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Somatic embryogenesis from floral tissues of feijoa (*Feijoa sellowiana* Berg)

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Abstract

The objectives of the present work were to study the embryogenic competence of floral tissues of *Feijoa sellowiana* and to investigate the influence of plant growth regulators on somatic embryo induction and development in order to establish a somatic embryogenesis protocol starting from somatic tissues. Petals, stamens and ovaries of floral buds were cultivated onto LPm basal medium supplemented with different levels of 2,4-D, Picloram, 2-iP, Kin and BAP. The highest embryogenic callus induction was obtained with Picloram (10 μM) and Kin (1 μM). Rates of embryogenic calluses induction in stamens and petals were significantly affected by PGRs. Embryogenic calluses were transferred to the same medium, supplemented with gradually reduced levels of PGRs-free medium. After 60 days in suspension cultures with 2,4-D (1 μM) and 2-iP (1 μM) calluses were transferred to PGR-free medium. After 30 days it was observed the development of globular somatic embryos on the surface of 18% of friable calluses previously induced with Picloram (10 μM) and Kin (1 μM). Only embryogenic calluses derived from stamens gave rise to this morphogenetic pattern.- Torpedo and cotyledonary somatic embryos transferred to PGR-free culture medium were converted

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-benzylaminopurine; 2-iP, N6 (2-isopentyl) adenine; Kin, kinetin; LPm, von Arnold and Eriksson (1981) modified medium; PGRs, plant growth regulators; Picloram, 4-amino-3,5,6-trichloropicolinic acid

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to complete plantlets. This is the first report of somatic embryogenesis in this species starting from somatic tissues.

Keywords: Myrtaceae; Plant growth regulators; Somatic embryos; Stamens

1. Introduction

Somatic embryogenesis is defined as the development of structures that follow histodifferentiation patterns resembling the events observed in zygotic embryogenesis (Emons, 1994; Dodeman et al., 1997). This in vitro morphogenetic pattern is a multi-step regeneration process starting with the induction of pro-embryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration, in response to physical and chemical treatments (von Arnold et al., 2002).

Feijoa sellowiana (*Acca sellowiana*) (Myrtaceae) is a native semi-woody fruit species of the southern Brazilian highlands that shows great potential for fruit production. This species, commercially exploited in several countries of the northern hemisphere, as well Australia and New Zealand, has been subjected to a program of domestication and improvement in its diversity (Canhoto and Cruz, 1996a).

In this species, the conventional methods of clonal propagation, such as cutting and grafting, have shown low efficiency because of the effects of phenolic oxidation. Micropropagation techniques based on organogenesis also present serious limitations (Bhojwani et al., 1987; Canhoto and Cruz, 1996a). The induction of somatic embryogenesis starting from mature and immature zygotic embryos has shown good results and efficiency for large-scale propagation of selected genotypes (Canhoto and Cruz, 1996b; Guerra et al., 1997; Dal Vesco and Guerra, 2001). However, the competence for somatic embryogenesis induction is limited to zygotic embryo explants, which develop somatic embryos from external cotyledonary tissue.

As this woody plant is allogamous, rapid clonal propagation from tissues of maternal origin of a known genotype would be useful for mass clonal propagation of selected clones. The capacity of leaves and flowers to induce regenerative structures is usually limited when compared with zygotic embryos. There are few papers that report on embryogenic patterns using floral explants. Canhoto and Cruz (1996a) investigated the embryogenic capacity of floral tissues and young leaves of feijoa. A low percentage (10%) of inoculated anthers produced calluses after 75 days in culture medium containing 2,4-D and BAP.

The main objective of the present work was to study the embryogenic competence of *F. sellowiana* floral tissues and to investigate the influence of plant growth regulators (PGR) on embryogenic induction and development.

2. Materials and methods

2.1. Plant material

Floral buds of the accession 441 of *F. sellowiana* Berg were collected before anthesis at the germoplasm bank of EPAGRI, Experimental Station of São Joaquim, State of Santa

Catarina, Brazil, and transported to the Laboratory of Developmental Physiology and Plant Genetics, Federal University of Santa Catarina, Florianópolis, Brazil. The sterilization procedures, inoculation and culture conditions were the same as described by Dal Vesco and Guerra (2001). After dissecting the bud tissues in an aseptic chamber, petals, stamen and ovaries were placed in test tubes (22 mm × 150 mm) containing 20 ml of LPm basal medium (von Arnold and Eriksson, 1981), Morel vitamins (Morel and Wetmore, 1951), sucrose (3%), phytagel® (0.2%) and supplemented with PGRs. Embryogenic calluses were transferred to Petri dishes (15 mm × 100 mm) containing 25 ml of LPm basal medium supplemented with PGRs. The cultures were incubated at 27 °C in the dark. For maturation and conversion the cultures were transferred to a culture room with a 16 h photoperiod (40 μmol m^{-2} s^{-1} of cool-white fluorescent light).

2.2. Induction

Petals, stamens and ovaries were evaluated for calluses induction using LPm basal medium supplemented with different types and levels of PGR. The experiment was arranged in a completely randomized design, with nine treatments. Two levels of 2,4-D (0 and 20 μM), alone and in combination with 5 μM of 2-iP, Kin or BAP and Picloram (10 μM) in combination with Kin (1 μM) were tested. Each experimental unit consisted of five explants (petals, stamens and ovaries) with three replicates. Data on percentages of callus induction were recorded after 60 days in culture.

2.3. Proliferation

Calluses induced in the previous treatments were transferred to Petri dishes containing the same gelled LPm basal medium, supplemented with 2,4-D (5 μM) plus BAP (1 μM), Kin (1 μM) or 2-iP (1 μM), with Picloram (5 μM) plus Kin (1 μM). The cultures were incubated in a culture room in the dark for 60 days. In sequence stamen, petal, and ovary-derived calluses (1 g/cluster) were subcultured for more 60 days to Petri dishes containing gelled LPm medium, supplemented with 2,4-D (1 μM) plus BAP (0.5 μM), Kin (0.5 μM) or 2-iP (0.5 μM), and Picloram (1 μM) in combination with Kin (0.5 μM). Data of embryogenic callus fresh weight (g) were recorded after 60 days in culture.

2.4. Establishment and proliferation of cell suspension cultures

Cell suspensions were initiated by inoculating 1 g fresh weight of stamen, petal, and ovary-derived calluses originated in the treatment with Picloram (1 μM) plus Kin (0,5 μM) in 1000 ml nipple-flasks, containing 100 ml of basal culture medium supplemented with 2,4-D (1 μM) and 2-iP (1 μM). The nipple-flasks were placed on a rotating orbital shaker (Steward apparatus) at 1 rpm and incubated at 27 °C in the dark.

2.5. Maturation

Cell fractions higher than 74 μm were collected using stainless steel tissue sieves (Celetor apparatus, Sigma) and re-inoculated on Petri dishes, containing 25 ml of gelled

LPm PGR-free culture medium. After 30 days, the cultures were subcultured on gelled LPm PGR-free culture medium or supplemented with Picloram (1 μM) plus Kin (0.5 μM).

2.6. Cytochemical analysis

The cultures were characterized by cytochemical analyses. Starch grains and lipid bodies in the suspension cultures were detected using Lugol and Sudan staining procedures (Johansen, 1940) respectively. The most important aspects were identified and microphotographed using an Olympus PM-20 camera coupled to an Olympus® stereomicroscope SZH-10, a BX-40 microscope, or an inverted IMT-2 microscope.

2.7. Statistical analysis

The percentage of calluses produced and values for fresh weight were submitted to F_{max}-test in order to verify the heterogeneity of the variance (S^2). These data were submitted to analysis of variance and mean separation was tested using SNK at the 0.05 level according to Compton (1994).

3. Results

3.1. Influence of PGR on callus induction and proliferation

The presence of Picloram (10 μM) and Kin (1 μM) in the basal medium significantly enhanced ($P < 0.01$) the percentage of callus induction from stamens (Table 1). Visual

Table 1
Percentage of embryogenic callus induction from stamen filaments, petals and ovaries of *Feijoa sellowiana*, cultured on LPm (von Arnold and Eriksson, 1981) basal medium supplemented with different types, levels and combinations of PGR after 60 days in culture

PGR (μM)	% Embryogenic callus		
	Stamens	Petals	Ovaries
Picloram (10) + Kin (1)	81.7 a	86.7 a	93.3 a
2,4-D (20) + 2-iP (5)	70.0 b	78.3 a	93.3 a
2,4-D (20) + BAP (5)	68.3 b	85.0 a	91.7 a
2,4-D (20) + Kin (5)	63.3 b	85.0 a	91.7 a
2-iP (5)	46.6 c	56.7 b	73.3 a
PGR-free	40.0 c	23.3 d	73.3 a
BAP (5)	30.0 d	44.4 c	73.3 a
Kin (5)	26.6 d	53.3 b	73.3 a
2,4-D (20)	16.6 e	80.0 a	85.0 a
Mean	50.3	65.9	82.8
CV (%)	11.3[a]	13.5[a]	13.8[a]

Means of three replicates. Values within columns with different letters (a, b, c, d, e) indicating significant differences according to the SNK test ($p = 0.05$). CV (%) = Coefficient of variation.

[a] Data transformed for analysis using $\log(x + 2)$.

evaluations using the stereomicroscope showed the presence of callus induction after 9 days in culture (Fig. 1a) when the basal LPm medium was supplemented with 2,4-D (20 μM) and Kin (5 μM).

Petals, stamens and ovaries cultivated on basal medium supplemented with different PGR showed an intense callus induction from the basal region of the explants. Distinctly visible calluses were observed after 60 days in culture according to the explant and PGR used. Calluses obtained from stamen filaments, petals and ovaries in the presence of 2,4-D plus cytokinins or with cytokinins alone were quite compact and yellowish (Fig. 1b). Calluses formed from petals and ovaries in medium with Picloram were compact and yellowish. Only calluses induced from stamens in basal medium supplemented with Picloram plus Kin were friable and whitish (Fig. 1c).

It was observed a clear influence of the auxins and cytokinins supplemented to the culture medium on calluses induction and proliferation (Table 1). The best treatments for stamen and petal callus induction were those with auxins plus cytokinins. However, SE formation was not observed in any of the calluses before the 60th day following the inoculation. Calluses obtained in the induction treatments continued proliferating after transference to culture medium with reduced levels of auxin and cytokinin. Fresh weight of embryogenic calluses increased significantly ($p < 0.01$) when the basal LPm medium was supplemented with 1 μM Picloram and 0.5 μM Kin (Table 2), but no SE development was observed. High levels of auxins combine or not with cytokinins were necessary to the callus induction which showed different features according to the source of PGRs used in the induction phase (Fig. 1b and c).

3.2. Cell suspensions

Cell suspensions originated from embriogenic calluses of petals, stamens and ovaries grew in LPm basal culture medium supplemented with 2,4-D (1 μM) and 2-iP (0.5 μM) but SE development was not observed. The suspension cultures derived from this treatment showed an increment in fresh weight up to ten times the initial value (Data not shown).

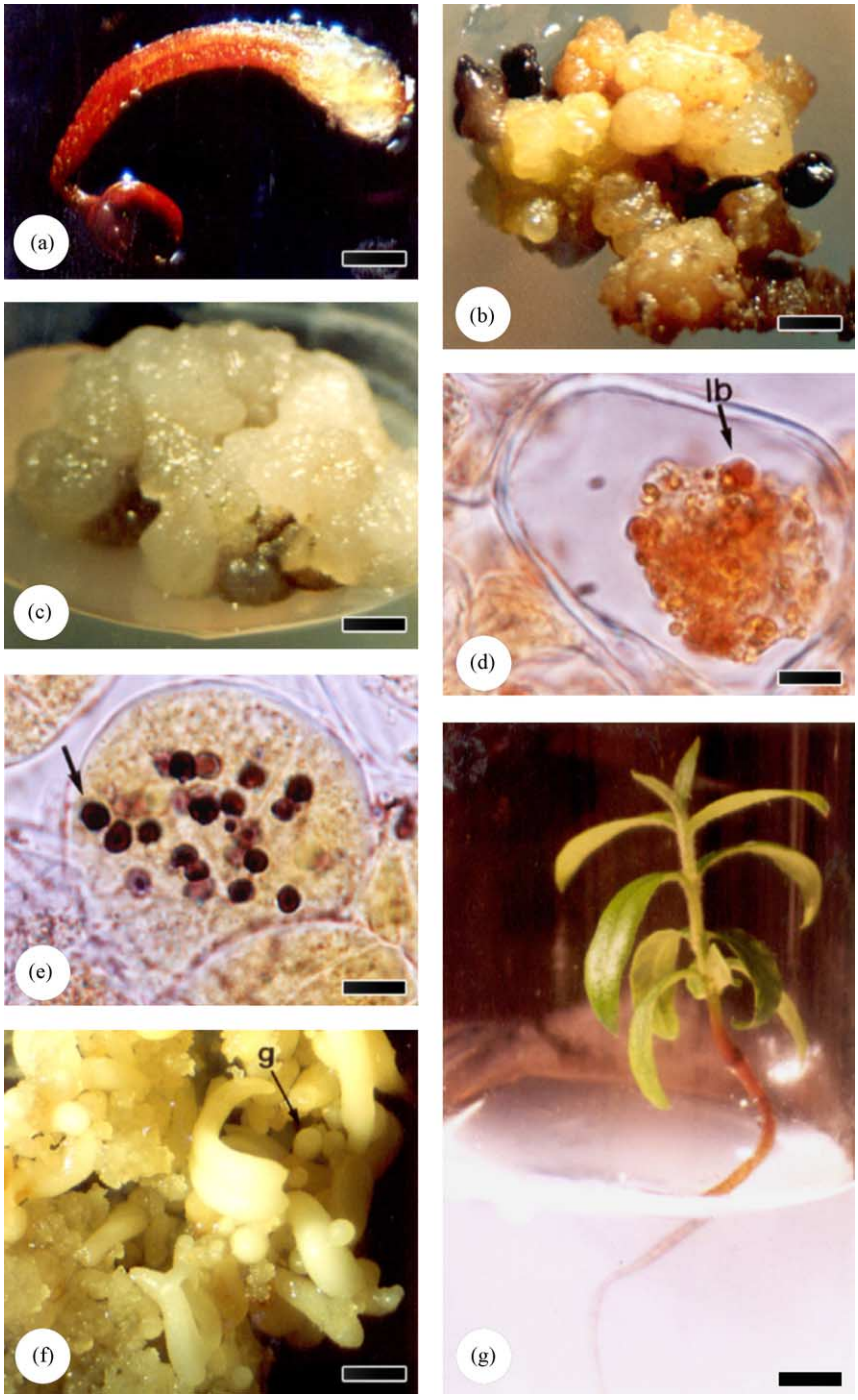
Cytochemical analyses showed two different structural characteristics. The cells from stamen-derived suspensions induced in Picloram plus Kin and further cultivated in basal medium with 2,4-D plus 2-iP were isodiametric, small (20–30 μm), and densely

Table 2
Increment in fresh weight (g) of embryogenic calluses of *Feijoa sellowiana* from an initial aliquot of 1 g, after 60 days in culture

PGRs (μM)	Stamens	Petals	Ovaries
Picloram (1) + Kin (0.5)	1.80 a	1.15 a	1.46 a
2,4-D (1) + 2-iP (0.5)	0.33 b	0.44 b	0.49 b
2,4-D (1) + BAP (0.5)	0.14 b	0.31 b	0.47 b
2,4-D (1) + Kin (0.5)	0.08 b	0.25 b	0.37 b
CV (%)	44.0[a]	28.7[a]	33.5[a]

Means of four replicates. Values within columns with different letters (a, b) indicating significant differences according to the SNK test ($p = 0.05$). CV (%): coefficient of variation.

[a] Data transformed for analysis using $\log(x + 2)$.


(a)
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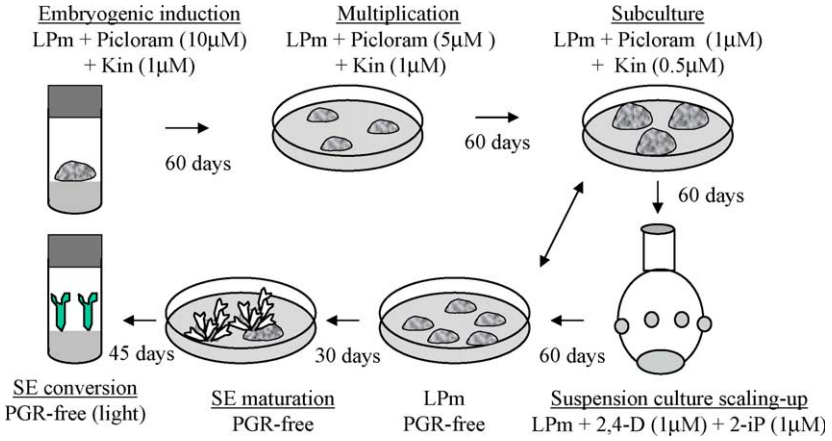

Fig. 2. Induction and developmental pathway in embryogenic cultures derived from stamens of *Feijoa sellowiana* Berg.

cytoplasmic, showing a high cellular division rate. Cells derived from petals and ovaries were elongated (70–80 μm). Lipid bodies (Fig. 1d) and starch grains (Fig. 1e) were mostly accumulated by the cells derived from stamen cultures than in cells derived from petals and ovaries.

3.3. Development of somatic embryos and embryo conversion

The development of globular SE from stamens was observed on the surface of 18% of the friable calluses previously induced with Picloram (10 μM) and Kin (1 μM), and further cultivated in suspension with 2,4-D (1 μM) and 2-iP (1 μM) (Fig. 1f), and finally transferred to Petri dishes containing gelled LPm basal culture medium free of PGRs. Other explant sources (petals and ovaries) did not originate somatic embryos. The SE development was not was not synchronized, and embryos in different developmental stages could be seen (Fig. 1f). Torpedo and cotyledonary somatic embryos transferred onto LPm PGR-free culture medium were capable of conversion into complete plantlets (Fig. 1g). Fig. 2 shows the induction and developmental pathway of stamen-derived embryogenic cultures of *F. sellowiana*.

In the present work, many of the somatic embryos were morphologically normal. However, morphologic abnormalities were also observed: occurrence of fused hypocotyl and cotyledon, lack of cotyledon and apical meristems, or presence of only one or three cotyledons.

Fig. 1. Induction of calluses derived from *Feijoa sellowiana* stamen filaments on basal medium supplemented with 2,4-D (20 μM) and Kin (5 μM): (a) 9 days after inoculation (bar: 2.4 mm); (b) 60 days after inoculation (bar: 2.4 mm); (c) 60 days after inoculation on medium supplemented with Picloram (10 μM) and Kin (1 μM) (bar: 2.4 mm); (d) cell suspension showing lipid bodies accumulation (bar: 6.7 μm); (e) cell suspension showing starch grain accumulation (bar: 6.7 μm) (f) different stages of SE development (bar: 1.8 mm); (g) SE germinated into plantlet (bar: 5 mm). lb: lipid bodies, g: globular embryo.

4. Discussion

Induction of embryogenesis depends on the tissue culture environment, e.g., condition for osmosis, concentrations of sucrose, amino acids and salts, and hormone balance (Emons, 1994). A number of studies have revealed the fundamental role of the exogenous application of auxins, mainly 2,4-D, which is considered to be one of the main inductive factors for somatic embryogenesis (Ammirato, 1993). This has been demonstrated in experiments on carrot (Borkird et al., 1986), *F. sellowiana* (Cruz et al., 1990), *Populus* spp. (Michler, 1995), *Theobroma cacao* (Alemanno et al., 1996), *Carya illinoinensis* (Rodriguez and Wetzstein, 1998), myrtle (Canhoto et al., 1999) and switchgrass (Dutta Gupta and Conger, 1999).

Picloram is used in tissue culture to induce and/or maintain calluses or cell suspensions, or to induce the formation of embryogenic calluses. In the present study, the addition of Picloram (10 μM) to the basal medium combined with Kin (1 μM) resulted in the highest rate of embryogenic callus induction, 81.7% for stamens and 86.7% for petals (Table 1). Canhoto et al. (1999) reported that an auxin (Picloram or 2,4-D) was required for the induction of somatic embryogenesis directly from epidermal cells or indirectly from groups of meristematic cells close to the adaxial surface of zygotic embryos of *Myrtus communis*. It is generally accepted that complete removal of auxin or a decrease in its level in the culture medium following the callus induction phase can serve as a trigger for SE development (Zimmerman, 1993). Small calluses could be seen on the surface of the stamen filament of or from cocoa (*Theobroma cacao*) when it was cultivated on MS medium with 2,4-D and Kin, and morphological differences could be observed among calluses from embryogenic and non-embryogenic genotypes. Clumps of SE at different stages of development were observed on nodular calluses. The white compact calluses expanded without producing SE (Alemanno et al., 1996).

The mechanism of action of auxins in physiological and regulatory processes is related to the presence of protein receptors located in the membrane, cytoplasm and nucleus. There is, in the latter, the activation of RNA-polymerase, which is specific to the transcription of genes involved in the regulation of cell division (Dudits et al., 1995).

Cytochemical analyses showed the presence of many starch grains dispersed within the cells derived from stamens that originated somatic embryos. Such features are characteristics of embryogenic cultures (Emons, 1994; Yeung, 1995). It was shown that the number of starch grains of *D. carota* embryogenic cells ranged from 5 to 25 by cell (Emons, 1994). The storage of lipid bodies is indicative of the embryogenic competence, and such accumulation occurs before and during embryo development, which may coincide with protein synthesis (Merkle et al., 1995). In the present work, the presence of starch grains and lipid bodies suggests the accumulation of reserves for embryo development. However, the development of SE was not observed in the suspension cultures.

Somatic embryogenesis can probably be achieved for all plant species provided the appropriate explant, culture medium and environmental conditions are employed. Somatic embryos are normally obtained indirectly mediated by a callus phase. The difference between direct and indirect somatic embryogenesis, however, is not very clear (von Arnold et al., 2002). Alemanno et al. (1996) showed that the somatic embryos from stamen filaments of *Theobroma cacao* originated from meristematic cells of nodular calluses.

In the present work, many of the somatic embryos were morphologically normal. However, morphologic abnormalities were also observed: occurrence of fused hypocotyl and cotyledon, lack of cotyledon and apical meristems, or presence of only one or three cotyledons. Morphological abnormalities have been observed in other plants such as carrot (Borkird et al., 1986), cacao (Alemanno et al., 1997) and pecan (Rodriguez and Wetzstein, 1998).

Abnormal somatic embryos are normally associated with the use of 2,4-D or other strong auxins in the induction medium. The same auxin, required in high concentrations or for long periods to start a sequence of events leading to embryogenesis, can also give rise to these abnormalities. Also, long-term cultures can increase the occurrence of somaclonal variation (von Arnold et al., 2002). A histological study of *Theobroma cacao* somatic embryos has shown clearly the deficit of storage reserves as compared to the zygotic embryos (Alemanno et al., 1997).

In the present study cells of stamen filaments demonstrated their competence to embark on a new developmental embryogenic program. The somatic embryo pattern obtained in this investigation is similar to that established for alfalfa (Cervelli and Senaratna, 1998) which involves several steps: production of the callus from explant material (induction), transfer of calluses into liquid medium (cell multiplication), sieving to isolate the fraction enriched with embryogenic cells (synchronization), and plating embryogenic cells in PGR-free medium for embryo development.

Even considering several reports regarding somatic embryogenesis in *F. sellowiana* (Canhoto and Cruz, 1996a; Dal Vesco and Guerra, 2001), this is the first work to actually describe the embryogenic pathway from maternal tissues. The importance of this achievement is associated with the establishment of a protocol for mass clonal propagation from elite genotypes, allowing the capture and fixation of genetic gains, resulting from the genetic improvement program in progress in Santa Catarina State (south of Brazil), the diversity center of this species.

5. Conclusions

The results obtained in the present work show that the production of SE from stamens filaments of *F. sellowiana* requires a three-step protocol which is dependent on: (i) callus induction in culture medium supplemented with Picloram and Kinetin; (ii) gradual decrease in the levels of these PGR, (iii) establishment of cell suspensions in a culture medium with 2,4-D and 2-iP, and (iv) further culture in PGR-free medium. Somatic embryos from stamens were observed on the surface of 18% of the friable calluses. Since dicotyledonous woody species are usually considered to be recalcitrant to somatic embryogenesis, the results obtained in this work provide additional support for a better understanding of this in vitro morphogenetic pattern and allow the establishment of a somatic embryogenesis protocol for mass clonal propagation of this species.

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