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Induction, biochemical trait and phytochemical screening of calluses of *Amburana cearensis* (Allemão) A.C. Smith

Jéssica Nascimento Costa Vasconcelos[1], Alone Lima Brito[1], Andressa Priscila Pianco Santos Lima[1], Jackson Roberto Guedes da Silva Almeida[2], Ana Paula de Oliveira[2], Taliane Leila Soares[1*] and José Raniere Ferreira de Santana[1]

[1]Universidade Estadual de Feira de Santana, Av. Transnordestina, s/n, 44036-900, Novo Horizonte, Feira de Santana, Bahia, Brazil. [2]Universidade Federal do Vale do São Francisco, Petrolina, Pernambuco, Brazil. *Author for correspondence. E-mail: talialeila@gmail.com

ABSTRACT. *Amburana cearensis* is an arboreal legume of the Fabaceae family, with high phytotherapic and medicinal potential due the presence of secondary metabolites. The objective of this study was to evaluate the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-2,5,6- trichloro-2-pyridinecarboxylic acid (picloram) on the *in vitro* induction of callogenesis of *A. cearensis* and analyze the biochemical and phytochemical potential of these calluses. For callus induction, leaf and cotyledon segments were used as explants, which were inoculated in woody plant medium (WPM) supplemented with different concentrations of 2,4-D (0, 5, 10, 20, 40 µM) or picloram (0, 5, 10, 20, 40, 80 µM). The callus growth curve was estimated based on fresh weight, measured at 7-day intervals until 28 days after inoculation. The calluses were analyzed by biochemical tests to quantify the reducing sugars and total proteins. Phytochemical screening and high-performance liquid chromatography were performed to establish the phytochemical profile of extracts from calluses. The concentrations of 21.94 µM and 26.46 µM of 2,4-D induced the greatest formation of compact and friable calluses from the leaf and cotyledon segments, respectively. The growth curve had two distinct phases (lag and exponential) for both types of calluses evaluated. The maximum levels of reducing sugars and total proteins in the calluses from leaf and cotyledon segments were obtained on the day of inoculation and after 28 days of cultivation, respectively. The results of the phytochemical analysis identified the presence of coumarin in all the extracts evaluated, this secondary metabolite has high pharmacological potential.

Keywords: medicinal plants; callogenesis; growth regulators; growth dynamic; cumarin.

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Introduction

The use of plants for medicinal, nutraceutical and phytotherapeutic purposes to replace synthetic medicines has increased greatly in the past three decades throughout the world (Ekor, 2014; Majumder et al., 2019; Grigoriadou et al., 2019), stimulating investments to study plants with pharmacological potential (Pereira et al., 2017; Welz, Klein, & Menrad, 2018; Rivera-Mondragón et al., 2019).

Among these medicinal plants is the species *Amburana cearensis* (Allemão) A.C. Smith, popularly known in Brazil as 'cumaru' (Almeida et al., 2017). Its bark and seeds are used for medicinal purposes due to the presence of secondary metabolites such as protocatechuic acid, coumarin, flavonoids (isokaempferide, kaempferol, afrormosin, 4''-methoxy-fisetin and quercetin) and phenolic glycosides (amburoside A and B), which variously have anti-inflammatory, antibiotic, antioxidant, antispasmodic and bronchodilator effects (Pereira et al., 2017; Oliveira et al., 2020).

Because of excessive extraction for wood and indiscriminate collection of seeds for commercial purposes, along with habitat loss, *A. cearensis* is classified as endangered according to the International Union for the Conservation of Nature (IUCN, 2020). Therefore, it is important to conduct studies to promote sustainable management to obtain raw material with the necessary quantity and quality for use by the pharmaceutical industry.

In this context, advanced biotechnological methods for culturing plant cells, tissues or organs can provide new strategies for the conservation and rapid propagation of valuable, rare and/or endangered medicinal plants and production of phytochemicals they produce (Isah, 2019). *In vitro* culture techniques, especially the culture of calluses can produce some active ingredients and specific medicinal compounds

that are equal to or better than those produced by intact plants, such catharanthine from *Catharanthus roseus*, and anthraquinone from *Cassia acutifolia* (Hussain et al., 2012). Therefore, it is crucial to develop a protocol for rapid callus growth to reduce the time necessary for production of secondary metabolites with phytotherapeutic potential from *A. cearensis*.

Among the factors affecting the biosynthesis and accumulation of secondary metabolites in *in vitro* cultures are the basal medium composition and the composition and concentrations of plant growth regulators (PGRs) (Adil, Ren, Kang, Thi, & Jeong, 2018; Kwiecień, Smolin, Beerhues, & Ekiert, 2018; Jeet et al., 2020). Previous research has shown that callogenesis mostly depends on exogenous supply of auxin and its type and concentration (Naik et al., 2017).

Among the growth regulators used most often for callus induction are the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-2,5,6- trichloro-2-pyridinecarboxylic acid (picloram) (Osman, Sidik, & Awal, 2016). The efficiency of 2,4-D in inducing the formation of calluses is associated with its main characteristics, which are to stimulate cell division of plant tissues and strongly suppress organogenesis (Osman et al., 2016; Abbas, El-Shabrawi, Soliman, & Selim, 2018). In turn, picloram is considered to be more effective in increasing callus induction than the growth regulator 2,4-D (Kiong, Thing, Gansau, & Hussein, 2008).

The objectives of this study were to evaluate the effect of 2,4-D and picloram on the *in vitro* induction of callogenesis in *A. cearensis* and analyze the biochemical and phytochemical potential of these calluses. To the best of our knowledge, this study is the first report of callus induction in *A. cearensis* and biochemical analysis.

Material and methods

Plant material and location of the experiment

To obtain *A. cearensis* plants *in vitro*, were used seeds from the germplasm collection of the Embrapa Semi-Árido, Petrolina, Bahia, Brazil (9°24”S, 40°26”W) and 365.5m altitude. The experiments were conducted in the Plant Tissue Culture Laboratory (LCTV), part of the Experimental *Horto Florestal* of *Universidade Estadual de Feira de Santana* - UEFS, located in Feira de Santana, Feira de Santana Bahia, Brazil (12°16′05.8″S, 38°56′19.7″W) and 286 m altitude.

In vitro establishment

Amburana cearensis seeds were washed in running tap water for 10 minutes and then disinfested in a laminar flow cabinet with immersion in 70% ethanol (Sigma-Aldrich, St. Louis, MO, USA) for 1 minute, followed by commercial bleach (2.5% sodium hypochlorite solution) (Anhembi SA, Osasco, São Paulo, Brazil) with two drops of neutral dish detergent (Quimica Amparo Ltda, Amparo, São Paulo, Brazil) for 10 minutes.

After these procedures, the seeds were washed four times in sterile distilled water and inoculated in test tubes containing 15 mL of culture medium, according to the method described by Campos, Brito, Gutierrez, Santana, and Souza (2013).

Culture medium

The culture medium used was woody plant medium (WPM), according to the formula of Lloyd and McCown (1980), containing 3% sucrose (Synth, Diadema, São Paulo, Brazil) and solidified with 0.7% agar (Himedia, Mumbai, India). The medium was supplemented with 2,4-D (Sigma-Aldrich, St. Louis, MO, USA) at various concentrations (0.0, 5.0, 10.0, 20.0, 40.0 µM) or picloram (Sigma-Aldrich, St. Louis, MO, USA) at concentrations (0, 5.0, 10.0, 20.0, 40.0, 80.0 µM). The medium’s pH was adjusted to 5.7 ± 0.1 with sodium hydroxide (Merck, Darmstand, Germany) or hydrochloric acid (Merck, Darmstand, Germany) at 0.1 N, followed by autoclaving for 15 minutes at a temperature of 121ºC and pressure of 1 atm.

Callus induction and culture conditions

Leaf and cotyledon segments with approximate surface area of 1 cm^2, obtained from 45-day-old plants produced by *in vitro* germination, were used as explants. They were inoculated in test tubes containing 15 mL of WPM, supplemented with different concentrations of 2,4-D or picloram, as described previously. The tubes were kept in a growth room with temperature of 26 ± 2ºC, 16:8 photoperiod and photosynthetically active radiation of 60 µmol m^{-2} s^{-1} supplied by white fluorescent lamps.

The experimental design was completely randomized in a 2 x 5 factorial scheme, for a total of 10 treatments, with 20 repetitions per treatment, where each repetition consisted of a test tube with a type of explant, making a total of 20 explants/treatment. Thirty days after the start of *in vitro* culture, the following parameters were evaluated: percentage of responsive explants, percentage of explant area covered with calluses, callus color and texture.

Callus growth curve

To determine the callus growth curve, approximately 200 mg of fresh weight of calluses obtained of leaf and cotyledon segments explants each and from the culture medium WPM supplemented with 21.94 µL and 26.46 µL of 2,4-D, respectively. The growth curve was plotted from day zero (explants on the day of inoculation), in seven-day intervals until the 28th day, represented by abscissa axis and the fresh weights by the ordinate axis. To obtain the fresh weight, the explants were carefully placed in sterile petri dishes, sealed with plastic wrap to preserve aseptic conditions so they could return to the culture medium, and weighed with a precision scale to determine their fresh weight. The growth percentage of the calluses was determined according to the equation [(FWf - FWi)/(FWf)] x 100, where FWi is initial fresh weight and FWf is final fresh weight of calluses in grams (g). Four repetitions were used, each one consisting of three experimental units (three calluses).

Biochemical analysis

The biochemical analysis of the calluses was carried out with the same intervals used to plot the growth curve. A total of 250 mg of callus sample was collected at 10-day intervals until 40 days after inoculation (0 - on the day of inoculation, 10, 20, 30 and 40 days), according to the method described by Nogueira et al., (2008). The plant extract was obtained by homogenization in a mortar of a 250 mg sample (fresh weight) diluted in 2 mL of distilled water. The extract was placed in a water bath at 40ºC for 30 minutes and then was centrifuged at 8000 x g for 30 minutes at 25ºC. The supernatant was collected and stored under refrigeration at a temperature of 4ºC for subsequent analysis of reducing sugars and crude protein.

Quantification of reducing sugars

The method described by Miller (1959) was employed to quantify the reducing sugars, utilizing 3,5-dinitrosalicylic acid (DNS, Sigma-Aldrich, St. Louis, MO, USA) as reagent. Supernatant aliquots of 500 µL were added to 1.0 mL of distilled water and 1.0 mL of DNS. This mixture was homogenized in an agitator and placed in boiling water for 5 minutes and then allowed to cool to room temperature. Finally, the samples were submitted to spectrophotometry at 540 nm, and the quantification was based on the standard curve for glucose.

Quantification to total proteins

The calluses obtained from cotyledon and leaf segments were macerated using a mortar and pestle and for each 250 mg of callus 2 mL of distilled water was added. The samples remained at 40ºC in a water bath for 30 minutes and centrifuged at 8000 x g for 30 minutes at 25ºC. The supernatant was collected and stored refrigerated for subsequent quantification of soluble protein content. The protein content in the different fractions was measured according to Bradford (1976). Three repetitions per collection date, as described above, were used to determine the concentrations of sugars and proteins.

Extraction and qualitative analysis of secondary metabolites

The qualitative analysis of the secondary metabolites was performed using callus material from leaf and cotyledon segments after *in vitro* culture for 30 days in WPM supplemented with 21.97 µM and 26.46 µM of 2,4-D, respectively (chosen due to higher percentage of responsive explants with this growth regulator). Material from the aerial part of the plants germinated under conventional conditions containing substrate soil + vermiculite (1:1), kept in a greenhouse was also evaluated.

To prepare the extracts, the different plant materials were placed in a forced-air oven and dried for seven days at a temperature of 40°C. Then the material was macerated in methanol (Merck, Darmstand, Germany) through consecutive extractions in the interval of 72h each. Finally, the solution was concentrated by eliminating the organic solvent to obtain the crude extract. The phytochemical evaluation was carried out according the method described by Wagner and Bladt (1996). An aliquot (0.05 mg) of each extract was

dissolved in 1 mL of chloroform (Himedia, Mumbai, India), and the samples were applied on a CCDA plate with the aid of a capillary tube. Then they were eluted in specific solvent systems for the classes of secondary metabolites to enable identification.

High-performance liquid chromatography (HPLC) was used for analysis using a Shimadzu® LC-20 chromatograph equipped with an LC-20ADVP quaternary pump system, DGU-20A degasser, SPD-20AVP photodiode array detector, CTO-20ASVP oven, SIL-20ADVP automatic injector and SCL-20AVP controller. The data were treated with the Shimadzu® LC Solution 1.0 software (Japan). In the initial method, the mobile phase was composed of two solvents: solvent A – 0.1% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) diluted in ultrapure water, and solvent B – 100% acetonitrile (Sigma-Aldrich, St. Louis, MO, USA), with flow of 0.8 mL min^{-1}. At the start, the gradient consisted of 100% A, and after 50 minutes this gradient reached 50% A and 50% B, and soon thereafter returned to the original condition. The stationary phase (5 µm) was contained in an Agilent Eclipse Plus® C18 Agilent column measuring 250 x 4.6 mm, maintained at 30ºC. The assays were performed in triplicate, in which 8 µL of each sample was injected. The chromatographic profile was monitored by the diode array detector (DAD) at wavelengths of 270 and 340 nm. In parallel, 23 analytic standards were analyzed individually to investigate their presence in the samples.

Statistical analysis

Percentage data were transformed to arcsine before statistical analysis for normalization and homogenization of the variances. Then the data were submitted to analysis of variance by the F-test ($p \leqslant 0.05$ or $p \leqslant 0.01$) and the means were compared by Scott-Knott test (explant types) and quantitative factors (2,4-D and picloram levels) by polynomial regression (linear and quadratic). Statistical analyses were performed using "agricolae" package implemented in the R program (R Development Core Team, 2016).

Results and discussion

Effect of the growth regulator 2,4-D on the induction of calluses in *A. cearensis* plants cultured from leaf and cotyledon segments

The choice of explant is the most important criterion for successful cell and tissue culture protocols (Jasdeep et al., 2019). In the present study, there was a marked difference in the duration for the callus induction first initiated in the two different explants tested. The cotyledon explants were more responsive in terms of time taken to induce callogenesis (6-7 days), whereas leaf explants required 10-14 days to show initial response. A possible explanation for this, may be that the young cotyledons are very active physiologically and are easily affected by exogenous plant growth regulators (Murkute, Patil, Patil, & Kumari, 2002; Naik & Chand, 2003).

The percentages of responsive explants and area of the explants covered with calluses were significantly influenced ($p \leqslant 0.05$) by explant type and by application of the growth regulator 2,4-D (Figure 1 a-b). There was a significant rising quadratic response ($p \leqslant 0.05$) of the percentage of responsive explants associated with the type of explant and concentration of 2,4-D (Figure 1a). When using leaf segments as explants, there was an increase in the percentage of responsive explants with rising concentration of 2,4-D in the culture medium. The maximum callus induction response from the leaf segments explant (99.4%) was obtained at a concentration of 21.94 µM 2,4-D. Similar percentage of responsive calluses obtained with leaf explants was also observed with cotyledon segments (99.4%) when grown in medium supplemented with 26.46 µM of 2,4-D (Figure 1a).

The quadratic behavior of the percentage of responsive explants indicates that 2,4-D concentrations in the interval between 21.94 µM and 26.46 µM had a tendency to reduce that percentage in the explants obtained from leaf and cotyledon segments, respectively. The 2,4-D is the one of the most commonly used auxin in tissue culture for induction of callogenesis and somatic embryogenesis in many plant species (Passamani et al., 2020).

The result found in the present study revealed that the presence of 2,4-D in the culture medium was essentially required to induce callus formation in *A. cearensis*, since the explants cultured in the medium without this growth regulator became oxidized and did not form calluses (Figure 1a). Yuan, Yue, Wu, and Gu (2013) also observed that the induction of calluses in Moso bamboo [*Phyllostachys heterocycla* var. pubescens (Mazel ex J. Houz.) Ohwi] varied in function of the presence and concentration of 2,4-D in the culture medium.

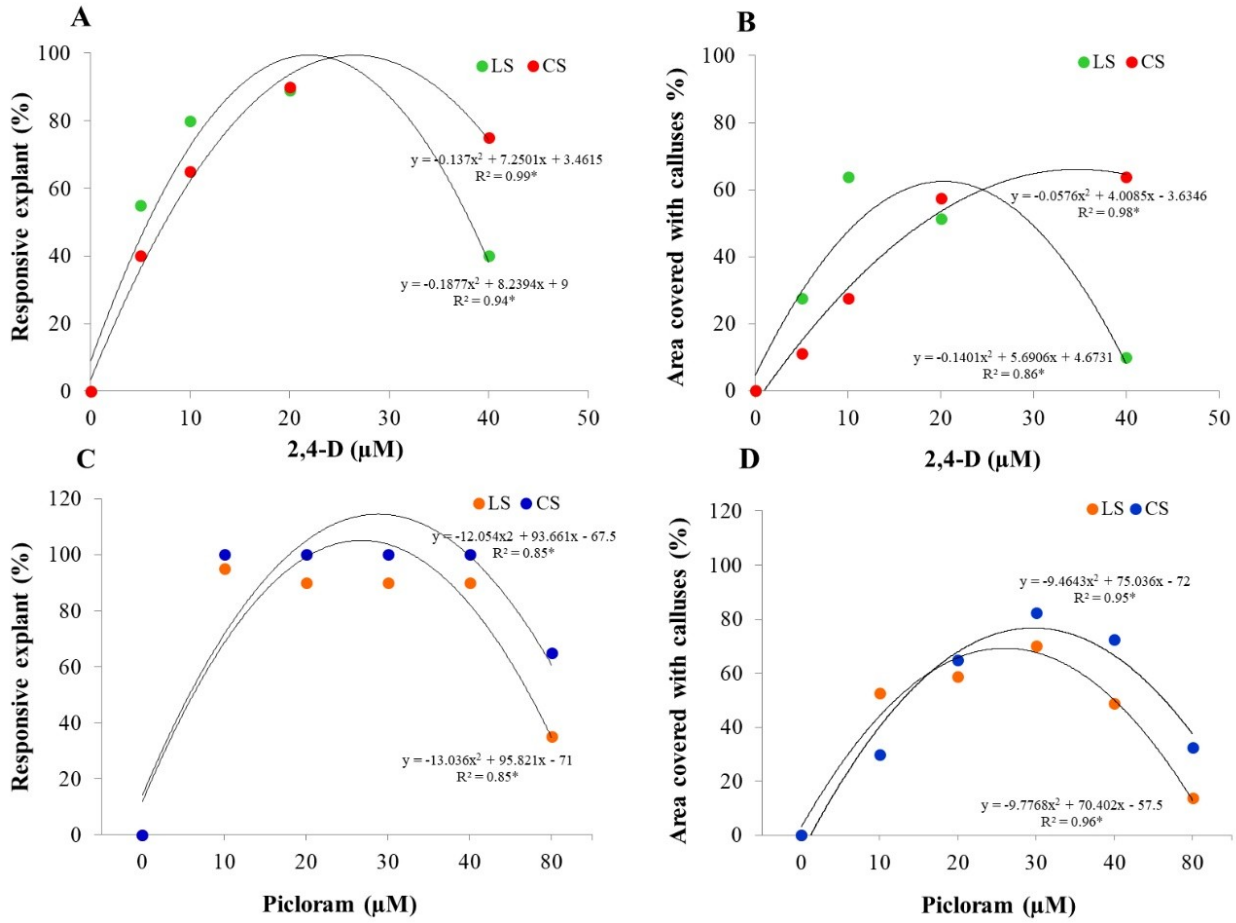

Figure 1. Percentage of responsive explants and percentage of explant area covered with calluses obtained from leaf (LC) and cotyledon segments (CS) of *Amburana cearensis* plants grown in WPM supplemented with different concentrations of 2,4-D (A-B) and picloram (C-D). *significant ($p \leq 0.05$) by the F-test. Means followed by the same letters, lowercase in the columns and uppercase in the rows, differ from each other by the Scott-Knott test at 5% probability.

With respect to the percentage of the explant area covered with calluses, there was a negative quadratic response ($p < 0.01$) in both explant types (Figure 1b). The concentration of 20.67 µM of 2,4-D in the culture medium induced the maximum area covered with calluses in the leaf explants (86.37%), while the 2,4-D concentration of 34.80 µM promoted the highest percentage in the cotyledon explants (66.11%) (Figure 1 b). Nogueira et al. (2008), investigating the induction of calluses in *Byrsonima intermedia* A. Juss., found that the concentrations of 2,4-D significantly influenced the explant area covered with calluses, where the concentration of 17.7 µM promoted the highest percentage (90.0%).

Effect of picloram on the induction of calluses in *A. cearensis* plants cultured from leaf and cotyledon segments

The interaction of picloram concentration x explant type was also significant ($p < 0.01$) for the variables percentage of responsive explants (RE%) and explant area covered with calluses (CA%) (Figure 1c-d). There was a significant rising quadratic response ($p \leq 0.05$) of the percentage of responsive explants associated with the type of explant and concentration of picloram (Figure 1c).

The addition of picloram to the culture medium induced a rate of responsive explants greater than 90.0%, except for the concentration of 80.00 µM of this regulator. This behavior was common for both explant types, although the highest percentage of responsive explants (100.0%) was observed for the cotyledon segments cultured in media containing four picloram concentrations (5.0, 10.0 20.0 and 40.0 µM) (Figure 1c). However, at the highest picloram concentration in the medium (80.00 µM), there was a phytotoxic effect on the leaf segments, since the percentage of responsive explants and area covered with calluses were significantly lower than in the cotyledon segments (Figure 1c-d). Picloram has been shown to be effective in inducing callogenesis in other plant species, such as *Zea mays* L. (Erkoyuncu, Yorgancilar, & Atalay, 2017) and *Papaver rhoeas* L. (Aghaali, Hoshino, Monfared, & Moieni, 2019).

With regard to the area covered with calluses, there was a rising quadratic effect for the cotyledon and leaf segments. As the picloram concentration increased to the maximum point (30 µM), the explant area covered with calluses increased as well, reaching 88.08% and 70.10% for cotyledon and leaf segments, respectively, (Figure 1d).

The success of callus induction and subsequent callus growth in subculture depends of explant type, of the exogenous supply of plant growth regulators (PGRs), as well as the type and concentration of PGR (Wójcikowska & Gaj, 2017; Adil et al., 2018).

Calluses growth

Five days after inoculation, the explants were larger and started to form calluses. This increase in size of the explants is related to the presence of auxins, which intensify the proton pump mechanism, causing acidification of the cell walls and elongation of cells (Perrot-Rechenmann, 2010). In this study, we observed that the period of 30 days was sufficient for all the explants to reach their maximum potential callus coverage. In general, both explant types grown in medium supplemented with 2,4-D developed friable calluses, with color varying from light yellow to dark brown, with smooth and regular texture and compact consistency (Figure 2).

The typical growth pattern of callus is sigmoidal, generally in five distinct phases: lag, exponential, linear, deceleration and stationary (Taimori, Kahrizi, Abdossi, & Papzan, 2016). However, the growth curve of the *A. cearensis* calluses showed only two growth phases (lag and exponential), represented by the regression equations $y = 0.0001x^2 + 0.0015x$ ($R2 = 0.98$) and $y = 0.0007x^2 + 0.0321x$ ($R2 = 0.99$) for those formed from leaf and cotyledon explant, respectively (Figure 3a-b). These results are in line with those reported by Oliveira et al. (2018), who also identified two distinct growth phases (lag and exponential) for calluses obtained from leaf explants of *Genipa americana* L.

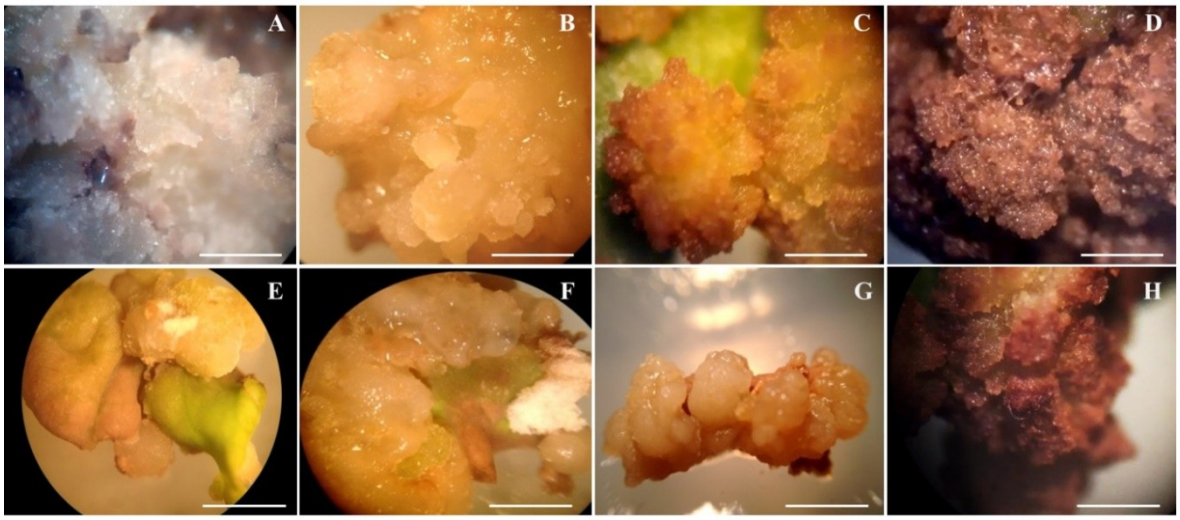

Figure 2. Callus aspects obtained from cotyledon segments (A- D) and leaf segments (E-H) grown on WPM supplemented with 21.94 µL of 2,4-D. A) Creamy white friable. B) Yellow friable. C) Yellowish green friable. D) Dark brown compact. E) Yellow compact. F) Yellowish green friable. G) Yellow friable. H) Dark brown friable. Bar: 0.5 cm.

The lag phase is characterized by accumulation of callus weight, without cell division (Daffalla, Elsheikh, Ali, & Khalafalla, 2019). In this work the lag phase occurred from the day of inoculation to the 7th day of cultivation, with increases of 0.002 g and 0.16 g for the calluses from the leaf (Figure 3a) and cotyledon segments (Figure 3b), respectively.

Some authors have demonstrated that the period of the lag phase varies according to the species studied (Abbas et al., 2018) and type of explant considered (Feitosa et al., 2013). Our results agree with those reported for other plant species, such as the pequi tree (*Caryocar brasiliense* Camb), in which the lag phase of the callus growth curve happened until the 7th day of cultivation (Landa, Paiva, Paiva, Bueno Filho, 2000).

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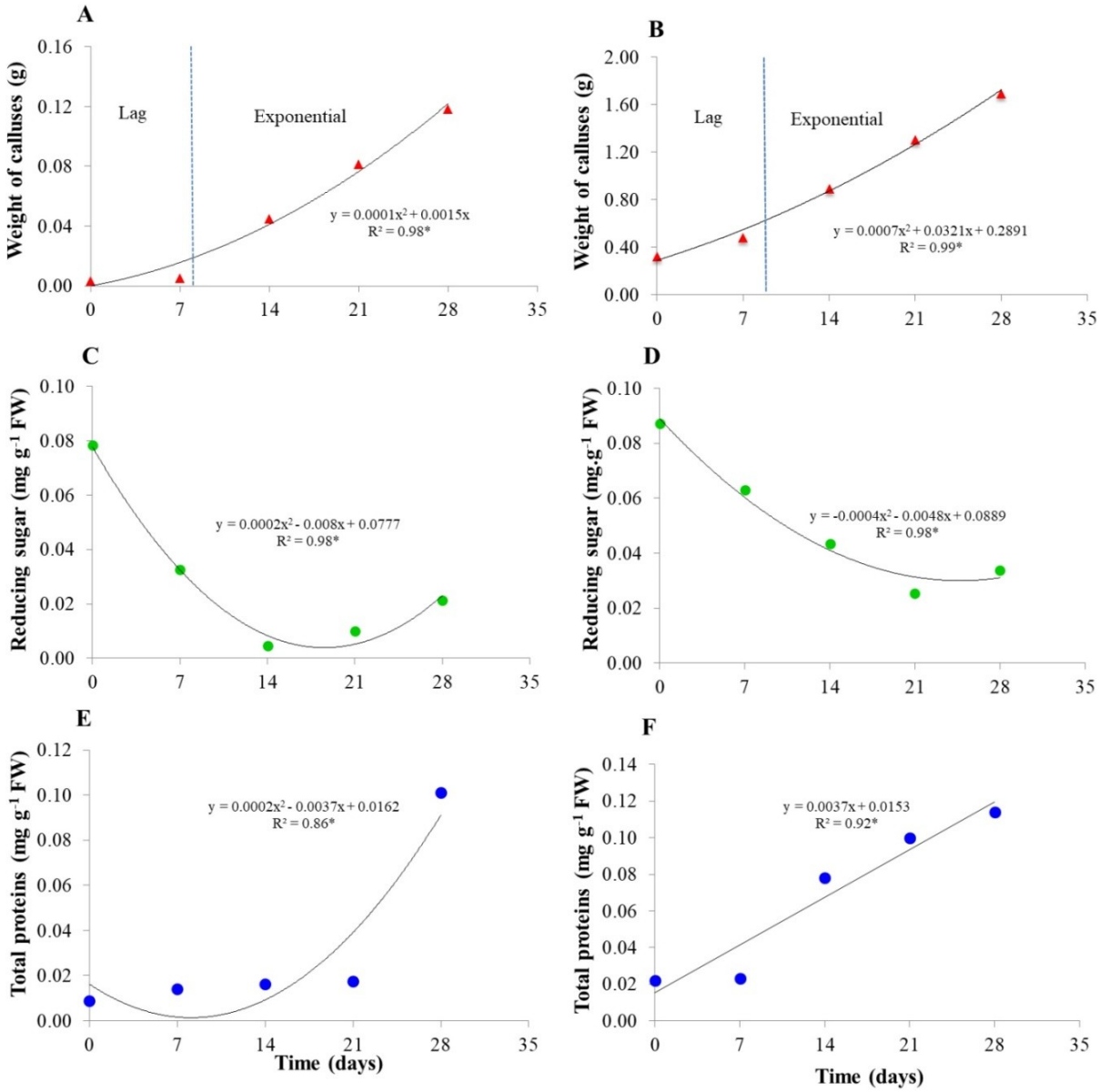

Figure 3. Callus growth curve of *Amburana cearensis* obtained from leaf (A) and cotyledon segments (B) inoculated in WPM supplemented with 21.94 µL and 26.46 µL of 2,4-D, respectively. Concentration of reducing sugars of the calluses from leaf segments (C) and cotyledon segments (D). Concentration of total proteins of calluses from leaf segments (E) and from cotyledon segments (F). *significant ($p \leq 0.05$) by the F-test.

Biochemical analyses

The biochemical analyses of the *A. cearensis* calluses revealed a significant reduction ($p \leq 0.05$) in the content of reducing sugars during the cultivation period, with the maximum level observed on the day of inoculation of the leaf and cotyledon segments (Figure 3c-d).

Regarding the levels of reducing sugars, high values of sugars were observed for both types of explants at the beginning of *in vitro* cultivation. On the other hand, at fourteen days after inoculation, the levels of reducing sugars had declined markedly in relation to the initial value in the calluses from the leaf segments, by 93.58% (Figure 3c), while in the calluses obtained from cotyledon segments this reduction was 71.26% 21 days after inoculation (Figure 3d). These decreases can probably be attributed to the increased levels of proteins, since the energy necessary for protein synthesis is released from the catalysis of carbohydrates. These results corroborate the findings reported for other authors who also observed a high reducing sugar content in calluses from different plant species, such as *Byrsonima intermedia* A. Juss. (Nogueira et al., 2008) and *Pyrostegia venusta* (Ker Gawl.) Miers (Coimbra, Chagas, Vilela, & Castro, 2019) on the day of inoculation.

The total proteins in the calluses produced by leaf segments increased substantially as of the 21st day of cultivation (Figure 3e), while for the cotyledon segments this increase occurred earlier, as of the seventh day

after inoculation (Figure 3f), comprising the exponential phase. It is interesting to note that the increase in the protein content as of the seventh day coincides with the exponential phase of the callus growth curve of *A. cearensis*. This elevation in the level of proteins certainly indicates changes in the cell metabolism, which requires specific proteins for the cell expansion and other functions (Costa et al., 2015). Other authors mentioned that the increase in the protein level may be related to the greater ammonium absorption of the culture medium by the explant (Mesquita et al., 2002). Previous studies have shown that the presence of ammonium in the culture medium results in increased synthesis of amino acids and proteins, which are usually produced using energy released in the carbohydrate catabolism (George, 1996).

Biochemical markers can assist in the early identification of morphogenic and physiological processes that occur during cell differentiation, growth, plant propagation and somatic embryogenesis (Cangahuala-Inocente, Steiner, Maldonado, & Guerra, 2009).

Phytochemical analysis

The results of the phytochemical screening performed on the ethanol extracts from the aerial parts of the plants grown under conventional conditions (*in vivo*) and the calluses obtained *in vitro* in general had the same phytochemical profile, despite the different cultivation conditions (Table 1).

Table 1. Qualitative phytochemical screening of plant materials of *Amburana cearensis* submitted to extraction by maceration.

Chemical Class	*Amburana cearensis*		
	In vivo	*In vitro* calluses	
	(Aerial part)	Leaf segments	Cotyledon segment
Flavonoids	++	-	-
Alkaloids	-	-	-
Lignans	-	-	-
Quinones	++	-	-
Cinnamic derivatives	+	-	-
Coumarins	+++	++	+
Mono, sesqui and diterpenes	-	-	-
Triterpenes and steroids	+	-	-

(-) Not detected; (+) Positive; (++) Moderately positive; (+++) Strongly positive.

The secondary metabolites were grouped into two distinct classes: terpenes and phenolic compounds. The extracts were negative for the presence of alkaloids, lignans, monoterpenes, sesquiterpenes and diterpenes, while all the extracts were positive for the presence of coumarin, with the intensity of the reaction of these metabolites varying in function of the type of plant material evaluated (Table 1). The concentration of coumarin was greater in the extract from the calluses obtained from leaf segments than those obtained from cotyledon segments, and lower in relation to the plant material cultivated under conventional conditions. The results obtained in this study are in line with those reported by Pereira et al. (2017), Oliveira et al. (2020) and Ferreira et al. (2020) who also identified the presence of coumarin in extracts of *A. cearensis* seeds and stem bark aqueous explant, respectively.

Plants that contain coumarin have attracted significant attention due to its wide pharmacological action, such as anti-inflammatory, antioxidant, antimicrobial and anticoagulant actions, besides acting as an adjuvant in the treatment of cancer (Al-Amiery, Kadhum, & Mohamad, 2012; Zhao et al., 2019; Maleki, Bahrami, Sadeghian, & Matin, 2020; Wang et al., 2020; Kasperkiewicz, Ponczek, Owczarek, Guga, & Budzisz, 2020).

It should be mentioned that the application of qualitative methods, such as phytochemical prospection, is relevant, since it enables initial screening quickly and at a lower cost (Mendonça et al., 2015), especially when the phytochemical profile of potential medicinal species is not yet known or has not been widely studied, as is the case of *A. cearensis*. This is particularly true of plants found in biomes where conservation of biodiversity is of relevant interest.

In our study, it was possible observed different chromatographic profiles between the extracts evaluated via high-performance liquid chromatography (HPLC) (Figure 4). The chromatogram of the extracts from the calluses obtained from the cotyledon (Figure 4a~c) and leaf segments (Figure 4d-f), showing a highly intense peak at retention time near 50 minutes, while in the extract from the aerial parts exhibited a more complex chromatographic profile and the retention time of this peak was shorter, near 23 minutes (Figure 4c). These results to indicate that the extract from the plants growth *in vivo* have more substances than extracts from the calluses grown *in vitro*.

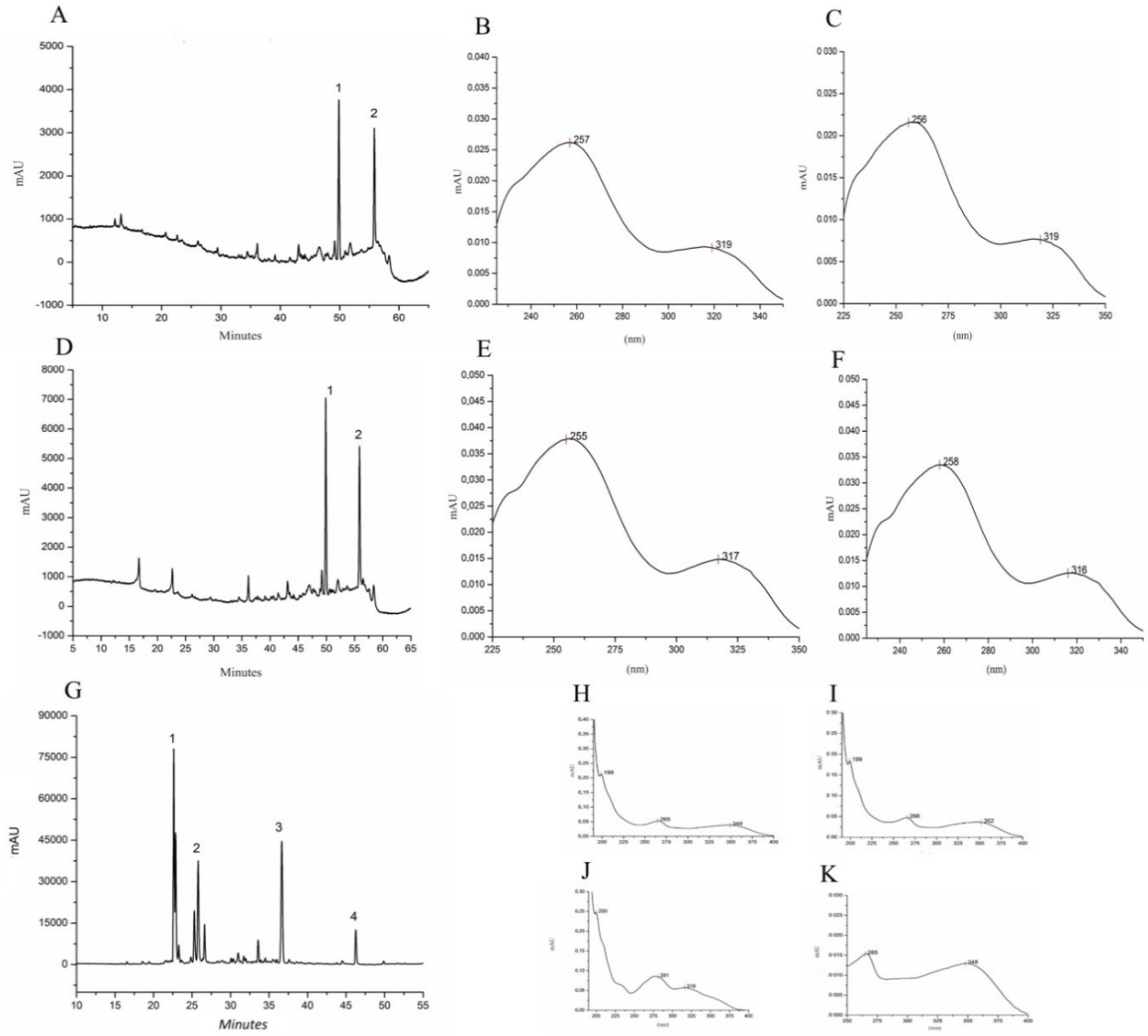

Figure 4. Chromatogram and UV spectra from of different extracts of *Amburana cearensis* with diode array detection at 340 nm obtained by the HPLC method. A-C) Extract from the calluses obtained from cotyledon segments. A) Chromatogram. B) Spectrum of peak 1. C) Spectrum of peak 2. D-F) Extract calluses obtained from leaf segments. D) Chromatogram. E) Spectrum of peak 1. F) Spectrum of peak 2. G-K) Extracts from the aerial parts of the plants grown under conventional conditions. G) Chromatogram. H) Spectrum of peak 1. I) pectrum of peak 2. J) Spectrum of peak 3. K) Spectrum of peak 4.

Some authors have mentioned that the synthesis and level of secondary compounds can be different between plants grown *in vitro* and *in vivo* (Dehghani, Mostajeran, & Asghari, 2011), since the *in vitro* environment has high relative air humidity, low irradiation, low content of carbon gas, and the presence of sugars and growth regulators in the culture medium (Anasori & Asghari, 2008).

However, in this study, there was virtually no variation in the composition of the secondary metabolites in the *in vitro* conditions tested, except for coumarin, which in the extracts from calluses obtained from leaf segments was detected with greater intensity than in the calluses from cotyledon segments. This result corroborates the findings of other researchers who also have reported that *in vitro* conditions were not effective for production of secondary metabolites, because the dedifferentiation of cells can lead to loss of the ability to produce metabolites of interest (Dehghani et al., 2011; Saiman et al., 2014).

The auxins 2,4-D and picloram can be used to induce callus formation in *A. cearensis*. In this respect 2,4-D induced a greater percentage of calluses at concentrations of 21.94 μM and 26.46 μM for leaf and cotyledon segments, respectively. The growth curve allowed identifying the development dynamics of the *A. cearensis* calluses, indicating that those obtained from leaf segments grew slower in relation to cotyledon segments. The biochemical analysis of the *A. cearensis* calluses revealed a decline in the levels of reducing sugars and increase of total proteins during the growth phase.

The results of the phytochemical analysis identified the presence of coumarin in all the extracts evaluated, which can be very useful to the pharmaceutical industry, since this secondary metabolite has high pharmacological potential. However, it is necessary to evaluate the growth for a longer period so as to analyze other phases of the growth curve of *A. ceaerensis* calluses and establish the best moment for subculture. Considering the large scale production of cells is a mandatory step to set up economically viable *in vitro* production of pharmacological compounds, additional studies starting from leaf and cotyledon segments explant of *A. cearensis* calluses, should also be conducted via bioreactor systems.

Conclusion

In conclusion, the results of this study indicate that the concentrations of 21.94 μM and 26.46 μM of 2,4-D induced greater formation of compact and friable calluses from the leaf and cotyledon segments, respectively. The highest percentage of calluses growth was observed during the exponential phase for both explant types. The biochemical analysis of the *A. cearensis* calluses revealed that the maximum concentrations of reducing sugars occurred on the day of inoculation for both types of explants assessed. With respect to the total proteins, the calluses obtained from the cotyledon explants presented a substantial increase as of the seventh day after inoculation, while for the calluses from the leaf explants this increase in proteins only occurred as of the 21st day after inoculation. The phytochemical screening identified the presence of coumarin in the extracts from the calluses grown *in vitro* and the aerial part of the plants grown under conventional conditions.

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