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In vitro callus induction and development of *Vernonia condensata* Baker with embryogenic potential

Indução de calos e desenvolvimento *in vitro* de *Vernonia condensata* Baker com potencial embriogênico

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ABSTRACT

Vernonia condensata Baker has been traditionally used in folk medicine for the treatment of several inflammatory and infectious processes. Overexploitation of this plant species has drastically reduced its population in its natural habitat (Cerrado). Therefore, tissue culture tools, such as somatic embryogenesis, can be used as an alternative method for rapid and large-scale plant regeneration. The objectives of this study were to induce callogenesis in *Vernonia condensata* from different types of explants and to evaluate the structural aspects of the development of pro-embryogenic masses of this species by means of histological analyses. The formation of calli was induced from leaf explants and internodal segments, which were inoculated in EME medium supplemented with 50 g L^{-1} sucrose, 0.5 g L^{-1} malt extract and 2.68 µM NAA, plus varying concentrations of BAP (0.00, 2.22, 4.44 or 8.88 µM). After 40 days, the following morphogenetic traits were evaluated: intensity of callus formation, intensity of oxidation, callus texture, and morphogenesis. The calli with embryogenic masses were analyzed by light and scanning electron microscopy. Both types of explants were responsive regarding callogenesis, with the BAP concentration of 4.44 µM promoting the formation of friable calli associated with a larger percentage of calli with embryogenic masses. Cells from leaf explants and internodal segments were able to dedifferentiate and change into embryonic structures.

Index terms: Alumã; *in vitro* culture of calli; anatomical analysis; scanning electron microscopy; growth regulators.

RESUMO

Vernonia condensata Baker tem sido tradicionalmente usada na medicina popular para o tratamento de vários processos inflamatórios e infecciosos. A exploração excessiva dessa espécie de planta tem reduzido drasticamente a sua população em seu habitat natural (Cerrado). Portanto, ferramentas de cultura de tecidos, como a embriogênese somática, podem ser usadas como um método alternativo para a regeneração de plantas de forma rápida e em larga escala. O objetivo do estudo foi induzir a calogênese de *Vernonia condensata* a partir de diferentes tipos de explantes e avaliar os aspectos estruturais do desenvolvimento de massas pró-embriogênicas desta espécie, por meio de análises histológicas. A formação dos calos foi induzida a partir de segmentos foliares e internodais inoculados em meio EME acrescido de 50 g L^{-1} de sacarose, 0,5 g L^{-1} extrato de malte e 2,68 µM ANA, variando-se as concentrações de BAP (0,00; 2,22, 4,44 e 8,88 µM). Após 40 dias avaliou-se os seguintes caracteres morfogenéticos: intensidade de calo formado, intensidade de oxidação, textura do calo e morfogênese. Os calos com massas embriogênicas foram analisadas por microscopia de luz e eletrônica de varredura. Ambos os tipos de explantes são responsivos quanto à calogênese, sendo que a concentração de 4,44 µM BAP promove formação de calos friáveis associada à maior porcentagem de calos com massas embriogênicas. Células de explantes foliares e segmentos internodais foram capazes de desdiferenciarem e se transformar em estruturas embrionárias.

Termos para indexação: Alumã; cultivo de calos *in vitro;* análise anatômica; microscopia eletrônica de varredura; reguladores de crescimento.

INTRODUCTION

Vernonia condensata Baker, popularly known in Brazil as "alumã", "figatil" or "necroton", is a medicinal plant belonging to the family Asteraceae, widely distributed in the country, traditionally used in folk medicine for its analgesic, anti-inflammatory, anti-fever, antibacterial and antiulcerogenic properties (Boeing et al., 2016; Thomas et al., 2016; Sharifi-Rad et al., 2018; Silva et al., 2018). Besides this, some secondary metabolites such as saponins, tannins, alkaloids, phenolic compounds and flavonoids have been detected in extracts of *V. condensata* (Risso; Scarminio; Moreira, 2010).

In general, *V. condensata* is propagated asexually through cuttings (Lorenzi; Matos, 2008). Overexploitation of this plant species due to its phytochemical potential has drastically reduced the population in its natural habitat. Therefore, it is important to conduct studies to promote sustainable management and develop alternative propagation methods, such as a regeneration system for rapid multiplication to produce elite plants. In this context, *in vitro* culture techniques, in particular somatic embryogenesis, a remarkable expression of cellular totipotency for clonal mass propagation and genetic improvement, especially of woody species that present a very long life cycle (Grzyb; Mikuła, 2019; Pais et al., 2019).

In this process, the induction of calli and subsequent regeneration of plants are the main steps necessary before undertaking any genetic improvement program. However, the establishment of callogenic masses and the biochemical characterization of calli can reveal the processes that trigger the development of explants, supplying important information about the *in vitro* morphogenetic process of plant tissues.

The analysis of somatic embryogenesis through histological observations is increasingly common, encompassing the use of various techniques that provide detailed knowledge of the events that occur when plant species are grown *in vitro*. Thus, for example, it is possible to monitor the cell and tissue development of the plant material during the different stages of the somatic embryogenesis process, as well as to identify the tissue that is most responsive and/or embryogenetic and/or the best cell marker of the start of the process (Gomes et al., 2017). Furthermore, histological analyses and examination by scanning electron microscopy can elucidate various aspects related to the *in vitro* morphogenesis of different plant species (Aslam et al., 2011*;* Alcantara et al., 2014*;* Delporte et al., 2014; Meira et al., 2018). However, to the best of our knowledge, no studies of this nature have been published involving *V. condensata*.

Due to the scarcity of studies related to the development of calli and somatic embryogenesis of *Vernonia condensata*, the objective of this work was to clarify for the first time cells of *V. condensata* with embryogenic potential from *in vitro* culture of calli, to better understand the structural events of the development of the pro-embryogenic masses formed, through anatomical analysis and scanning electron microscopy.

MATERIALS AND METHODS

Plant material

To obtain the calli, we used as explants leaf and internodal segments of plants after *in vitro* growth for 40 days in MS medium (Murashige; Skoog, 1962). The experiment was performed in the Plant Tissue Culture Laboratory of Maria Milza College, in Mangabeira, Bahia, Brazil. Voucher specimens of *V. condensata* were deposited in the herbarium of UFES, under number HUEFS 150499.

Induction of calli

The leaf and internodal segments were grown in EME culture medium (Grosser; Gmitter Junior, 1990) supplemented with 50 g L^{-1} sucrose, 0.5 g L^{-1} malt extract, 2.68 µM NAA (naphthaleneacetic acid), and variable concentrations of BAP (benzylaminopurine): 0.00, 2.22, 4.44 or 8.88 µM, solidified with 0.2% Phytagel™. The medium's pH was adjusted to 5.7 ± 0.1 and then was sterilized by autoclaving for 20 minutes at temperature of 121º C and pressure of 1 atm.

The culture medium was distributed in Petri dishes (9 cm diameter and 1 cm height) and after 24 hours the dishes with the explants were placed in a growth room in the dark with temperature of 27 ± 2 ºC for 40 days. The experimental design was completely randomized in a 2 x 4 factorial scheme (2 explant types x 4 BAP concentrations), with 10 repetitions. Each experimental plot was composed of one Petri dish containing five explants.

Morphogenetic traits evaluated

After *in vitro* growth for 40 days, the following characteristics were evaluated: a) intensity of callus formation, by assigning scores from 1 to 4: score 1- absence of calli, score 2 – small calli (from 0.5 to 0.8 cm), score 3 – medium calli (from 0.8 to1.0 cm), and score 4 – large calli (> 1.0 cm), b) intensity of callus oxidation (score of 1-3): 1 – absence of oxidation, 2 – partially oxidized calli, 3 – totally oxidized calli, c) callus texture (friable, partially compact and compact), and d)

morphogenesis – percentage of calli with embryogenic masses and percentage of calli with roots.

Callus morphology

Fixation of the samples

For anatomical analysis, callus samples (n = 10) from treatments that were responsive to embryogenic masses formation were fixed in modified Karnovsky's solution (Karnovsky, 1965) [glutaraldehyde (2%), paraformaldehyde (2%), CaCl2 (0.001 M), sodium cacodylate buffer (0.05 M), pH 7.2] for 48 hours and observed by light and scanning electron microscopy.

Callus analysis by scanning electron microscopy (SEM)

After fixation, the samples (n = 5 calli) were dehydrated in increasing concentrations of ethanol (35-100%) for 20 minutes each. The samples were dried in hexamethyldisilazane, and the dried samples were then mounted on metallic supports (stubs), and metallized with gold for 180 s using a sputter coater (MED 010, Balzers Union, Balzers, Liechtenstein). The images were obtained using a LEO 435 VP variable pressure scanning electron microscope (Carl Zeiss, Jena, Germany), at 20 kv.

Callus analysis by light microscopy (LM)

For anatomical analyses, the calli (n =5) were fixed in the same modified Karnovsky's solution for one week, dehydrated in an ethyl alcohol series (35–100%) for 9 h, and infiltrated and embedded using the Historesin kit (hydroxyl methacrylate, Leica, Heidelberg, Germany). The samples were blocked and the polymerization occurred at room temperature for 48 h. Serial histological sections (5 µm) were obtained with a Leica RM 2155 rotary microtome (Nussloch, Germany), arranged in histological slides and stained with acid fuchsin (0.1% w/ v), followed by toluidine blue (0.05% w/v) (Feder; O'Brien, 1968), covered with Entellan® and cover slips. The sections were then analyzed and digital images obtained with an Axioskop 2 photomicroscope (Carl Zeiss, Jena, Germany).

Statistical analysis

To assess the significance of the treatments, the data were submitted to analysis of variance by the F-test and the means were compared by the Tukey test at 5% significance, except for data without normal distribution, which were submitted to the nonparametric Kruskal-Wallis test. The mean values of the explant types were compared by the Student-Newman-Keuls test at 5% probability. To complement the statistical analyses, regression analysis was applied to the quantitative factors related to the BAP concentrations and mathematical models were chosen according to the equations with the best fit, confirmed by the higher coefficients of determination (R^2) and the F-test, both at 5% probability. The data were analyzed by the SAS 9.2 program (SAS Institute 2009).

RESULTS AND DISCUSSION

Embryogenic callus induction and multiplication of leaf and internodal segments of *V. condensata*

The morphogenetic responses based on the intensity of callus formation, intensity of oxidation, texture and *in vitro* morphogenesis varied according to the treatment employed and are presented in Tables 1, 2, 3 and 4, respectively. The intensity of callus formation was significantly influenced by the type of explant and concentration of the growth regulator BAP (Table 1). According to Kumlay and Ercisli (2015), the application of cytokinins affects callogenesis, resulting in decrease of the cell wall lignification, facilitating callus initiation and growth *in vitro*.

A previous study has demonstrated that callus proliferation usually starts from the cut surface of the explant and finally covers the whole explant (Hoque et al., 2006). In our study, the calli started to appear 10 days after the induction treatment, with varying sizes, color, consistency and morphogenesis. The results observed in this study corroborate the findings for *Stevia rebaudiana* Bertoni, where the time of callus induction was six days using 1 mg L^{-1} BAP + 1 mg L^{-1} NAA for both media (MS and B5) and explant types (leaf and internode) (Keshvari et al., 2018). On the other hand, other authors have reported no significant difference between interaction effects of explant type × hormone concentration on callus induction percentage in *Cuminum cyminum* L. (Kazemi; Kahrizi; Mansouri, 2016) and *Mentha pulegium* L. (Jafari; Kahrizi; Mansouri, 2016).

The culture medium without the addition of cytokinin BAP (control) did not cause any response to the callus induction in leaf segment explants, with formation only of adventitious roots (Figure 1a). In contrast, the internodal segments were more likely to form calli compared to the leaf explants in the culture medium without BAP (Table 1, Figure 1b-c). This variation between the responses displayed by two explant types can be attributed to the physiological state of the donor organs, mainly related to the level of endogenous phytohormones and sensitivity to auxins due to variations in the number of receptors or receptor affinity (Jiménez, 2005).

Table 1: Evaluation of the intensity of callus formation of *Vernonia condensata* explants cultured with different concentrations of BAP, according to the attribution of scores –score 1: absence of calli, score 2: small calli (0.5 to 0.8 cm), score 3: medium calli (0.8 to 1.0 cm), score 4: large calli (> 1.0 cm). Explant types: IS = internodal segment, LS: leaf segment.

BAP (μM)	Callus Formation Intensity											
	Calli (score: 1)			Calli (score: 2)			Calli (score: 3)			Calli (score: 4)		
	IS	LS	P	IS	LS	P	IS	LS	P	IS	LS	P
0.00	2.80b	5.00a	**	2.20a	0.00b	*	0.00a	0.00a	ns	0.00a	0.00a	ns
2.22	0.00a	0.78a	ns	1.89a	2.89a	ns	2.00a	0.67b	*	1.11a	0.44a	ns
4.44	0.10a	0.70a	ns	2.90a	1.60b	*	1.50a	1.70a	ns	0.50a	1.00a	ns
8.88	0.00a	1.70a	ns	3.00a	2.70a	ns	1.60a	0.70a	ns	0.40a	0.00a	ns
P	**	ns		ns	*		*	**		**	**	

Means followed by the same lowercase letters in the rows do not differ from each other by the Kruskal-Wallis test at 5% probability. ns = not significant at 5% probability. ** highly significant ($p \leq 0.01$) and * significant ($p \leq 0.05$).

Table 2: Evaluation of the oxidation intensity of explants of Vernonia condensata cultured with different concentrations of BAP, according to the attribution of scores – score 1: absence of oxidation, score 2: partially oxidized calli, score 3: totally oxidized calli. Explant types: IS: internodal segment and LS: leaf segment.

BAP (μM)	Callus Oxidation Intensity								
	Calli (score: 1)			Calli (score: 2)			Calli (score: 3)		
	IS	LS	P	IS	LS	P	IS	LS	P
0.00	2.70a	0.00b	2.70a	0.00a	0.00a	ns	0.10a	0.00a	ns
2.22	4.56a	0.67b	4.56a	0.33b	3.56[a]	*	0.11a	0.11a	ns
4.44	4.20a	0.50b	4.20a	0.80b	4.00a	*	0.00a	0.50a	ns
8.88	3.90a	0.20b	3.90a	1.10b	3.50[a]	*	0.00b	3.18a	*
P	ns	**		**	ns		**	**	

Means followed by the same lowercase letters in the rows do not differ from each other by the Kruskal-Wallis test at 5% probability. ns = not significant at 5% probability. ** and * significant at 1% and 5% probability, respectively.

Table 3: Evaluation of callus texture of explants of *Vernonia condensata* grown with different concentrations of BAP. (score 1 – friable calli, score 2 – partially compact calli, score 3 – totally compact calli). Explant types: IS – internodal segment and LS – leaf segment.

BAP (μM)	Callus Texture								
	Calli (score: 1)			Calli (score: 2)			Calli (score: 3)		
	IS	LS	P	IS	LS	P	IS	LS	P
0.00	2.20a	0.00b	*	0.00a	0.00a	Ns	0.00a	0.00a	ns
2.22	5.00a	2.00b	*	0.00b	1.67a	*	0.00a	0.44a	ns
4.44	4.80a	1.20b	*	0.20b	2.50a	*	0.00b	1.30a	*
8.88	5.00a	0.40b	*	0.00b	2.70a	*	0.00b	1.90a	*
P	ns	**		**	ns		**	**	

Means followed by the same lowercase letters in the rows do not differ from each other by the Kruskal-Wallis test at 5% probability. ns = not significant at 5% probability. ** and * significant at 1% and 5% probability, respectively.

Table 4: Evaluation of morphogenesis *in vitro* morphogenesis of explants of *Vernonia condensata* grown with different concentrations of BAP. Explant types: IS – internodal segment and LS – leaf segment.

BAP (μM)	Calli with embryogenic masses (CEM)			Calli with roots (CR)		
	IS	LS	P	IS	LS	
0.00	6.00a	0.00a	Ns	6.00a	0.00a	ns
2.22	75.56a	13.33b	*	8.89a	11.11a	ns
4.44	80.00a	16.00b	*	2.00a	4.00a	ns
8.88	58.00a	16.00b	*	2.00a	2.00a	ns
P	**	ns		**	**	

Means followed by the same lowercase letters in the rows do not differ from each other by the Kruskal-Wallis test at 5% probability. ns = not significant at 5% probability. ** and * significant at 1% and 5% probability, respectively.

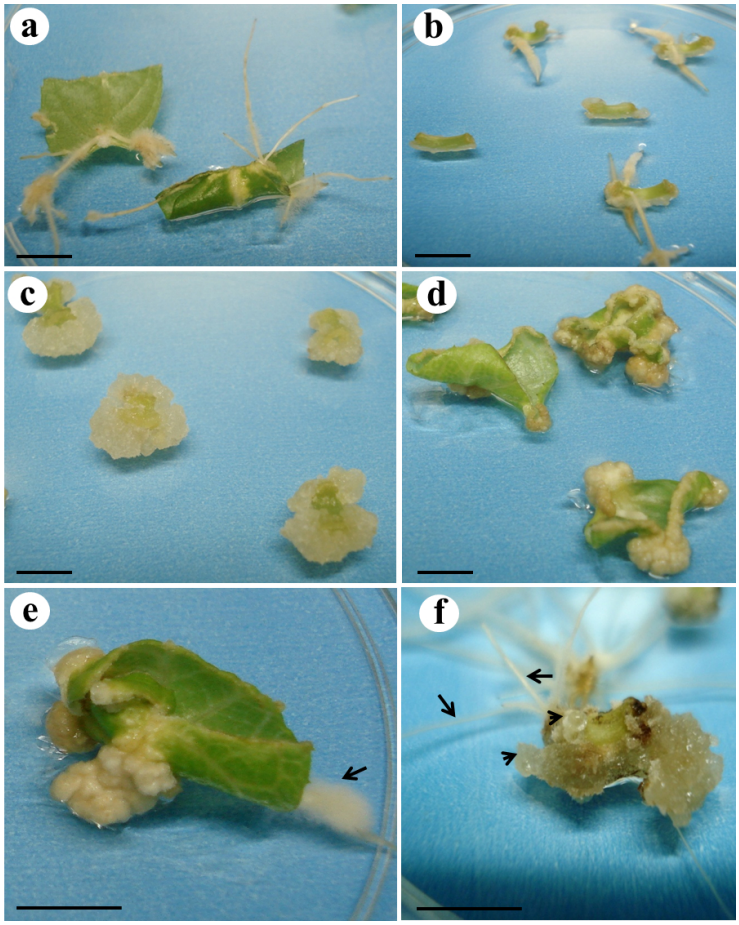

Figure 1: a) Absence of embryogenic calli, with formation only of adventitious roots from leaf segments in culture medium without BAP. b) Start of formation of embryogenic calli from internodal segments in the absence of cytokinin. c) Greater formation of embryogenic calli from internodal segments in the culture medium without BAP. d) Development of compact calli from leaf segments in grown in culture medium supplemented with 4.44 μM BAP. e) Formation of roots from embryogenic calli obtained from leaf segments (arrow). f) Rhizogenic callus from internodal segment (arrow) and formation of somatic embryos (arrowhead) grown in culture medium without de BAP. Bars: 1 mm.

In the control treatment, root development was observed in 100% of the leaf explants, due to the supplementation of the medium with 2.68 μM NAA, which through interaction with the endogenous level of auxin was sufficient to promote rhizogenesis. NAA, an auxin analogue, is extensively applied as a rooting medium for several plants species, such as *Arabidopsis thaliana* (Chen et al., 2014) and *Pinus massoniana* Lamb. (Wang; Yao, 2019).

Observations from other authors indicate that the type of medium and explant are important factors in quantity and quality of calli obtained from explants (Keshvari et al., 2018). Kumlaya and Ercisli, (2015) also noted that the callus induction percentage and friability of the produced calli varied widely between explants. According to these authors, the internodal segments as explant sources proved to be the best for callus induction in potato when compared to the callus formation from leaf explants. In turn, Kazemi, Kahrizi and Mansouri (2016) observed similar behavior between explant types regarding callus induction percentage.

The internodal explants grown in culture media containing 0.00 and 4.44 μM BAP produced larger quantities of small calli (score 2, 0.5 to 0.8 cm) than the corresponding leaf explants. In a previous study, Ribeiro et al. (2007) also reported larger average numbers of small calli formed from leaf explants of *Dieffenbachia* sp. cultured in MS medium supplemented with 2.22 μM BAP plus 16.11 μM NAA.

The medium supplemented with 2.22 μM BAP favored the development of more calli with score 3, classified as medium (0.8 to 1.0 cm) from internodal segments. Donato et al. (2000), evaluating somatic embryogenesis *in vitro* in common cabbage (*Brassica oleracea* L., var. *leucocephala*) did not observe a significant difference regarding the intensity of callus formation, both in the treatments that combined 2,4-D and KIN and in the plants treated with the association of 2,4-D and BAP.

For calli with score 4 (large, >1.0 cm), no significant differences were observed between the explants used. Kumlaya and Ercisli, (2015) induced a larger percentage of callogenesis in *Solanum tuberosum* L. grown in MS culture medium (Murashige; Skoog, 1962) supplemented with 3.0 mg L^{-1} benzylaminopurine (BAP) and 2.0 mg L^{-1} naphthaleneacetic acid (NAA).

With respect to oxidation intensity (Table 2), the calli with score 1 presented a different response between the two explant types, with the largest numbers without oxidation being produced by explants from internodal segments at all the BAP concentrations tested.

For the calli with a score of 2 (partially oxidized), only in the absence of BAP was no difference observed between the two explant types. However, when the cytokinin was added to the culture medium, there was a significant increase in the number of partially oxidized calli when the leaf segments were used as explants. Similar behavior was observed for the calli with score of 3 (totally oxidized), since the leaf explants produced a smaller number of that callus type, especially when grown in medium containing 8.88 μM BAP.

Another morphogenetic trait evaluated was callus texture (Table 3), for which there was a significant difference between the two explant types, with the average textures of the calli from internodal segments being significantly superior with all the BAP concentrations tested. On the other hand, Costa, Loureiro and Pereira, (2008), investigating the influence of auxins and types of explants on the induction of friable calli in *Piper hispidinervum* C. DC. observed the highest percentage of friable calli formed came from leaf explants cultured in medium supplemented with NAA. In turn, Rodrigues and Almeida (2010), utilizing different concentrations of BAP, found that the addition of 26.64 μM of this cytokinin to the culture medium and the performance of two subcultures after the *in vitro* culture was necessary for callogenesis in *Cissus sicyoides* L. from leaf segments.

Regarding the number of partially compact calli, we observed significant differences between the two explant types when grown in culture medium supplemented with BAP. The largest values of this variable were produced by leaf segments grown in medium with cytokinin.

With respect to the number of totally compact calli, there was a significant difference between the treatments only when using the BAP concentrations of 4.44 and 8.88 μM, with the average number of calli from the leaf explants being significantly higher (Figure 1d).

The evaluation of the *in vitro* morphogenesis revealed a significant difference between the two explant types. The average values of the calli obtained from internodal segments were significantly higher than the calli with embryogenic masses, by 75.56%, 80.00% and 58.00% when grown in culture medium EME + 2.68 μM NAA supplemented with BAP at concentrations of 2.22, 4.44 and 8.88 μM, respectively. Both cytokinins and auxin can interact and promote the development of callogenesis and shoot formation, which play a role in cell the cycle and morphogenic competence in plant growth (Mohd Din. et al., 2016). The balance between auxin and cytokinins played a major role in the initiation of regeneration induced in the calli (Binte; Wagiran, 2018).

Several researchers have used exogenous application of plant growth regulators such as NAA and BAP for callus induction and regeneration in different plant species. Gow, Chen and Chang, (2010), investigating somatic embryogenesis of *Phalaenopsis amabilis*, evaluated the effect of BAP and NAA concentrations on the species' regeneration and found that the use of 2.22 μM BAP promoted the development of embryos with the greatest regeneration rate (90%), results that were near those of the present study. Liu, Gao and Guo, (2008), studying the regeneration of *Erigeron breviscapus* (vant.) Hand. Mazz. a species with medical importance, found a significant difference between the treatments in the regeneration of shoots when the leaf explants were cultured in medium with 5.0 or 10.0 μM BAP plus 5.0 μM NAA, with production of about 17 buds per explant after 30 days. With respect to the percentage of calli with roots, no significant differences were observed between the two types of explants (Table 4).

Callus histological analysis

The histological analysis showed that the cells from both leaf and internodal segments, in the culture media used, were able to dedifferentiate, modifying their embryonic structures (Figure 2 a-h). Cells that differed in pro-embryonic complexes were small and isodiametric, with absence of meatus and dense cytoplasm, all of which are characteristic of embryonic cells. These cells initially formed pro-embryonic cell complexes, as shown in Figure 2 b, c, f. Some of these cell complexes changed into globular embryonic structures that were small and translucent (Figure 2 a, d, e, h). In our study, although different explants were used, the morphology and histology of the observed structures were similar, such as the presence of small and organized cells, with isodiametric shapes and somatic embryo in the globular stage.

However, we did not observe whether these embryos developed to the next stages (cordiform, torpedo and cotyledonary), and thus whether they grew into plants. Probably the absence of complete protoderm formation in internodal segments (Figure 2c) and absence of cauline apical meristem primordium in leaf segments (Figure 2g) were factors that contributed to the development of other embryonic stages.

The analysis of histological sections is fundamental to characterize the morphogenetic regeneration pathway *in vitro* (Almeida et al., 2006). Gow, Chen and Chang, (2010), observed via scanning electron microscopy that somatic embryos of *Phalaenopsis* directly form explants, without previous callus formation, thus characterizing a regeneration pathway via direct embryogenesis. In the present study, even without conversion into plants, the examination of the histological sections allowed characterizing the morphogenetic pathway as occurring by indirect somatic embryogenesis, based on the formation of globular embryonic structures obtained from the calli induced in the explants (Figure 2 b, g).

According to Yeung (1995), one of the most important aspects of embryogenesis, which characterizes the start of histodifferentiation of the somatic embryo, is the formation of the protoderm. Further according to the author, the development of somatic embryos of carrot can be interrupted by abnormal protoderm formation. In our study, we observed vascular independence of the globular structure in relation to the initial explant (Figure 2g). Furthermore, we noted both complete and incomplete protoderm formation, respectively with outermost layer of the structure intact (Figure 2g) and not intact (Figure 2c), in different structures of the globular stage. It is possible that the incomplete protoderm contributed to the absence of the other phases of embryonic development, causing failure of conversion into plants. It was not possible to observe the unicellular origin of the somatic embryos, because the fixation of the material was not done since the introduction of the material *in vitro* (day zero), and also none of them presented a structure similar to a suspender, from either explant type or any culture medium.

According to Dornelas, Vieira and Appezzato-Da-Gloria, (1992), in somatic embryos of multicellular origin, the base of the embryonic axis tends to be extensively linked to the explant without the formation of the suspender or equivalent structure, as also was the case in the present study. Therefore, it was not possible to confirm the uni- or multicellular origin of the embryogenic structures observed. In contrast, Grzyb and Mikula, (2019) demonstrated that somatic embryos of *Cyathea delgadii* Sternb. are of multicellular origin whose leaf primordia had begun to split at the tip. Quiroz-Figueroa et al. (2006) evaluated somatic embryogenesis in *Typha domingensis* Pers. (southern cattail) and observed that the presence of a suspensor was essential to determining the unicellular origin of the SE and its degree of development.

The low initiation frequency of somatic embryogenesis technique, low number of embryos generated and low frequency of plant conversion due the some factors such as low quality, defective in somatic embryos maturation and germination (Choudhary et al., 2009; Rai; Jaiswal; Jaiswal, 2009) are the leading problems that hamper the efficient large-scale production of some species and limit their widespread practical uses (Maruyama; Hosoi, 2019).

Studies of somatic embryogenesis carried out in several plant species have demonstrated this low or non-existent rate of conversion of embryos to seedlings, such as, *Oplopanax elatus* (Nakai) Nakai (Moon et al., 2013), *Manihot esculenta* Crantz (Vidal et al., 2014), *Medicago sativa* L. (Amini; Deljou;

Nabiabad, 2016), *Crataegus* spp. (Taimori et al., 2016) and *Coffea arabica* and *C. canephora* (Etienne et al., 2018).

The main abnormalities exhibited in somatic embryogenesis in different plant species are: fusion of two or more embryos, lack of apical and radical meristems, translucent embryos, multiple cotyledons, and loss of bipolarity (Hashemloian, 2008; El Dawayati et al., 2012; Ruffoni; Savona, 2013). In this study, the majority of embryos obtained did not develop further than the globular phase, thus not attaining complete maturity. Some of these embryos formed a sub-apical meristem of the root (Figure 1d), but did not form a stem tip meristem. Therefore, while we observed the development of roots from both explant types, particularly in the culture medium without BAP, we did not observe the formation of the aerial part of the plantlets (Figure 1 e-f).

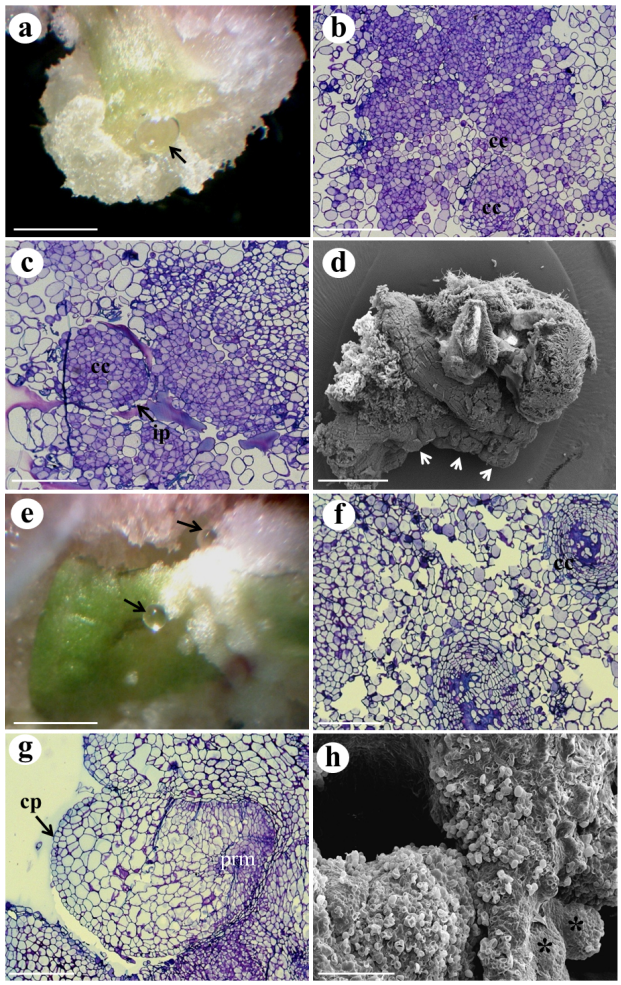

Figure 2: Induction of embryogenic calli from internodal and leaf segments of V. condensata, grown in vitro. a) Somatic embryo in globular stage (arrow) from an internodal segment grown in EME culture medium supplemented with 4.44 µM BAP. b) Histological section indicating pro-embryonic cell complexes from an internodal segment. c) Pro-embryonic cell complex with incomplete formation of the protoderm (arrow) from an internodal segment. d) SEM image of somatic embryos in globular stages (arrows), from an internodal segment. e) SEM image of somatic embryos in globular stages (arrows) from a leaf segment. f) Histological section indicating a pro-embryonic cell complex from a leaf segment. g) Histological section indicating a somatic embryo in globular stage with complete protoderm (arrow) and start of formation of root meristem from a leaf segment. h) SEM image of somatic embryos in globular stages (asterisks) from a leaf segment. cc = pro-embryonic cell complex, cp = complete protoderm, ip = incomplete protoderm, prm = primary root meristem. Bars = 1 mm (a, d, e, h); 200 µm (b, c, f and g).

The main abnormalities exhibited in somatic embryogenesis in different plant species are: fusion of two or more embryos, lack of apical and radical meristems, translucent embryos, multiple cotyledons, and loss of bipolarity (Hashemloian, 2008; El Dawayati et al., 2012; Ruffoni; Savona, 2013). In this study, the majority of embryos obtained did not develop further than the globular phase, thus not attaining complete maturity. Some of these embryos formed a sub-apical meristem of the root (Figure 1d), but did not form a stem tip meristem. Therefore, while we observed the development of roots from both explant types, particularly in the culture medium without BAP, we did not observe the formation of the aerial part of the plantlets (Figure 1 e-f).

The polar auxin transport in the initial stage of the globular embryo is essential for the establishment of bilateral symmetry, a process that can be impaired by homogeneous distribution of auxin in the embryo itself (Venkatesh et al., 2009). Some authors mention that the auxin polar transportation in SE when disrupted induces embryo abnormalities (Verma et al., 2018). It is possible that the endogenous level of auxin in the initial explant, together with the addition of NAA in the culture media, contributed to the formation of a monopolar structure, with only a root meristem. Some authors have reported the formation of somatic embryos without shoot apical meristem (Filippi; Appezzato-da-Gloria; Rodriguez, 2001).

The embryos formed from both explant types and all the culture media used in this study did not develop into plantlets. However, the embryos of both explant types did form roots, especially those from internodal segments grown in culture medium without BAP. We stress that the malformation during the *in vitro* embryogenesis process in this experiment might have been related to the use of auxin and cytokinin in the culture medium. Therefore, more detailed studies should be conducted to try to obtain embryos with better quality, with successful conversion into plantlets. Obtaining somatic embryos with good quality depends on the correct choices of the sources and concentrations of auxins and cytokinins, induction time and maturation stage, all of which are fundamental for the efficiency of somatic embryogenesis.

CONCLUSIONS

The results of this work showed that both types of *V. condensata* explants were responsive to callogenesis, but the internodal segments presented greater capacity to produce friable calli and a higher percentage of calli with pro-embryogenic masses. The BAP concentration of 4.44 µM promoted the formation of friable calli associated with the highest percentage of calli with embryogenic masses. The cells of the leaf and internodal explants were able to dedifferentiate, changing into embryonic structures. Further studies are necessary to determine the best concentrations and combinations of growth regulators for regeneration of *Vernonia condensata* plants. There is also a need to identify the changes that occur in the different phases of callogenesis through biochemical analyses. Finally, future studies investigating somatic embryogenesis of calli of *V. condensata* by culturing cells in suspension can be manipulated in different aspects, aiming to increase the biosynthesis of secondary metabolites, such as the flavonoids that are common to this species, for medicinal uses.

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