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CELLULAR AND MOLECULAR BIOLOGY

Embryogenic cultures and somatic embryos development from mature seeds of jabuticaba (*Plinia cauliflora* (Mart.) Kausel)

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Abstract: *Plinia cauliflora* is an important Brazilian species that produces highly appreciated fruits, with a great potential of commercialization. However, the high cost of seedlings is a bottleneck for the expansion of commercial orchards. The present study aimed to investigate somatic embryogenesis as a propagation method for *P. cauliflora* using seeds as explants. To induce embryogenic mass (EM) and somatic embryo (SE) development we evaluated the supplementation of culture medium with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), combined or not with activated charcoal (AC). For the embryo maturation, we investigated the effects of AC, polyethylene glycol (PEG), Gelzan®, 6-benzylaminopurine and gibberellin supplementation. For the EM induction, the best results were obtained in MS culture medium supplemented with 300 µM 2,4-D and 1 g L^{-1} AC. During the first maturation phase, the supplementation of 30 g L^{-1} PEG improved the somatic embryo formation at the torpedo and cotyledonary stages, whereas the maturation treatments did not result in the conversion of the embryos into plantlets. The anatomical analysis showed that the 2,4-D presence for 60 days may have been deleterious for embryonic development. These results represent the first report of *P. cauliflora* somatic embryogenesis and its feasibility for mass propagation.

Key words: 2,4-Dichlorophenoxyacetic acid, auxin, micropropagation, Myrtaceae, osmotic stress.

INTRODUCTION

The *Plinia* (ex *Myrciaria*) genus is a member of the Myrtaceae family and contains several species and hybrids such as *Plinia peruviana*, *P. jaboticaba* and *P. cauliflora*, among others, popularly known as jabuticaba. The fruits present great potential for commercialization, being greatly appreciated for *in natura* consumption (Balerdi et al. 2006). In addition, the fruit skin has a high content of flavonoids and anthocyanins (Danner et al. 2011), which can be used by the pharmaceutical and food industries.

This fruit tree can be an alternative for plantation, especially for small family farmers. However, the high cost of seedlings is a bottleneck for the expansion of *Plinia* sp. commercial orchards. Considering the long period that this species needs to enter the reproductive phase, which oscillates between 8 and 15 years after seed germination, the use of vegetative propagation techniques could be interesting. These techniques can anticipate its reproductive period, directly contributing to a more representative economic exploitation (Sasso et al. 2010).

Even considering the progress obtained in the asexual propagation of *Plinia* sp. (Duarte et al. 1997, Danner et al. 2006, Sasso et al. 2010),

the main method for obtaining seedlings is still through seeds, since the rooting rate of the cuttings is low (Scarpare Filho et al. 1999, Sasso 2009). Despite the success of sexual propagation, the seeds of *Plinia* sp. could rapidly lose their viability (Valio & Ferreira 1992) and are characterized as recalcitrant seeds.

When compared to any other propagation process, somatic embryogenesis offers several advantages such as high multiplication rate, possibility of production staggering, no-tillage of the obtained seedling, no need of grafting and lower production cost. For these reasons, somatic embryogenesis has been used as a biotechnological tool in studies of plant development, clonal propagation, and plant breeding (Carvalho et al. 2006).

Several studies focusing on explant disinfestation and *in vitro* establishment have been reported for fruit trees of the Myrtaceae family such as *Acca sellowiana* (O.Berg) Burret and *Eugenia uniflora* L. (Souza et al. 2006b), *Psidium cattleianum* Sabine (Souza et al. 2006a) and *Plinia* sp. (Picolotto et al. 2007). Protocols of somatic embryogenesis have been developed for *Psidium guajava* (Kamle & Baek 2017) and *Psidium cattleianum* (Freire et al. 2018).

The best described somatic embryogenesis protocols for Brazilian native species belonging to the Myrtaceae family were developed for *Acca sellowiana* (Cruz et al. 1990, Canhoto & Cruz 1996, Cangahuala-Inocente et al. 2007, Pescador et al. 2008, 2012, Fraga et al. 2012). Several of these studies focused on morphoanatomical, biochemical, and molecular studies of the somatic embryos (SE) obtained. Studies of the somatic embryogenesis process in *Plinia peruviana* have already been performed in our research group (Silveira et al. 2020). However, similar studies investigating this process in *Plinia cauliflora* have not been reported yet.

Therefore, studies focusing on an efficient technique for *in vitro* establishment of *P. cauliflora* explants and on the best conditions for embryogenic masses (EM) and SE formation and development are necessary. The present study reports, for the first time, the different phases of *P. cauliflora* somatic embryogenesis using mature seeds as initial explants until the formation of somatic embryos at different stages. Morphoanatomical analyses were combined with somatic embryogenesis protocol development at each phase of the process.

MATERIALS AND METHODS

Plant material

The somatic embryogenesis experiments were performed at the Laboratory of Plant Micropropagation and the anatomical analyses at the Laboratory of Anatomy and Biomechanics of the Department of Botany, Biological Sciences Center, Federal University of Parana, Curitiba-PR, Brazil. Ripe fruits of *P. cauliflora* were collected from a tree located at Vitorino-PR, Brazil (52º46' W; 26º19' S; altitude of 820 meters). The fruits were transported to the laboratory in a styrofoam box containing ice and manually pulped.

Surface disinfection procedures and in vitro introduction

The seeds were submitted to a superficial sterilization with 70% ethanol for 1 min, followed by immersion in 5% sodium hypochlorite plus 0.01% Tween-20® (10, 15 and 20 min). Afterwards, the seeds were rinsed three times in sterile distilled water. Then the seeds were opened and the cotyledons were separated and individually introduced together with the embryonic axes (the seeds are polyembryonic) into test tubes (15 x 2.5 cm) (one cotyledon per tube), containing 10 mL of culture medium.

In vitro culture conditions

The basal culture medium consisted of the MS salts and organic compounds (Murashige & Skoog 1962), supplemented with 30 g L^{-1} of sucrose and gelled with 0.6% agar (Vetec®), except otherwise stated. Plant Preservative Mixture™ (PPM) (1 mL L^{-1}) was supplemented or not to the culture medium prior to autoclaving. The pH of all culture media was adjusted to 5.8 and the gelling agent was added prior to autoclaving at 121 °C for 20 min.

During the SE induction and the first stage of maturation, cultures were maintained in the dark at 25 ± 2 ºC. During the second phase of maturation, the cultures were maintained at the same temperature, under cool white fluorescent light with a photosynthetic photon flow density of approximately 30 $\mu mol.m^{-2}.s^{-1}$ and a 16 h photoperiod.

Induction of EMs

In the first experiment, the basal culture medium was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 5, 10 or 15 µM and 1 mL L^{-1} of PPM™. In the second experiment, the culture medium was supplemented with activated charcoal (AC) (0.5; 1 or 2 g L^{-1}), 2,4-D (100, 200, 300 or 500 µM) and 3 mL L^{-1} of PPM™.

Forty cotyledons (with embryonic axes) were used per treatment and cultured in test tubes. Sixty days after inoculation, the percentage of oxidized explants and explants forming EM were evaluated.

First phase of somatic embryo maturation

After 60 days of induction, the EMs were subcultured in Petri dishes containing the basal culture medium supplemented with AC (1 g L^{-1}) or PEG-4000 (30, 60 or 90 $g.L^{-1}$). Cultures were maintained in the dark at 25 ± 2 °C.

After 30 days of culture, the oxidation and the percentage of EMs bearing SE at each stage (globular, torpedo and cotyledon) were evaluated. Forty tubes were used per treatment.

Second phase of somatic embryo maturation

Torpedo embryos obtained in the previous phase were maintained under light. Four experiments were implemented, evaluating the effect of the following compounds: AC (1 g L^{-1}) or PEG-4000 (30 g L^{-1}), Gelzan® (2, 2.5 or 3 g L^{-1}) instead of agar, 6-benzylaminopurine (BAP) (2, 4 or 8 µM) and combinations of BAP (0.5 µM) and gibberellic acid (GA_3) (1 or 2 µM). GA_3 was filtrated on a 20 µm filter and added to the culture medium after autoclaving.

The cultures were evaluated after 30 days of culture for partial oxidation, survival (green color) and conversion to plantlets (radicle emission and shoot formation).

Experimental design and statistical analysis

In all experiments, the experimental design was completely randomized. For the induction experiments 40 test tubes (16 x 150 mm) with explants were used per treatment. For the maturation experiments, 5 embryos were inoculated per Petri dish (10 x 2 cm) and 10 dishes per treatment, reaching a total of 50 embryos per treatment.

A bifactorial analysis was applied to the data of the experiments of EM induction. The data obtained were submitted to Bartlett´s test to verify the homogeneity of the treatment variances; then all data were transformed by $\sqrt{(x + 1)}$. The means of each treatment were compared by Tukey´s test at 1 and 5% probability. The statistical software used was Assistat, version 7.7pt. (Silva & Azevedo 2009).

Anatomical studies

Three samples of each phase (induction and subsequent phases of maturation) were collected for anatomical studies. The samples were fixed in FAA_{70} (Johansen 1940) and dehydrated in increasing ethanol series. The samples were included in methacrylate according to the manufacturer's recommendations (Leica Historesin®). The embedding material was sectioned in a rotary microtome at 10 µm and stained with toluidine blue (O'Brien et al. 1964). Temporary slides were assembled with water and analyzed under a light microscope. The photographs were taken with an Olympus SC30 camera attached to an Olympus BX41 microscope.

The experimental design was carried out with three samples of each phase of development, and, for each sample, ten permanent slides were made with twelve cuts in each slide.

RESULTS

Seed surface disinfection

The best treatment to avoid contamination was PPM supplementation to the culture medium. The percentages of contamination were lower in PPM treatments, without statistical differences between them but the longer exposure time to sodium hypochlorite induced the highest oxidation percentages (data not shown). Therefore, the recommended disinfection treatment is ethanol 70% for 1 min followed by 5% NaClO for 10 min and 1 mL L^{-1} PPM in the culture medium.

Embryogenic masses and somatic embryo formation

EM formation started 10 days after the inoculation of the cotyledons and embryonic axes *in vitro*. The best treatment for EM induction was the addition of 15 µM 2,4-D to the culture medium. Despite the high EM formation (70%), the oxidation of these masses was also high, and reached 52.5% in the 15 µM 2,4-D treatment (data not shown). After 60 days in culture, EMs from 2,4-D-supplemented treatments showed an asynchronous formation of SE, with different embryo stages in the same EM (Figure 1- c).

In media supplemented with a combination of 2,4-D and AC, we observed that EM formation preceded SE development in globular, heart-shaped, torpedo and cotyledonary stages (Fig. 1b, c, d). However, the time required for the onset

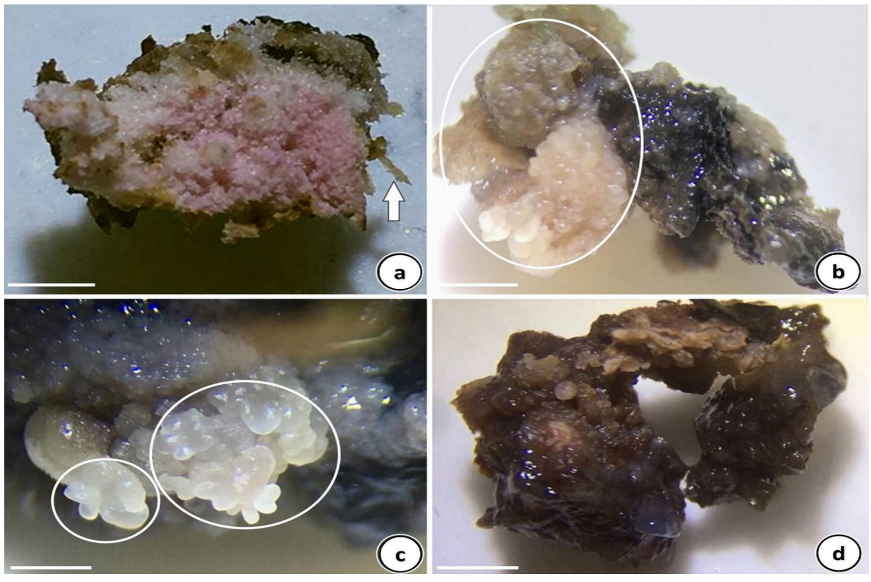

Figure 1. Embryogenic cultures of *Plinia cauliflora* 60 days after introduction in MS culture medium. (a) Formation of masses and roots (arrow) in the culture medium containing 100 µM 2,4-D and 1 g L^{-1} activated charcoal; (b) initiation of embryogenic mass formation (circled in white), in the culture medium containing 200 µM 2,4-D and 1 g L^{-1} activated charcoal; (c) somatic embryos (circled in white) obtained in the presence of 300 µM 2,4-D and 1 g L^{-1} activated charcoal; (d) oxidized explant in the culture medium containing 500 µM 2,4-D and 0.5 g L^{-1} activated charcoal. Bar: 0.1 cm.

of EM formation was increased from 10 days to 20 days when AC was supplemented, in average.

Comparing the "AC" and "2,4-D concentration" factors and their effects on oxidation, no significant interaction between them was observed (Table I). However, when analyzed separately, the differences between treatments were significant. A direct relationship between increasing 2,4-D concentration and oxidation rates was observed. In the case of AC concentrations, there were no significant differences between the treatment with 1 and 2 g L^{-1} of AC (Table I). For EM formation, the interaction between the factors AC and 2,4-D was significant. The highest rate of EM formation was 82.5% (Table I) in culture medium containing 300 µM 2,4-D and 1 g L^{-1} AC (Figure 1-c) but did not differ from the rate obtained with 200 µM 2,4-D and 1 g L^{-1} AC and the oxidation, in turn, was less than 3%.

First phase of somatic embryo maturation

For the initiation of SE maturation in the dark (Figure 2a), the masses were transferred to a culture medium supplemented with AC (1 g L^{-1}) or PEG-4000 (30, 60, 90 g L^{-1}).

As previously mentioned, SE formation in *Plinia cauliflora* is asynchronous (Fig. 2b, c and d). For the success of the first maturation phase, it is desirable that the embryos reach the torpedo stage, since maturation continues from that stage in the presence of light.

After 30 days in these culture media, most SE were in the torpedo stage (Fig. 2c), except in the AC-supplemented treatments. The highest percentages of torpedo embryos (77.5 and 65%) were obtained in the treatment with 30 g L^{-1} and 60 g.L^{-1} PEG, respectively (Table II, Figure 2). Therefore, a concentration of 30 g L^{-1} PEG could be used with a good formation of torpedo embryos and at a lower cost.

Second phase of maturation and conversion of somatic embryos

Effect of PEG and activated charcoal

The torpedo embryos obtained in the first phase of maturation were transferred to new culture media for the final maturation.

No statistical difference was observed among the results obtained in the culture media supplemented with AC or PEG (30 g L^{-1}), either

Table I. Oxidation and embryogenic mass formation in *Plinia cauliflora* seeds cultured in MS culture medium, according to 2,4-D and activated charcoal concentration, 60 days after *in vitro* introduction.

ACTIVATED CHARCOAL (g.L^{-1})	OXIDATION^{ns}(%)					EMBRYOGENIC MASSES**(%)				
	2,4-D (µM)									
	100	200	300	500	Averages	100	200	300	500	Averages
0.5	6.07	8.35	9.07	9.65	8.28a	17.5aB	57.5aA	37.5bAB	17.5aB	32.5a
1.0	2.23	2.25	2.43	5.66	3.39b	17.5aC	45.0abAB	82.5aA	30.0aBC	43.75a
2.0	1.41	2.64	3.25	5.81	3.28b	20.0aA	30.0bA	47.5bA	30.0aA	31.87a
Averages	3.24c	4.75b	4.9b	7.04a		18.33b	44.16a	55.83a	25.83b	
CV (%)	38.52					41.88				

Means followed by the same capital letter in the row and lowercase in the column do not differ by Tukey´s test at 1% probability. ** Significant at 1% probability level (p <0.01), by Tukey´s test. ^{ns} Not significant (p ≥0.05).

for oxidation or for survival (Figure 3a). There was no embryo conversion into plant after 60 days and approximately 30% of the embryos remained green in the presence of light (Figure 3-a).

Effect of Gelzan®

No statistical difference regarding SE oxidation was observed among the media gelled with different concentrations of Gelzan® (2, 2.5 or 3 g L^{-1}). Survival was low, with the highest averages (18 to 25%) obtained in the culture medium supplemented with 2.5 or 3 g L^{-1} (figure 3b). No somatic embryo conversion could be observed in these treatments.

Effect of BAP alone or combined with GA3

Embryos grown in media containing BAP (2, 4 or 8 µM) (Figure 3c) had a high percentage of oxidation (> 50%). The higher the BAP concentration, the greater the oxidation, so survival also decreased with increasing BAP concentration, with the highest mean value of survival (23.33%) at 2 µM BAP (Figure 3c). When the SEs were transferred to culture media supplemented with GA_3 (1 or 2 µM) combined with 0.5 µM BAP, there was no significant difference between the means of oxidation and survival (Figure 3d). In the presence of GA_3 and BAP the survival of somatic embryos was less than 3%.

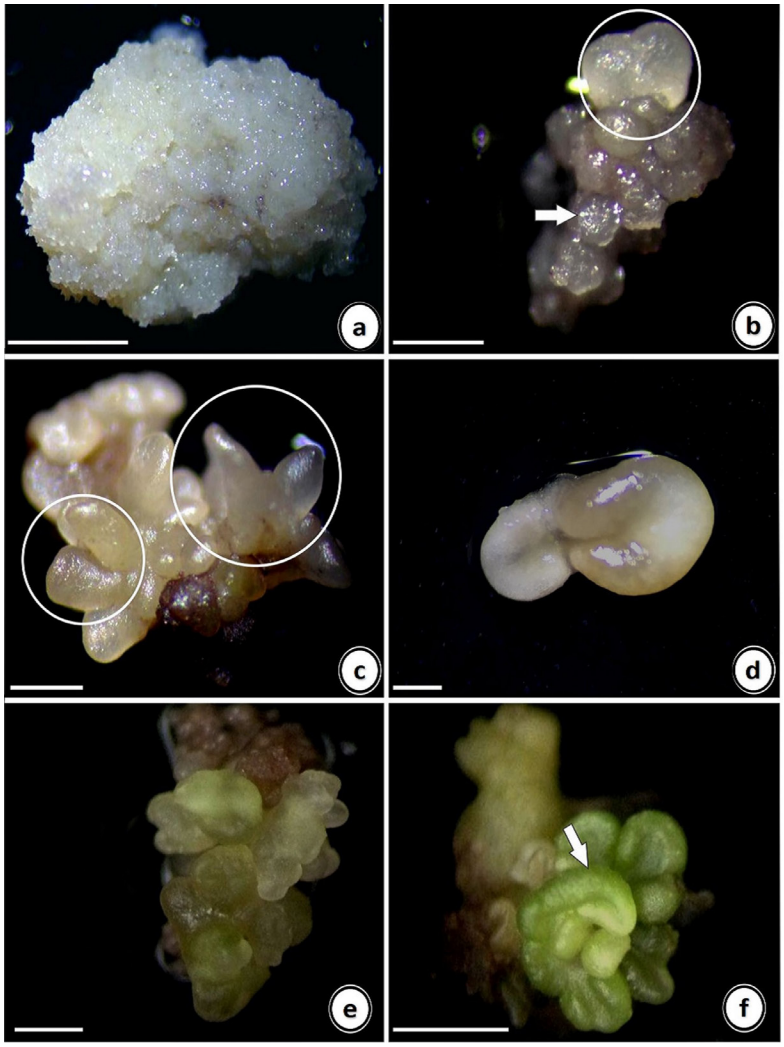

Figure 2. Somatic embryos of *Plinia cauliflora*, 30 days after transfer of the embryogenic masses into the maturation culture media. (a) Embryogenic mass in multiplication; (b) formation of globular (arrow) and heart (circle) embryos in the culture medium containing 30 g L^{-1} PEG-4000; (c) torpedo embryos (circles) in the culture medium with 60 g L^{-1} PEG; (d) fused cotyledonary embryos in 90 g L^{-1} PEG; (e) asynchronous maturation of somatic embryos under light; (f) cotyledonary embryos fused under light. Bar: 1 cm.

Table II. Effect of polyethylene glycol (PEG-4000) and activated charcoal (AC) on the somatic embryo formation in embryogenic masses from *Plinia cauliflora* seeds after 30 days in culture.

Treatment	Oxidation^{ns} (%)	Globular stage* (%)	Torpedo stage* (%)	Cotyledon stage ^{ns} (%)
1 g.L^{-1} CA	2.5 a	50.0 a	20.0 c	1.0 a
30 g.L^{-1} PEG	5.0 a	18.0 b	77.5 a	3.05 a
60 g.L^{-1} PEG	12.0 a	20.0 b	65.0 ab	2.23 a
90 g.L^{-1} PEG	15.0 a	27.5 b	50.0 b	2.43 a

Means followed by the same letter do not differ by Tukey´s test. * Significant by Tukey´s test at 1% probability level (p <0.01). ^{ns} Not significant (p> = 0.05).

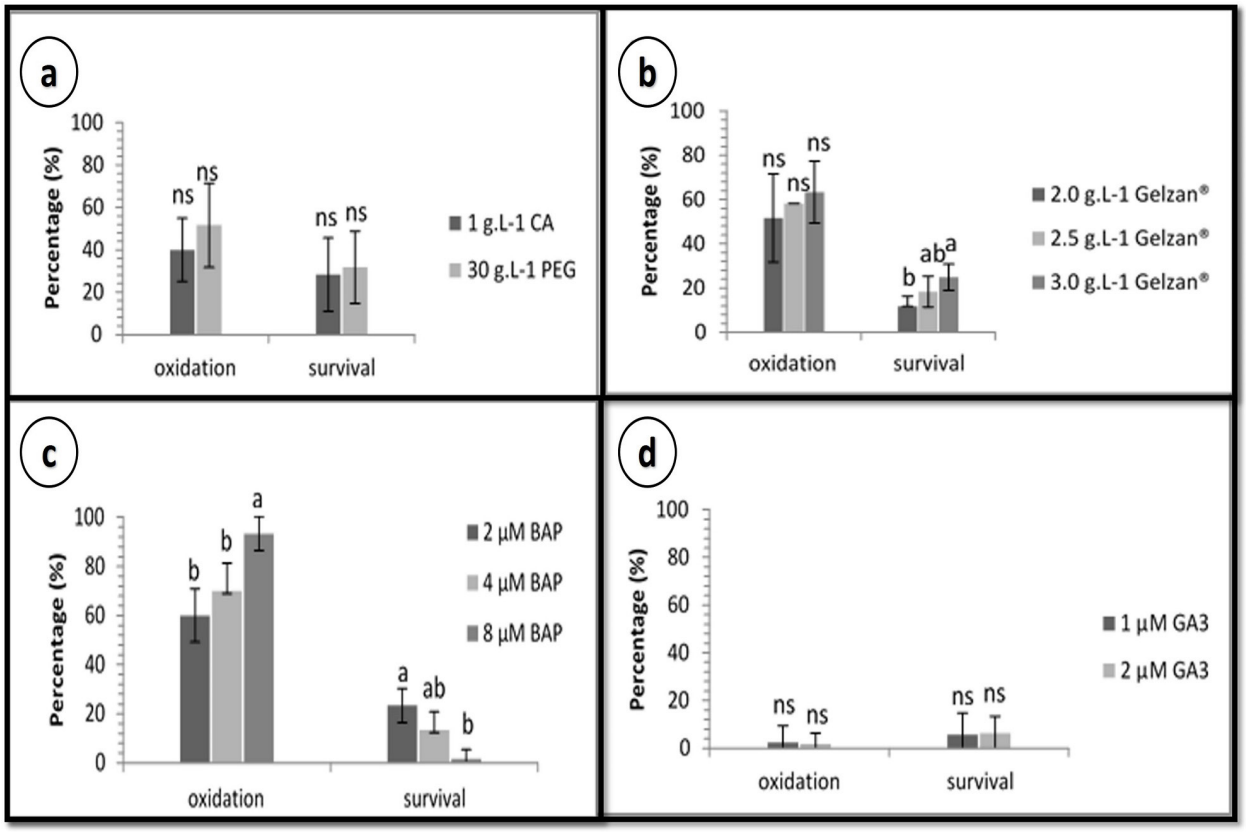

Figure 3. Oxidation and survival of somatic embryos of *Plinia cauliflora* cultivated in MS medium for 30 days in function of the addition to the maturation culture medium of AC or PEG (a), Gelzan (b), BAP (c) and BAP (0.5 µM) with GA_3 (d). Columns with the same letter do not differ significantly by Tukey´s test at 5% probability.

Anatomical analyses

During the induction phase, the formation of the EMs was observed, with cicatrization tissue between the explant and the masses (Figure 4a). Through the histochemical tests, it is also possible to visualize phenolic compounds, stained in dark blue, in areas of cell death or in the cicatrization tissue (Figures 4a, c and h).

In the EMs cultured in an AC-free culture medium (Figure 4b), cell division was observed.

The cells were well-vacuolated and no other meristematic characteristics such as dense cytoplasm were observed. When these EMs were cultured in a medium containing AC, embryos began to develop, with a conspicuous procambium and without the thickening of xylem cell walls (Figure 4c and d).

As mentioned earlier, the formation of the somatic embryos of *P. cauliflora* during the first phase of maturation occurred in an asynchronous way, i.e. there were several stages of development (globular, torpedo, cotyledonary) at the same time (Figure 4e).

During the maturation phase, the somatic embryos survived and had a differentiated vascular system, with the conspicuous thickening of the xylem wall (Figure 4f). However, the formation of the apical meristem was interrupted by a phenolic zone, as shown in the detail of Figure 4e. In the torpedo embryo (Figure 4g), a procambium was formed, as well as a well-defined epidermis.

DISCUSSION

Based on our results, it can be concluded that an efficient disinfection procedure for *in vitro* introduction of seeds was established using 70% ethanol (1 min) + 5% NaClO (10 min) and supplementing the culture medium with 1 ml.L^{-1} PPM.

During SE induction, the cells of the explants are first dedifferentiated and then redifferentiated into specific cells, acquiring embryogenic competence. Auxins are responsible for mediating this transition from somatic cells to embryogenic cells and for reprogramming the genes involved in embryogenesis (Yang & Zhang 2010). 2,4-D is a synthetic compound analogous to auxins, commonly used to induce the somatic embryogenesis process for several species (Isah 2016). In the present study 2,4-D was added to the culture medium and induced the formation of EMs. After 60 days on such a medium, EMs and SEs at different stages were visible (Figure 4e). In Myrtaceae species other authors also observed asynchronous development of SEs, for instance in *Myrciaria aureana* (Motoike et al. 2007) and *Psidium guajava* (Nasim 2010).

The addition of AC along with 2,4-D to the culture medium was also tested. In addition to the delay in the formation of EMs when AC was added to the culture medium, the percentage of oxidation was significantly reduced and there was an increase in the percentage of formation of EMs. AC is commonly used in tissue culture to darken culture media and adsorb compounds that are inhibitory or toxic to plant tissues (Moshkov et al. 2008). For example, it was used at all stages of somatic embryogenesis of *Pinus taeda* to increase initiation frequencies (Pullman & Johnson 2002), to improve yield and quality of somatic embryos during maturation (Capuana & Debergh 1997, Caraway & Merkle 1997, Pullman et al. 2005, Lelu-Walter et al. 2006) and more frequently during germination of somatic embryos (Vooková & Kormuák 2001, Salaj et al. 2004, Andrade & Merkle 2005). However, it had a negative effect on the maturation of *Myrciaria aurea* somatic embryos (Motoike et al. 2007).

The promoter or inhibitory role of AC depends on several factors. It is believed that the nonselective adsorption of some compounds, especially plant growth regulators present in the culture media, often influences the response. Furthermore, it is not clear which compounds naturally present in AC are released in the culture medium. Hence it is necessary to focus more studies on the exact mechanism of action of AC in plant tissue culture (Thomas 2008).

During the first phase of embryo maturation, the presence of 30 g L^{-1} PEG-4000 in the culture medium favored the maturation of SE until the torpedo stage. Similarly PEG-3350 (50 g L^{-1})

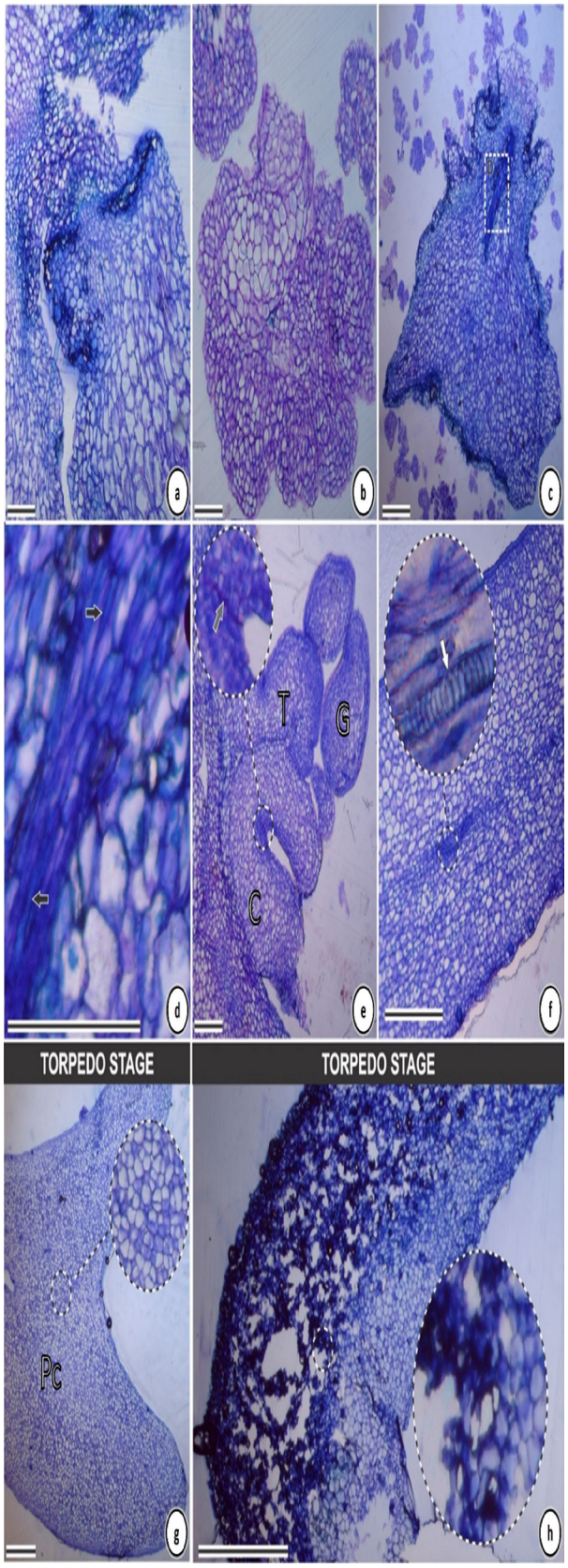

Figure 4. Histology of EMs and somatic embryos of *Plinia cauliflora*. (a) Formation of EMs from seeds in the medium containing 15 µM 2,4-D. (b) In the medium containing 2,4-D, without activated charcoal. (c) In the medium containing 2,4-D and activated charcoal. (d) Detail of C, arrows indicate the elongated procambial cells. (e) Somatic embryos during maturation, in several stages: G = globular, T = torpedo, C = cotyledonary. Gray arrow indicates phenolic compounds in the apical meristem. (f) Globular embryo. White arrow: xylem cell wall thickening. (g and h) Torpedo embryo during the maturation phase, Pc = procambio. Details: healing zones and cell death in the region of the vascular elements (g and h). Bars: 50 µm.

stimulated the development and maturation of SEs of *Myrciaria aureana*, another Myrtaceae species (Motoike et al. 2007). Bajpai et al. (2016) used the same concentration for SE maturation of *Psidium guajava* and obtained 74.38% of cotyledonary embryos. Norgaard (1997) reported that supplementing the culture medium with PEG-4000 increased the SE formation yield for *Abies nordmanniana*. The use of PEG as an osmoticum in SE maturation media has proved effective in increasing germination and conversion (Capuana & Debergh 1997).

PEG is also reported to improve the quality of white spruce SE by promoting normal differentiation of the embryonic shoot and root (Stasolla et al. 2003). PEG molecules are too large to move through the cell wall and do not cause plasmolysis. Non-plasmolysing osmotic compounds are effective in promoting SE maturation (Linossier et al. 1997, Walker & Parrott 2001). Similarly, Rai et al. (2009) reported the use of PEG (0.5–2.0%) in guava (*Psidium guajava* L.) embryo maturation, resulting in 42.0 and 59.6% germination of mature somatic embryos, respectively.

In the present study, there was no conversion of embryos into plantlets. The survival rates for mature embryos did not exceed 25% when using gellan (Gelzan®), AC and PEG. Some

studies of conifer species have shown that the supplementation of culture media with gellan (Phytagel®) in the appropriate concentration had a positive effect on SE development and growth (Garin et al. 2000, Klimaszewska et al. 2000, Lelu-Walter & Pâques 2009, Teyssier et al. 2011, Morel et al. 2014). Hazubska-Przybył et al. (2016) stated that control of the osmotic pressure of the culture medium through varying concentrations of gellan gum also affected the accumulation of starch in SEs of *Picea* spp. When the concentration of Phytagel® increased from 4 to 8 g L^{-1}, the starch accumulation process was inhibited because of the difficulty of absorbing sucrose when osmotic pressure of the culture medium was rising.

In the present study, BAP alone and combinations of BAP and GA_3 were detrimental to the process and few embryos survived. For Myrtaceae, there are no records in the literature of tests with BAP alone during the maturation of somatic embryos as it is always combined with other regulators. In studies performed with torpedo and pre-cotyledonary embryos of *Acca sellowiana* obtained on a maturation culture medium containing glucose, the same combinations of BAP and GA_3 were added to LPm culture medium for conversion of the embryos into plants. In that case, the rate of conversion was 25.4%, after 15 days (Cangahuala-Inocente et al. 2007). The synergistic effect of cytokinin and gibberellin on the promotion of somatic embryo conversion into plantlets has also been reported in other species such as *Vitis* sp. (Zlenko et al. 2002), *Eryngium foetidum* (Martin 2004), *Centella asiatica* (Paramageetham et al. 2004), *Catharanthus roseus* (Junaid et al. 2007) and *Hoya wightii* (Lakshmi et al. 2013).

It is important to mention that the somatic embryos were translucent and showed secondary somatic embryo formation (Figure 2c), indicating that the 2,4-D present in the culture medium during the induction phase still affected the maturation phase of the embryogenesis process. Bajpai et al. (2016) also observed secondary SE in *Psidium guajava*, and concluded that this repetitive somatic embryo formation may be favorable to large-scale production and also to breeding programs, referring to the cloning of elite genotypes.

Once the embryogenic cells are formed, they continue to proliferate in the form of EMs. These masses can proliferate in a culture medium similar to that used for the initiation of cultures (Von Arnold et al. 2002). In some species, even without plant regulators, the masses continue to multiply. Auxins have an inhibitory effect on the maturation and development of SEs. Therefore, for maturation the masses need to be transferred to an auxin-free culture medium (von Arnold et al. 2002). The accumulation of reserve products occurring during maturation is necessary for the subsequent conversion of somatic embryos into plantlets (von Arnold et al. 2002). Protein synthesis and reserve accumulation are related to water stress (Zimmerman 1993).

In the present study, two days after transfer to light conditions, cotyledonary embryos acquired a greenish color (Figure 2e, f). However, after 30 days, none of them emitted radicles and shoots. The somatic embryos were transferred every 30 days to a fresh culture medium but even after 60 days they did not show any sign of conversion, remaining green throughout the period. This lack of conversion into plants could be related to morphophysiological abnormalities of the SEs (fused cotyledons or with more than two cotyledons) after exposure to 2,4-D for an extended period of time (60 days).

From seed explants, it was possible to obtain EMs in MS culture medium supplemented with 2,4-D (300 µM) and 1 g L^{-1} of activated charcoal. During the first maturation phase, the addition of 30 g L^{-1} PEG to the culture medium allowed

somatic embryo formation at the torpedo and cotyledonary stages, whereas the maturation treatments did not result in the conversion of the embryos into plantlets.

Studies of the anatomy of *P. cauliflora* SEs showed the reaction of the cells after their exposure to 2,4-D, resulting in cicatrization zones with accumulation of phenolic compounds and cell death. This tissue may have formed in response to the stress caused by the exposure to high concentrations of 2,4-D, which is a herbicide, and also by the long period of culture in the presence of this auxin under *in vitro* conditions (60 days). According to Tuffi Santos et al. (2009), the appearance of a cicatrization tissue in *Eucalyptus* clones submitted to glyphosate acts as a barrier that prevents the progression of necrosis to other regions of the leaf. The differentiation of this tissue results from the ability of plants to form new tissues from parenchyma cells (Dickison 2000). Moreover, phenolic compounds were present in the cicatrization tissues observed in the SE. Studies by other authors have shown that herbicides and pollutants may induce accumulation of phenols in plant cells (Sant'Anna-Santos et al. 2006, Tuffi Santos et al. 2008, 2009).

Similarly to our findings in experiments of EM induction in medium containing AC and 2,4-D, when analyzing the anatomy of SEs of *Acca sellowiana*, Fraga et al. (2012) observed dense meristematic formations with high cell division when globular somatic embryos were formed.

The experiments performed were important in showing that high exposure to a synthetic auxin (2,4-D) in the induction phase, despite the formation of somatic embryos, was detrimental to cells once the embryos formed were abnormal and showed zones of cicatrization with the presence of phenolic compounds. Further experiments are required in order to find the appropriate time of exposure of the explants to 2,4-D during the induction phase and to define the best conditions that lead to the maturation of somatic embryos and their conversion into seedlings.

It is important to emphasize that this study is the beginning of the development of a somatic embryogenesis process for a native Brazilian woody species, which presents difficulties of propagation, and that there is no report in the literature of a somatic embryogenesis protocol for this species.

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FLRO performed all experiments and wrote the first draft of the manuscript. FLRO, HPFF, MQ designed the experiments. FLRO, HPFF, JD performed in vitro experiments. FLRO, BFSS performed the anatomical analyzes. FLRO, BFSA, HPFF contributed to data analysis. MQ and HPFF supervised this study. All authors revised the manuscript and approved the submission.