Somatic embryogenesis induction in cubiu (*Solanum sessiliflorum* Dunal)

Indução de embriogênese somática em cubiu (*Solanum sessiliflorum* Dunal)

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ABSTRACT

Cubiu (*Solanum sessiliflorum* Dunal) is a solanaceous shrub native to the Amazon region that has been attracting interest from researchers and producers of other Brazilian regions. The objective of this study *in vitro* was to evaluate the effect of growth regulators and explants sources on the somatic embryogenesis of *S. sessiliflorum* Dunal. Hypocotyl and cotyledon explants from seedlings were transferred to MS culture medium supplemented with combinations of NAA (0, 5, 10 and 20 mg L^{-1}) and KIN (0 and 1 mg L^{-1}). Immature zygotic embryos were cultivated in MS culture medium with different concentrations of NAA (0, 2.5, 5 and 10 mg L^{-1}) and KIN (0 and 1 mg L^{-1}) or 2,4-D (0, 2.5, 5 and 10 mg L^{-1}) and KIN (0 and 0.1 mg L^{-1}). All the cotyledon and hypocotyl explants developed calluses after 30 days in the culture medium with NAA. These responses were independent of supplementing the culture medium with KIN. Embryo-like structures at several stages of development were found on the cotyledons of immature zygotic embryos growing in the MS medium supplemented with 2,4-D at 5 and 10 mg L^{-1}. After the subcultures, new calluses originated either from pro-embryonic clusters or cellular masses cultivated in the regulator-free medium. The isolation of these regions and their transference to the regulator-free MS medium permitted the differentiation of the pro-embryonic masses into globular and heart-shaped embryos.

Index terms: Solanaceae; *in vitro* culture; somatic embryos; plant growth regulators.

RESUMO

O cubiu (*Solanum sessiliflorum* Dunal) é uma solanácea arbustiva nativa da região Amazônia que vem despertando interesse de pesquisadores e produtores em outras regiões do Brasil. O objetivo deste trabalho foi avaliar o efeito de reguladores de crescimento e fontes de explante sobre a indução de embriogênese somática *in vitro* em *S. sessiliflorum* Dunal. Hipocótilos e cotilédones de plântulas germinadas *in vitro* foram transferidos para meio de cultura MS suplementado com combinações de ANA (0, 5, 10 e 20 mg L^{-1}) e de KIN (0 e 1 mg L^{-1}). Embriões zigóticos imaturos foram cultivados em meio de cultura MS com diferentes concentrações de ANA (0, 2.5, 5 e 10 mg L^{-1}) e KIN (0 e 1 mg L^{-1}) ou 2,4-D (0, 2.5, 5 e 10 mg L^{-1}) e KIN (0 e 0.1 mg L^{-1}). Após 30 dias de cultivo, todos os explantes cotiledonares e de hipocótilo formaram calos na presença ANA independentemente da suplementação ou não do meio de cultura com KIN. Diretamente sobre os cotilédones de embriões zigóticos imaturos cultivados em meio de cultura MS suplementado com 5 e 10 mg L^{-1} de 2,4-D foram observadas estruturas semelhantes a embriões somáticos em diferentes estádios de desenvolvimento. Na superfície desses embriões formaram-se calos que após subcultivo em meio isento de reguladores de crescimento, originaram complexos ou massas celulares pró-embriogênicas. O isolamento dessas regiões e transferência para meio de cultura MS sem reguladores de crescimento permitiu o avanço dos pró-embriões para o estádio globular e cordiforme.

Termos para indexação: Solanaceae, cultura *in vitro*, embriões somáticos, reguladores de crescimento vegetais.

INTRODUCTION

Cubiu (*Solanum sessiliflorum* Dunal) is an Amazonian plant with potential to supply the food, cosmetic and pharmaceutical industry with the primary products which are supplying the regional free market with fresh fruit only (Andrade Júnior; Andrade, 2014; Andrade Júnior; Andrade; Costa., 2016; Barriuso et al., 2016; Cardona et al., 2013; Gonçalves et al., 2013).

In the Amazonian region, cubiu is an easy plant to cultivate, but it produces seeds with non-uniform germination that have early losses in viability during storage conditions (Pereira et al., 2012). Thus, finding alternatives to the usual production system are of fundamental importance to preserve the current germplasm. Currently, cell and tissue cultures, and developments in genetic engineering have been the most useful technologies (Rai; Shekhawat, 2014) to permit the

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large scale multiplication of clones, and promote the establishment of large field crops (Guerra et al., 2016).

The success of these methods, however, depends on several factors associated to the induction and control of morphogenesis through organogenesis and somatic embryogenesis for plant regeneration (George, 1993). *In vitro* organogenesis is the monopolar formation of roots, leaves and shoot apex by explants and calluses. In somatic embryogenesis, single cells or a small group of somatic cells starts the development of bipolar structures with no vascular connection to the original tissue. This development occurs in a sequence of events similar to zygotic embryogenesis (von Arnold et al., 2002). Although plant cells in general have the capacity for embryogenesis, the acquisition of embryogenic competence is dependent on many circumstances mainly determined by the given physiological state of the cell which is determinate by its genetic and developmental or environmental conditions (Fehér, 2008).

Although regenerative protocols via organogenesis from *S. sessiliflorum* were investigated (Boufleuher et al., 2008; Schuelter et al., 2009), there is no report about the cubiu regeneration through somatic embryogenesis.

Thus, the objective of the present experiment was to evaluate *in vitro* the effects of growth regulators and sources of explants on the induction of the somatic embryogenesis of *S. sessiliflorum* Dunal.

MATERIAL AND METHODS

Plant material and cultivation conditions

Seeds of *S. sessiliflorum* cv ´Santa Luzia` were disinfested with ethanol at 70% (2 minutes) and sodium hypochlorite at 1% (20 minutes) and then rinsed three times in distilled and autoclaved water. The seeds were incubated in vials containing 50 mL MS culture medium (Murashige; Skoog, 1962) supplemented with 30 g L sucrose, 200 mg L myo-inositol, vitamins (0.5 mg L nicotinic acid, 0.5 mg L pyridoxine and 0.1 mg L thiamine), 2 mg L glycine and 6. 5 g L agar. The pH was adjusted to 5.8 before autoclaving. The cultures were maintained in a growth chamber with a photoperiod of 16 h, under 40 mmoL $m^{-2}s^{-1}$ light radiation from two fluorescent lamps (20 W, Osram, Brazil). The temperature in the growth chamber was maintained at 24 ± 2 °C. The hypocotyls and cotyledons of seedlings that grew for 20 days were excised to be used as explants.

Zygotic embryos (ZE) were extracted from immature seeds collected at the 80th day after the anthesis. The fresh fruit were initially washed in tap water. Thereafter, these fruit were first disinfested with ethanol at 70% for 2 minutes and sodium hypochlorite at 1% for 20 minutes, and then rinsed three times in distilled and autoclaved water.

Hypocotyl and cotyledon explants

Cotyledons and hypocotyl segments with 1 cm in length were horizontally incubated in Petri dishes (100 x 15 mm) containing 20 mL MS culture medium supplemented with NAA at 0, 5, 10 and 20 mg L^{-1} and KIN at 0 and 1 mg L^{-1}, and the Petri dishes were then stored under dark conditions.

The experimental design was completely randomized in the factorial arrangement 2x4x2 with two sources of explants (hypocotyl and cotyledon), four levels of NAA (0, 2.5, 5 and 10 mg L^{-1}) and two levels of KIN (0 and 1 mg L^{-1}). Three Petri dishes were inoculated with each one of the culture media with these combinations. Every experimental unit consisted of ten explants.

The percentage of explants with calluses, the texture and color of these calluses, the presence of roots and embryonic structures were all evaluated at the 30 and 60th day. Thereafter, these calluses were divided into two groups and subcultivated for more 30 and 60 days in basal regulator-free culture media. One group was subcultivated under dark conditions, and the other was cultivated under alternate cycles of light with irradiance at 40 µmol m^{-2} s^{-1} for 16 hrs when the development was visually evaluated.

Zygotic embryos explants

Two experiments were designed to evaluate the capacity of the zygotic embryos to induct somatic embryogenesis. In the first, ZE were incubated in Petri dishes containing 20 mL MS culture medium supplemented with hydrolyzed casein at 100 mg L^{-1} and combinations of NAA (0, 2.5, 5 and 10 mg L^{-1}) and KIN (0 and 1 mg L^{-1}) and stored under dark conditions.

The experimental design was completely randomized in a factorial arrangement of treatments 2x4. Every experimental unit had ten ZE per Petri dish with four

replications. Data from the percentage of explants with calluses, root formation, and the presence of embryonic structures developed under darkness were collected at the 30 and 60th day after the inoculation. Thereafter, the material was transferred to growth regulator-free MS culture medium for more 30 days.

In the second experiment, the effects of 0, 2.5, 5 and 10 mg L^{-1} 2,4-D were evaluated in combination with 0 and 0.1 mg L^{-1} KIN in the MS culture medium supplemented with hydrolyzed casein (100 mg L^{-1}). The experimental design was completely randomized in the factorial arrangement of treatments 2x4 with four replications. Every experimental unit had ten ZE per Petri dish. Under darkness, data from the percentage of explants with calluses, percentage of explants with somatic embryos (SE) and number of somatic embryos per explant were collected at the 30, 45, 60 and 75th day after the inoculation. Thereafter, the material was also transferred to regulator-free culture medium for more 90 days, but now under light conditions, and subcultivated for more 30 days.

The cell cultures were also characterized by using cytochemical methods. Thus, they were dyed with Blue Evans at 0.1% and carmine acetic at 2% (Durzan, 1988). The relevant aspects were identified under optical microscope before taking micro-photographical images.

Forty-five days after the induction on the culture media with 2,4-D, these materials were fixed into solution of F.A.A. 50 (Johansen, 1940) and preserved in the ethanol 70 % for preparing the permanent laminas. Thereafter, they were immersed into 2-hydroxyethyl methacrylate-hystoresin, Leica Instruments (Gerrits, 1991).

Slices between 7 and 10 µm in thickness were sectioned in rotary microtome, dyed with toluidine blue, and the laminas were prepared with the synthetic resin Permount. The microphotography was taken under the photonic microscope Olympus BX50 attached to a digital camera Canon Power Shot A95, and the images were acquired through the Zoom Browser EX program.

Data from explant sources and growth regulators were described by their means and standard deviations.

RESULTS AND DISCUSSION

In seven days, the development of calluses was detected on the sectioned regions of hypocotyls and cotyledons. In 30 days, all the explants cultivated on the culture media with 5, 10 and 20 mg L^{-1} NAA developed semi-friable, filamentous and yellow-pale calluses, except in the regulator-free culture medium. These responses were independent of supplementing the culture medium with KIN.

All the hypocotyl surfaces had calluses, and small adventitious roots started developing from some of these explants (Figure 1a) in which the percentage of roots increased with the time (Table 1). In this period, most of the cotyledonary explants also developed calluses (Figure 1b). In 60 days, therefore, 100% of the explants developed roots on regulator-free culture media (Table 1). The calluses from hypocotyls were whitish and foamy. In 30 days, even after the subculture on regulator-free medium, there was no embryogenesis induction.

The different treatments induced the formation of calluses in both hypocotyl and cotyledonary explants, similar to those obtained by Schuelter et al. (2009) for cubiu with a spongy and whitish appearance, but did not induce adventitious shoots or embryogenic structures. Callus induction in tomato (*Lycopersicon esculentum*) var. Moneymaker (Chaudhry et al., 2010) was also obtained from hypocotyls cultivated in MS culture medium however supplemented with KIN (4 mg L^{-1}), BAP (5 mg L^{-1}) and AIA (2 mg L^{-1}).

In the present investigation, higher concentrations of NAA increased the percentage of roots in the hypocotyls. In the cotyledons, the roots developed on the regulator-free or on the culture media supplemented with 5 mg L^{-1}, but they were absent in those media supplemented with 10 and 20 mg L^{-1} NAA (Table 1). In regulator-free culture media, the high percentage of roots suggests that these explants have contents of endogenous auxin prone to induce the rhizogenesis (Taiz et al., 2017).

These responses are in disagreement with Picoli et al. (2000) who detected somatic embryos in eggplant cotyledons (*Solanum melongera*) cultivated on MS culture media supplemented with 2.5, 5.0, 7.5 and 10.0 mg L^{-1} NAA. These authors reported linear decrease in the callogenesis consistent with the increases in the NAA concentrations. In this case, the embryogenesis was induced in all these treatments. Similarly, Huda et al. (2007) reported the callus induction and somatic embryos in eggplant cotyledons (*S. melongera*) cultivated on MS culture media supplemented with NAA (2.0 mg L^{-1}) and BAP (0.05 mg L^{-1}).

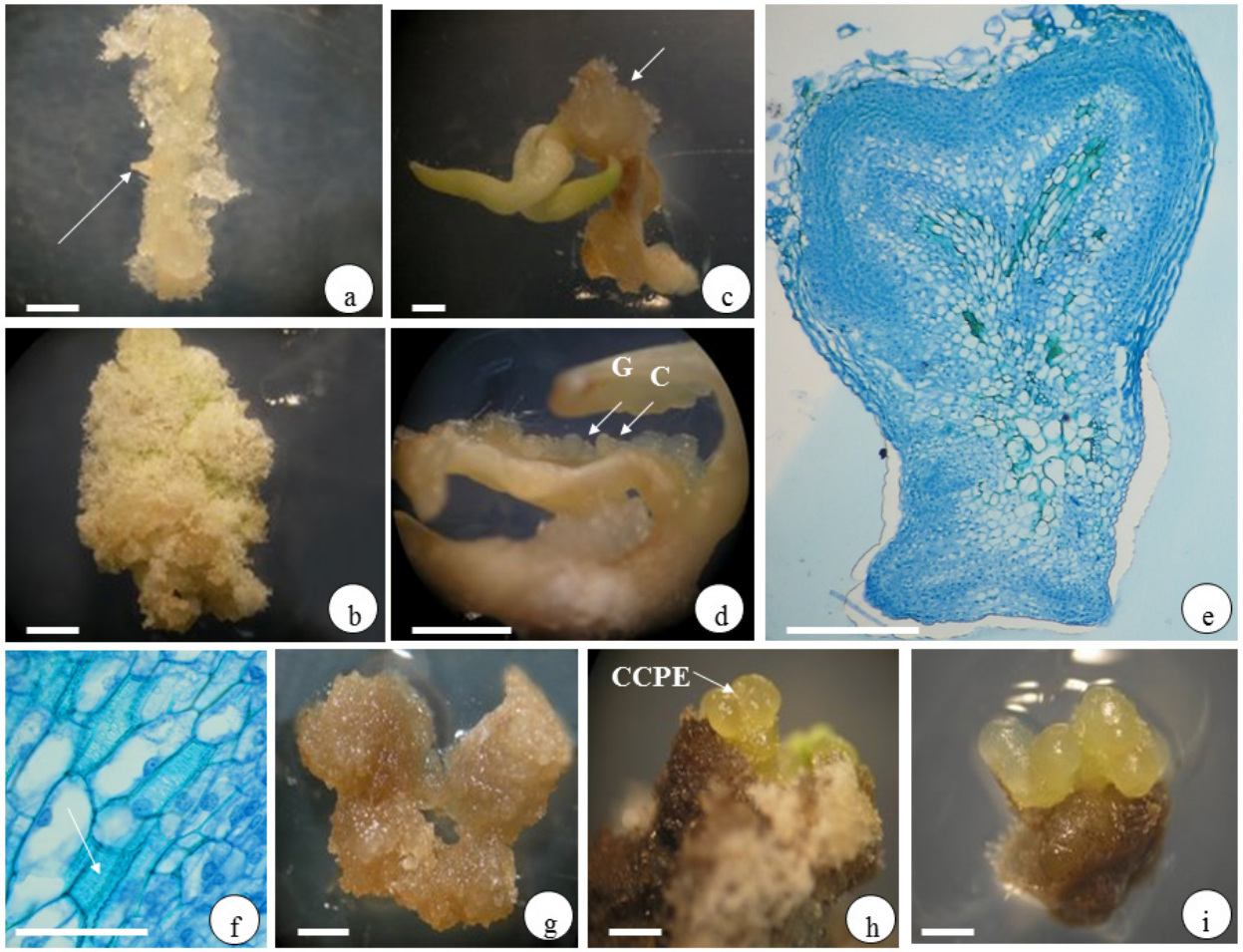

Figure 1 – Morphogenetic responses of *S. sessiliflorum*: a) calluses and roots formation (arrow) on the hypocotyl (NAA at 5 mg L^{-1}); b) semi-friable callus on cotyledons, bar = 2.5mm; c) callus on hypocotyl-radicle regions (arrow); d) globular (G) and heart-shaped (C) structures on the cotyledonary leaves of ZE after 45 days (10 mg L^{-1} 2,4-D), bar = 25 mm; e) longitudinal section showing the SE in the closed vascular system (10 mg L^{-1} 2,4-D), bar = 300 µm; f) differentiation of xylem cell in the vascular tissue (arrow), bar= 35 µm.; g) friable callus (10 mg L^{-1} 2,4-D), bar = 1 mm; h) pro-embryonic cell complexes (CCPE) developed on culture medium supplemented with 10 mg L^{-1} 2,4-D and 0.1 mg L^{-1} KIN after 150 days, bar = 0.5 mm; i) SE heart-shaped after 180 d, bar = 1 mm. This figure is in color in the electronic version.

Table 1 – Percentage of explants with root induction from hypocotyls (H) and cotyledons (C) of *S. sessiliflorum* cultivated in the MS culture media.

	DAY	Without KIN				With KIN			
		NAA (mg L^{-1})				NAA (mg L^{-1})			
		0	5	10	20	0	5	10	20
H	30	33 ± 6	13 ± 6	10	17 ± 6	10 ± 0	10 ± 0	23 ± 11	23 ± 21
	60	33 ± 6	30 ± 10	57 ± 6	60 ± 10	23 ± 15	27 ± 11	47 ± 31	57 ± 15
C	30	83 ± 29	10	0	0	7 ± 6	3 ± 6	0	0
	60	100	17 ± 6	0	0	20 ± 0	20 ± 17	0	0

In the embryo axis, the calluses were formed at the 7th day, but just after the cotyledonary opening and a small elongation of the hypocotyl. The average of calluses from EZ varied according to the concentrations of NAA and KIN. Increases in the NAA concentration in KIN-free culture media reduced the calluses proliferation. Conversely, there were increases in calluses formation under KIN at 1 mg L^{-1} (Table 2).

Table 2 – Percentage of calluses from ZE of *S. sessiliflorum* cultivated in culture media with two growth regulators for 30 days.

		NAA (mg L^{-1})			
		0	2.5	5	10
KIN	0	0	85 ± 5.77	72.5 ± 9.57	47.5 ± 9.57
(mg L^{-1})	1	0	47.5 ± 9.57	50 ± 8.16	75 ± 5.77

In most explants, the calluses developed in those treatments with auxins which are plant hormones capable of starting cellular division and controlling the growth, and lengthen the plant cells (George, 1993). In other species, otherwise, the development of calluses (Guo et al., 2011) and somatic embryos is also influenced by the cytokinin (Jayapadma; Bhat; Kuruvinashetti, 2008). Callus induction in several plant species occurs through the exogenous application of auxins and cytokinins (Ikeuchi; Sugimoto; Iwase, 2013). Godishala, Mangamoori and Nanna (2011) reported the possibility of establishing embryogenic callus culture from cotyledon explants of *Solanum lycopersicon* using culture media supplemented with auxin and cytokinin.

Otherwise, calluses proliferation were observed on immature ZE of *S. sessiliflorum* developing on treatments with 2,4-D independently of supplementing these culture media with KIN for 30 days (Table 3). In those treatments free of 2,4-D, independently of supplementing the culture media with 0.1 mg L^{-1} KIN, there was 100% of ZE germination, which started upon one week after the inoculation.

At the 7th day and after a small elongation of the hypocotyl, callogeneses were detected in the axis hypocotyl-radicle (Figure 1c). In 30 days, small protuberances were observed on the cotyledonary leaves of the ZE inoculated on culture media with 5 and 10 mg L^{-1} 2,4-D. All the explants from culture media supplemented with 2.5 mg L^{-1} 2,4-D developed calluses in a way similar to the responses detected at the 60th day when these same culture media were supplemented with KIN (Table 3). Increases in the concentration of 2,4-D decreased the percentage of calluses, and these responses were higher in those culture media free of KIN.

In 45 days, the first structures similar to somatic embryos (SE) were then observed. At first, these structures in the adaxial epidermis of cotyledons were globular and had color appearance between the white and straw-yellow. These responses were observed on the MS culture medium supplemented with 2,4-D (5 and 10 mg L^{-1}) and free of KIN, or 2,4-D (10 mg L^{-1}) combined with KIN (0.1 mg L^{-1}). Most of the regenerated ES were globular, heart-shaped, and similar to the same globular and heart-shaped stage of the SE (Figure 1d). In this period, the highest percentage of explants with embryo-like structures was found in the treatment with only 10 mg L^{-1} 2,4-D and free of KIN. In this treatment, we found the highest number of SE per explant. In those treatments where the induction was positive, there was significant increment in the number of embryos in the period of 75 days (Table 4).

Table 3 – Percentage of calluses developed from ZE inoculated on MS culture media supplemented with two growth regulators for four periods.

	With KIN				Without KIN			
	2,4-D (mg L^{-1})				2,4-D (mg L^{-1})			
DAYS	0	2.5	5.0	10.0	0	2.5	5.0	10.0
30	0	95.0 ± 5.8	85.0 ± 10	70.0 ± 20.0	0	100.0	75.0 ± 31.1	62.5 ± 9.6
45	0	95.0 ± 5.8	87.0 ± 5.0	72.5 ± 15.0	0	100.0	75.0 ± 31.1	62.5 ± 9.6
60	0	100.00	95.0 ± 5.8	72.5 ± 15.0	0	100.0	87.5 ± 15.0	62.5 ± 9.6
75	0	100.00	95.0 ± 5.8	72.5 ± 15.0	0	100.0	87.5 ± 15.0	62.5 ± 9.6

Table 4 – Percentage of explants with embryogenic structures (%) and the number of embryos per explant (NE) from the MS culture medium supplemented with two growth regulators for four periods.

Trait	Days	With KIN				Without KIN			
		2,4-D (mg L^{-1})				2,4-D (mg L^{-1})			
		0	2.5	5.0	10.0	0	2.5	5.0	10.0
%	30	0	0	0	0	0	0	0	0
	45	0	0	0	15 ± 5.8	0	0	5 ± 10	22.5 ± 9.6
	60	0	0	0	22.5 ± 9.6	0	7.5 ± 9.6	12.5 ± 15	25 ± 12.9
	75	0	0	12.5 ± 12.6	22.5 ± 9.6	0	7.5 ± 9.6	22.5 ± 15	35 ± 12.9
NE	30	0	0	0	0	0	0	0	0
	45	0	0	0	3.2 ± 2.22	0	0	0.7 ± 1.5	7.2 ± 5.6
	60	0	0	0	5.5 ± 2.9	0	2.7 ± 3.8	3.7 ± 5.7	7.7 ± 5.1
	75	0	0	4 ± 6.7	7.5 ± 1.3	0	2.7 ± 3.8	6.5 ± 5.4	11 ± 2.2

In the present study, the induction of somatic embryogenesis was observed from ZE cultivated in culture media with 2,4-D. The acquisition of embryogenic competence is influenced by different factors (Namasivayam, 2007). The agents used to induce *in vitro* embryogenesis in somatic plant cells are highly variable from various plant hormones to stress treatments (Fehér; Pasternak; Dudits, 2003). Auxin has so far been considered the most prominent plant hormone related to cell division and differentiation, as well as in the triggering of SE (George, 1993) and 2,4-D the most widely used inducer (Fehér, 2008; Xu et al., 2014). Explants with high levels of endogenous auxin may be more responsive (Nascimento-Gavioli et al., 2017). The highest response in the induction of *Capsicum baccatum* somatic embryos was obtained when the explants (cotyledon and leaf) were cultured on MS medium with 2,4-D (2.0 mg L^{-1}) and KIN (0.5 mg L^{-1}) (Venkataiah et al., 2016).

Anatomical studies of embryo-like structures from the treatments with 2,4-D showed independent structures, and suggested closing vascular system (Figure 1e and 1f), which means the presence of SE. In 60 days, there was no development of ontogenetic stages as torpedo and cotyledons. In these structures and nearby them, there was calluses proliferation instead. Although there are benefits from high concentrations or long-term treatments with growth regulators that induce somatic embryogenesis, the responses can be quite different from the initial aims. Long-term embryonic cultures with 2,4-D, for example, induce genetic variation capable of affecting the embryogenic potential (George, 1993).

Similarly, the induction of embryogenesis from ZE cultivated in culture media with 2,4-D was reported by several authors (Fehér, 2008). Guerra et al. (2016) reported that in *Acca sellowiana* culture medium supplemented with 2,4-D and subsequent culture in regulator- free medium resulted in somatic embryogenesis induction starting from ZE as explants. The 2,4-D has definitively been the most applied auxin (Fehér, 2008).

According to Jiménez (2005), when the 2,4-D is removed from the culture media or the doses are reduced, the endogenous levels of IAA are also reduced to permit the establishment of the polar gradient by the auxins. Thus, the continuous growth in culture medium with this hormone impedes the reduction of the endogenous levels of auxin. In most species, in which growth regulators are required to induce somatic embryogenesis, auxins and cytokinins are essential to induce the embryogenesis because they may be important in regulating the cell cycle and dividing the cells (Fehér; Pasternak; Dudits, 2003; Jiménez, 2005). Auxins promote cellular dedifferentiation, and the cellular division is reactivated via the coordination of gene expression and the transcriptional modifications of regulatory proteins which are involved in the cellular cycle (Jiménez, 2005).

In 75 days, the explants developed two types of calluses: the first with filamentous and dark-colored appearance was formed on the ZE cultivated in culture

media with 2.5 mg L^{-1} 2,4-D, the next developed on the ZE cultivated with 5 and 10 mg L^{-1} 2,4-D. They were friable, whitish and had the granular texture. They were transferred to light conditions and cultivated in regulator-free culture media for more 90 days, but subcultivated for periods of 30 days. In the first subculture, the cytochemical evaluation with Blue Evans and acetic carmine revealed the presence of elongated cells with light-colored cytoplasm in the filamentous calluses. These calluses were incapable of dyeing by using the carmine acetic, and the typical globular pro-embryogenic structures were not detected. Friable calluses with granular texture (Figure 1g) developed on the ZE, and had small isodiametric cells that strongly reacted with the carmine acetic because the presence of glycoprotein which are associated to the embryogenesis.

Calluses of *Caesalpinia echinata* regenerated from young leaflets also had the filamentous appearance, but they did not show embryogenic origin (Pessotti et al., 2007). According to Cid (1992), these filamentous embryogenic cultures are composed of differentiated elements as fibers and sclereideos which indicate the presence of non-dedifferentiated portion in the calluses, and consequently their origins were not embryogenic. According to Jong, Schimidt and Vries (1993), these typical embryogenic cells are small (20-30 mm) and isodiametric; they have dense cytoplasm and high capacity of division. Calli induced by 2,4-D observed in *Cleome rosea* (Simões et al., 2010) presented a nodular appearance which is considered a typical physical feature of embryogenic calli.

In 150 days, the *in vitro* conditions permitted the development of complex or pro-embryogenic cell masses (Figure 1h) in 7.5% of the calluses cultivated in the culture media supplemented with 10 mg L^{-1} 2,4-D and 0.1 mg L^{-1} KIN. These complexes have friable appearance and develop embryos in their surface when the conditions are appropriate. The isolation of these regions and their transference to regulator-free MS culture media permitted the formation of globular and heart-shaped pro-embryonic structures after the period of 30 days (Figure 1i). The formation of somatic embryos was asynchronous, so there were embryos in different stages of development. Somatic embryos did not progress to other advanced embriogenic stages.

The present investigation showed the first responses of embryogenic cells from pro-embryogenic masses, and embryogenesis induction from ZE treated with 5 and 10 mgL^{-1} 2,4-D. Similar responses were reported by Carvalho et al. (2004) who obtained the indirect path of the *Diospyros kaki* embryogenesis which was firstly induced through pro-embryogenic cellular mass with the SE developing in their surface. The SE of *Diospyros kaki* developed on the ZE cultivated for 150 days in the MS culture medium supplemented with 2.21 mg L^{-1} 2,4-D and 0.43 mg L^{-1} KIN. Pessotti et al. (2007) verified that young leaflets of *Caesalpinia echinata* developed calluses on culture medium supplemented with 5, 10 and 20 mg L^{-1} 2,4-D. Their transference to regulator-free culture medium stimulated the development of pro-embryogenic cellular masses in 95% of the calluses. The long period on the regulator-free culture medium impeded the conversion of these cellular masses into SE.

In the present study, the induction of somatic embryogenesis either by direct or indirect way was verified just when the ZE was used as explant source. The use of ZE have some advantages in comparison with the others sources. First, the responses are fast. The ZE, therefore, can be an excellent source of explants for investigating the physiology involved in the somatic embryogenesis. As the cells have meristematic characteristics, immature tissues as the ZE are naturally embryogenic, and require less exogenous stimulus from the culture medium (Litz; Gray, 1995). Second, the cells from mature tissues have heterogeneous specificity, and they differentiate slowly. This case requires re-determination through the indirect path for calluses induction or embryogenic masses (Fehér; Pasternak; Dudits, 2003).

The use of ZE as explant sources of cross pollinated species can impose some limits to the method because these explants represent unknown genotypes. According to Litz and Gray (1995), plants from seeds must be meiotic recombinants from two parents that are not genetically similar, except in a few cases. However, the control of the pollination can be a strategy to reduce the effects from the double fertilization.

The protocol from clonal propagation via somatic embryogenesis is a complex procedure because it involves

the knowledgement of biological factors that affect several stages of the embryonic development.

Although synchronized development was not yet attained, and the regeneration frequency is still low, this investigation is a compelling argument for studying better conditions to achieve the maturation of somatic embryos through growth regulators capable of overcoming the constraints during the somatic embryogenesis of *S. sessiliflorum*. Synchronized development and SE maturation are fundamental to attain the massive production of embryogenic crops.

CONCLUSIONS

This study describes, for the first time, *S. sessiliflorum in vitro* induction of SE differentiated directly from ZE cultivated in culture media with 2,4-D (5 e 10 mg L^{-1}) and the induction of embryogenic cells derived from pro-embryogenic masses transferred to regulator-free culture medium. The results obtained provide additional support for a better understanding of this *in vitro* morphogenetic pattern. Further studies will be necessary to identify conditions of maturation and conversion of somatic embryos into complete plantlets of this species.

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