500 μm; **e** and **f** 200 μm

(Fig. 4c, arrow). As observed using light microscopy, the embryogenic callus was formed by small and isodiametric clumps of cells containing prominent nuclei and dense cytoplasm; these clumps were round structures bounded by a layer of organized cells (Fig. 4e, asterisks).

In contrast, non-embryogenic callus was friable or soft and translucent (Fig. 4b, d) and comprised large, highly vacuolated, elongated cells with lower nucleus: cytoplasm ratios than those of cells within the embryogenic callus (Fig. 4e). Histological analyses showed that some cells in the internal portions of this callus exhibited meristematic characteristics, and regions containing these cells were designated as meristematic centers (Fig. 4f). However, these cells were dispersed and did not form organized structures like those observed in the embryogenic callus.

Histomorphological analyses were also performed on embryogenic and non-embryogenic sugarcane callus during the 28 days of incubation with the control and maturation treatments. Using scanning electron microscopy and histological analyses, we observed that embryogenic callus incubated in the maturation treatment contained somatic embryos that were more globular than those observed in the control treatment (Fig. 5a–d). These results suggest that the addition of 1.5 g L^{-1} AC is required for somatic embryo organization and progression in the embryogenic callus of sugarcane var. SP79-1011 (Fig. 5c, d, asterisks).

In contrast, non-embryogenic callus (Fig. 5e–h) incubated in both treatments exhibited a morphology similar to that observed before incubation (Fig. 5f, h). Scanning electron microscopy revealed that the structure of the callus surface was similar in both treatments. Histological analyses revealed the presence of meristematic centers in the internal portion of the callus (Fig. 5f–h); however, these cells did not allow somatic embryos to develop despite incubation in maturation treatments (Fig. 5h), and the cells maintained a similar structure to that observed in the control treatment (Fig. 5f).

The morphological evolution of somatic embryos was monitored at various developmental stages by histological analysis of sugarcane var. SP79-1011 embryogenic callus.

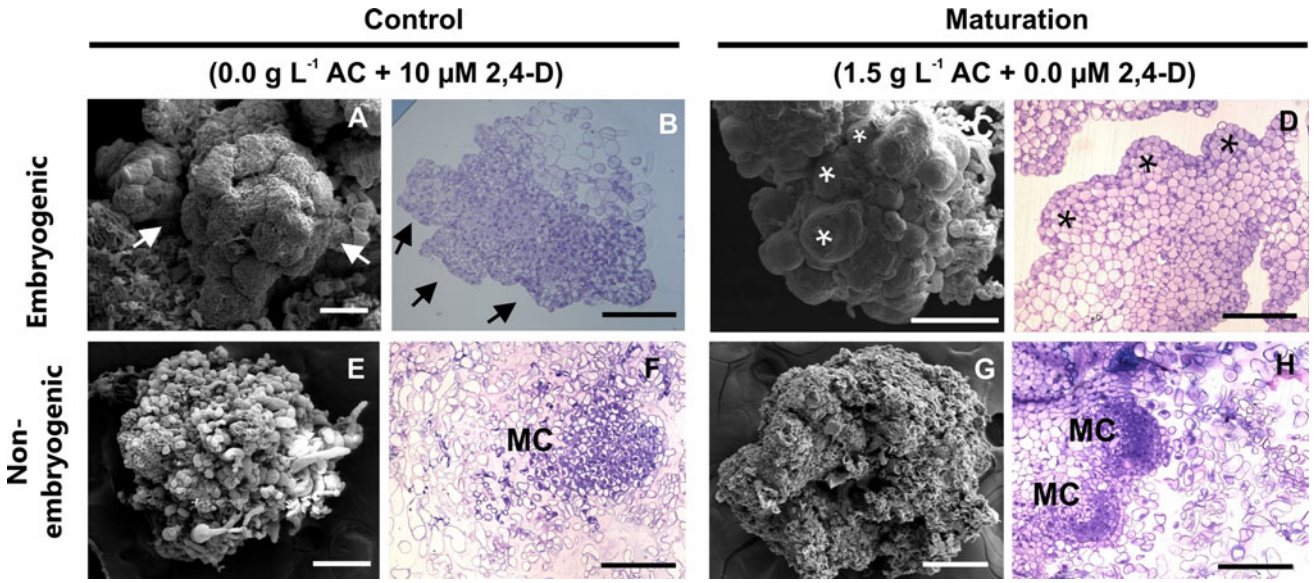

Fig. 5 Histomorphological aspects of embryogenic (**a**–**d**) and non-embryogenic (**e**–**h**) sugarcane callus var. SP79-1011 after 28 days of incubation with control and maturation treatments. *Arrows* indicate that the embryogenic callus exhibits protuberances on the surface. *Asterisks* (*) indicate more organized cells that eventually form globular structures on the surface of embryogenic callus. **a**, **c**, **e** and **g** scanning electron micrographs. **b**, **d**, **f** and **h** images of histological staining. *MC* meristematic center. *Bars* = 200 µm

During 28 days of incubation in the maturation treatment, somatic embryos were observed and characterized at various developmental stages (Fig. 6).

First, we observed the formation of pro-embryos from a group of embryogenic cells in the embryogenic callus (Fig. 6a); this formation was followed by the development of globular somatic embryos (Fig. 6b). The globular somatic embryos of monocots differ slightly from those of dicots, the former of which do not exhibit bilateral symmetry but have a more evident suspensor, as observed in

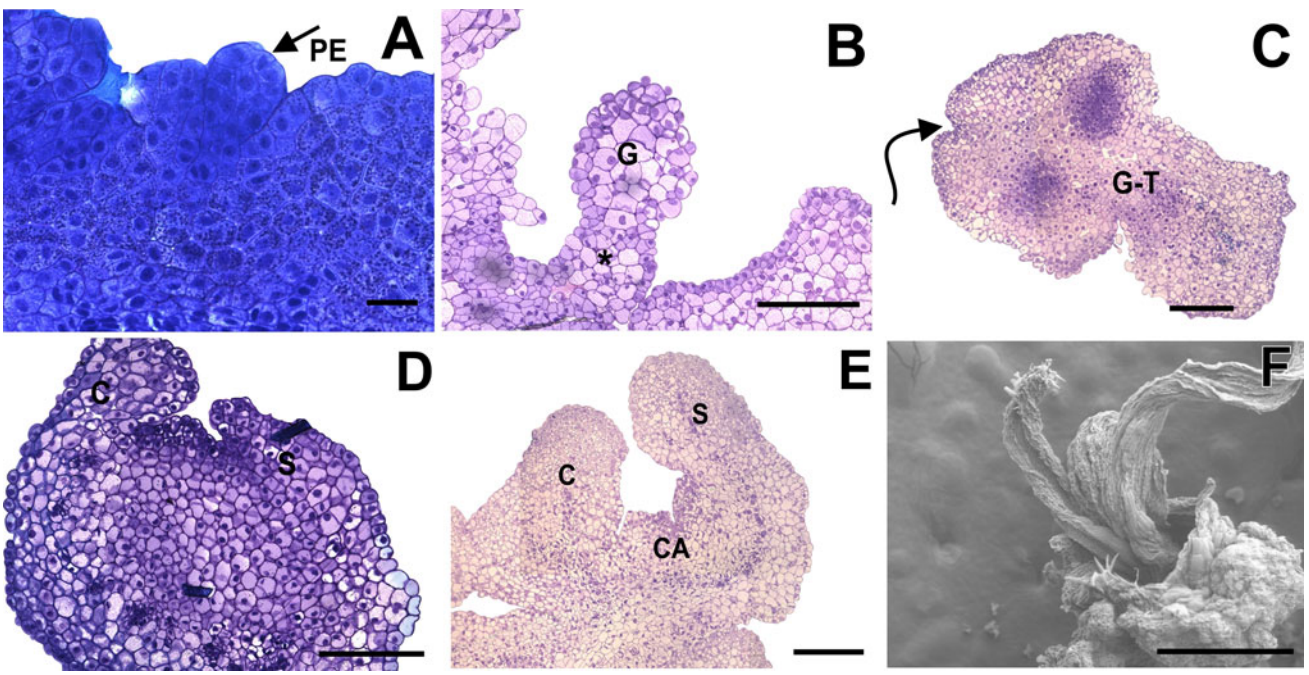

Fig. 6 Histomorphology during somatic embryo evolution in the embryogenic callus of sugarcane var. SP79-1011 showing the pro-embryo (*arrow*, PE) **a**, globular (G) (**b**), globular-torpedo (G-T) (**c**), torpedo (**d**) and cotyledonary-mature (**e**) stages of somatic embryo development and a plantlet (**f**) after somatic embryo germination. The *arrow* in **c** indicates the scutellum gap. *C* coleoptile, *S* scutellum, *CA* caulinar apex. The *asterisk* (*) in **b** indicates suspensors. *Bars* = 200 µm

the present work (Fig. 6b). Additionally, sugarcane embryogenesis does not include a cordiform stage, which occurs during the transition from the globular to the torpedo and cotyledonary stages. In this sequence, a gap is formed in the globular structure, which is observed during the globular-torpedo stage transition (Fig. 6c, arrow). This gap becomes larger and develops into the scutellum and coleoptile structures that are observed in the torpedo (Fig. 6d) and cotyledonary stages (Fig. 6e). Between the scutellum and the coleoptile, a region composed of meristematic cells exists from which the shoot apex meristem develops (Fig. 6e). The cotyledonary embryo will mature and develop into a plantlet after germination (Fig. 6f).

The effects of maturation treatment on the endogenous levels of free PAs in embryogenic and non-embryogenic callus

The effect of maturation treatment on the presence of endogenous free PAs in embryogenic and non-embryogenic sugarcane var. SP79-1011 callus was analyzed. These analyses were performed at 7-day intervals during the 28 days of incubation in both the control and maturation treatments.

The levels of total free PAs (Fig. 7), Put, Spd and Spm (Fig. 8) were affected by the type of callus used (embryogenic or non-embryogenic) and the treatment (control or maturation). In both types of callus exposed to both treatments, Put was generally the most abundant polyamine followed by Spd and Spm, respectively (Fig. 8).

Embryogenic callus (Fig. 7) in the control (containing 2,4-D) and maturation (containing AC) treatments presented a similar pattern during incubation; no statistically significant changes were observed until day 21, and a significant reduction in the total free PAs was noted at 28 days of incubation. Non-embryogenic callus incubated in the control treatment exhibited an increase in free PAs during the first 7 days, whereas no significant differences were observed for up to 14 days of incubation in maturation treatment (Fig. 7). In both treatments, non-embryogenic callus exhibited a significant reduction in total free PAs after 14 days of incubation (Fig. 7). The level of total free PAs appears to be modulated moreso by the type of callus than by the treatment applied. Once the callus has acquired (or not acquired) embryogenic competence, the level of total free PAs exhibits a similar trend during incubation; the unique exception occurs in the non-embryogenic callus at 28 days of culture.

Differences in Put, Spd and Spm levels were observed among embryogenic and non-embryogenic callus incubated with the control and maturation treatments (Fig. 8). Embryogenic callus inoculated with the control and maturation treatments exhibited similar patterns in the endogenous levels of free Put for up to 21 days of culture with no significant difference observed; however, a significant reduction in Put was noted at 28 days of incubation with the maturation treatment (Fig. 8a). In contrast, Put levels in non-embryogenic callus incubated for 7 days in the control treatment were higher than those in callus incubated in the maturation treatment, and subsequent decreases were noted in non-embryogenic callus incubated in the control treatment for 21 and 28 days (Fig. 8a). These results suggest that Put may not be required to induce changes in the development of somatic embryos in the embryogenic callus of sugarcane var. SP79-1011.

Spd levels followed a similar pattern in embryogenic callus that was incubated in both control and maturation treatments, exhibiting no significant changes for up to 21 days of culture with a significant reduction after this period in both treatments (Fig. 8b). The Spd levels differed significantly between embryogenic and non-embryogenic callus at the beginning of the incubation and at 7 and 21 days of culture in both treatments (Fig. 8b).

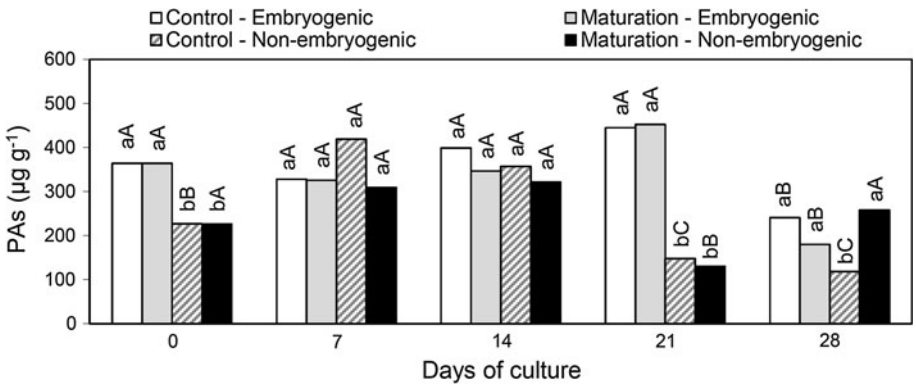

Fig. 7 Levels of total free PAs in embryogenic and non-embryogenic callus of sugarcane var. SP79-1011 during 28 days of incubation with control (with 10 µM 2,4-D) and maturation (with 1.5 g L^{-1} AC) treatments. *Lowercase letters* denote significant differences between treatments for each day of culture. *Capital letters* denote significant differences among days of culture with the same treatment. Means followed by *different letters* are significantly different ($P < 0.05$) according to the SNK test (n = 3; coefficient of variation = 4.2 %)

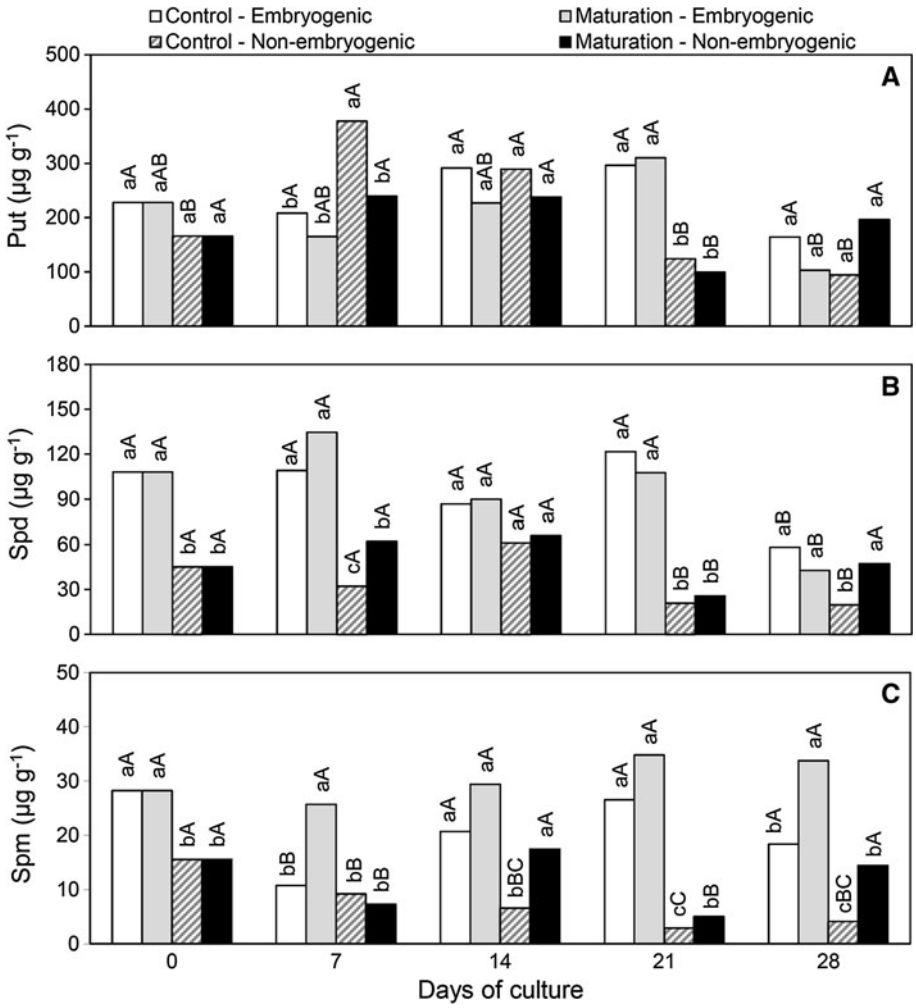

Fig. 8 Put (**a**), Spd (**b**) and Spm (**c**) levels in the embryogenic and non-embryogenic callus of sugarcane var. SP79-1011 during 28 days of incubation with control (with 10 µM 2,4-D) and maturation (with 1.5 g L^{-1} AC) treatments. *Lowercase letters* denote significant differences between treatments for each day of culture. *Capital letters* denote significant differences among days of culture with the same treatment. Means followed by *different letters* are significantly different ($P < 0.05$) according to the SNK test (n = 3; coefficient of variation for Put = 5.3 %; coefficient of variation for Spd = 5.2 %; coefficient of variation for Spm = 9.0 %)

In the present study, Spm levels were lower than those of Spd or Put (Fig. 8). At 28 days of culture in the maturation treatment, embryogenic callus exhibited significantly higher levels of free Spm when compared with embryogenic callus incubated in the control treatment and non-embryogenic callus incubated in both treatments (Fig. 8c). At inoculation, the endogenous level of Spm in non-embryogenic callus was significantly lower than that in embryogenic callus (Fig. 8c). These results suggest that Spm could be important for the acquisition of embryogenic competence and somatic embryo maturation in sugarcane var. SP79-1011.

Discussion

The initiation and maintenance of embryogenic callus is an important step for somatic embryo development and plantlet regeneration. Thus, methods for obtaining embryogenic and non-embryogenic callus contribute to a useful experimental system for studying the mechanism of morphogenesis in vitro, as described here for sugarcane var. SP79-1011.

Both genetic and physiological factors trigger in vitro embryogenesis in various types of plant somatic cells (Karami et al. 2009; Karami and Saidi 2010); these factors trigger the replacement of the existing pattern of gene expression in explant tissue with a new embryogenic gene expression program (Chugh and Khurana 2002; Zeng et al. 2007). This process is only possible if (1) the cells are competent and (2) they receive the appropriate inductor stimuli. Among the factors involved, plant growth regulators, especially auxins, are key components because their exogenous application recapitulates the embryogenic potential of mitotically quiescent somatic cells (Karami et al. 2009); the auxin 2,4-D is an important growth regulator that is most commonly used for induction in embryogenic cell and tissue culture systems (Fehér et al. 2003; Santa-Catarina et al. 2004).

Plant regeneration from sugarcane callus is a well-established process (reviewed by Lakshmanan 2006); however, few studies have attempted to understand the factors that lead to differentiation between embryogenic and non-embryogenic callus. Auxin (mainly 2,4-D) was reported to be

necessary for the induction of sugarcane embryogenic callus (Lakshmanan 2006). However, embryogenic callus induction was also observed in the absence of auxin (Verdeil et al. 2007). In our case, the addition of 2,4-D (10 μM) was essential for obtaining embryogenic callus by indirect somatic embryogenesis induction in sugarcane var. SP79-1011 (data not shown). Several studies have shown that 2,4-D is the main growth regulator used to induce embryogenic potential in sugarcane (Ho and Vasil 1983; Gallo-Meagher et al. 2000; Lima et al. 2001; Lakshmanan 2006). The effect of adding 2,4-D to the culture medium may be twofold in that 2,4-D acts as an auxin (either directly or by interfering with the metabolism of indoleacetic acid [IAA]) and as a stress promoter (Fehér et al. 2001, 2002). The physiological and molecular actions of 2,4-D that affect somatic embryogenesis have been associated with the regulation of endogenous IAA metabolism (Michalczuk et al. 1992; Sasaki et al. 1994; Pan et al. 2010), the control of DNA methylation (LoSchiavo et al. 1991; Pan et al. 2010; Karami et al. 2009; Karami and Saidi 2010) and induction-specific proteins (Hadrami and D'Auzac 1992; Pan et al. 2010) as well as gene expression regulation (Fehér et al. 2003; Santa-Catarina et al. 2004; Karami et al. 2009; Steiner et al. 2012).

The callus induced in sugarcane var. SP79-1011 contained some cells that were embryogenesis competent and others that were not; these cells were separated into embryogenic and non-embryogenic callus (Fig. 4) during in vitro maintenance. These 2 types of callus are morphologically distinct (Figs. 2, 4). These results suggest that 2,4-D-induced embryogenic callus already contains certain groups of cells that exhibit embryogenic characteristics; unlike the cells in non-embryogenic callus, these cells were able to develop somatic embryos if an adequate stimulus was provided. Several studies have indicated that auxin signaling and stress may be key events in the genetic reprogramming of somatic cells during early embryogenesis, which is necessary for the dedifferentiation required to acquire embryogenic status (Quiroz-Figueroa et al. 2006; Patade et al. 2008; Zavattieri et al. 2010; Karami et al. 2009; Karami and Saidi 2010; Pan et al. 2010). Although several studies have been performed, the relationship between auxin and stress conditions and the acquisition of somatic embryogenesis competence requires further study. In this sense, knowledge regarding cellular morphology as well as biochemical and molecular changes could be important for understanding the acquisition of embryogenic competence in the callus of sugarcane var. SP79-1011.

After the appropriate stimulus with auxin, the induced competent cells begin to develop into somatic embryos during the maturation process, which usually occurs together with the removal of the auxin and the addition of maturation promoters. Consequently, desiccation occurs, and reserves of carbohydrates, proteins and lipids are accumulated, resulting in somatic embryo germination (Jimenez 2001; Steiner et al. 2008; Zimmerman 1993; Ceccarelli et al. 2000). In the embryogenic callus of sugarcane var. SP79-1011, the removal of 2,4-D and the addition of AC (1.5 g L^{-1}) led to somatic embryo maturation; in contrast, adding both 2,4-D and AC decreased somatic embryo maturation (Figs. 1, 2, 5 and 6).

Non-embryogenic callus of sugarcane var. SP79-1011 was unable to produce somatic embryos even when subjected to maturation treatments, showing that competent cells and the appropriate inductor stimulus are both necessary for differentiation into somatic embryos. Incubation of callus in the absence of growth regulators is required to inhibit cell proliferation during the multiplication phase and stimulate the formation of somatic embryos (Gahan and George 2008). In addition to the removal of 2,4-D, a stimulus is required to induce maturation once embryogenic competence is expressed at the level of the single cell; these cells are then capable of differentiating into embryos if they receive inducers of differentiation such as maturation inducers (Fehér et al. 2003).

AC has been used for the in vitro culture of various plant species in small concentrations because it supports plant growth and development (Lameira et al. 1997; Thomas 2008); this usage includes its role as a promoter of maturation in sugarcane (Blanco et al. 1997). The role of AC in promoting maturation has not yet been elucidated; however, studies have shown that the effect is indirect and is most likely the result of its ability to adsorb substances in the culture medium (either those released by the explant or others), thereby removing certain components and inducing a maturation response (Thomas 2008).

Cultures of embryogenic callus from sugarcane var. SP79-1011 exhibited greater growth than non-embryogenic callus in the control (10 μM 2,4-D) and maturation (1.5 g L^{-1} AC) treatments mainly at the end of the culture period (Fig. 3). Similar results were observed for embryogenic sugarcane callus of 2 American varieties, CP70-321 and CP65-357, and one South African variety, NCo310 (Gandonou et al. 2005), as well as for cotton (Zhang et al. 2000). These results demonstrate that embryogenic callus has greater growth potential than non-embryogenic callus when stimulated with 2,4-D and AC.

Additionally, non-embryogenic callus exposed to maturation treatment exhibited more oxidation; the cells were somewhat brown compared with the cells in embryogenic callus (Fig. 2). The oxidation of hydroxycinnamic residues leads to the formation of bridges between phenolic polysaccharide chains as well as between polysaccharides and either lignins or structural proteins, decreasing the phenolic extensibility of the cell wall and thereby interrupting cell growth (Arnaldos et al. 2001). This finding may explain the reduced rate of cell growth observed in non-embryogenic

callus (Fig. 3). Studies have also shown that quinones produced by bridging during phenolic oxidation may impede the in vitro morphogenesis of plant tissues by affecting regeneration (Dalal et al. 1992).

The formation of embryogenic cells can be correlated with characteristic morphological changes (Fehér et al. 2003). The embryogenic callus of sugarcane var. SP79-1011 was characterized by its smoothness, compact nature and somewhat yellow color, whereas non-embryogenic callus was friable, translucent and mucilaginous (Fig. 4); these differences confirm the characterization of embryogenic and non-embryogenic callus reported by Lakshmanan (2006). Histological changes associated with the position and activity of cells during the acquisition of competence has been the subject of many studies in plants, including sugarcane (Rodriguez et al. 1995).

In the present study, embryogenic callus was histologically characterized as possessing embryogenic characteristics, being small and isodiametric, having cytoplasm-rich cells and forming globular structures; in contrast, non-embryogenic callus was not organized, presenting elongated and highly vacuolated cells that were dispersed throughout the callus (Fig. 4) as previously demonstrated in several plant systems (reviewed by Fehér et al. 2003). Initially, the callus induced in sugarcane var. SP79-1011 contained both embryogenic and non-embryogenic cells (Supplementary Figure 2), which were separated during the subculture into embryogenic and non-embryogenic callus. An earlier study reported similar results in CUBA 87-51 sugarcane callus (Rodriguez et al. 1995). The acquisition of embryogenic competence in tissue culture may require the presence of non-embryogenic cells that produce and secrete molecules into the culture medium (Hecht et al. 2001). These molecules could then be perceived by other cells, which in turn could become competent and develop into embryos (Pennell et al. 1992). Several plant-produced molecules that may have a role in cell–cell signaling have been identified, including chitinases and arabinogalactan proteins (McCabe et al. 1997; Toonen et al. 1997, Hecht et al. 2001). Recently, embryogenic competence in sugarcane callus has been associated with the presence of dehydrins, indicating that specific proteins might be necessary for the induction and/or maintenance of embryogenesis (Burrieza et al. 2012).

In maturation medium containing AC (1.5 g L^{-1}), somatic embryos at various developmental stages were formed from sugarcane embryogenic callus (Fig. 6), and the characteristic somatic embryogenesis structures described for monocots (including sugarcane) were identified in these embryos. A typical bipolar orientation with a scutellum and a coleoptile was observed during somatic embryo differentiation from the globular to cotyledonar forms as previously described for sugarcane (Falco et al. 1996). At the mature stage, the cell vacuoles exhibit specialized behaviors and accumulate storage compounds such as proteins (Dodeman et al. 1997), carbohydrates and lipids; this accumulation results in the storage of reserve compounds for use in the subsequent conversion of embryos into plantlets (Cangahuala-Inocente et al. 2004).

In addition to morphological alterations, genetic and physiological factors are also important for triggering in vitro embryogenesis in various plant somatic cells (Karami et al. 2009). Among several physiological factors, PAs could be used as markers for the acquisition of plant developmental and somatic embryogenesis competence (Minocha et al. 1999, 2004; Shoeb et al. 2001; Santa-Catarina et al. 2004, 2007; Silveira et al. 2006; Steiner et al. 2008; Paul et al. 2009; Wu et al. 2009; Alcázar et al. 2010).

The embryogenic and non-embryogenic callus of sugarcane var. SP79-1011 exhibit distinct patterns in their levels of total free PAs when incubated in the control and maturation treatments (Fig. 7). These results suggest that the level of total free PAs is modulated moreso by the type of callus (embryogenic or non-embryogenic) than by the treatment (control or maturation). After a certain amount of time, the total free PA levels are similar between control and maturation treatments regardless of whether embryogenic competence was achieved. Additionally, an increase in the level of total free PAs in embryogenic callus at 21 days of incubation could be an important factor associated with somatic embryo development in var. SP79-1011. The higher total PA levels in embryogenic callus compared with non-embryogenic callus might be associated with high levels of PAs in dividing meristematic cells because these levels increase when embryos are formed (Li and Burrit 2003).

Among the PAs studied, free Put was observed at higher concentrations than Spd or Spm (Fig. 8); however, similar Put concentrations were observed in embryogenic and non-embryogenic callus, and higher concentrations were observed at the beginning of the culture period, suggesting that Put is not an essential PA in sugarcane callus morphogenesis. Several studies have demonstrated a higher concentration of Put in embryogenic cultures during multiplication and noted its importance in the events that precede cell differentiation (Silveira et al. 2004; Minocha et al. 2004; Paul et al. 2009). In *Pinus taeda* (Silveira et al. 2004) and *Picea rubens* (Minocha et al. 2004), free Put was the most abundant PA and was found to be necessary for the progression of somatic embryogenesis. Additionally, compared with Spd and Spm, larger amounts of Put were observed during the induction of somatic embryogenesis in *Momordica charantia*, and this PA is a prerequisite for cell division (Paul et al. 2009). However, in cell aggregates of *Ocotea catharinensis* that were incubated with 2,4-D, lower levels of Put were detected in potentially embryogenic

cultures using somatic embryogenesis receptor kinase (SERK) expression (Santa-Catarina et al. 2004).

The higher levels of Spd and Spm in sugarcane var. SP79-1011 embryogenic callus at the beginning of incubation (Fig. 8) could be an important signaling mechanism related to the acquisition of embryogenic competence that induces the maturation of somatic embryos and ensures their conversion into plantlets.

Spd and especially Spm appear to be more important than Put during the evolution of morphogenetic somatic embryos. Whereas cell division (and therefore an increase in the extensibility of the cell wall) is prevalent under multiplication conditions, cell elongation, the formation of new structures such as the scutellum and the coleoptile and the early vascularization of mature embryos are all events that occur during the maturation of sugarcane var. SP79-1011.

Several studies have suggested the importance of Spm or Spd during the evolution of zygotic and somatic embryo development (Minocha et al. 1999; Santa-Catarina et al. 2006, 2007; Steiner et al. 2007), and a recent study demonstrated that free Spd and Spm were involved in glycerol-mediated promotion of somatic embryogenesis in *Citrus sinensis* (Wu et al. 2009). Additionally, the role of Spd and Spm during the morphogenetic evolution of somatic embryos in *Oenothera catharinensis* may be related to alterations in endogenous nitric oxide levels (Santa-Catarina et al. 2007).

Conclusions

During somatic embryogenesis in sugarcane var. SP79-1011, 2 types of callus—embryogenic and non-embryogenic—were characterized; however, only the embryogenic callus enabled the formation of somatic embryos. The morphological and biochemical aspects of callus under control and maturation treatments were determined. Supplementing the medium with AC after 2,4-D elimination was necessary for somatic embryo maturation and evolution. Compared with non-embryogenic callus, embryogenic callus exhibited a characteristic morphological pattern at induction, and an adequate stimulus was necessary for somatic embryo maturation.

Our results suggest that Spm could be important for the acquisition of embryogenic competence and somatic embryo maturation in sugarcane var. SP79-1011. These novel observations regarding morphological and biochemical changes provide preliminary information for further studies on embryogenic capacity in sugarcane callus. Furthermore, an understanding of the factors that affect the morphogenic competence of embryogenic and non-embryogenic callus can be helpful for genetic transformation work in sugarcane.

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