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Somatic embryogenesis as an alternative for *in vitro* multiplication of *Butia odorata* from mature zygotic embryos

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Abstract: *Butia odorata* is a palm native to southern Brazil and Uruguay, not domesticated, much appreciated for its fruits and economic potential. However, the extractivism and the difficulty of propagation have led to the decline of natural populations. The objective of this work was to prove the possibility of induction of somatic embryogenesis in *B. odorata*. Mature zygotic embryos were induced in two media, MS and Y3, combined with auxin 2,4-D and picloram in five concentrations (2,4-D: 0, 361.99, 452.49, 542.99 and 633.48 µM/L, picloram: 0, 50, 150, 300 and 450 µM/L). The results promising during induction with the formation of embryogenic calli and somatic embryos, however the regeneration of them was not efficient, this may be due to the occurrence of somatic embryos fused during its development. The roots were formed, but the aerial part remained molten, not completing its development. Auxin picloram and Y3 medium provided the most adequate conditions for calogenesis, formation of embryogenic callus and somatic embryos, with concentrations of 150, 300 and 450 µM/L. This is the first description of somatic embryogenesis in *B. odorata* that will serve as the basis for future research and adjustments of the methodology proposed here.

Key words: Arecaceae, picloram, pindo palm, somatic embryo, 2,4-D.

INTRODUCTION

Butia odorata, also known as pindo palm, is a palm (Arecaceae) native to the Pampa biome in Brazil and Uruguay (Noblick 2011). The fruits, rich in phenolic compounds, carotenoids, anthocyanins, L-ascorbic acid, flavonoids, and fibers (Beskow et al. 2014), are typically consumed in natura or processed into jelly, sweets, juices, and ice creams. Native pindo palm populations are diminishing due to urban and agricultural expansion, and the inadequate management cattle ranching, which compromises the regeneration of populations due to trampling and grazing of the seedlings (Rivas & Barbieri 2014).

B. odorata is propagated exclusively by seeds, which present dormancy and uneven germination results in poor seedling production, thus hindering the effectiveness of conservation. Fior et al. (2011) and Schlindwein et al. (2013) suggested several alternatives to overcome dormancy, as opening the seed embryonic cavity or through dry and warm stratification. However, these methods are labor-demanding and require specific conditions. Plants remain juvenile for between six to 15 years (Barbieri et al. 2014), extending the amount of time required for conventional propagation. Also, pindo palm only have one apical meristem, which limits asexual propagation. In this context, tissue culture techniques can contribute significantly to the

propagation of the species, with a consequent reduction in the time of production of plantlets and optimization of the period between planting and production (Nguyen et al. 2015, Naik & Al-khayri 2016).

Somatic embryogenesis in these palm species has been induced from zygotic embryos with promising results in the formation of somatic embryos and conversion to plantlets. Zygotic embryos were found to be excellent explants for somatic embryogenesis induction. Protocols made for palms such as *Cocos nucifera* (Fernando & Gamage 2000), *Bactris gasipaes* (Steinmacher et al. 2007), and *Elaeis guineensis* (Balzon et al. 2013) showed promising success that could be applied to other palm species.

Somatic embryogenesis occurs through a morphogenic route that demonstrates high plasticity and totipotency of plant cells. In this route, somatic cells are induced to differentiate into embryogenic cells that undergo morphological and biochemical changes to produce somatic embryos at the end of the process (Quiroz-Figueroa et al. 2006). In fact, the explant cells undergo dedifferentiation acquire meristematic identity to form somatic embryos, regardless of the type of explant used, however depending on the imposed culture environment. Analysis of proteomes and transcriptomes has led to the molecular identification and functional characterization of many genes involved in the initiation and development of somatic embryos (Elhiti et al. 2013, Santos et al. 2018). These proteins are predicted to be involved in epigenetic regulation and expression of key genes (Mahdavi-Darvari et al. 2015). Most published somatic embryogenesis protocols for palm trees required high (generally above 200 µM) exogenous auxins, such as 2,4-D or picloram and activated charcoal to stimulate somatic embryogenesis induction (Steinmacher et al. 2007, Balzon et al. 2013). Guerra & Handro (1998) tested the auxins 1-Naphthalene acetic acid (NAA), picloram and 2,4-D, but only the concentrations of 226.24 and 454.48 µM of 2,4-D were effective for *Euterpe edulis*. For *E. oleracea*, Ledo et al. (2002) induced the formation of somatic embryos at concentrations of 339.36 and 454.48 µM of 2,4-D, while Scherwinski-Pereira et al. (2012) concluded that 225 µM picloram was the concentration that favored the greatest induction of embryogenic calli. The concentration of 600 µM of 2,4-D stimulated the production of embryogenic calli from zygotic embryo and plumule of *C. nucifera* (Pérez-Núñez et al. 2006).

In this context, the objective of this work was to induce somatic embryogenesis from mature zygotic embryos, to identify the factors related to the process and to provide information for the development of protocols for *in vitro* multiplication of *B. odorata*.

MATERIALS AND METHODS

Plant material and cultivation conditions

Mature *Butia odorata* fruits were collected from a wild open-pollinated population in the city of Barão do Triunfo, RS. The endocarps were broken with bench vises to isolate seeds and then disinfested in 70% ethanol for one minute and 2.5% sodium hypochlorite (NaClO) for 20 minutes. The seeds were then rinsed three times inautoclaved water. Under aseptic conditions, the zygotic embryos were placed in petri dished containing 25 ml culture medium. In all phases, except for regeneration, cultures were kept in a dark growth room at 25±2°C. Two basic media, one containing MS salts (Murashige & Skoog 1962) and the other Y3 salts (Eeuwens 1976) were used. Both media were supplemented with EDTA iron, MS vitamins, glutamine (200 mg/L), asparagine (100 mg/L), arginine (100 mg/L), sucrose (30 g/L), and solidified with 2.3 g/L of Phytagel™.

The medium pH was adjusted to 5.8±0.1, prior to autoclaving at 121°C and pressure at 1.3 atm for 20 minutes.

Induction of somatic embryogenesis

For induction, five concentrations of the auxins 2,4-D (0; 361.99, 452.49, 542.99 and 633.48 µM) and picloram (0, 50, 150, 300 and 450 µM) were tested combined with the basic medium (MS or Y3) and activated charcoal (2.0 g/L). Cultures were subcultured after 90 days post inoculation. After 150 days, the calli were transferred to a second culture medium for multiplication of the embryogenic cultures.

Multiplication of embryogenic cultures

After the induction process, the calli were maintained in the same basic culture medium, but two concentrations of auxin picloram (20 and 40 µM), associated with cytokinin 2-isopentenyladenine (2iP) (10 µM) were tested, without activated charcoal. After 90 days (240 days from the beginning of the experiment), the embryogenic calli were transferred to the somatic embryo differentiation and maturation medium.

Differentiation and maturation of somatic embryos

At this stage, the calli were maintained in the same basic medium, but picloram was replaced by NAA (0.54 µM), combined with 2ip (12.3 µM), without activated charcoal. After 90 days under these conditions (330 days from the beginning of the experiment), the somatic embryos were transferred to the plantlets conversion medium.

Conversion of somatic embryos to plantlets

Somatic embryos were converted on half-strength MS medium with 2.0g/L activated charcoal and no growth regulators. The somatic embryos were incubated in test tubes (25 x 150 mm) containing 15 mL culture medium and kept in a growth room with a photoperiod of 16h, light intensity of 100 $\mu mol.m^{-2}s^{-1}$, provided by LED lamps, at 25±2°C. The somatic embryos remained for up to seven months in this medium, with subcultures at 90 day intervals.

Morphological, anatomical and histochemical characterization of calli

Samples were collected from all treatments after 45, 90, 180 and 270 days, and their morphology was observed using under a Leica stereomicroscope (M205FA) with a coupled camera (DFC310FX). The samples were also processed for anatomical analyses, which they were fixed in 1% glutaraldehyde and 4% formaldehyde (McDowell & Trump 1976), dehydrated in a growing ethanol series (10-100°G) and methacrylate infiltrated (Historesin Leica) according to the manufacturer. The material was cut into a Leica manual rotating microtome (DM 2125RT) with a thickness of 3 to 5 micrometers. The sections were stained with toluidine blue (0.5%) (O'Brien et al. 1964) and subjected to the Lugoltest for starch detection (Johansen 1940), periodic-Schiff acid (PAS) for neutral carbohydrates (O'Brien & McCully 1981), and Xylidin Posseau (XP) for proteins (Vidal 1970). The slides were assembled in Entelan and the anatomical sections were analyzed and recorded under Leica optical microscope (DM 750) with coupled camera (ICC50 HD).

Statistical analyses

The experimental design was completely randomized and each treatment was composed of 10 replicates with five zygotic embryos per Petri dish (15 x 90 mm). The evaluations were performed at 150 days for primary callus formation, germinated zygotic embryos, undeveloped zygotic embryos and oxidation. Evaluation for the formation of embryogenic callus was done after 240 days and the mean

number of somatic embryos were evaluated on the 330th day. The data were submitted to analyses of variance (ANOVA) and the means were compared by the Scott-Knott test with the statistical program Assistat (Silva & Azevedo 2016).

RESULTS AND DISCUSSION

Induction of somatic embryogenesis

Formation of primary calli took place in the treatments with 361.99 µM2, 4D, and 50 µMicloram; however, zygotic embryos tended to germinate in treatments without auxin. The germination of zygotic embryos in induction medium was observed in *Euterpe edulis* with 226.24 µM 2,4-D (Guerra & Handro 1988) and in *E. oleracea* with 113.12 and 226.24 µM 2,4- D (Ledo et al. 2002).

The primary calli presented different morphologies (smooth, nodular or friable), colors (white, cream or yellow) and consistencies (compact or mucilaginous) (Figure 1). The explants in culture showed changes in their morphology during the first week in all treatments, initially with zygote embryo swelling (Figure 1c).

Table I shows the percentages of formation of primary and embryogenic calli in the

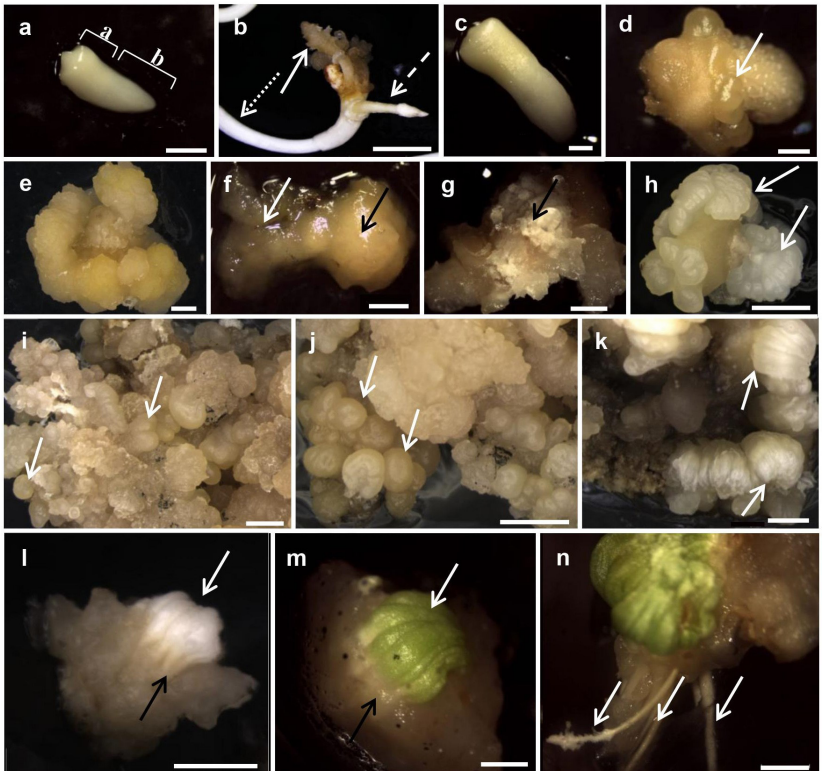

Figure 1. Morphology of callus obtained during the process of somatic embryogenesis in zygoticembryos (ZE) of B. odorata. (a) Mature ZE at the time of inoculation, proximal region (a), distal region (b). (b) Seed lings with callus formation (arrow) with leaf (dotted arrow) and root (dashed arrow). (c) Mature swollen ZE. (d) Primary callus with translucent embryogenic structures (arrow). (e) Embryogenic callus in Y3 medium (induced with picloram). (f) Mixed mucilaginous callus (white arrow) and compact (black arrow). (g) Crispycallus (arrow) and mucilage. (h) Embryogenic callus. (i) Embryogenic callus in MS medium with somatic embryos (arrows). (j) Globular somatic embryos (arrows). (k) More developed and fused somatic embryos (arrows). (l) Somatic embryos (SE) isolated from the embryogenic callus with aerial part (White arrow) and root zone (black arrow). (m, n) SE in regeneration medium MS without growth regulators: m SE with greenish cotyledons (white arrow) and root region (black arrow) n Roots formed). Bar: 1 mm (a), 5 mm (b, l), 2 mm (c, d, e, f, g, h, i, j, k, m, n).

induction phase, at the end of 150 and 240 days, respectively. At 45 days, the presence of primary callus was observed. In some treatments, in addition to the primary calli, the formation of embryogenic callus was also observed in the induction medium, those characterized by yellow color, being compact (hard) and nodular. Some explants did not respond to the induction of callogenesis or even germination, remaining with their initial morphology without signs of oxidation or injury by manipulation, and were classified as undeveloped. These undeveloped embryos were possibly non-viable since similar behavior was observed in the control treatments.

We observed a higher nodular growth in the median region of the zygotic embryo until the formation of the primary callus after 45 days of culture (Figure 1d). These and other modifications during the callogenesis process were also observed in zygotic embryos of other palms with an initial period of primary callus formation varying among species, including the same species. In macaw palm (*Acrocomia aculeata*), Moura et al. (2009) observed the formation of spongy and nodular callus at

Table I. Percentage of germination of zygotic embryos (GZE), undeveloped zygote embryo (UZE) and primary callus (PC) at 150 days; embryogenic callus (EC) at 240 days of cultivation in the MS or Y3 culture medium with 2,4-D or picloram auxins at five different concentrations and somatic embryo (SE) at 330 days of culture.

Culture medium	2,4-D (µM)	picloram (µM)	GZE (%)	UZE (%)	PC (%)	EC/PC (%)	SE/EC
MS	-	-	56.00	44.00	0.00c	0.00	0.00
MS	361.99	-	24.00	36.00	40.00b	7.50	21.00
MS	452.49	-	8.00	38.00	54.00b	19.50	4.80
MS	542.99	-	4.00	36.00	60.00b	33.00	2.18
MS	633.48	-	0.00	8.00	92.00a	20.50	1.30
MS	-	50	6.00	48.00	46.00b	0.00	0.00
MS	-	150	0.00	50.00	50.00b	12.33	2.60
MS	-	300	0.00	48.00	52.00b	31.50	1.00
MS	-	450	0.00	30.00	70.00a	46.00	4.71
Y3	-	-	60.00	40.00	0.00c	0.00	0.00
Y3	361.99	-	36.00	50.00	14.00c	0.00	0.00
Y3	452.49	-	36.00	32.00	32.00c	2.50	2.00
Y3	542.99	-	4.00	38.00	58.00b	8.50	12.75
Y3	633.48	-	0.00	38.00	62.00b	16.42	2.00
Y3	-	50	4.00	24.00	72.00a	0.00	0.00
Y3	-	150	0.00	28.00	72.00a	37.00	3.00
Y3	-	300	0.00	34.00	66.00a	31.17	2.67
Y3	-	450	0.00	16.00	84.00a	36.33	1.06

Lowercase vertical letters differ from one another by the Scott-knott test at 5% probability.

60 days in induction medium and Luis & Scherwinski-Pereira (2014) observed it after 30 days. In açaí palm (*E. oleracea*), Scherwinski-Pereira et al. (2012) verified the presence of callus after 30 days and Freitas et al. (2016) observed the formation of primary callus after 60 days of induction and after 90 days classified three types of callus: 1) embryogenic callus; 2) nodular and yellow callus; and 3) spongy, whitish (or pale) non-embryogenic callus. In this study, different types of callus were formed, and they were classified as primary callus, embryogenic callus (Figure 1e and h) and non-embryogenic callus (Figure 1g).

Zygotic embryos placed on MS medium with 633.48 µM of 2,4-D (92%) and 450 µM picloram (70%) and in Y3 medium with picloram at the concentrations of 50 µM (72%), 150 µM (72%), 300 µM (66%) and 450 µM (84%) showed the highest rates of primary callus formation. These results are in agreement with those of other studies, in which the auxins or their absence determined either the callus formation or the zygotic embryos germination (Steinmacher et al. 2007, Balzon et al. 2013).

Picloram excelled at primary callus induction compared to 2,4-D for *B. odorata*. The use of picloram with activated charcoal has been shown more effective results than 2,4-D for some palm trees. For *E. oleraceae*, Scherwinski-Pereira et al. (2012) compared the use of picloram and 2,4-D in MS medium and the concentration of 450 µM picloram was the most effective, with 44.8% of primary callus formation, while in 2,4-D was 21.2%. For *E. guineenses*, Balzon et al. (2013) obtained 97.5% of embryogenic callus in MS medium with 450 µM picloram and 79.4% with 450 µM 2,4-D. For *B. odorata* the treatments with 633.48 µM of 2,4-D induced callogenesis in MS (92%) and Y3 (62%), but only 20.5% and 16.42%, respectively, became embryogenic (Table I). The percentage of embryogenic calli in relation to the primary calli formed was above 30% in MS treatments with 542.99 µM 2,4-D and 300 µM picloram, and in Y3 with 150, 300 and 450 µMpicloram. Treatment with 450 µM picloram in MS medium was the most expressive with 46% of embryogenic calli formation. Thus, it was concluded that despite the high rates of primary callus, not all calli presented or acquired embryogenic competence.

In spite of the morphological differences presented, during the formation of the primary callus, it was possible to observe a common morphology in some of these calli represented by the yellow compact nodular callus observed up to 90 days (Figure 1d). Another common feature observed was that the callus formed in MS medium with 2,4-D presented smaller size, smooth and mucilaginous appearance (Figure 1c), and callus formed in MS with picloram were nodular (Figure 1e). Generally, the calli induced by picloram showed intense yellow color, nodular appearance and rapid growth, visually observed by increasing callus size in MS and Y3 medium, but not all became embryogenic. The combination of 2,4-D auxin in MS and Y3 medium provided greater development of organogenesis, expressed by root formation in primary calli, when compared to the picloram combination in MS or Y3, which did not form roots.

Multiplication of embryogenic cultures

The calli formed in the induction media were transferred to the multiplication medium in order to maintain the embryogenic lines in repetitive cycles of cell division, and allow the multiplication of the embryogenic calli. During the transfer, compact calli were observed, a characteristic described in the literature for embryogenic calli (Balzon et al. 2013). We observed oxidation in some calli possibly caused by the removal of the activated charcoal from the culture medium after 180 days in culture

(30 days in multiplication medium). Possibly the stressdue to high concentrations of auxin and in the absence of an antioxidant such as activated charcoal to adsorb these compounds resulted in the oxidation of some callus. However, oxidation was most notably observedin 2,4-D treatments in MS and Y3 medium, whereas picloram induced callus did not oxidize in the same way (Figure 1e).

Data from the literature show that after the formation of embryogenic calli, the reduction of auxin or its elimination from the culture medium is recommended, as it may have an inhibitory effect on the formation of somatic embryos. According Fehér (2015), in order to allow the establishment of cell or tissue polarity and embryonic development, 2,4-D should be removed from the culture medium. Fehér (2015) reports studies with *Arabidopsis* showing that 2,4-D removal induces the establishment of auxin synthesis and the transport of polar auxins is a key step in the formation of the meristem underlying embryonic development. According to Guerra & Handro (1998), successive subcultures in culture medium with high concentrations of auxin for the multiplication of embryogenic calli can affect negatively and even prevent the development of somatic embryos. Balzon et al. (2013) observed that the reduction of the auxin concentration from 450 to 40 µM was important to establish repetitive cycles of cell division, thus enabling the multiplication of the embryogenic callus of *E. guineensis*. The same observations were described when the auxin is reduced in *C. nucifera* (Fernando & Gamage 2000) and *A. aculeata* (Luis & Scherwinski-Pereira 2014). In this work, the reduction in the concentration of auxin to 20 µM plus the addition of 10 µM of 2iP maintained the visual growth of the calli and allowed the development of embryogenic zones. At 210 days, some calli began to show globular structures with a smooth surface (Figure 1j).

Differentiation and maturation of somatic embryos

The embryogenic calli with globular somatic embryos and/or whitish and elongated structures formed in the multiplication medium (Figure 1i-k) were transferred to the differentiation and maturation medium. The purpose of this medium is to provide conditions favorable for the proembryos todifferentiate into somatic embryos and to complete their development. Therefore, the auxin NAA and the cytokinin 2iP were added to the culture medium, removing picloram or 2,4-D that could prevent the development of the somatic embryos. The combination NAA and 2iP have shown positive effects on the development and maturation of somatic embryos until the torpedo phase in palm species (Guerra & Handro 1988, 1998, Balzon et al. 2013).

The embryogenic callus presented somatic embryos at different stages of development. Among them, globular somatic embryos were observed, with yellow coloration and smooth texture in different sizes, as well as more developed somatic embryos, presenting a more elongated white structure with yellow coloration in its base (Figure 1l), but the aerial part was fused. Based on the somatic embryogenic description of other palm species, it can be said that the pindo palm fused somatic embryos (Figure 1m) would be near to the torpedo stage, but the fusion did not allow its individual development. Fused somatic embryos were observed during somatic embryogenesis in *Accasellowiana* (Pescador et al. 2008) and *C. nucifera* (Montero-Córtes et al. 2010).

Conversion of somatic embryos to plantlets

The somatic embryos (Figure 1l) obtained after 90 days in the differentiation and maturation medium were isolated and transferred to the MS medium, with half strength salts and devoid

of growth regulators. Ledo et al. (2002) reported somatic embryos germination of açaí palm in MS medium without growth regulators at 210 days. The exposure to light induced the production of chloroplasts making the cotyledons green, yet these embryos collapsed and did not develop. However, some cotyledon fused somatic embryos presented root formation (Figure 1n). Luis & Scherwinski-Pereira (2014) observed somatic embryo germination in regeneration medium, starting at 45 days, with a 31.9% frequency of plant regeneration. These authors also observed abnormal somatic embryos with root development, in this case, the apical meristem appeared to be absent or underdeveloped, which affected the development of the aerial part. The somatic embryos of pindo palm did not develop possibly due to the fusion of the cotyledons that did not allow the individualization of the seedlings, once roots were formed.

On the other hand, Freitas et al. (2016) used cytokinin BAP (N6-benzylaminopurine) and gibberellin GA_3 (gibberellic acid) in the regeneration medium at concentrations of 1.0 µM and 0.5 µM, respectively. Somatic embryos started germination after 60 days and most of the regenerated plants presented aerial part and developed roots. Even without fully understanding the process of somatic embryogenesis, the major bottleneck appears to be the conversion of somatic embryos into plants. Low conversion is reported as one of the major difficulties in developing efficient protocols (Pérez-Núñez et al. 2006, Sáenz et al. 2006).

In general, the morphogenic route of somatic embryogenesis, under the conditions studied, can be characterized as indirect and asynchronous for *B. odorata* species. The same model has been described for other palm trees that also have used the zygotic embryo as explant, for example açaí palm (Ledo et al. 2002), peach palm (Steinmacher et al. 2007), oil palm (Balzon et al. 2013) and macaw palm (Moura et al. 2009, Luis & Scherwinski-Pereira 2014). Embryogenic tissues have been tested as a source of explant for somatic embryogenesis, because tissues of zygotic embryos are highly responsive to *in vitro* induction, cause minimal damage to the parent plant and are naturally pathogen free (Ree & Guerra 2015). The zygotic embryo, which originates from allogamous plants, used as an explant for somatic embryogenesis, will not only produce clones of the mother plant but will help in the conservation of the species, facilitating its propagation and the production of plantlets, besides being able to contribute to future breeding programs with controlled crosses.

Morphological, anatomical and histochemical characterization of callus

At 90 days in culture, most of the primary calli were yellow, compact and nodular (Figure 2a-c). Regardless of the treatment, the callus presented the formation of meristematic centers and/or embryogenic structures. And from each treatment, except the controls and the treatments 50 µM picloram in MS, 361.99 µM 2,4-D and 50 µM picloram in Y3, at least one callus presented embryogenic characteristics. The anatomical analyses allowed the identification of meristematic zones formed by agglomerates of meristematic cells in the majority of the primary calli from 60 days on (Figure 2d-f). At 150 days of the induction process, embryogenic cells and proembryos were observed in the embryogenic callus (Figure 2j-k). According to Verdeil et al. (2007), the nuclear architecture and chromatin structure differentiate meristematic cells from embryogenic cells. The meristematic cell has a spherical nucleus containing one or more nucleoli, dense cytoplasm and fragmented vacuoles. The embryogenic cell presents a thick wall, an evident nucleus with one nucleolus,

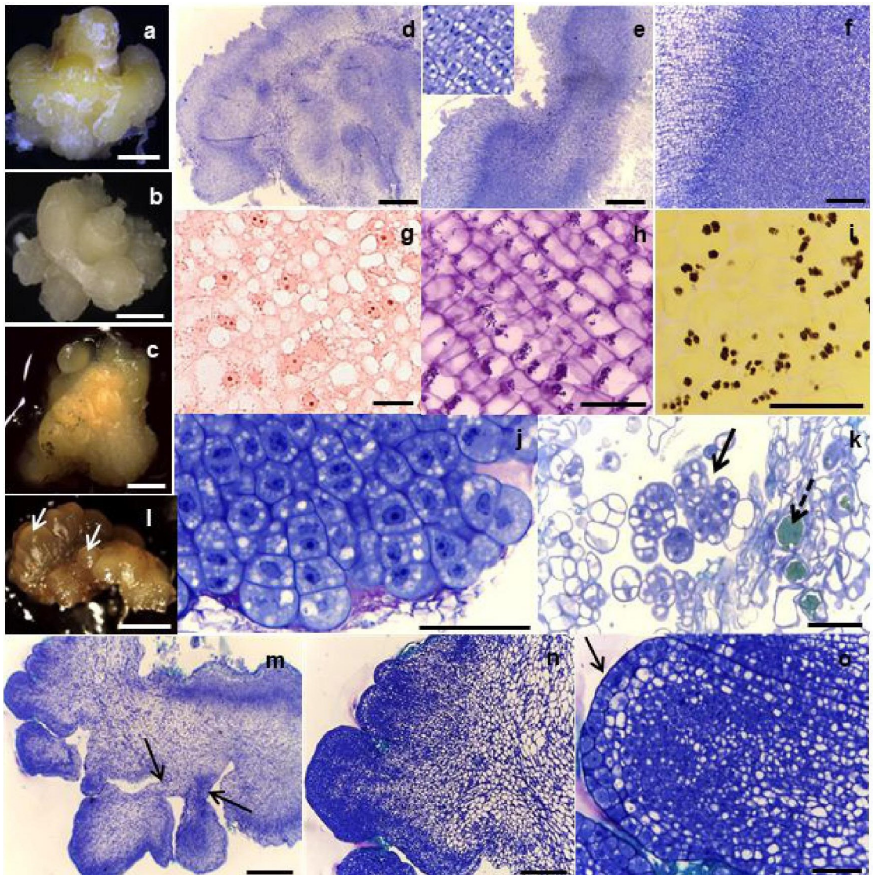

Figure 2. Anatomy and histochemistry of embryogenic calli and somatic embryos of *B. odorata*. (a-i) Morphology and anatomy of primary callus at 90 days in induction medium to somatic embryogenesis. (a) Primary callus in MS medium with 300 µM picloram. (b) Primary callus in Y3 medium with 300 µM / L picloram. (c) Primary callus in MS medium with 633.48 µM 2,4-D. (d) Anatomic section of the callus in a. (e-f) Anatomic section of the callus in b. (g) Protein-free cells stained with XP. (h) Cells with carbo hydrates stained with PAS. (i) Cells with starch grains, Lugol'sreagent. (j-o) Morphology and anatomy of embryogenic callus at 150 days. (j) Embryogenic cells. (k) Isolated proembryos (arrow) and phenolic compounds (dashed arrow). (l) Callus with somatic embryos (SE) (arrows). (m-o) Callus anatomy in l. (m) SE fused to the tissue of origin (arrows). (n) SE delimited by the protoderm. (o) Detail of the protoderm (arrow). Bar: 1 mm (a), 2 mm (b, c, l), 500 µm (d, e, m, n), 200 µm (f), 20 µm (g), 50 µm (h, i, j, k, o).

dense cytoplasm and vacuoles in reduced number or absent.

Histological sections showed meristematic zones as more stained regions, indicating intense cell division and forming bands with meristematic cells lined up in some callus (Figure 2e-g). The cells in the meristematic regions are small, isodiametric, with a dense cytoplasm (Figure 2e- detail). The presence of meristematic centers is a hallmark characteristic of embryogenic calli. According to Fehér et al. (2003), the presence of these primary centers, followed by the formation of an organ, is related to the ability of a cell to respond to specific signals, such as those generated by the action of hormones. For example, in *C. nucifera* it was observed that somatic embryogenesis originated from meristematic regions (Sáenz et al. 2006).

Starch grains and carbohydrates were detected (Figure 2i-J), both are indicative of meristematic and embryogenic cells, because

they are energy reserve structures for the development of tissues and organs, such as somatic embryos, aside from indicating high activity of cell division. Proteins were not detected in the embryogenic calli (Figure 2h). Phenolic compounds were observed in several primary, embryogenic calli, close to the proembryos (Figure 2k). The production of phenolic compounds appears to be a response to stress (Taiz & Zaiger 2009), in this case possibly caused by exposure to highly concentrated auxin or lignin deposition.

On the surface of the embryogenic calli, globular structures were observed and classified as globular somatic embryos (Figure 2l). The analyses of the histological sections showed the absence of vascular attachment of the somatic embryo to the callus and fusion of the base of this embryo to the tissue of origin (Figure 2m), which confers multicellular origin to the somatic embryo. According to Williams & Maheswaran (1986), the multicellular origin can produce somatic embryos fused to the original tissue, because single or multiple-cell origin for somatic embryos are directly related to coordinated behavior of neighboring cells as a morphogenetic group. So, if the cell is alone in its state of readiness it may act independently and express embryogenic potential from the usual single cell initiation point- i.e. that point of the program normally entered by a zygote.It was also observed a defined protoderm (Figure 2o), meristematic cells and parenchyma cells. Somatic embryos are characterized mainly by the presence of defined protoderm, bipolarity and procambium. These characteristics were described for *C. nucifera* (Fernando et al. 2003), *P. dactylifera* (Sané et al. 2006), *E. guineensis* (Silva et al. 2014) and *E. oleracea* (Freitas et al. 2016). During the analyses we do not detect procambium. This may indicate the need for the embryos to remain longer in the maturation medium until fully developed.

Future studies may focus on numerous possible factors that inhibited plantlet conversion, such as time of exposure to auxin and phases of the process (induction, multiplication, maturation and conversion). Growth regulators such as N6-benzylaminopurine (BAP), abscisic acid (ABA) or gibberellic acid (GA_3), may play an important role in the development and germination of somatic embryos (Sané et al. 2012, Bawis et al. 2015, Freitas et al. 2016). The conversion of the somatic embryo to plantlet requires studies on the time required to complete its development between the globular stage (Figures 1i-j and 2l) and the torpedo stage until germination. The presence of fused somatic embryos is common during primary and secondary embryogenesis of *C. nucifera*, although it is less frequent in secondary embryogenesis (Pérez-Núñez et al. 2006). Thus, secondary embryogenesis may be an alternative in the induction of somatic embryogenesis in *B. odorata*.

In conclusion, the present work presents, for the first time, a description of the induction of somatic embryogenesis in *B. odorata*. The mature zygotic embryos were responsive to the induction treatments in an indirect and asynchronous manner in the presence of auxin and absence of light. The auxin picloram at concentrations of 150, 300 and 450 µM in Y3 culture medium was the most effective in the induction of embryogenic calli and somatic embryos in *B. odorata*.

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