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Stimulation of 6-benzylaminopurine and meta-topolin-induced *in vitro* shoot organogenesis and production of flavonoids of *Amburana cearensis* (Allemão) A.C. Smith)

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ABSTRACT

Amburana cearensis (cumarú) is a tree species native to the semi-arid region of northeastern Brazil. Extracts obtained from its bark, seeds and leaves are widely used in folk medicine. The growing demand for phytotherapic preparations from this species makes it necessary to develop techniques for commercial cultivation to supply inputs to the pharmaceutical industry. In this respect, *in vitro* culture is a viable alternative to meet this demand. The objective of this study was to evaluate the effect of two cytokinins, 6-benzylaminopurine (BAP) and meta-topolin (Mtop), on the direct organogenesis and to establish the phytochemical profile of *A. cearensis*. *In vitro* multiplication was tested from leaf and cotyledon segments, inoculated in woody plant medium (WPM) supplemented with varying concentrations of BAP (0.00, 2.22, 4.44, 6.66 or 8.88 μM) or Mtop (0.0, 2.0, 4.0, 6.0, 8.0 or 10.0 μM). Phytochemical screening and high-performance liquid chromatography were carried out to establish the phytochemical profile of extracts from the aerial part of cumarú, obtained from plants resulting from conventional conditions in greenhouse, clonal shoots and from *in vitro* culture. The results allowed establishing a new protocol for *in vitro* regeneration of *A. cearensis* using Mtop. The extracts these plants grown *in vitro* contained a higher quantity of flavonoids than those grown under conventional conditions. The results obtained from this study provide valuable new information to support future works, so that the medicinal properties and the secondary metabolites produced by this plant can be further investigated in benefit to human health in sustainable form.

1. Introduction

Amburana cearensis (Allemão) A. C. Smith is a species of the family Fabaceae, native to the Caatinga (shrubland) biome (Pereira et al., 2017), found in the Brazilian semi-arid region (Araruna et al., 2013). Extracts from this leguminous tree, popularly known as cumaru, are often used in traditional medicine, especially to treat respiratory diseases like colds, bronchitis and asthma (Araruna et al., 2013). These preparations have anti-inflammatory, antimicrobial, anticoagulant, vasodilatory, antispasmodic and antithrombotic activities, due to the presence of secondary metabolites such as coumarins, flavonoids and phenolic glycosides (Canuto and Silveira, 2010; Pereira et al., 2017).

Besides medicinal uses, *A. cearensis* is widely extracted for its wood. This extractivisim, along with environmental degradation and habitat loss, has drastically reduced the population of the species. The production of plants via direct organogenesis of shoots utilizing the normal ontogenetic pathway for development of branches from axillary meristems is one of the most important *in vitro* propagation methods (Gahan and George, 2008). This tissue culture technique allows the production of uniform plantlets with high quality, in turn able to produce

Abbreviations: BAP, benzylaminopurine; HPLC, high-performance liquid chromatography; Mtop, meta-topolin; WPM, woody plant medium; UV, ultraviolet.

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metabolites with medicinal potential without depending on planting in fields. This is advantageous both from the ecological and economic perspectives (Oksman-Caldentey and Inzé, 2004).

Various species of medicinal plants have been successfully micropropagated to increase the production of metabolites with phytotherapeutic properties (Kwiecień et al., 2018; Shasmita et al., 2018; Chauhan et al., 2018). The biosynthesis of secondary metabolites via tissue culture and organogenesis is influenced by many factors, among them the type of nature of the explants, the culture medium (Praveen and Murthy, 2010), and the types and concentration of growth regulators (Amoo et al., 2013).

In a previous study, Campos et al. (2013) observed that 6-benzylaminopurine was the most efficient regulator for the *in vitro* multiplication phase of *A. cearensis,* although the number of shoots formed was only 2.36 per explant. Therefore, it is important to find an alternative cytokinin to induce a reasonable shoot development rate and sufficient plant quality under *in vitro* conditions.

Meta-topolin is a naturally occurring aromatic cytokinin that was originally isolated from leaves of *Populus* x *canadensis* Moench, cv. robusta (Horgan et al., 1975). Several research groups have shown that it promotes the *in vitro* proliferation of shoots with high quality of various plant species (Bairu et al., 2008; Aremu et al., 2013; Gentile et al., 2017; Ahmad and Anis, 2019), besides reducing physiological disorders such as hyperhydricity (Magyar-Tábori et al., 2010).

Due to the chemical, toxicological and pharmacological characteristics of *A. cearensis*, as well as its economic value for the pharmaceutical industry and the dearth of studies of its *in vitro* propagation (Campos et al., 2013), the objective of this study was to investigate the effects of concentrations of the cytokinins BAP and Mtop and types of explants, to establish a protocol for direct organogenesis of *A. cearensis*, as well as to determine the best culture conditions to maximize the production of secondary metabolites by this species.

2. Material and methods

2.1. Initial in vitro culture

To obtain the plants *in vitro*, *A. cearensis* seeds were washed under running tap water for 10 min, disinfested in a laminar flow cabinet and immersed in 70% ethanol for 1 min, followed by a sodium hypochlorite solution - NaOCl [commercial sanitary water (Qboa®) – 2.5% active chloride] with 2 drops of neutral detergent (Ypê®) for 10 min. Then the seeds were washed four times in sterile distilled water and inoculated in test tubes (25 × 150 mm) containing 15 mL of Woody Plant Medium (WPM, Lloyd and McCown, 1980) and kept in a growth room at temperature of 26 ± 2 °C, 16 h photoperiod and photosynthetically active radiation of 60 μmol m^{-2} s^{-1} supplied by cool white fluorescent lamps.

Table 1
Elution systems and reagents used to characterize the main secondary metabolites in the extracts of *A. cearensis* by thin-layer chromatography.

Phytochemical	Elution system	Reagent
Alkaloids	Toluene:ethyl acetate: diethylamine (70:20:10, v/v)	Dragendorff
Quinone	Toluene:formic acid (99:1, v/v)	10% ethanolic KOH
Coumarins	Toluene:ethyl ether: (1:1 saturated with acetic acid 10%, v/v)	10% ethanolic KOH
Flavonoids, Cinnamic acid derivatives	Ethyl acetate:formic acid: glacial acetic acid:water (100:11:11:26, v/v)	NEU
Lignans	Chloroform:methanol:water (70:30:4, v/v)	Vanillin sulfuric
Monoterpenes, sesquiterpenes and diterpenes	Toluene:ethyl acetate (93:7, v/v)	Vanillin sulfuric
Triterpenes and steroids	Toluene:chloroform: ethanol (40:40:10, v/v)	Liebermann-Burchard

2.2. In vitro multiplication

For the multiplication phase, nodal segments (NS) and cotyledon segments (CS) were used, obtained from plantlets with age of 45 days, resulting from *in vitro* germination. Woody plant medium (WPM), as described by Lloyd and McCown (1980), was used in all the experiments, supplemented with 3% sucrose (Synth®) and solidified with 0.7% agar (Himedia®). The medium's pH was adjusted to 5.7 ± 0.1 before autoclaving. Then portions of the medium were distributed in test tubes (25 × 150 mm) and sterilized by autoclaving for 15 min at temperature of 121 °C and pressure of 1 atm.

2.3. Application of benzylaminopurine (BAP) for in vitro shoot regeneration of nodal and cotyledon segments

As mentioned, two explant types were used, nodal segments and cotyledon segments, inoculated vertically in the culture medium, supplemented with different concentrations of BAP (0.00, 2.22, 4.44, 6.66, 8.88 μM). The experimental design was completely randomized in a 2 × 5 factorial scheme (2 explant types x 5 BAP concentrations), with 5 repetitions, each one composed of 4 test tubes, and each tube composed of one explant, for a total of 20 explants. The cytokinin 6-benzylaminopurine (BAP) was purchased from Sigma-Aldrich, Italy.

2.4. Application of meta-topolin (Mtop) for in vitro shoot regeneration of nodal and cotyledon segments

Nodal and cotyledon segments were used, inoculated vertically in culture medium supplemented with different concentrations of Mtop (0.00, 2.07, 4.14, 6.21, 8.28 μM). The experimental design was completely randomized in a 2 × 5 factorial scheme (2 explant types x 5 Mtop concentrations), for a total of 10 treatments, with 5 repetitions, each one composed of 4 test tubes, and each tube composed of one explant, for a total of 20 explants. The cytokinin Mtop 6-(3-hydroxylbenzylamino) purine was produced by PhytoTechnology Laboratories, USA.

2.5. Traits evaluated after in vitro growth

After growth of the plantlets for 45 days in culture medium (WPM) containing BAP or meta-topolin, the following variables were evaluated: explant response percentage (ER%); number of shoots per explant (NS); and length of the shoot aerial part (APL).

2.6. Assay of phytochemical compounds using HPLC-DAD (high-performance liquid chromatography)

2.6.1. Preparation of extracts

The extracts were prepared from aerial parts of the plants that were germinated under conventional conditions containing substrate soil + vermiculite (1:1), kept in a greenhouse; aerial parts of the plants obtained *in vitro* germinated seedlings, grown on WPM supplemented with 3% sucrose (Synth ®) and solidified with 0.7% agar (Himedia®) and aerial parts of 45 days old from clonal shoots obtained *in vitro* multiplication via cotyledon segments and cultivated in WPM supplemented with 3% sucrose (Synth ®) and 4.5 μM of Mtop, and solidified with 0.7% agar (Himedia®). All samples of aerial parts were collected after 45 days of cultivation and were dried in a forced air oven at 40 °C for 3 days at and then pulverized in a knife mill. To produce the crude extract, samples of the dried material (10 g) were macerated with hexane (Hex) and then with methanol (MeOH) not acidified with renewal of the extractor fluid every 72 h. Then the solution was concentrated by elimination of the organic solvent to obtain the crude extract. After the extraction, the extractive solution was concentrated under vacuum in a

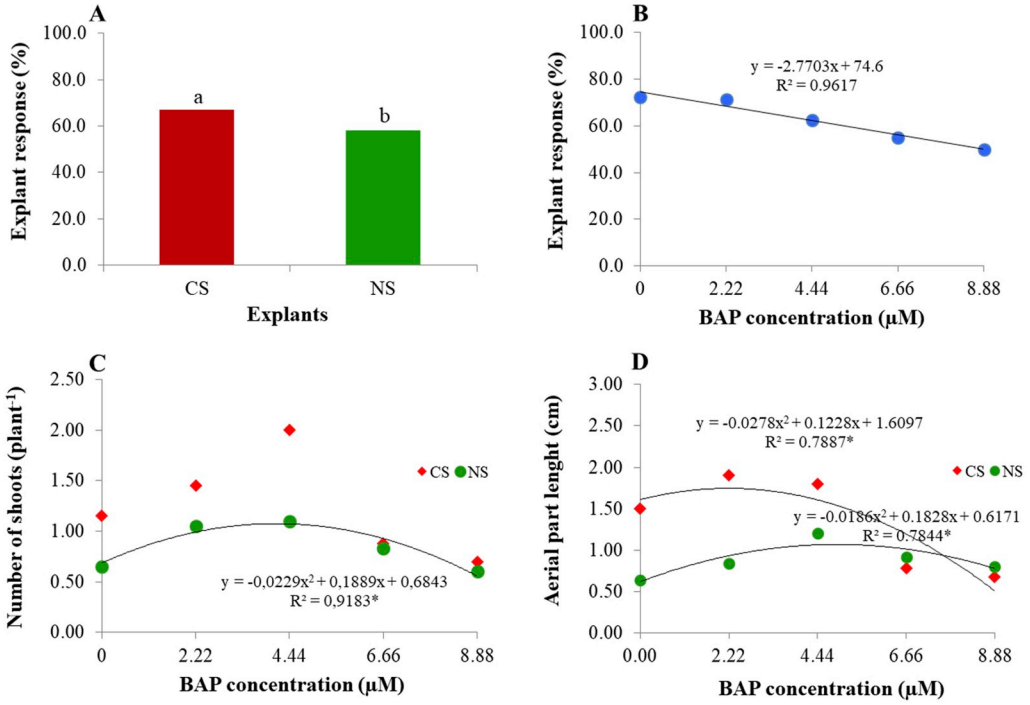

Fig. 1. Explant response percentage in the *in vitro* proliferation of *A cearensis* shoots in function of explant types (a) and different BAP concentrations (b). Number of shoots (c) and aerial part length (d) in function of the BAP concentration x explant type interaction. CS: Cotyledon segment and NS: Nodal segment * Significant ($p <$ 005) by the F-test.

rotary evaporator, yielding 10 g of crude hexane extract (Av-HexC) and 83 g of crude methanol extract (Av-MeOH). A portion of the methanol extract (80 g) was suspended in a 3:7 (v/v) mixture of methanol (MeOH) and water (H_2O) and partitioned with hexane, chloroform ($CHCl_3$) and ethyl acetate (AcOEt) in ascending order of polarity to obtain the respective extracts (Av-Hex 9.15 g; Av-$CHCl_3$ 4.35 g; Av-AcOEt 5.45 g; and Av-H_2O 40.0 g).

2.6.2. Qualitative analysis of phytochemicals

The qualitative analysis of the extracts to detect the presence of secondary metabolites in *A. cearensis* was performed in the Biochemistry Laboratory of Vale do São Francisco Federal University (UNIVASF). Each sample (0.05 mg) was dissolved in 1 mL of chloroform. The sample was placed individually on thin-layer chromatography (TLC) plates of silica gel 60 F_{254} aluminum supports, applied with a micropipette and eluted in different solvent systems as suitable for each class of secondary metabolite as described by Wagner and Bladt (1996) (Table 1).

The chromatographic analyses were performed on a High-performance liquid chromatography (HPLC-DAD) from Shimadzu® LC-20 coupled to a diode array detector (DAD) and a C18 column with dimensions of 250 × 4.6 mm, 5 µm (Hypersil ThermoScientific®) with guard column, and the temperature was kept stable at 30 °C throughout the analysis. Two solutions were used as mobile phase: Solution A consisted of ultrapurified water + trifluoroacetic acid 0.1% (v/v) and Solution B – 100% acetonitrile, with flow of 0.8 mL min^{-1} flow. At the start, the gradient was composed of 100% A, and after 50 min this gradient reached 50% A and 50% B, soon thereafter returning to the initial condition.

The samples were injected in the volume of 8 µL and the detection was performed in DAD at a wavelength of 270 nm and 340 nm. In parallel, 23 analytical standards were analyzed individually to investigate their presence in the samples. All the test were analyzed in triplicate. The data obtained were treated with the software LCSolution 1.0 (Shimadzu®, Japan) was used for the data analysis.

2.7. Statistical analysis

The data were submitted to analysis of variance (ANOVA) by the F-test ($p \leq 0.05$ or $p \leq 0.01$). Percentage data were transformed to arcsine before statistical analysis. The quantitative factors related to the BAP and Mtop levels were evaluated by polynomial regression (linear and quadratic). In turn, the factors related to explant types were analyzed by comparing the means with the Tukey test at 5% probability. All the analyses were performed with the "agricolae" package implemented in the R software (R Development Core Team, 2016).

3. Results and discussion

3.1. Effect of BAP on shoot regeneration from the nodal and cotyledon segments

With respect to the capacity for shoot regeneration of *A. cearensis*, there was a significant effect ($p < 0.001$) of the individual factors explant type and BAP concentration (Fig. 1 a-b).

The greatest shoot regeneration was attained when using cotyledon segments, with an explant response percentage of 67.0% (Fig. 1a). This result can be attributed to the youth of this tissue and hence its high morphogenic capacity. Various authors have reported the efficiency of using cotyledon segments for *in vitro* multiplication of shoots of several plant species (Nayak et al., 2013; Pandey and Tamta, 2016).

In this study, when using nodal segments as the explant source, the response percent was 58.0% (Fig. 1a) This result should be considered when intending to use different explants from a single donor plant as a strategy to maximize the *in vitro* multiplication rate of a superior genotype. Various micropropagation protocols of plants, including woody species and those with medicinal properties, indicate the use of nodal segments to induce shoots *in vitro* due to their high organogenic capacity and production of "true to type" clones (Purkayastha et al., 2008; Romyanon et al., 2015; Khanam and Anis, 2018), because of the presence of axillary buds in these explants.

Fig. 2. Growth and development of *A cearensis* after 30 days of culture with different BAP concentrations. a-e) cotyledon segments. f-j) nodal segments. a and f (0.00 μM BAP), b and g (2.22 μM BAP), c and h (4.44 μM BAP), d and i (6.66 μM BAP), e and j (8.88 μM BAP) Bar = 0.5 cm (a–e), Bar = 0.25 cm (f–j).

The regeneration of *A. cearensis* shoots occurred with both explant types and all BAP concentrations tested, including zero (Fig. 1b; Fig. 2), suggesting the existence of an endogenous cytokinin concentration in these type of explants. The results observed in this study are corroborated by other authors in different species, such as mandarin cultivars, who also reported the *in vitro* shoot regeneration existence in the absence of BAP (Soriano et al., 2019).

The regression curve of the explant response percentage in relation to the BAP concentrations can be seen in Fig. 1b. There was a significant negative linear relation ($p < 0.05$) as the BAP concentration in the culture medium increased. A possible explanation why the response percentage declined at higher BAP concentrations is the phytotoxic effect of cytokinin, caused by combination of the highest endogenous concentrations of BAP with the exogenous BAP cytokinin supply in the culture medium. Previous studies performed by other authors also have shown that the application of exogenously applied plant growth regulators (PGRs) can interact with phytohormones and change their concentrations (AyilGutiérrez et al., 2013).

A study involving *in vitro* propagation of another medicinal plant species (*Vernonia condensata* Baker) also demonstrated an inhibitory effect on the explant response percentage with higher BAP concentrations (Vicente et al., 2009).

With respect to the number of shoots per explant, there was a significant interaction ($p < 0.05$) between explant and BAP concentration (Fig. 1c). The largest number of shoots was formed from cotyledon segments grown in culture medium supplemented with 4.44 μM of BAP, with inducement of an average of 2.0 shoots/explant. The beneficial effect of BAP on the multiplication of shoots is related to the influence of this growth regulator on cell division and release of axillary buds from inhibition by apical dominance (Pozo et al., 2005). Our findings are in accordance with earlier results reported by Campos et al. (2013), who investigated the influence of the growth regulator BAP (0.0, 2.22, 4.44, 8.88 and 17.76 μM) on the *in vitro* multiplication of different explants (nodal, apex, cotyledon and stem segments) of *Amburana cearensis*. These authors observed that the WPM medium supplemented with 4.44 μM BAP provided a larger number of shoots from the explants from cotyledon and nodal segments, with 2.36 and 1.24 shoots/explant, respectively.

Regarding the aerial part length (APL), there was a rising and significant quadratic effect ($p < 0.05$) for the two explant types in function

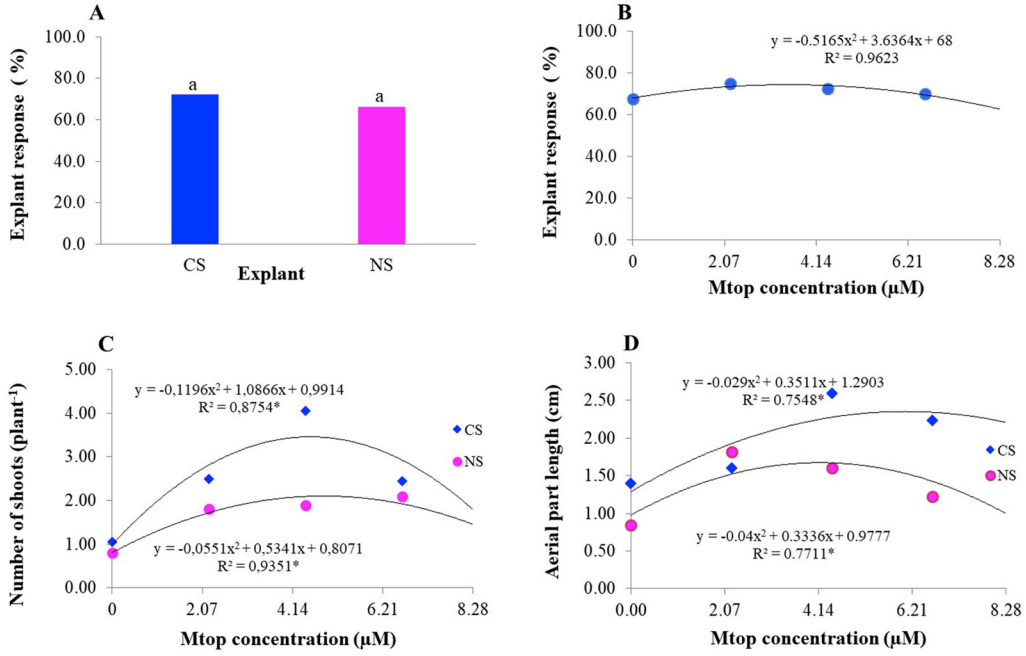

Fig. 3. Explant response percentage in the *in vitro* proliferation of *A cearensis* shoots in function of explant types (a) and different Mtop concentrations (b). Number of shoots (c) and aerial part length (d) in function of the Mtop concentration x explant type interaction. CS: Cotyledon segment and NS: Nodal segment * Significant ($p < 005$) by the F-test.

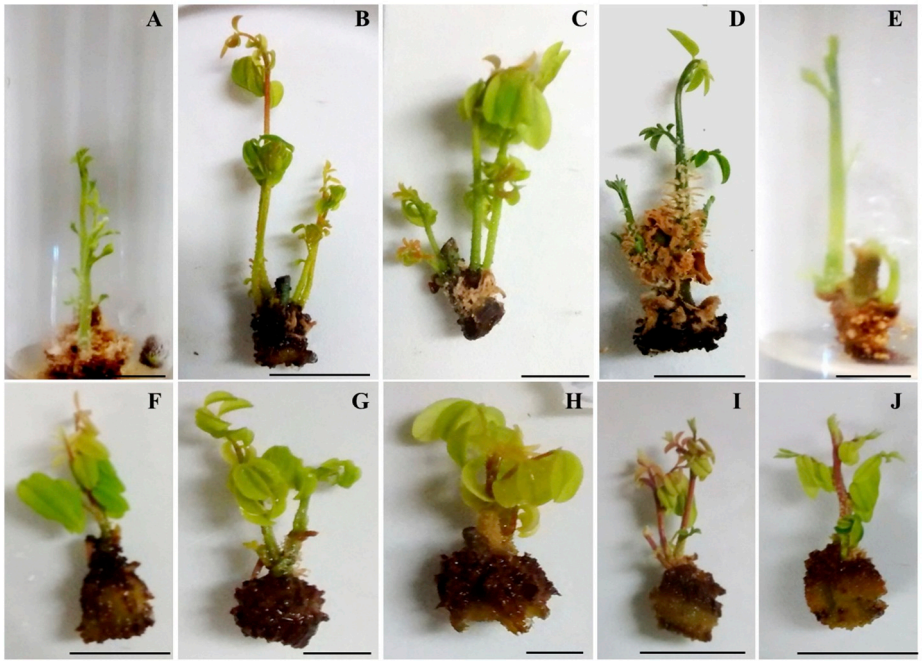

Fig. 4. Growth and development of *A cearensis* after 30 days of culture with different Mtop concent rations a-e) cotyledon segments. f-j) nodal segments. a and f (0.00 μM Mtop), b and g (2.07 μM Mtop), c and h (4.14 μM Mtop), d and i (6.21 μM Mtop), e and j (8.28 μM Mtop). Bar = 0.5 cm.

of exogenous application of BAP (Fig. 1d). The largest average APL values were 1.90 cm and 1.20 cm at the concentrations of 2.22 and 4.44 μM, observed in the plants from cotyledon and nodal segments, respectively. In a previous study, Purkayastha et al. (2008) observed that a higher concentration of cytokinins in the culture medium caused a shorter average shoot length. Other authors have stated that the smaller lengths of shoots as of a certain concentration of cytokinin can result from a possible cytotoxic effect of this hormone (Grattapaglia and Machado, 1998) and also for nutrient competition, can even stimulate the occurrence of hyperhydricity, chlorosis and formation of abnormal leaves (Naaz et al., 2019). In our study, we observed these occurrences in the treatments containing BAP above of the concentration of 4.44 μM (Figs. 2d, e, i, j).

3.2. *Effect of Mtop on shoot regeneration from nodal and cotyledon segments*

In general, there were no significant differences of the plants obtained from cotyledon and nodal segments in relation to the explant response percentage when grown in culture medium containing meta-topolin (Fig. 3a).

There was a significant quadratic response ($p < 0.05$) to meta-topolin in the shoots induction of *A. cearensis* (Fig. 3b). The best response was obtained with 2.07 μM of Mtop in the WPM, with an explant response percentage of 75.0% (Fig. 3b). Our results corroborate those observed for some other plants species, such as Manihot esculenta Crantz (Chauhan and Taylor, 2018). These researchers observed a higher percentage of responsive explants grown in WPM supplemented with Mtop than with BAP.

Considering the number of shoots in relation to the Mtop concentrations and explant type, there was a significant rising quadratic effect ($p < 0.05$) for both explant types (Fig. 3c). For the nodal segments, the response curve showed a tendency for the number of shoots to increase with higher concentrations of meta-topolin, up to a maximum of 6.21 μM, where the average number of shoots per explant was 2.1. However, when using explants from cotyledon segments, the highest average number of shoots per explant was 4.05 observed in WPM supplemented with 4.14 μM of meta-topolin. Some studies have demonstrated that meta-topolin is more effective than BAP to induce proliferation of shoots, in *Pelargonium* sidoides D.C (Wojtania, 2010), *Musa* spp. (Bairu et al., 2008).

The aerial part length was represented by a negative quadratic model significant ($p < 0.05$) for the two explant types in function of exogenous application of Mtop (Fig. 3d). The culture medium supplemented with meta-topolin concentration of 4.14 μM induced the largest average aerial part growth (2.60 cm) of the plants obtained from cotyledon segments. In turn, for the plants obtained from nodal segments, the Mtop concentration of 2.07 μM promoted the highest mean aerial part growth, of 1.82 cm (Fig. 3d). These results demonstrate that the addition of Mtop to the culture medium was beneficial for the aerial part length in comparison with the use of BAP.

The positive effect of Mtop on the other conventional purine-based cytokinins (BAP) and its potential as substitute for plant growth regulators (PGR) is well documented in several plant species due to the high number of multiple shoots produced, proving physiological and biochemical traits, successful rooting and easy acclimatization and proliferation capacity and lesser residual toxicity (Aremu et al., 2012; Gentile et al., 2017; Ahmad and Anis, 2019; Naaz et al., 2019). We also found that plants treated with meta-topolin were superior in quality (Fig. 4) and quantity (explant response percentage, number of shoots, and length of the shoot aerial part) compared to plants treated with BAP. Wojtania (2010) also demonstrated that the addition of meta-topolin in the culture medium resulted in higher shoot quality in *Pelargonium* that the BAP. These authors observed that all Pelargonium cultivars when grown in medium containing Mtop produced juvenile well developed shoots with high chlorophyll content. Moreover, this cytokinins was able to prevent the senescence of *Pelargonium* shoots.

Some authors have mentioned that the combination Mtop in equimolar concentrations with BAP can promote lower toxicity and lesser occurrence of physiological disorders (Amoo et al., 2010), a significantly smaller necrosis rate and somaclonal variation, and greater potential for rooting and acclimatization *ex vitro* (Bairu et al., 2008; Adeyemi et al., 2012).

Our results show that for establishment of an efficient regeneration system, meta-topolin can act as a new source of cytokinins, so that it can be recommended for *in vitro* regeneration of *A. cearensis*, as also reported

Table 2
Phytochemical screening of the plant materials of *A cearensis* submitted to extraction by maceration.

Phytochemical	*A cearensis plants*		
	Conventional conditions	*In vitro*	Clonal shoots
Alkaloids	–	–	–
Flavonoids	++	++	++
Cinnamic derivatives	+	+	++
Lignans	–	–	–
Coumarins	+++	+++	+++
Quinones	++	++	++
Mono, sesqui and diterpenes	–	–	–
Triterpenes and steroids	+	+	+

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

for other plant species (Amoo et al., 2015). It is interesting to note that the establishment of *in vitro* cultures for the clonal multiplication of medicinal plants offers an alternative strategy for the conservation and production of phytochemicals they produce (Isah, 2019).

3.3. Phytochemical constituents

The qualitative analysis of the ethanol extracts of *A. cearensis* revealed the presence of secondary metabolites grouped in two distinct classes: terpenes and phenolic compounds. All the extracts reacted positively for the presence of coumarins, with varying quantities. Other studies have also found the presence of coumarins in extracts from the seeds (Pereira et al., 2017) and seeds and trunk bark (Canuto and Silveira, 2010) of *A. cearensis*.

The extracts from all three types of plant material tested were positive for flavonoids, cinnamic derivatives and quinones while negative results were observed for the presence of alkaloids, lignans, monoterpenes, sesquiterpenes and diterpenes (Table 2).

Our results reveal the presence of the main classes of secondary metabolites found by other researchers in plants of the genus *Amburana*. Pereira et al. (2017), evaluating an extract of the seeds of *A. cearensis*, identified the various compounds with antioxidant properties, with the majority of peaks corresponding to the isoflavone coumarin. This secondary metabolite have different potential pharmacotherapeutic applications, due to their antioxidant, anti-inflammatory, antimicrobial and anticoagulant activities, as well as possible use as adjuvants in cancer therapy (Al-Amiery et al., 2015; Achar et al., 2019).

Other authors also demonstrated the presence of several compounds, including isokaempferide, kaempferol, afrormosin, coumarin and other phenols compounds including amburoside in the ethanolic extract of the trunk bark of *Amburana cearensis* (Canuto and Silveira, 2010; Araruna et al., 2013). These metabolites have several pharmacotherapeutic applications, variously due to antinociceptive, analgesic, anti-inflammatory, and anticoagulant activities (Araruna et al., 2013; Lei et al., 2015; Pereira et al., 2017).

The phytochemical screening of the *A. cearensis* extracts showed that the extracts from the plants grown in conventional conditions and *in vitro* had the same phytochemical profile despite the different cultivation conditions (Table 2). Synthesis of similar phytochemicals in naturally and *in vitro* growth plants was also reported by Mukhia et al.

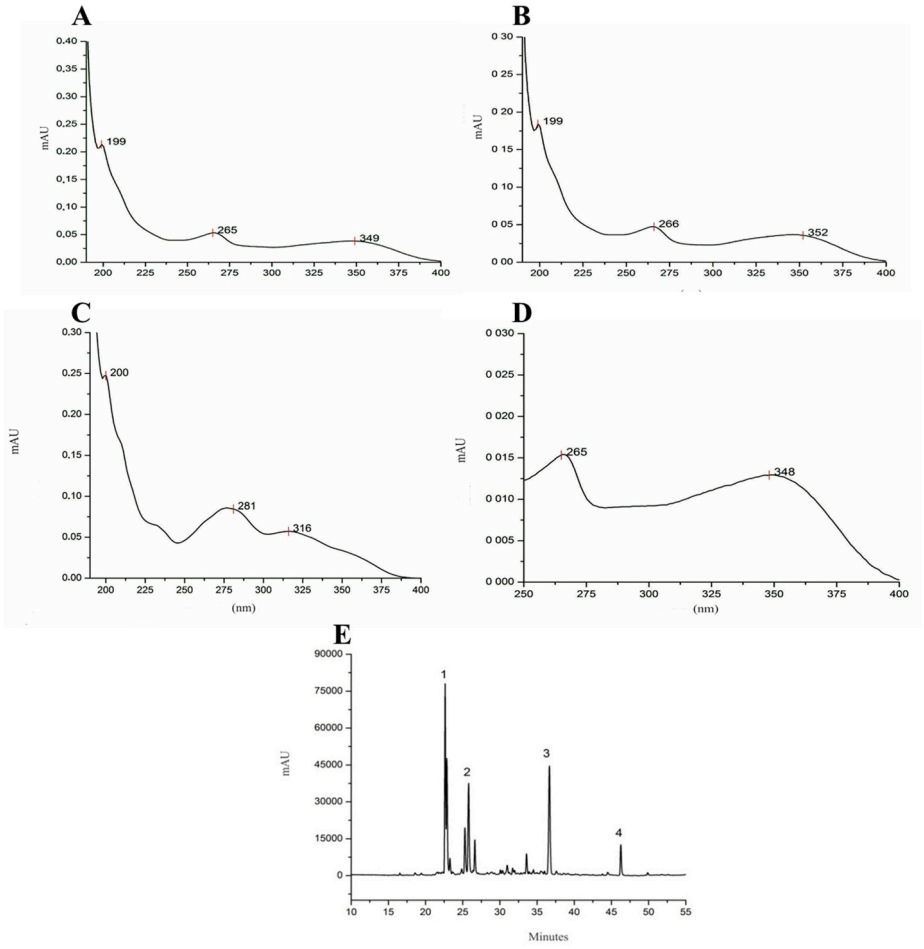

Fig. 5. Comparison of the UV spectra of the extract and the chromatogram at 340 nm of the extract using HPLC of the *A cearensis* plants grown under conventional conditions. a) Spectrum of peak 1. b) Spectrum of peak 2. c) Spectrum of peak 3. d) Spectrum of peak 4. e) HPLC Chromatogram.

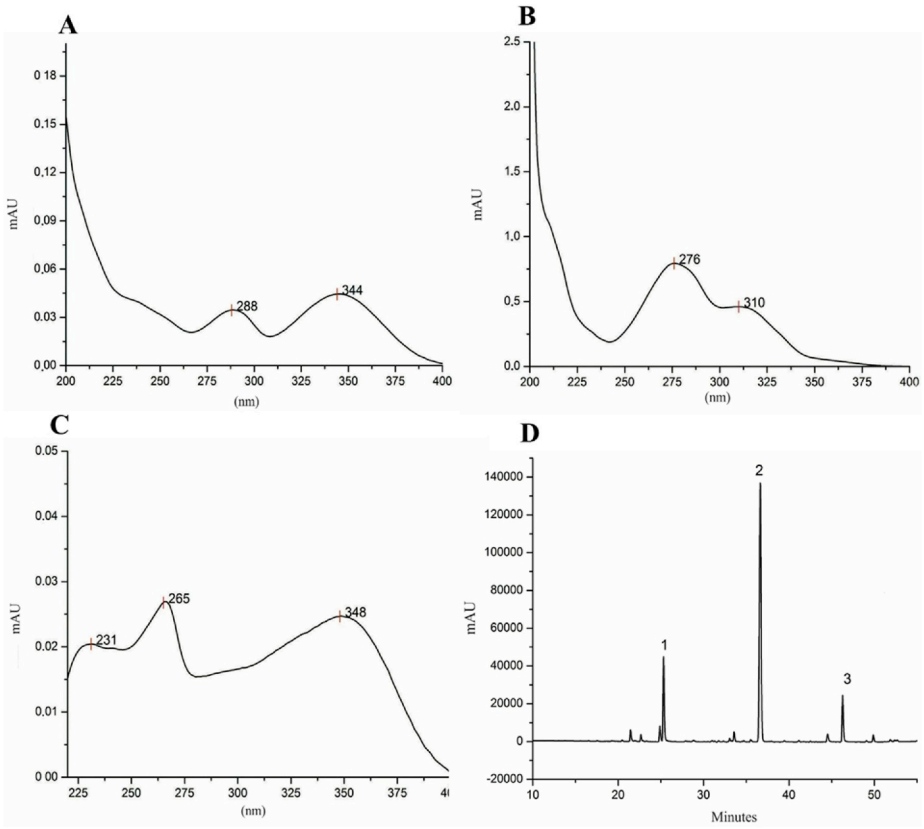

Fig. 6. Comparison of the UV spectra of the extract and the chromatogram at 340 nm of the extract using HPLC from *A cearensis* plants grown *in vitro*. a) Spectrum of peak 1. b) Spectrum of peak 2. c) Spectrum of peak 3. d) Chromatogram.

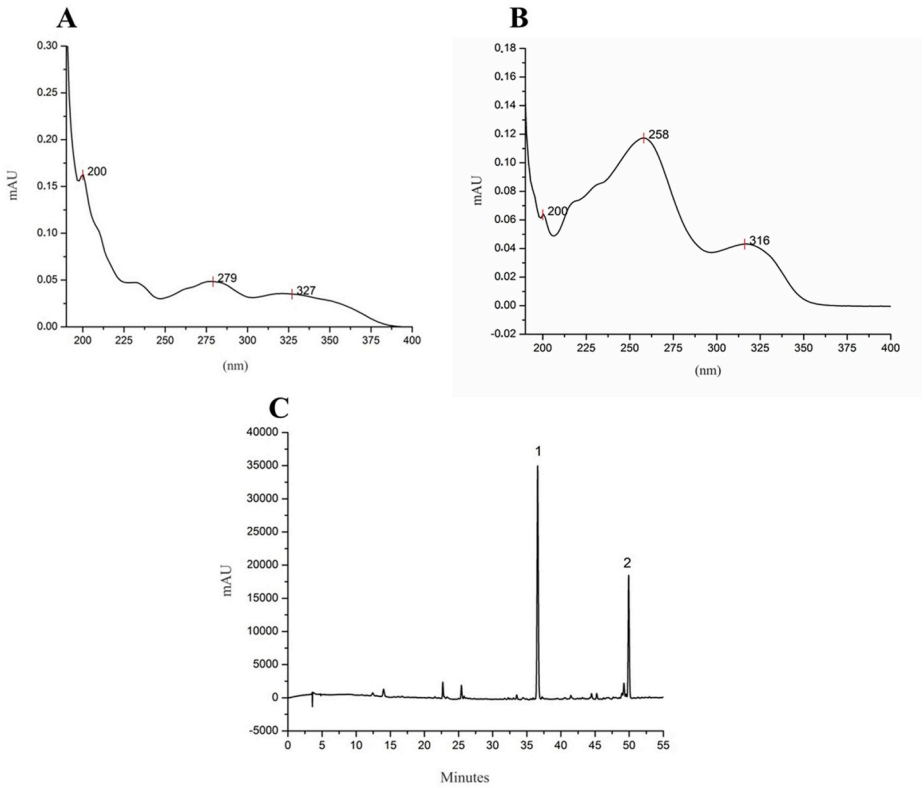

Fig. 7. Comparison of the UV spectra of the extract and the chromatogram at 340 nm of the extract using HPLC from *A cearensis* clonal shoots. a) Spectrum of peak 1. b) Spectrum of peak 2. c) Chromatogram.

(2019). In other plant species, such as *Arnica montana* L., Nikolova et al. (2013) observed by quantitative analysis that there was no difference in the synthesis of surface flavonoids among *in vitro*, *ex vitro* and *in vivo* grown plants.

It is interesting to note that although the extracts of different parts of *A. cearensis* plants present the same qualitative profile, there can be substantial quantitative differences. In the present study, we observed higher production of some metabolites in the plants germinated *in vitro* than in the plants from the two other cultivation conditions. Probably, the differences in the content of these secondary metabolites might be due to the different growth conditions and stages of plant development. Studies carried out by other authors have shown also higher level of total phenolic and flavonoid contents in extracts of *in vitro* samples compared to extracts of field grown plants (Thiruvengadam and Chung, 2015).

According to Lucchesini et al. (2009), the different conditions used for *in vitro* culturing can influence the production of metabolites, both in quantity and quality, since the *in vitro* environment has high relative humidity, low irradiation, low carbon gas concentration, and presence of sugars and growth regulators on the culture medium (Hussain et al., 2012; Thiruvengadam and Chung, 2015; Rodrigues et al., 2019).

For the majority of plants, external factors or variables (light, temperature, soil moisture, soil fertility and salinity) can significantly affect processes associated with plants' growth and development, including their capacity to synthesize secondary metabolites, leading to general changes in the phytochemical profiles, which are important for production of bioactive substances (Ferrandino et al., 2014; Verma et al., 2015; Griesser et al., 2015).

The chromatographic profiles of the extracts submitted to HPLC analysis are shown in Figs. 5–7). At the selected wavelength (340 nm), a large difference can be perceived among the three types of extracts evaluated. The number of peaks present shows that the extracts from the aerial part of the plants grown under conventional conditions (Fig. 5a–e) have a more complex profile (Fig. 5e), indicating the presence of a larger number of substances compared to the extracts from plants grown *in vitro (*Fig. 6) and the shoots (Fig. 7). Besides this, the different retention times observed when comparing the extracts from the plants grown *in vitro* (Fig. 6a–d) and the shoots (Fig. 7a–c) grown *in vitro*, on the one hand, with the extracts from the aerial part of the plants cultivated under conventional conditions (Fig. 5a–e) indicate that the substances composing these three extracts are different.

The chromatographs obtained for the extracts from the plants grown *in vitro* (Fig. 6d) and the shoots (Fig. 7c) have an intense peak at a retention time near 36 min, while for the extract from the aerial part of the plants grown under conventional conditions this retention peak is 23 min (Fig. 5e).

We were unable to identify any of the analytical standards in the samples. However, the UV spectra indicated that the major peaks corresponded to the class of flavonoids (Figs. 5–7), since the UV spectra had an absorption profile with two peaks, characteristic of flavonoid compounds, one in the range 240–280 nm (band II) and the other in the range 300–400 (band I). In general, band II can be attributed to the A ring of the benzoyl system and band I to the B ring of the flavonoid structure (Fonseca et al., 2007).

The phytochemical analyses produced relevant information regarding the presence of secondary metabolites in the different extracts of the *A. cearensis* plants. From the pharmaceutical perspective, our most important finding is that the extracts from the *A. cearensis* plants grown *in vitro* contained a higher quantity of flavonoids than the extracts from plants grown under conventional conditions. This evidence supports the great current demand for the use of natural products with medicinal purposes added to the small quantities normally existing in the plants and the worrisome environmental implications generated by the predatory collection of plants in their natural environment and renews interest in the use of *in vitro* methods for the production of these secondary metabolites. In addition, this is an advantage in practical terms because micropropagation allows obtaining material of better quality for medicinal purposes (Isah, 2019). The results presented here can be used in future investigations in the areas of pharmacology, phytochemistry and biology to discover new drugs.

4. Conclusions

The findings of the study showed that the *in vitro* regeneration response was influenced by the explant type (nodal or cotyledon segments) and growth regulators (BAP or Mtop) supplemented in the culture medium. Meta-Topolin in the medium was found to be superior in enhancing the response of explants as compared to BAP. The best multiplication rate via direct organogenesis was obtained with explants from cotyledon segments inoculated in the WPM medium integrated with meta-topolin in the concentration of 4.14 μM. This result, to the best of our knowledge, is the first report of successful application of Mtop to promote *A. cearensis* organogenesis. However, the culture processes described have yet to be fully optimized, but it is considered that use of Mtop brings important new opportunities for development of simple and rapid plant regeneration systems in *A. cearensis* with potential applications in genetic transformation and gene editing for this important species. This is the first study to provide data on the production of secondary metabolites from of different *A. cearensis* extracts via HPLC revealed several bioactive chemical constituents, including coumarins, flavonoids, cinnamic derivatives, quinones, triterpenes and steroids, which have excellent potential for use to produce medicinal compounds. It is important to highlight the need to expand this study, exploring other factor that influence the biosynthesis of secondary metabolites in *A. cearensis* through tissue culture, including use of other approaches such as for example temporary immersion bioreactor system to produce the yield of shoots that will be required for adequate amounts of compound extraction, aiming to increase the biosynthesis of secondary metabolites, such as the coumarin that are common to this species, for medicinal uses.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101408.

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