2014-05-22

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Acta Physiologiae Plantarum, Heidelberg, v.36, p.1853-1865, 2014
http://www.producao.usp.br/handle/BDPI/45262

Acta Physiol Plant (2014) 36:1853–1865
DOI 10.1007/s11738-014-1560-6

ORIGINAL PAPER

The transition of proembryogenic masses to somatic embryos in *Araucaria angustifolia* (Bertol.) Kuntze is related to the endogenous contents of IAA, ABA and polyamines

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Received: 3 March 2014 / Revised: 8 April 2014 / Accepted: 28 April 2014 / Published online: 22 May 2014

Abstract In somatic embryogenesis (SE) of conifers, the inability of many embryogenic cell lines to form well-developed somatic embryos may results from failure and constraints during the transition of proembryogenic masses (PEMs) to early somatic embryos. In the present work, we propose the inclusion of a preculture and prematuration steps looking at enhancing PEM III-to-early somatic embryos transition. It was further hypothesized that these results would correlate with the contents of endogenous indole-3-acetic acid (IAA), abscisic acid (ABA) and polyamines (PA). To test these hypotheses, the embryogenic culture was subjected to preculture with fluridone (FLD) and prematuration treatments with different combinations of carbon source and polyethylene glycol (PEG). The frequency of PEM III was increased after FLD preculture and the contents of IAA and ABA decreased, while the contents of PA increased. Putrescine (Put) was the most abundant PA present at this stage, followed by spermidine (Spd) and spermine (Spm). In early embryogenesis, prematuration treatments supplemented with maltose or lactose plus PEG enhanced the PEM III-to-early somatic embryos transition. IAA and ABA contents increased at this stage, while a decrease of the total free PA levels was observed. Put was the most abundant PA, followed by Spd and Spm, mainly in the treatment supplemented with PEG. This resulted in a decrease of PA ratio (Put/Spd + Spm) and, hence, PEM III-to-early somatic embryos transition. It was concluded that the preculture with FLD and prematuration treatments promote the PEM III-to-early somatic embryos transition throughout the whole early developmental process in *Araucaria angustifolia*.

Keywords In vitro conservation · Somatic embryogenesis · Morphology · Phytohormones

Introduction

Araucaria angustifolia (Bertol.) Kuntze is one of the most important Brazilian conifer species (Guerra et al. 2008),

Communicated by S. Werbrouck.

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and nowadays, it is endangered, since extensive exploitation has reduced its natural occurrence area to about 3 % of the original area (Guerra et al. 2002).

A. angustifolia somatic embryogenesis (SE) has been studied for many years, and the induction and proliferation of embryogenesis cultures are well established (Astarita and Guerra 1998; Guerra et al. 2000; Steiner et al. 2005, 2007). However, late and mature somatic embryos are normally obtained at low frequency (Steiner et al. 2008). To improve the regenerative protocol, a prematuration step has been suggested (Steiner 2009). According to this author, the main bottleneck of *A. angustifolia* SE is the transition of PEMs-to-early somatic embryos.

Phytohormones play an important role in a wide range of plant developmental processes, including embryogenesis. The auxin indole-3-acetic acid (IAA) is involved, among others, in the establishment and maintenance of cell polarity, which plays a key role in signaling and directional intercellular communication (Kleine-Vehn and Friml 2008). Polarization depends on the concentration gradient created by the directional movement of auxin, which is essential to the establishment of the polar axis, the patterning of plant body, and morphological adaptations to the environment (De Smet and Jurgens 2007). From the first cell division in plant embryogenesis, auxin is preferentially accumulated in the apical cell of the zygote and plays an important role in proembryonic cell fate (Friml 2003).

Abscisic acid (ABA) is mainly associated with the synthesis of storage substances during maturation phase and, therefore, has been employed in SE protocols to promote the maturation of somatic embryos (Dodeman et al. 1997; von Arnold et al. 2002). ABA not only promotes the transition of proliferating cells to somatic embryos (Langhansova et al. 2004), but it also improves their quality by increasing desiccation tolerance, preventing precocious germination (Robichaud et al. 2004; Vahdati et al. 2008), and reducing secondary embryogenesis (von Arnold et al. 2002). Su et al. (2013) suggested that ABA plays an important role during initial formation of *Arabidopsis thaliana* somatic embryos, mediating auxin biosynthesis as its transport and providing the establishment of embryonic polarity. It is well documented that the induction of somatic embryos in the presence of exogenous ABA results in increased levels of endogenous auxin (Rai et al. 2010).

Fluridone (1-methyl-3-phenyl-5-[3-trifluoromethyl (phenyl)]-4-(1H)-pyridinone) (FLD) is an inhibitor of the enzyme phytoene desaturase which converts the carotenoid phytoene to phytofluene (Fong and Schiff 1979). Gamble and Mullet (1986) showed that FLD inhibited carotenoid accumulation in barley seedlings grown in the dark without altering plastid biogenesis or protein composition. Since ABA is derived from carotenoids (Schwartz and Zeevaart 2010), the inhibition of the carotenoids could also indirectly prevent the synthesis of this hormone, and thus FLD has been known as an inhibitor of ABA synthesis (Stewart and Voetberg 1987; Rudus et al. 2009). Some detrimental effects of ABA in embryogenic cultures may be overcome by the use of FLD (Saab et al. 1990). On the other hand, some works have shown negative effects of FLD on the development of somatic embryos of different species (Kikuchi et al. 2006; Rudus et al. 2009; Su et al. 2013). Finally, in those embryogenic systems where high levels of ABA were associated with developmental failure, FLD did allow shoot formation (Srinivasan and Vasil 1986; Zaghmout and Torello 1990).

Polyamines (PA) are considered a class of compounds linked to cellular proliferation (Bais and Ravishankar 2002; Jiménez et al. 2005). In plants, the three most common PA are the diamineputrescine (Put), the triaminespermidine (Spd), and the tetraminespermine (Spm) (Bouchereau et al. 1999). They are free or conjugated with other low molecular weight substances, such as phenolics, or macromolecules, such as proteins and nucleoproteins (Malá et al. 2012), in cell walls, vacuoles, mitochondria, chloroplasts and cytoplasm (Kaur-Sawhney et al. 2003).

It has been shown that PA plays a key role in the regulation of somatic and zygotic embryogenesis (Kong et al. 1998; Thorpe and Stasolla 2001; Silveira et al. 2004a, b). High contents of PA in somatic embryos contribute to the accumulation of reserve substances, particularly proteins and triglycerides, which are then used during embryonic germination (Baron and Stasolla 2008). Changes in the contents of PA have been shown in embryogenic cultures of *Picea abies* (Serapiglia et al. 2008; Vondráková et al. 2010), *P. rubens* (Minocha et al. 1993), and *Pinus taeda* (Silveira et al. 2004a). Furthermore, many studies have shown the importance of exogenous PA on improving SE (Silveira et al. 2006; Steiner et al. 2007; Dutra et al. 2013). However, the mechanism by which polyamines regulate the process of cell differentiation is still not clear (Malá et al. 2012).

In other conifers, such as *P. abies*, the PEMs-to-early somatic embryos transition is triggered by changes in the levels of plant growth regulators (PGRs) in the culture medium, where both auxins and cytokinins were depleted in the prematuration step. However, by adding ABA to the culture medium, the progression of somatic embryos was stimulated (Filonova et al. 2000; von Arnold et al. 2002, 2005).

It seems likely that the inability of many embryogenic cell lines to form well-developed somatic embryos is, in large part, associated with disturbed or arrested PEM-to-early somatic embryos transition (von Arnold et al. 2002). In *A. angustifolia* SE, late embryogenesis is rarely observed, but when it does occur, a low number of

abnormal somatic embryos are formed (Steiner et al. 2008). It is believed that this problem results from failure in the PEM-to-early somatic embryos transition during early embryogenesis (Steiner 2009), which has been related to the morphology of PEMs before of maturation treatment with ABA in *Picea abies* (Filonova et al. 2000).

Taking this into account, in the present work, we included a preculture step with FLD to modify the ABA content and evaluated the effect of prematuration treatments, which combined different sources of carbon and polyethylene glycol (PEG) on the PEM-to-early somatic embryos transition, in turn, correlating the results with the contents of endogenous IAA, ABA and PA in *A. angustifolia*.

Materials and methods

Plant material

Megastrobili of *A. angustifolia* were collected in December 2009 and January 2010 from an open-pollinated tree of a natural population located in the Experimental Station of Santa Catarina's Research and Extension Agency, located in the county of Lages (884 m altitude 27° 48′58″ South, and 50° 19′34″ West), Santa Catarina State, Brazil. Seeds were isolated, and the immature embryos were excised. The embryogenic culture was obtained following the developmental route summarized in Fig. 1.

Induction of embryogenic culture

Embryogenic culture 91002 was induced in December 2009, according to procedures described by Steiner et al. (2005). In brief, immature zygotic embryos were excised from the seeds and then inoculated into borosilicate glass dishes containing 20 ml of PGRs-free ½ LP medium (von Arnold and Erikson 1981) supplemented with 10 g l^{-1} sucrose, 0.45 g l^{-1} L-glutamine and 2 g l^{-1} phytagel® (Sigma-Aldrich, St. Louis, MO, USA). The L-glutamine was filter-sterilized and added to the culture medium after autoclaving at 121 °C for 15 min. The pH of the culture medium was adjusted to 5.8 before adding phytagel®. The cultures were incubated in the dark at 25 ± 2 °C (Fig. 1a).

Proliferation of embryogenic culture

Embryogenic culture 91002 was proliferated during 5 months with subcultures every 3 weeks to a PGRs-free BM medium (Gupta and Pullman 1991) supplemented with 30 g l^{-1} sucrose, 0.5 g l^{-1} casein hydrolysate, 1.0 g l^{-1} myo-inositol and 1.0 g l^{-1} L-glutamine in alternating cycles between gelled (2 g l^{-1} of phytagel®) and liquid medium (Fig. 1a). The pH of the culture medium was

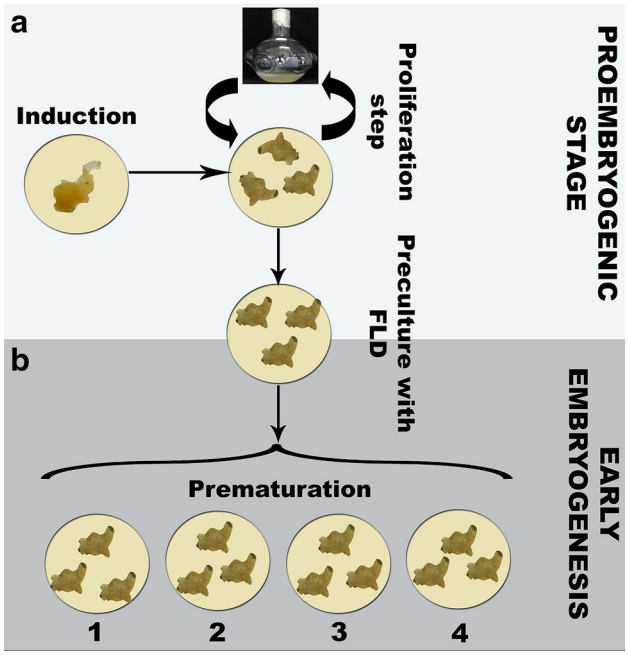

Fig. 1 Schematic representation of the developmental route of embryogenic culture 91002 of *Araucaria angustifolia*. **a** Proembryogenic stage: embryogenic culture induced in PGRs-free ½ LP medium (von Arnold and Erikson 1981) (induction step); proliferation of embryogenic culture in gelled and liquid PGRs-free BM medium (Gupta and Pullman 1991) (proliferation–prematuration step). Preculture in DKM medium (von Arnold and Clapham 2008) supplemented with 30 μM of fluridone (FLD) for 4 weeks (preculture with FLD; step between proembryogenic stage and early embryogenesis). **b** Early embryogenesis: transition of proembryogenic masses (PEMs) to early somatic embryos obtained in the prematuration treatments in DKM medium supplemented with 9 % of maltose (*1*); 9 % of maltose and 7 % of polyethylene glycol 3350 (PEG) (*2*); 9 % of lactose (*3*) and 9 % of lactose and 7 % of PEG 3350 7 % (*4*) (prematuration step)

adjusted to 5.8 before autoclaving and adding phytagel®. Casein hydrolysate, myo-inositol and L-glutamine were filter-sterilized and added to the culture medium after autoclaving at 121 °C for 15 min, followed by the distribution of 20 ml culture medium to borosilicate glass dishes previously autoclaved. The cultures were incubated in the dark at 25 ± 2 °C. In suspension cultures, 100 ml of the medium containing casein hydrolysate, myo-inositol and L-glutamine was sterilized by autoclaving at 121 °C during 15 min in adapted nipple flasks. Embryogenic culture 91002 was dispersed in the nipple flasks in the proportion of 2 g of fresh weight (FW) of cells to 100 ml of medium and then placed on a Stewart apparatus rotating at 1 rpm in the dark at 25 ± 2 °C.

Preculture and prematuration of embryogenic culture

Embryogenic culture 91002 was precultured (Fig. 1a, b) for 4 weeks in DKM medium (von Arnold and Clapham 2008) supplemented with 0.5 g l^{-1} casein hydrolysate,

0.1 g l^{-1} myo-inositol, 30.0 g l^{-1} sucrose, 2.0 g l^{-1} phytagel® and 30 μM FLD (Riedel-de Haën, Seelze, Germany) diluted in Dimethyl sulfoxide (DMSO). The casein hydrolysate and myo-inositol were filter-sterilized and added to the culture medium after autoclaving at 121 °C for 15 min. FLD was added directly to the culture medium without filter-sterilization. The culture medium (20 ml) was dispensed to borosilicate glass dishes previously autoclaved. The pH of the culture medium was adjusted to 5.8 before adding phytagel®, and the cultures were incubated in the dark at 25 ± 2 °C. The cultures were transferred to the same fresh culture medium once after 2 weeks. After this, the embryogenic culture was subcultured for 4 weeks in four different treatments (Fig. 1b). The culture medium combinations were as follows: PGRs-free DKM basal salts supplemented with 0.5 g l^{-1} casein hydrolysate, 0.1 g l^{-1} myo-inositol, filter-sterilized, and added after autoclaving at 121 °C for 15 min for sterilization; 2 g l^{-1} phytagel® with the following modifications (Fig. 1b): (1) 90.0 g l^{-1} maltose (*D*-(+)-maltose monohydrate, Sigma-Aldrich, St. Louis, MO, USA) (maltose 9 %), (2) 90.0 g l^{-1} maltose and 70.0 g l^{-1} polyethylene glycol 3350 (PEG) (maltose 9 % and PEG 7 %), (3) 90.0 g l^{-1} lactose (α-lactose monohydrate, Sigma-Aldrich, St. Louis, MO, USA) (lactose 9 %) and (4) 90.0 g l^{-1} lactose and 70.0 g l^{-1} polyethylene glycol 3350 (PEG) (lactose 9 % and PEG 7 %). The pH of the culture medium was adjusted to 5.8 before adding phytagel®, followed by autoclaving and distribution of the 10 ml medium in disposable Petri dishes. The cultures were incubated in the dark at 25 ± 2 °C. The cell aggregates were subcultured to the same culture medium once after 2 weeks.

Morpho-cytochemical analyses

Every 4 weeks, at the end of each step (Fig. 1), samples with approximately 30 mg FW of each treatment were analyzed by means of double staining with acetocarmine and Evans blue (Gupta and Durzan 1987). The number of PEM III and early somatic embryos was recorded. Three samples were taken randomly, and at least 100 aggregates were analyzed in each sample. The size of early somatic embryos was determined using an Olympus BX 40 microscope equipped with the image capture system Olympus DP 40 and DP Controller Software.

IAA and ABA determination

Free IAA and ABA contents were extracted and determined according to the methodology described by Silveira et al. (2004b) and Steiner et al. (2007). Every 4 weeks, at the end of each step (Fig. 1), two samples (0.5 g FW) of each treatment were collected and stored at −20 °C. Samples were ground in a 5 ml extraction buffer (80 % ethanol + 1 % polyvinylpyrrolidone-40). Extracted samples were transferred to a Falcon type tube (15 ml), and both [^{3}H] IAA and [^{3}H] ABA were added as internal radioactive standards. After 1.5 h of incubation, samples were centrifuged during 15 min at 15,500×*g*, at 4 °C. Supernatants were concentrated in a 'speed vac' at 45 °C until 20 % of the initial volume (1 ml) was reached. Volumes were elevated (Milli-Q water type) to 3 ml and the pH adjusted to 2.5, using HCl (1 N). Samples were partitioned twice, using ethyl ether as an organic solvent. Organic layers were combined, and the aqueous residue was eliminated by freezing the base of the tubes during 5 s in liquid nitrogen, with transference of the organic phase to a clean tube. The organic phase containing IAA and ABA was dried in a 'speed vac' at 45 °C, dissolved in 300 μl of 100 % methanol, transferred to micro tubes, and stored at −20 °C until analysis. Aliquots (40 μl) of stored samples were analyzed by reverse phase high performance liquid chromatography (HPLC) using a 5 μm C18 column (Shimadzu Shin-pack CLC ODS). The gradient was developed by mixing increasing proportions of absolute methanol to 10 % methanol plus 0.5 % acetic acid in water. The gradient of absolute methanol was programmed to 20 % over the first 15 min, from 20 to 45 % between 15 and 22 min, from 45 to 54 % between 22 and 33 min, from 54 to 100 % between 33 and 34 min, and 100 % between 34 and 50 min, with 1 ml min^{-1} flow at 40 °C. IAA content was determined using a fluorescence detector at 280 nm (excitation) and 350 nm (emission). ABA content was determined using a UV–VIS detector at 254 nm. A mixture of IAA and ABA was used as a standard. Fractions containing IAA and ABA were collected and analyzed in the Packard Tri-Carb liquid scintillation counter to estimate losses.

PA analysis

Every 4 weeks, at the end of each step (Fig. 1), three samples (0.5 g FW) of each treatment were collected and stored at −20 °C until the time of analysis. Samples were ground in 1.6 ml of 5 % (v/v) perchloric acid. Free PA was extracted, dansylated, and identified by reverse phase HPLC, according to the procedures described by Steiner et al. (2007). PA content was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with standard PA: Put, Spd and Spm.

Data analysis

The endogenous IAA and ABA analyses were carried out in duplicate, while the PA analysis was performed in triplicate. All experiments were repeated four times with

similar results, and the resulting data were presented as means of a single representative experiment. Data were analyzed by ANOVA ($P < 0.05$), followed by the SNK test (Student–Newman–Keuls) ($P < 0.05$).

Results

Morphology from PEM-to-early somatic embryos

In the proembryogenic stage (Fig. 1), PEMs proliferated in untreated culture, evolving through three specific developmental stages, PEM I, II and III (Fig. 2a, b), according to the features noted by Filonova et al. (2000) in *P. abies* and previously in *A. angustifolia* (Steiner 2009) (Fig. 2a, b). Using double-staining analysis with acetocarmine and Evans blue (Fig. 2), the presence of two typical conifer cells was identified in PEM: embryogenic cells (ECs), which were isodiametric and densely cytoplasmic, and suspensor-like cells (SCs), which were elongated and vacuolated (Fig. 2a, b). PEM III differed from PEM II and PEM I by the number of ECs and SCs (Fig. 2a). The prematuration step (Fig. 1) stimulated the differentiation of early somatic embryos from PEM III (Fig. 2c–f). The PEM III-to-early somatic embryos transition was characterized by embryonic individualization, in which compact clusters of ECs grew from PEM III, albeit still connected to it by SCs (Fig. 2c–e). This stage is the starting point of polarization and individualization of early somatic embryos with two regions, the dense globular embryonal mass (EM) in the apical part and suspensor (S) in the basal part (Fig. 2f). This is the first report describing the morphology of PEM III-to-early somatic embryo transition (Fig. 2c–f) in *A. angustifolia*.

Preculture and prematuration treatments increase the frequency of PEM III and early somatic embryos

The frequency of PEM I and II was quite similar when comparing control and embryogenic culture precultured with FLD. However, a significant increase in the frequency of PEM III (60 %) was observed as result of the

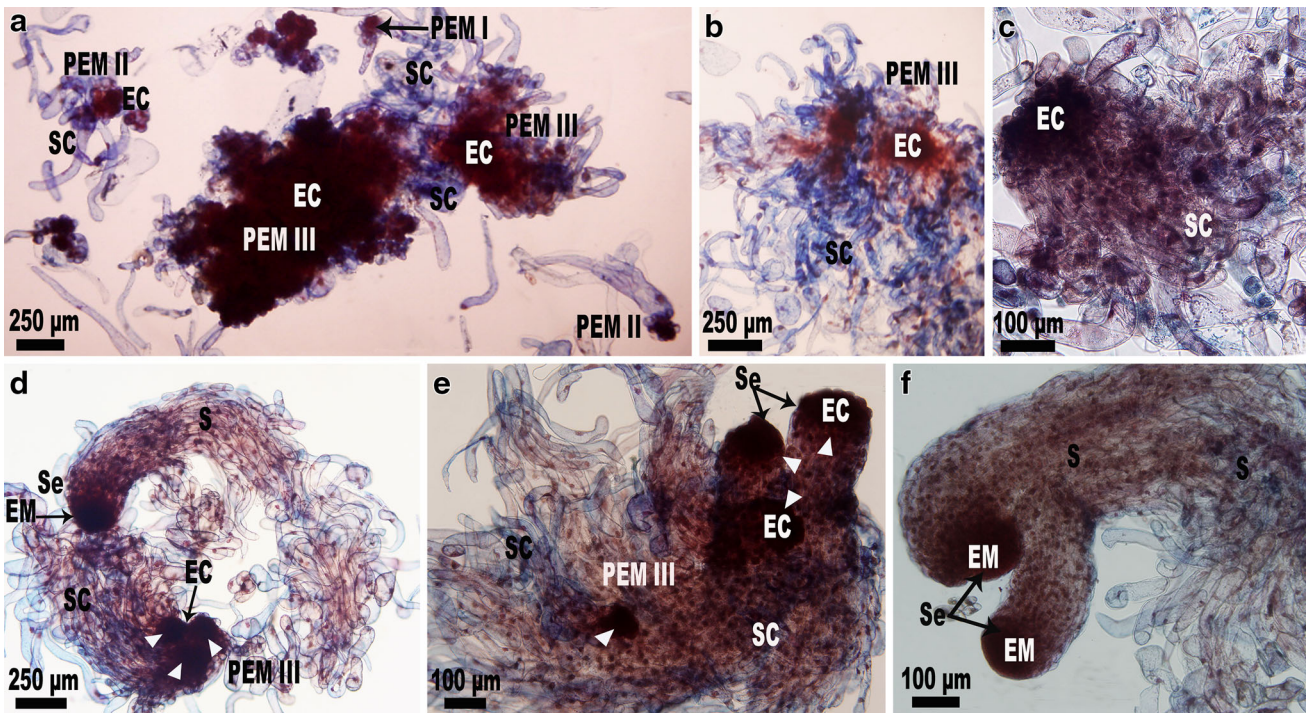

Fig. 2 Light microscopy of the cytochemical analysis with acetocarmine and Evans blue of embryogenic culture 91002 of *Araucaria angustifolia*. **a**, **b** Proembryogenic stage. Control (**a**): FLD-free culture and FLD (**b**): culture treated for 4 weeks in DKM basal medium supplemented with 30 µM of FLD. Note the presence of proembryogenic masses at three developmental stages: I (PEM I), II (PEM II) and III (PEM III); embryogenic cells (EC) stained with acetocarmine and suspensor-like cells (SC) stained with Evans blue (**a** and **b**). **c**–**f**. Early embryogenesis. PEM III showing the start of polarization and individualization of early somatic embryos in the prematuration medium supplemented with 9 % of maltose (**c**) and 9 % of lactose and 7 % of PEG (**d**). Note the presence of the embryogenic cells (EC), suspensor-like cells (SC) (**c** and **d**) and early somatic embryo (Se) with embryonal mass (EM) and suspensor (S) (**d**). Multiple somatic embryos (Se) emerging from PEM III in prematuration medium supplemented with 9 % of lactose and 7 % of polyethylene glycol (3350) (PEG) (**e**). Somatic embryos (Se) individualized and polarized from the prematuration medium supplemented with 9 % of maltose and 7 % of PEG. Note the presence of the embryonal mass (EM) and suspensor (S) organized at two different poles (**f**)

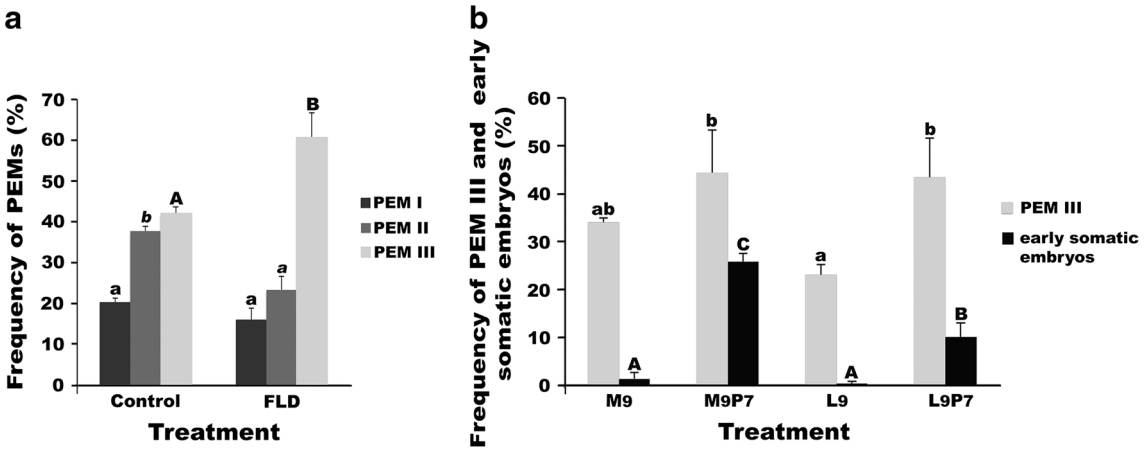

Fig. 3 Frequency of proembryogenic masses (PEMs) at stages I, II and III (PEM I, II and III) and early somatic embryos formed in embryogenic culture 91002 of *Araucaria angustifolia*. **a** Frequency of PEM I, II and III in the control (FLD-free culture) and FLD (culture treated for 4 weeks in DKM basal medium supplemented with 30 μM of FLD). **b** Frequency of PEM III and early somatic embryos in the prematuration treatments. The prematuration treatments consisted of DKM basal medium supplemented with 9 % of maltose (M9); 9 % of maltose and 7 % of polyethylene glycol 3350 (PEG) (M9P7); 9 % of lactose (L9) or 9 % of lactose and 7 % of PEG (L9P7). Presented values are mean percentage ± standard error of the total number of PEM I, II, III and early somatic embryos formed per treatment based on three independent replicates of 100 aggregates of each treatment ($n = 3$). Means followed by the *same letters* are not significantly different, according to the SNK test ($P < 0.05$). **a** $CV_{PEM\ I} = 14.21$; $CV_{PEM\ II} = 10.02$; $CV_{PEM\ III} = 10.52$; PEM I *lowercase letters*; PEM II *italics* and *lowercase letters* and PEM III *uppercase letters*. **b** $CV_{PEM\ III} = 20.95$; $CV_{early\ somatic\ embryos} = 23.96$; PEM III *lowercase letters*; early somatic embryos *uppercase letters*

FLD preculture when compared to control (40 %) (Fig. 3a). When embryogenic culture was transferred to prematuration treatments, an increase in the frequency of PEM III-to-early somatic embryos was observed (Fig. 3b). This transition was more effective in response to maltose or lactose combined with PEG (Fig. 3b). In these treatments, the number of both PEM III and early somatic embryos was enhanced. In PEG-free treatments, only a few polarized and individualized early somatic embryos were observed (Fig. 3b); however, the number of PEM III decreased in comparison to the FLD preculture. In addition, more early somatic embryos developed in response to maltose plus PEG than those developing in culture medium supplemented with lactose plus PEG (Fig. 4).

Endogenous IAA and ABA levels

The endogenous contents of IAA and ABA between pro-embryogenic stage and early embryogenesis showed slight modifications (Fig. 5). In the proembryogenic stage, the preculture with FLD promoted a decrease in both IAA and ABA contents, as compared to the levels found in the control (Fig. 5a). In the embryogenic culture submitted to FLD preculture, the decrease in IAA and ABA levels was coincident with an increase in the frequency of PEM III (Fig. 3a). Moreover, a higher number of SCs (Fig. 2b) were observed as compared to the control (Fig. 2a).

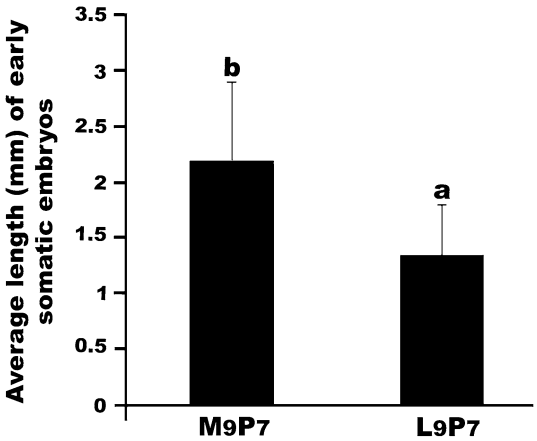

Fig. 4 Average length (mm) of early somatic embryos formed in embryogenic culture 91002 of *Araucaria angustifolia* during the transition of PEMs-to-early somatic embryos in early embryogenesis. The early somatic embryos were formed in DKM basal prematuration medium supplemented with 9 % of maltose and 7 % of polyethylene glycol 3350 (PEG) (M9P7) and 9 % of lactose and 7 % of PEG (L9P7). Presented values are mean percentage ± standard error of length (mm) of 50 early somatic embryos of each treatment ($n = 50$). Means followed by the *same letters* are not significantly different, according to the SNK test ($P < 0.05$). CV = 31.94

At early embryogenesis, the IAA levels were higher in response to maltose plus PEG, followed by maltose, lactose plus PEG, and finally by lactose only (Fig. 5b). Maltose treatment enhanced ABA levels when compared to those levels resulting from the treatment with lactose (Fig. 5b).

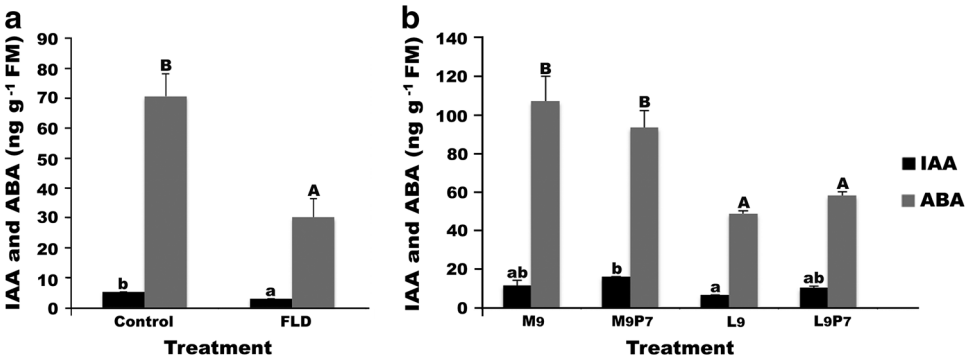

Fig. 5 Endogenous IAA and ABA contents [IAA and ABA ng g^{-1} of fresh weight (FW)] in embryogenic culture 91002 of *Araucaria angustifolia*. **a** Proembryogenic stages. Control: FLD-free culture and FLD: Culture treated for 4 weeks in DKM basal medium supplemented with 30 μM of FLD. **b** Early embryogenesis, when transition of proembryogenic masses (PEMs) to early somatic embryos occurs. The prematuration treatments consisted of DKM basal medium supplemented with 9 % of maltose (M9); 9 % of maltose and 7 % of polyethylene glycol 3350 (PEG) (M9P7); 9 % of lactose (L9) or 9 % of lactose and 7 % of PEG (L9P7). Presented values are mean percentage ± standard error of two replicates of IAA and ABA endogenous contents of each treatment ($n = 2$). Means followed by the *same letters* are not significantly different, according to the SNK test ($P < 0.05$). **a** CV_{IAA} = free; $CV_{ABA} = 15.55$. **b** $CV_{IAA} = 18.8$; $CV_{ABA} = 14.43$. IAA *lowercase letters*; ABA *uppercase letters* (**a** and **b**)

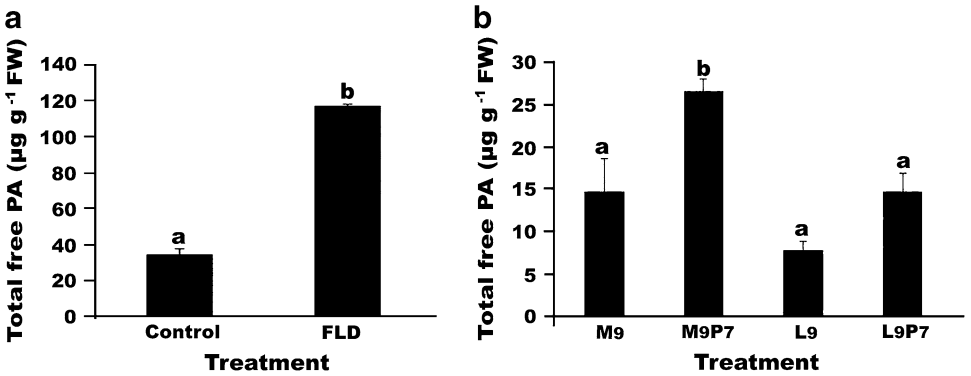

Fig. 6 Total free polyamines [total free PA μg g^{-1} of fresh weight (FW)] in embryogenic culture 91002 of *Araucaria angustifolia*. **a** Proembryogenic stages. Control: FLD-free culture and FLD: Culture treated for 4 weeks in DKM basal medium supplemented with 30 μM FLD. **b** Early embryogenesis, when transition of proembryogenic masses (PEM) to early somatic embryos occurs. Prematuration treatments consisted of DKM basal medium supplemented with 9 % of maltose (M9); 9 % of maltose and 7 % of polyethylene glycol 3350 (PEG) (M9P7); 9 % of lactose (L9) or 9 % of lactose and 7 % of PEG (L9P7). Presented values are mean percentage ± standard error of three replicates of each treatment ($n = 3$). Means followed by the *same letters* are not significantly different, according to the SNK test ($P < 0.05$). **a** $CV_{PA} = 4.36$. **b** $CV_{PA} = 19.83$

However, PEG was not associated with ABA accumulation (Fig. 5b).

Endogenous PA levels

Endogenous levels of PA ranged from the proembryogenic stage to early embryogenesis. Total free PA levels (Fig. 6a) and PA ratio (Fig. 7c) increased in response to FLD preculture, and the same pattern was observed for the endogenous contents of Put (Fig. 7a). We postulate that the increase of both total free PA and PA ratio probably resulted from the increase in Put levels after FLD preculture. The content of Spd also increased after preculture with FLD, but no variation in the content of Spm at this stage was observed (Fig. 7a).

Enhanced total free PA levels resulted from prematuration treatment with maltose plus PEG, followed by the treatment with maltose, lactose plus PEG, and, finally, lactose (Fig. 6b). The higher total free PA level in the treatment with maltose plus PEG was associated with the higher frequency of PEM III and early somatic embryos. More specifically, Put was more evident in early embryogenesis (Fig. 2d–f), followed by Spd and Spm, respectively (Fig. 7b). Different from the other PA, the content of Spm showed the highest values in the treatments supplemented with PEG, followed by lower values in PEG-free treatments with maltose and lactose, respectively (Fig. 7b).

PA ratio (Put/Spd + Spm) was higher in response to the treatment with PEG-free lactose, followed by PEG-free maltose. The treatments supplemented with PEG showed

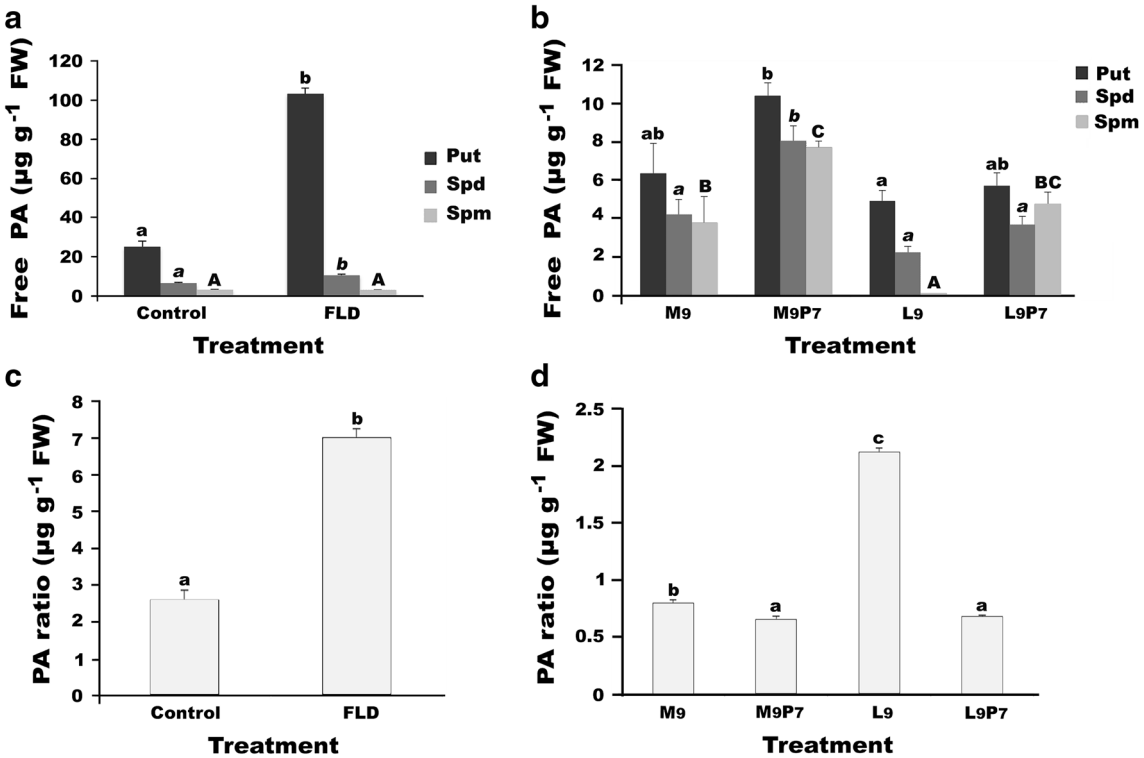

Fig. 7 Free polyamines [free PA µ g^{-1} of fresh weight (FW)] and polyamine ratio (Put/Spd + Spm) [PA ratio µg g^{-1} fresh weight (FW)] in embryogenic culture 91002 of *Araucaria angustifolia*. **a** Free polyamines in the proembryogenic stages. Control: FLD-free culture and FLD: Culture treated for 4 weeks in DKM basal medium supplemented with 30 µM of FLD. **b** Free polyamines in early embryogenesis. The prematuration treatments consisted of DKM basal medium supplemented with 9 % of maltose (M9); 9 % of maltose and 7 % of polyethylene glycol 3350 (PEG) (M9P7); 9 % of lactose (L9) or 9 % of lactose and 7 % of PEG (L9P7). **c** Polyamine ratio in the proembryogenic stages. **d** Polyamine ratio in early embryogenesis. Presented values are mean percentage ± standard error of three replicates of total of PA and PA ratio of each treatment ($n = 3$). Means followed by the *same letters* are not significantly different, according to the SNK test ($P < 0.05$). **a** $CV_{Put} = 4.82$; $CV_{Spd} = 6.78$; $CV_{Spm} = 6.55$. **b** $CV_{Put} = 19.68$; $CV_{Spd} = 19.79$; $CV_{Spm} = 27.07$

the lowest PA ratio (Fig. 7d). In these prematuration treatments, an increase in the Spd and Spm levels was observed, resulting in a decrease in the PA ratio.

Discussion

Current hypothesis about SE of *A. angustifolia* proposes that low numbers of early somatic embryos are ascribed to disturbances in the transition from PEM III-to-early somatic embryos. These hypotheses are based entirely on previous work with this species where the prematuration step was not considered in the regenerative protocol (Steiner et al. 2005, 2008; Steiner 2009) as compared to *P. abies* SE (Filonova et al. 2000; von Arnold et al. 2002). During the prematuration step in *P. abies*, PEM III-to-early somatic embryos transition was correlated with developmental decisions in late embryogenesis (Filonova et al. 2000). To verify whether these models also apply to SE in *A. angustifolia*, we have included the preculture and prematuration step in this paper, which has allowed us to analyze the effect of them on PEM III-to-early somatic embryos transition and correlate the results with the content of endogenous IAA, ABA and PA at specific developmental stages.

In *A. angustifolia*, the proembryogenic stage was characterized by the proliferation of PEMs with variations in size and morphology (Steiner et al. 2005), ranging from PEM I, II and III (Steiner 2009). In *P. abies*, PEM I was composed of a small compact clump of ECs adjacent to a single SC showing a tendency to elongation; PEM II was similar to PEM I, but had more than one SC. Finally, PEM III consisted of enlarged clumps of ECs appearing loose, rather than compact, and showing disturbed polarity (Filonova et al. 2000).

FLD is a nonspecific inhibitor of de novo ABA synthesis (Kikuchi et al. 2006). In the present work, the preculture of embryogenic culture with FLD increased the frequency of PEM III as compared to the control (Fig. 3a). To the best of our knowledge, this is the first report showing positive effect of FLD on the PEM progression in conifers. Furthermore, preculture of embryogenic culture with FLD

decreased the levels of IAA and ABA. However, ABA was not completely depleted in the embryogenic culture, and this threshold seems to play an important role in the transition from PEM III-to-early somatic embryos. In angiosperms, FLD showed a negative effect on the development of early somatic embryos as a result of the marked decrease of ABA levels in the embryogenic cultures (Kikuchi et al. 2006; Su et al. 2013).

In the present work, the decrease in ABA levels in response to FLD enhanced the transition of PEM III-to-early somatic embryos. This suggests that ABA endogenous levels in embryogenic culture before FLD treatment were detrimental to this transition. The results of the present work were confirmed by morphological analyses of *P. abies* PEM where the collapse of PEM I and II occurred when the cultures were directly submitted to ABA treatment (Filonova et al. 2000). Thus, in conifers, the responsiveness of PEMs to ABA seems to be related to the developmental stage. These results show the importance of the ABA in the early developmental stages of conifer SE; however, the threshold level of ABA is yet to be determined

In the prematuration treatments, along the transition from PEM III-to-early somatic embryos, an increase of IAA and ABA levels was observed, mainly in response to maltose (Fig. 5b). In plants, IAA is involved in the establishment of the polar axis plan, as well as in the patterning of plant body, and in *P. abies*, ABA plays a key role in the transition of PEM III-to-early somatic embryos (Filonova et al. 2000). It seems likely that ABA is required for spatial auxin response and the initiation of somatic embryos through both auxin biosynthesis and its polar transport (Su et al. 2013).

Even during early embryogenesis, the prematuration treatments with PEG improved the polarization and individualization of early somatic embryos (Figs. 2d–f, 3). In plants, PEG is known to simulate drought stress by the water potential in a manner similar to that of soil drying (Larher et al. 1993). The osmotic stress caused by PEG induced normal development, accumulation of storage nutrients, and desiccation tolerance in *P. glauca* somatic embryos (Attree et al. 1991, 1992). According to Stasolla et al. (2003), PEG affects the initial phases of embryonic development, possibly by altering the responsiveness of the tissues to IAA and ABA. Tissue responsiveness to ABA is essential for the establishment of the embryonic body plan, which occurs through the coordination of an apical-basal and radial growth (Stasolla et al. 2003). These authors revealed an increase in the activity of the *ZLL* gene in the early developmental stages of PEG-treated somatic embryos. This gene was associated with the maintenance of stem cell identity within the shoot apical meristem of *A. thaliana* in an undifferentiated state (Moussian et al. 1998).

In the present work, lactose and maltose plus PEG improved the development of early somatic embryos. Lactose was shown to enhance SE in *Citrus* spp (Tomaz et al. 2001; Kayim and Koc 2006), as well as in the conifer *Abies alba* Mill. (Schuller and Reuther 1993; Schuller et al. 2000). Therefore, we tested the effect of this sugar on the prematuration step of *A. angustifolia* embryogenic culture in comparison with maltose. The early somatic embryos resulting from maltose plus PEG were larger than those resulting from treatment with lactose plus PEG (Fig. 4). This fact may be related to the absence of the lactase enzyme in plants. Button (1978) suggested that part of lactose is hydrolyzed during autoclaving, but the relatively strong growth and prolific organization of *Citrus* spp ovular callus led the author to suggest that the necessary enzyme systems existed for its utilization. In *A. abies*, the maturation of somatic embryos was attributed to the effect of lactose combined with a deficiency in carbohydrate supply (Schuller and Reuther 1993; Schuller et al. 2000) in that the analytical data of the lactose uptake and metabolism in somatic embryos at different stages revealed lactose uptake without metabolic degradation (Schuller and Reuther 1993).

The morphology of *A. angustifolia* embryogenic culture was associated with high and low levels of Put and Spd/Spm, respectively (Figs. 6, 7). Furthermore, the increase in the contents of Put and Spm in response to FLD (Fig. 7) was associated with increases in the frequency of PEM III. In this same species, addition of Spm to the culture medium increased the size and number of PEM III (Dutra et al. 2013). These authors also showed that Spd and Spm promoted the transition of PEM I to PEM III stage. Steiner et al. (2007) showed increased growth of *A. angustifolia* embryogenic cultures when Put was added to the culture medium. The role of Put in cellular growth is not yet totally elucidated (Silveira et al. 2004a), but according to Feirer (1995), Put stimulated cell division in embryogenic cultures of *P. oocarpa* and *P. patula*, as well as the initiation of embryogenic cultures in *P. gerardiana* (Malabadi and Nataraja 2007). In accordance with our results (Fig. 7b), increased levels of Spd may be considered as a marker of SE competence in several species, such as *Oryza sativa* (Shoeb et al. 2001), *Medicago sativa* (Huang et al. 2001) and *Panax ginseng* (Monteiro et al. 2002). In *Daucus carota* cell cultures, only Put sustained cell proliferation, but Spd and Spm were essential in promoting differentiation (Galston and Kaur-Sawhney 1990). Furthermore, in *A. angustifolia*, PA supplemented with a PGRs-free BM medium increased the endogenous IAA and ABA levels, showing a direct relationship between PA levels and ABA accumulation (Steiner et al. 2007).

The results of the present work also showed that the PA ratio influenced the transition from PEM III-to-early

somatic embryos. In *A. angustifolia* early zygotic embryos, the PA ratio was higher during early seed developmental stages, corresponding to cellular multiplication and elongation, and then a reduction in the final stages, corresponding to stabilization of the dry matter content (Astarita et al. 2003). In the present work, values of PA ratio in response to the treatment with maltose and PEG were similar to those observed by this author.

Conclusion

In the present work, the inclusion of a preculture step and prematuration treatments was essential for the PEM III-to-early somatic embryos transition throughout the whole early developmental process. Furthermore, FLD preculture promoted the differentiation of PEM I and II to PEM III, and this enhanced the responsiveness of embryogenic culture to the prematuration treatments with PEG, maltose and lactose. Also, in the proembryogenic stage, FLD decreased the endogenous levels of ABA and IAA, but increased PA levels, mainly Put. This suggests the existence of a threshold of these substances, especially ABA and IAA, in the PEMs as a requirement for subsequent progress to the early embryogenesis stage. We propose that the regulation of the transition from PEM III-to-early somatic embryos is dependent on a reduction in the ABA levels in the proembryogenic stage. In early embryogenesis, this transition is enhanced by prematuration treatments with maltose or lactose plus PEG, along with an increase in the levels of IAA and ABA and a decrease of PA levels, mainly Put. The content of Put decreased, while the contents of Spd and Spm increased during the prematuration phase, leading to a reduction of PA ratio and enhancing transition from PEM III-to-early somatic embryos. This strategy overcomes a major bottleneck in the protocol of SE in *A. angustifolia* associated with the transition from PEM III-to-early somatic embryos.

Based on our results, we propose a hypothetical model to somatic embryogenesis in *A. angustifolia* (Fig. 8). This schematic representation of the modulation encompasses two cycles. The Cycle A included induction, proliferation, FLD preculture and prematuration. The first step of the trigger in PEM III-to-early somatic embryos transition is the withdrawal of PGRs of culture medium following by FLD preculture, which proved to be efficient in the endogenous changes of IAA, ABA and PA levels. After, cultures submitted to pre maturation step in the presence of maltose or lactose and PEG began to polarize resulting in the individualization of early somatic embryos. In the cycle

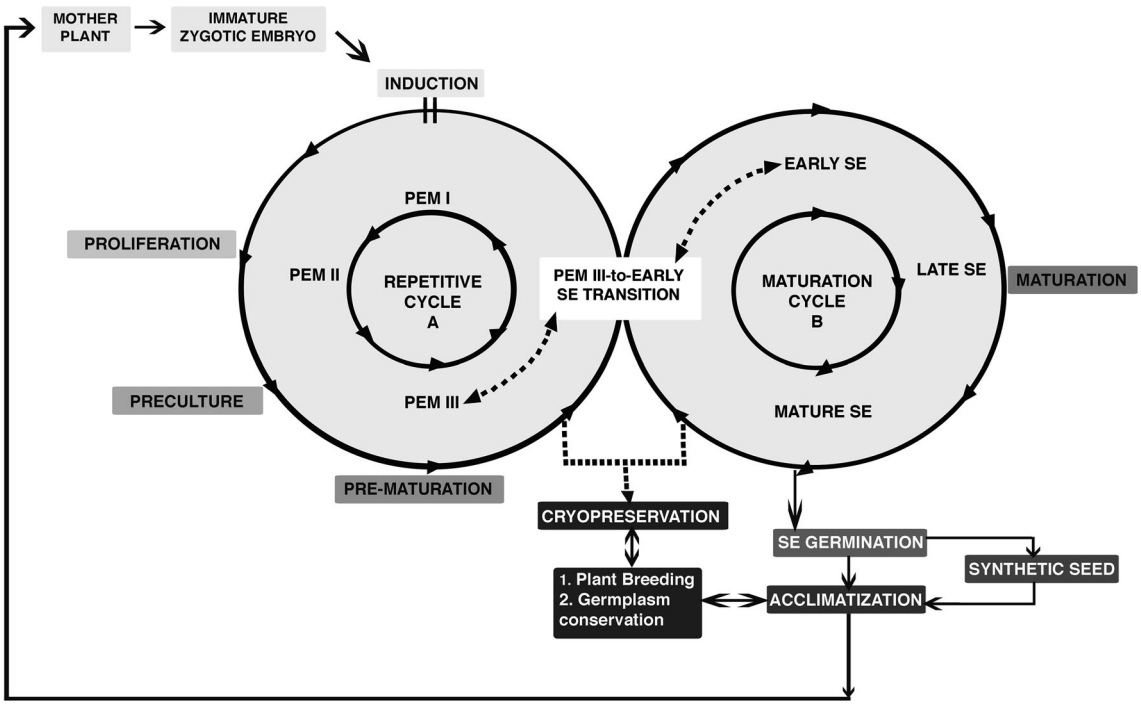

Fig. 8 Schematic representation of the modulation of somatic embryogenesis of *A. angustifolia*. The repetitive Cycle A comprises the induction, establishment and proliferation of embryogenic culture (Steiner et al. 2005). Based on our results, we propose in this Cycle the inclusion of preculture and prematuration steps, as a trigger of PEM III-to-early somatic embryos transition. The maturation Cycle B comprised the conditions required for the progression and maturation of the somatic embryos. After that, the mature somatic embryos can be germinated. The cryopreservation can be used in the two Cycles, both as strategy of conservation or plant breeding

B, starts the maturation step, where early somatic embryos are able to develop in late somatic embryos. At this stage should occur an intensive histogenesis including establishment of the root and shoot meristems. Based on this model, future efforts should be employed to develop an efficient method of maturation of early somatic embryos. The somatic embryos obtained can be germinated or, alternatively, encapsulated into synthetic seeds. Cryoconservation can be used in both cells from Cycle A and somatic embryos from Cycle B, enabling, in this manner, the integration of the technique into breeding and/or germplasm conservation programs.

Author contribution Cell culture and morphological analysis were performed by Francine L. Farias-Soares, Neusa Steiner, Éder C. Schmidt, Maria L. T. Pereira, Gladys D. Rogge-Renner, Zenilda L. Bouzon and Miguel Pedro Guerra. Biochemistry analyses were performed by Francine L. Farias-Soares, Neusa Steiner and Eny S. I. Floh. Writing was performed by Francine L. Farias-Soares, Neusa Steiner, and Miguel P. Guerra.

Acknowledgments This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) and Fundação de Apoio à Pesquisa Cientifica e Inovação Tecnológica do Estado de Santa Catarina (FAPESC). This study is part of the Ph.D. thesis of the first author.

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