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Callus induction and bioactive phenolic compounds production from *Byrsonima verbascifolia* (L.) DC. (Malpighiaceae)[1]

Indução de calos e produção de compostos fenólicos bioativos em *Byrsonima verbascifolia* (L.) DC. (Malpighiaceae)

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ABSTRACT - This study developed a methodology for callus induction in leaf segments of *B. verbascifolia* and evaluated the bioactive phenolic compounds production. Leaf explants were cultured in MS medium with 30 g L^{-1} sucrose, solidified with 7 g L^{-1} agar supplemented with 2,4-D (0; 4.52; 9.05; 18.10 μM) and BAP (0; 4.44; 8.88; 17.75 μM) in the presence and absence of light. Forty-five days after inoculation we assessed the percentage of callus induction, color, consistency, fresh and dry matter, total phenols, flavonoids, tannins contents, and chromatographic profile by HPLC-DAD method. Callus induction occurred only in medium with growth regulators. Maximal induction (100%) was found in medium containing 2,4-D combined with BAP in the presence and absence of light. We obtained friable and compact callus in yellow, green, and red. Culture media containing 4.52 μM 2,4-D + 4.44 μM BAP induced 100% of friable callus with higher fresh and dry weight in the absence of light. The callus produced higher amounts of total phenols and flavonoids than the initial explant. Total tannins were detected only in callus induced in media containing 17.75 μM BAP and 4.52 μM 2,4-D + 17.75 μM BAP, and were not found in the initial explant. The bioactive phenolic compounds detected are derived from benzoic, *p*-coumaric, cinnamic, gallic acids, and catechins.

Key words: Cerrado. Medicinal plant. *In vitro* culture. Secondary metabolites.

RESUMO - Objetivou-se por este trabalho desenvolver uma metodologia para indução de calos em segmentos foliares de *B. verbascifolia* e avaliar a produção de compostos fenólicos bioativos. Explantes foliares foram inoculados em meio MS com 30 g L^{-1} de sacarose, solidificados com 7 g L^{-1} de agar e suplementados com 2,4-D (0; 4,52; 9,05; 18,10 μM) e BAP (0; 4,44; 8,88; 17,75 μM), na presença e ausência de luz. Após 45 dias da inoculação foram avaliados a porcentagem de indução de calos, coloração, consistência, matéria fresca e seca, teores de fenóis, flavonóides e taninos totais e o perfil cromatográfico por HPLC-DAD. A indução de calos ocorreu apenas nos meios com reguladores de crescimento. A indução máxima (100%) foi observada em meios com 2,4-D e BAP combinados, na presença e ausência de luz. Foram obtidos calos friáveis e compactos com coloração amarela, verde e vermelha. Meios de cultivo contendo 4,52 μM 2,4-D + 4,44 μM BAP, na ausência de luz induziram 100% de calos friáveis, com maiores pesos fresco e seco. Os calos produziram quantidades significativas de fenóis e flavonóides totais, superiores aos teores detectados no explante inicial. Taninos totais foram detectados apenas em calos induzidos em meios contendo 17,75 μM de BAP e 4,52 μM de 2,4-D + 17,75 μM de BAP e não foram observados no explante inicial. Os compostos fenólicos bioativos detectados são derivados de ácido benzóico, *p*-cumárico, cinâmico, gálico e catequinas.

Palavras-chave: Cerrado. Planta medicinal. Cultivo *in vitro*. Metabólitos secundários.

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INTRODUCTION

Many plant species are capable of producing bioactive compounds, which can play an important role in maintaining human health. However, seasonal and environmental variations, soil type, and plant age associated with difficulties in the propagation of certain species may limit the availability of many bioactive compounds and restrict a more thorough investigation of their properties (CHERDSHEWASART; SUBTANG; DAGKAB, 2007). Furthermore, the chemical synthesis of several herbal substances is often impractical due to their structural complexity (OKSMAN-CALDENTEY; INZÉ, 2004).

Different bioactive compounds have been found in *in vitro* culture, especially callus culture such as flavonoids and stilbenes (MANEECHAI *et al.*, 2012), sterols (LOREDO-CARRILLO *et al*., 2013), cardenolides (SAHIN; VERMA; GUREL, 2013) and phenolic acids (SZOPA; EKIERT, 2014). *In vitro* plant cell culture has advantages for providing bioactive compounds with a defined production system and short culture periods, which ensures a continuous supply of metabolites of interest; also, cells are free of diseases and are not exposed to seasonal variations. Furthermore, it allows the production of new compounds not normally found in the mother plant (LOREDO-CARRILLO *et al*., 2013).

Auxins and cytokinins are usually employed to induce callus, as they promote cell growth by stimulating cell division and elongation through synergistic, antagonistic, and additive interactions (COENEN; LOMAX, 1997). Auxin 2,4-dichlorophenoxyacetic acid (2,4-D) has been widely used either alone or combined with cytokinins, especially 6-benzylaminopurine (BAP), to stimulate callus induction and obtain bioactive compounds *in vitro*. However, the concentration and combination of these regulators must be defined for each species (LOREDO-CARRILLO *et al*., 2013).

Byrsonima verbascifolia (L.) DC. (Malpighiaceae) or "murici-cascudo" is a species of the Brazilian Cerrado used in traditional medicine to treat diarrhea, intestinal infections, wound healing, inflammation, Chagas disease, and infections of the female genital tract (ALMEIDA *et al*., 1998). Recent studies have confirmed some traditional uses and have demonstrated that different parts of plant have antimicrobial (CECÍLIO *et al*., 2012), antimutagenic and antigenotoxic activities (GONÇALVES *et al*., 2013). *B. verbascifolia* is a natural source of bioactive phenolic compounds such as aromatic esters, naphthoquinones and amentoflavones (GUILHON-SIMPLICIO; PEREIRA, 2011), tannins and coumarins (CECÍLIO *et al*., 2012), and flavonoids and saponins (GONÇALVES *et al*., 2013).

The use of *B. verbascifolia* in traditional medicine is based on the extraction of leaves and barks. The main problem related to the species propagation is a low percentage of seed germination, due to the presence of a pyrene and a highly lignified seed coat, which acts as a mechanical barrier preventing both the input of water and/or oxygen and root protrusion (SILVA *et al*., 2012). The difficulties in propagating *B. verbascifolia* and its medicinal potential make this species a good candidate for biotechnological studies, which are rare for species in the Malpighiaceae family and virtually nonexistent for *B. verbascifolia*. So far, there are no reports on the establishment of callus cultures of *B. verbascifolia* and its ability to produce bioactive compounds of interest *in vitro*. In this study, we described the conditions for inducing callus cultures and analyzed the effect of growth regulators 2,4-D and BAP on callus induction and bioactive phenolic compounds production.

MATERIAL AND METHODS

The following reagents and materials were employed in the analysis: chlorogenic, *trans*-cinnamic, ferulic, caffeic and tannic acids, apigenin, rutin, (+)-catechin hydrate, luteolin, chrysin, quercetin, 2,4-dichlorophenoxyacetic acid, 6-benzilaminopurine, methanol, formic acid and agar were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The Folin-Dennis reagent was prepared according to Association of Official Analytical Chemists (1995). Water was treated in a Purite water purification system (Purite, Oxon, UK).

The leaf explants were obtained from *Byrsonima verbacifolia* (L.) DC. (Malpighiaceae) seedlings *in vitro* cultured. *Byrsonima verbascifolia* seeds were collected in the Cerrado region in Ijaci, Southern Minas Gerais State, Brazil (21º11'36.97" S; 44º55'59.07" W GRW and altitude 908 m). Fertile samples were collected and the vouchers were deposited in the Herbarium PAMG (PAMG 56309) at the Agricultural Research Company of Minas Gerais (EPAMIG).

For the callus induction, the leaf segments were placed on basal medium MS (MURASHIGE; SKOOG, 1962) with 30 g L^{-1} sucrose plus 2,4-D (4.52; 9.05 and 18.10 μM) or BAP (4.44; 8.88 and 17.75 μM) and their possible combinations, to induce callus formation and solidified with 7 g L^{-1} agar. The pH was adjusted to 5.8 ± 0.1 after adding plant growth regulators and medium was sterilized at 120 °C (1.37×10^5 Pa) for 20 min. The explants were transferred to a growth chamber and kept at 27 ± 2 °C under presence of light (16:8 h light/dark regime, with a light intensity of 40 μmol m^{-2} s^{-1}) and absence of light.

After 45 days of inoculation, the callus induction (%), color, consistency, fresh and dry weights of calli, total phenols, flavonoids and tannins contents were evaluated. The chromatographic profile of phenolic compounds was determined by Hight Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD). A completely randomized design in factorial arrangement 15x2 (fifteen concentrations of 2,4-D or/and BAP and two conditions of light, presence and absence) was performed. The controls were represented by the absence of growth regulators in the media. Each treatment was composed by 20 replicates.

The hydromethanolic extracts was prepared with approximately 200 mg of dried callus samples were extracted with 10 mL methanol:water (1:1) solution, according Castro *et al*. (2009). The extract was filtered and the final volume was completed to 10 mL with methanol: water (1:1). The extract was utilized for determination of total phenols, flavonoids and tannins contents.

Phenols were quantified with 100 µL of hydromethanolic extract, following the Association of Official Analytical Chemists procedure (1995). Total phenols content was calculated by a calibration curve with 100 mg L^{-1} tannic acid solution as standard. Determinations were performed in triplicate and the result was expressed in micrograms of tannic acid equivalents per milligram of dry matter (µg TAEq mg^{-1} DW).

Total flavonoids assay was performed according to Woisky and Salatino (1998) using 300 µL of hydromethanolic extract. The flavonoid content was calculated by a calibration curve with 100 µgmL^{-1} rutin in a methanol solution of 2% aluminum chloride as standard. Determinations were performed in triplicate and the result was expressed in microgram of rutin equivalents per milligram of dry matter (µg REq mg^{-1} DW).

Total tannins assay was performed with 5 mL of hydromethanolic extract. The extract was concentrated at 50 °C and resuspended with 500 µL of methanol: water (50%). After, 15 µL was used for determination by Radial Diffusion Method, described by Hagerman (1987). Determinations were performed in triplicate and the result was expressed in microgram of tannic acid equivalents per milligram of dry matter (µg TAEq mg^{-1} DW).

Chromatographic profiles of samples from the initial explant and callus produced by the treatments 6 (BAP 17.75 µM) in the presence and absence of light and 7 (4.52 µM 2,4-D + BAP 4.44 µM) were obtained by HPLC-DAD. Samples were analyzed by a modular system liquid chromatography Shimadzu Prominence HPLC (Shimadzu Corp., Kyoto, Japan) comprising two LC-20AD pumps, diode array detector SPD M20A and computer system operated by LC WorkStation software (LC Solution 1.25). The separation of compounds was performed with a reversed-phase column Gemini C18 (4.6 X 250 mm, 5 µm, Phenomenex®, Torrance, CA, USA) conditioned at 35 °C. The mobile phases comprised A: water:formic acid (99.9:0.1) and B: methanol:formic acid (99.9:0.1) at the proportion of 0% B (0–5 min.); 0–100% B (5–30 min.); 100% B (30–35 min.). A 20 µL injection volume and a flow rate of 1.0 mL min^{-1} were employed. Separations were monitored at three wavelengths to detect phenolic substances; 254 nm and 328 nm for phenolic acids and flavan-3-ols and 350 nm for flavones, flavonols and chalcones (SAKAKIBARA *et al*., 2003). The determination of phenolic compounds in the samples was performed by comparing retention times and UV spectrum of standards previously injected and by data from the literature (ABAD-GARCÍA *et al*., 2009; SAKAKIBARA *et al*., 2003).

Data were subjected to analysis of variance by F test at $p \leq 0.05$ significance level using the Variance Analysis System of Balanced Data SISVAR 5.1 Software (FERREIRA, 2011) Mean rates were further separated by Tukey's Test when differences were significant.

RESULTS AND DISCUSSION

Callus induction started nine days after inoculation in media containing 2,4-D and / or BAP in the presence and absence of light and callus formation was observed only in the presence of plant growth regulators. There was no interaction between presence and absence of light.

Maximal induction (100%) was found in all media supplemented with different concentrations of 2,4-D and BAP in the presence and absence of light (Tables 1 and 2), whereas the lowest induction (7%) occurred in media with 4.44 µM BAP in the presence of light (Table 1). These results demonstrate the need for exogenous supplementation of medium with plant growth regulators and the importance of joint use of auxin and cytokinin for better establishment of callus cultures of *B. verbascifolia*. According to Kakani and Peng (2011), the interplay between auxin and cytokinin is essential to the process of growth and development of plants. Auxins and cytokinins control cell division in undifferentiated cells; however, interaction efficiency depends on the species and plant tissue (COENEN; LOMAX, 1997).Callus color varied between red, yellow, and green shades. In the presence of light, callus were predominantly green with reddish parts and had compact consistency whereas yellow friable callus were found only in the presence of isolate 2,4-D (Table 1). In the absence of light, callus were yellow or yellow with reddish parts, and had compact consistency (Table 2). We obtained yellow friable callus by employing

Table 1 - Induction, fresh and dry weight, color and consistency of *Byrsonima verbascifolia*'s callus, in the presence of light after 45 days cultivation

Treatment	Growth regulators	Induction (%)	Fresh weight (g)	Dry weight (g)	Color	Consistency
Control	Absence of regulator	-	-	-	-	-
1	4.52 µM 2,4-D	53d	0.71 ± 0.09 b	0.07 ± 0.01 b	Yellow/Red	Friable
2	9.05 µM 2,4-D	87b	0.77 ± 0.08 b	0.04** c	Yellow/Red	Friable
3	18.10 µM 2,4-D	68c	0.70 ± 0.07 b	0.04** c	Yellow/Red	Friable
4	4.44 µM BAP	7f	0.47 ± 0.09 c	0.03** c	Green/Red	Compact
5	8.88 µM BAP	35e	0.48 ± 0.10 c	0.08 ± 0.01 b	Green/Red	Compact
6	17.75 µM BAP	94b	0.21 ± 0.05 d	0.04** c	Green/Red	Compact
7	4.52 µM 2,4-D + 4.44 µM BAP	100a	1.77 ± 0.09 a	0.17 ± 0.04 a	Green	Compact
8	4.52 µM 2,4-D + 8.88 µM BAP	100a	1.58 ± 0.12 a	0.15 ± 0.05 a	Green/Red	Compact
9	4.52 µM 2,4-D + 17.75 µM BAP	100a	0.69 ± 0.07 b	0.07 ± 0.01 b	Red	Compact
10	9.05 µM 2,4-D + 4.44 µM BAP	100a	1.55 ± 0.15 a	0.14 ± 0.05 a	Green/Red	Compact
11	9.05 µM 2,4-D + 8.88 µM BAP	100a	0.92 ± 0.20 b	0.07 ± 0.01 b	Green	Compact
12	9.05 µM 2,4-D + 17.75 µM BAP	100a	0.99 ± 0.21 b	0.09 ± 0.02 b	Green	Compact
13	18,10 µM 2,4-D + 4.44 µM BAP	100a	0.77 ± 0.10 b	0.08 ± 0.02 b	Green/Red	Compact
14	18.10 µM 2,4-D + 8.88 µM BAP	100a	1.02 ± 0.25 b	0.08 ± 0.01 b	Green/Red	Compact
15	18.10 µM 2,4-D +17.75 µM BAP	100a	0.28 ± 0.06 d	0.03** c	Green/Red	Compact
F		9.456*	5.227*	7.253*		

* Significant value at 5% level by F test; means in the column followed by the same letter are not significantly different at $p \leq 0.05$ by the Tukey test; **Standard deviation values for these data were omitted, since they are less than 0.009. Legend: - (no callus induction)

Table 2 - Induction, fresh and dry weight, color and consistency of *B. verbascifolia*'s callus, in the absence of light after 45 days cultivation

Treatment	Growth regulators	Induction (%)	Fresh weight (g)	Dry weight (g)	Color	Consistency
Control	Absence of regulator	-	-	-	-	-
1	4.52 µM 2,4-D	71 c	0.68 ± 0.16 b	0.06 ± 0.01 b	Yellow	Friable
2	9.05 µM 2,4-D	73 c	0.85 b ± 0.11	0.04** c	Yellow/Red	Friable
3	18.10 µM 2,4-D	82 b	0.34 ± 0.05 c	0.03** c	Yellow	Friable
4	4.44 µM BAP	53 d	0.15 ± 0.05 d	0.02** c	Yellow/Red	Compact
5	8.88 µM BAP	74 c	0.19 ± 0.04 d	0.03** c	Yellow/Red	Compact
6	17.75 µM BAP	59 d	0.32 ± 0.07 c	0.04** c	Yellow	Compact
7	4.52 µM 2,4-D + 4.44 µM BAP	100 a	1.66 ± 0.18 a	0.15 ± 0.05 a	Yellow	Friable
8	4.52 µM 2,4-D + 8.88 µM BAP	100 a	1.49 ± 0.15 a	0.14 ± 0.05 a	Yellow/Red	Compact
9	4.52 µM 2,4-D + 17.75 µM BAP	100 a	0.88 ± 0.10 b	0.06 ± 0.01 b	Yellow/Red	Compact
10	9.05 µM 2,4-D + 4.44 µM BAP	100 a	0.81 ± 0.09 b	0.04** c	Yellow/Red	Compact
11	9.05 µM 2,4-D + 8.88 µM BAP	100 a	1.58 ± 0.10 a	0.15 ± 0.05 a	Yellow	Compact
12	9.05 µM 2,4-D + 17.75 µM BAP	100 a	1.43 ± 0.10 a	0.13 ± 0.02 a	Yellow	Compact
13	18,10 µM 2,4-D + 4.44 µM BAP	100 a	0.84 ± 0.05 b	0.07 ± 0.01 b	Yellow	Compact
14	18.10 µM 2,4-D + 8.88 µM BAP	100 a	0.87 ± 0.09 b	0.05 ± 0.01 c	Yellow/Red	Compact
15	18.10 µM 2,4-D +17.75 µM BAP	100 a	0.34 ± 0.05 c	0.04** c	Yellow/Red	Compact
F		10.778*	6.534*	8.123*		

* Significant value at 5% level by F test; means in the column followed by the same letter are not significantly different at $p \leq 0.05$ by the Tukey test; ** Standard deviation values for these data were omitted, since they are less than 0.009. Legend: - (no callus induction)

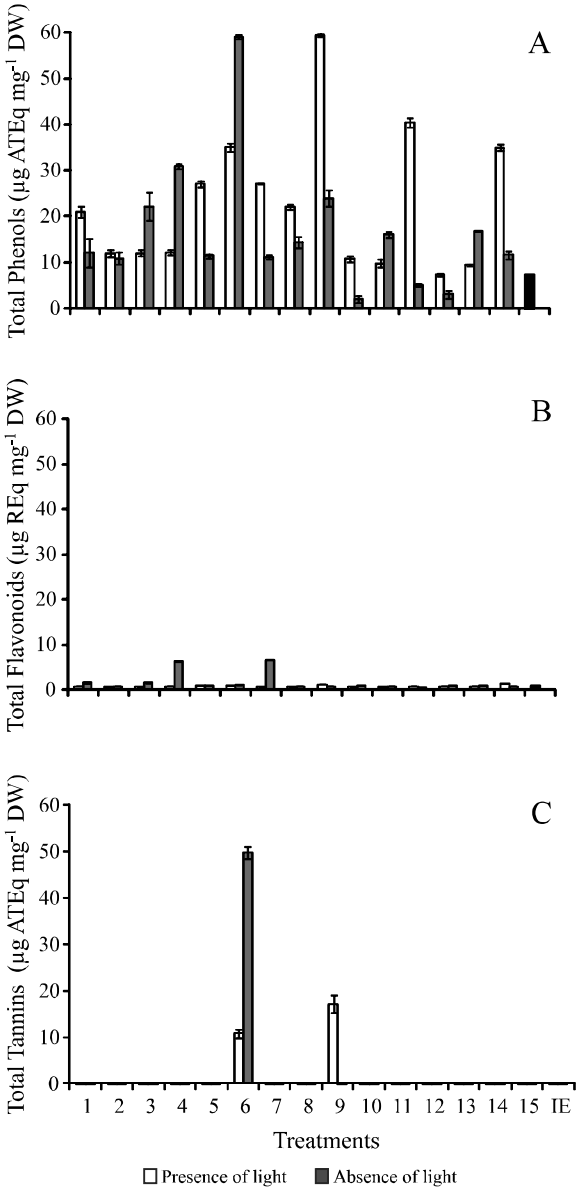
2,4-D alone or low concentrations of 2,4-D associated with BAP (Table 1). Red or reddish callus parts indicate the presence of anthocyanins (MEYER; PE´PIN; SMITH, 2002). Yellow friable callus are considered organogenic and, according to Arunyanart and Chaitrayagun (2005), translucent yellowish parts indicate the embryogenic potential of callus.

Overall, the combination of different concentrations of 2,4-D and BAP enabled a direct relationship between callus induction (%) and callus weight. Maximal induction and highest fresh and dry weight were found in callus induced by treatments 7 and 8 (in the presence and absence of light), treatment 10 (presence of light), treatments 11 and 12 (absence of light) (Tables 1 and 2). However, considering the formation of friable callus alone, only the medium supplemented with 4.52 µM 2,4-D + 4.44 µM BAP (Treatment 7) in the absence of light was effective in promoting the establishment of 100% friable callus with higher fresh and dry weights. Therefore, light was not a limiting factor for inducing callus in *B. verbascifolia*. However, light provided the formation of green callus indicating the presence of chloroplasts, whose synthesis is directly influenced by light (FUKUDA *et al*., 2008). Compact consistency of callus can be attributed mainly to the presence of isolate BAP and 2,4-D associated with BAP at higher concentrations.

In general, all callus produced significant amounts of phenols and flavonoids, regardless of the presence or absence of light, or type and concentration of regulators ($p<0.05$). Total phenols and flavonoids contents ranged from 10.1 µg TAEq mg^{-1} DW to 59.2 µg TAEq mg^{-1} DW, and from 0.78 µg REq mg^{-1} DW to 9.1 µg REq mg^{-1} DW, respectively, and were higher than those found in the initial explant (7.2 µg TAEq mg^{-1} DW and 0.98 µg REq mg^{-1} DW, respectively) (Figure 1A and 1B). Tannins were not detected in the initial explant; however, these compounds were produced in high amounts in callus induced by the treatment with 17.75 µM BAP (treatment 6) in the absence of light, and in smaller amounts in established callus in the presence of light, in medium with 17.75 µM BAP (treatment 6) and 4.52 µM 2,4-D + 17.75 µM BAP (treatment 9) (Figure 1C).

These results indicate that *in vitro* techniques both promote and increase the production of phenolic compounds in callus of *B. verbascifolia*, especially tannin, in intact plants. Palacio *et al*. (2012) reported that production *in vitro* of secondary compounds in medicinal plants is possible due to variation of culture conditions, including changes in types and concentrations of plant growth regulators. Callus induced in medium containing 17.75 µM BAP (treatment 6), in the absence of light, had the highest levels of total phenols and tannins (around 59.2 µg TAEq mg^{-1} DW and 51 µg TAEq mg^{-1} DW) (Figures 1A and 1C), respectively, with phenol levels approximately 8 times higher than those in the initial

Figure 1 - Total phenols (A), flavonoids (B) and tannins (C) contents in *B. verbascifolia*'s callus. The bar represents the mean±standard deviation of 3 observations. Legend: 1) 4.52 µM 2,4-D; 2) 9.05 µM 2,4-D; 3) 18.10 µM 2,4-D; 4) 4.44 µM BAP; 5) 8.88 µM BAP; 6) 17.75 µM BAP; 7) 4.52 µM 2,4-D + 4.44 µM BAP; 8) 4.52 µM 2,4-D + 8.88 µM BAP; 9) 4.52 µM 2,4-D + 17.75 µM BAP; 10) 9.05 µM 2,4-D + 4.44 µM BAP; 11) 9.05 µM 2,4-D + 8.88 µM BAP; 12) 9.05 µM 2,4-D + 17.75 µM BAP; 13) 18,10 µM 2,4-D + 4.44 µM BAP; 14) 18.10 µM 2,4-D + 8.88 µM BAP; 15) 18.10 µM 2,4-D +17.75 µM BAP; IE = initial explant



explant (7.2 µg TAEq mg^{-1} DW). Considerable total flavonoids contents (9.1 µg REq mg^{-1} DW) were found in callus induced in media with 4.44 µM BAP and 4.52 µM 2,4-D + 4.44 µM BAP, in the absence of light (treatments 4 and 7). These values were 9 times higher than those in the initial explant (0.98 µg REq mg^{-1} DW) (Figure 1B). According to Coenen and Lomax (1997), the accumulation of secondary compounds correlates negatively with cell growth, which was also found in this study. Generally, callus with higher concentrations of phenolic compounds showed lower fresh and dry weight and compact consistency (Tables 1 and 2 and Figure 1). The oxidative action of phenolic compounds is a limiting factor for growth of *in vitro* cultures (ARUNYANART; CHAITRAYAGUN, 2005).

Bioactive phenolic compounds produced in callus of *B. verbascifolia* were identified in hydromethanolic extracts of samples from the initial explant and callus from treatment 6 (17.75 µM BAP) in the presence and absence of light, and treatment 7 (4.52 µM 2,4-D + 4.44 µM BAP) in the absence of light by HPLC-DAD analysis (Figure 2). As shown in Table 3, the analysis indicated different derivatives of phenolic acids and catechins, by comparison with retention times and UV spectra of previously injected standard solutions and data found in literature (ABAD-GARCÍA *et al.*, 2009; MÄÄTTÄ; KAMAL-ELDIN; TÖRRÖNEN, 2003; SAKAKIBARA *et al.*, 2003).

Three phenolic substances were detected in the initial explant: one benzoic acid derivative and two *p*-coumaric acid derivatives (Figure 2A and Table 3). In hydromethanolic extracts of callus induced in treatment 6 (17.75 µM BAP) in the presence and absence of light, and in treatment 7 (4.52 µM 2,4-D + 4.44 µM BAP) in the absence of light we found benzoic acid, cinnamic acid, gallic acid, *p*-coumaric acid and *m*-coumaric

Figure 2 - Chromatograms obtained by HPLC-DAD analysis of hydromethanolic extracts by detection at 254 nm. A. Chromatographic profile of the Initial Explant - Peak 1 = benzoic acid derivative, Peaks 2-3 = p-coumaric acid derivative. B. Chromatographic profile of callus from treatment 6 (17.75 µM BAP) in the presence of light - Peaks 1-3 = benzoic acid derivatives, Peaks 4-6 = catechins, Peaks 7-8 = m-coumaric acid derivatives. C. Chromatographic profile of callus from treatment 6 (17.75 µM BAP) in the absence of light - Peaks 1-3 = benzoic acid derivatives, Peaks 4-7 = gallic acid derivatives, Peaks 8-12 = catechins, Peaks 13-15 = m-coumaric acid derivatives. D. Chromatographic profile of callus from treatment 7 (4.52 µM 2,4-D + 4.44 µM BAP) in the absence of light - Peaks 1-2 = cinnamic acid derivatives, Peak 3 = benzoic acid derivative, Peak 4 = gallic acid derivative, Peak 5 = benzoic acid derivative, Peaks 6-7 = gallic acid derivatives, Peaks 8-9 = benzoic acid derivatives, Peaks 10-11 = p-coumaric acid derivatives

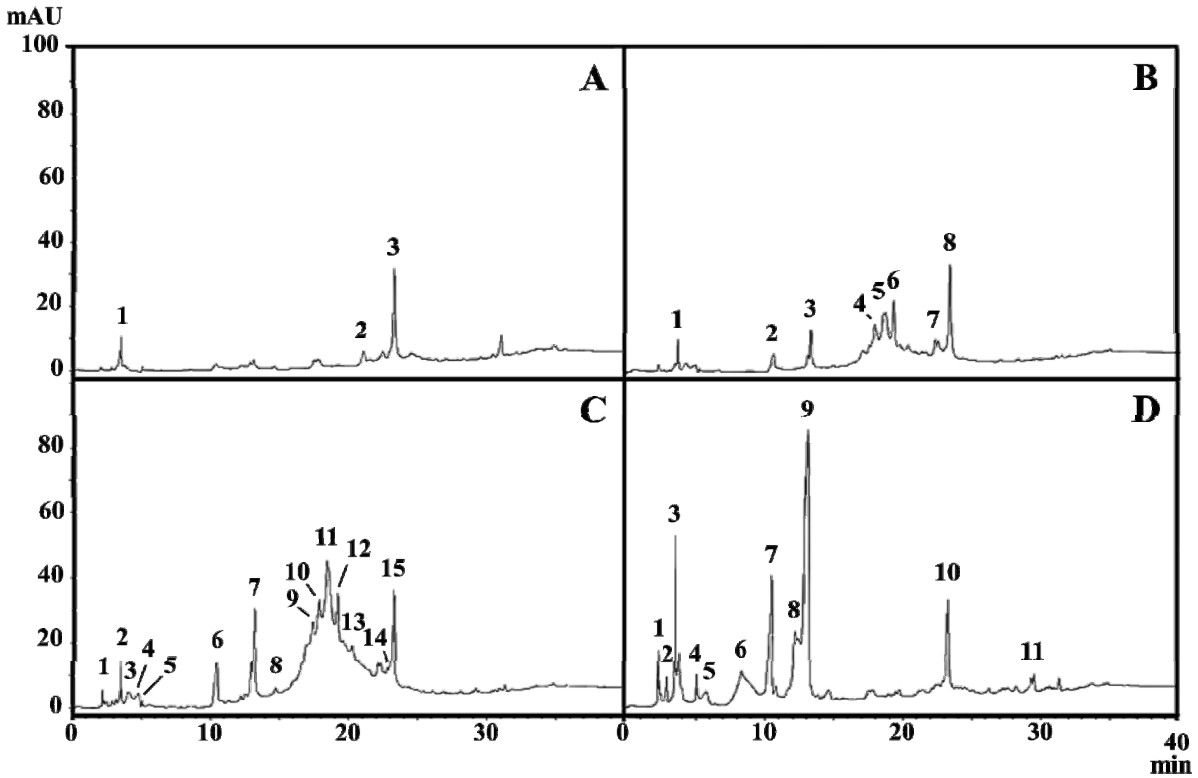


Table 3 - Phenolic compounds in hydromethanolic extracts in *Byrsonima verbascifolia*'s callus using HPLC-DAD

Sample	Peak number	Rt (min.)	λmax (nm)	Compound	Reference
Initial explant	1	3.491	264	Benzoic acid derivative	Määttä; Kamal-Eldin; Törrönen (2003)
	2	21.037	326	*p*-cumaric acid derivative	Sakakibara *et al.* (2003)
	3	23.264	326		
Treatment 6 (presence of light)	1	3.506	265	Benzoic acid derivative	Sakakibara *et al.* (2003)
	2	10.433	258		
	3	13.146	258		
	4	17.780	279	Cathechin	Reference substance; Sakakibara *et al.* (2003)
	5	18.393	279		
	6	19.125	279		
	7	22.149	270	*m*-cumaric acid derivative	Sakakibara *et al.* (2003)
	8	23.226	270		
Treatment 6 (absence of light)	1	2.163	274	Benzoic acid derivative	Sakakibara *et al.* (2003)
	2	3.509	272		
	3	4.096	259	Gallic acid derivative	Abad-Garcia *et al*. (2009)
	4	4.780	262		
	5	5.055	262		
	6	10.462	261		
	7	13.164	257	Cathechin	Reference substance; Sakakibara *et al.* (2003)
	8	14.721	278		
	9	17.351	279		
	10	17.791	279		
	11	18.384	279		
	12	19.122	279		
	13	22.127	273	*m*-cumaric acid derivative	Sakakibara *et al.* (2003)
	14	22.313	273		
	15	23.195	273		
Treatment 7 (absence of light)	1	2.225	277	Cinamic acid derivative	Sakakibara *et al.* (2003)
	2	2.878	278		
	3	3.477	269	Benzoic acid derivative	
	4	5.051	261	Gallic acid derivative	
	5	5.761	271	Benzoic acid derivative	
	6	8.303	260	Gallic acid derivative	Sakakibara *et al.* (2003)
	7	10.426	261		
	8	12.899	255	Benzoic acid derivative	
	9	13.092	258		Sakakibara *et al.* (2003)
	10	23.256	327	*p*-cumaric acid derivative	
	11	29.490	308		

Legend: Rt = retention time in minutes; $\lambda_{máx}$ (nm) = maximum wavelength

acid derivatives, and catechins (Figures 2B-D and Table 3). Gallic acid derivatives were detected only in callus induced in the absence of light, suggesting the influence of light during synthesis and/or accumulation of these substances in *B. verbascifolia*. The presence of gallic acid derivatives and catechins is also reported by Gonçalves *et al*. (2013) in hydromethanolic extracts of mature leaves of *B. verbascifolia* along with quercetin

and epicatechin. These authors demonstrated that the extracts did not cause mutagenicity, did not alter the reproductive function of female Swiss mice, and did not affect embryo development.

Derivatives of benzoic and cinnamic acids are therapeutically important and thus well described in literature. Hydroxybenzoic derivatives are found in small amounts in plants and are components of complex structures such as hydrolyzable tannins (gallotannins and ellagitannins). Hydroxycinnamic derivatives are more easily found and consist primarily of *p*-coumaric, caffeic and ferulic acids. Derivatives of *p*-coumaric acid are described as neuroprotective, antibacterial agents, potent inhibitors of human tyrosinase and hypopigmentation agents (AN; KOH; BOO, 2010).

Derivatives of gallic and benzoic acids have different biological activities. Studies on the bioactivity of gallic acid and its derivatives show activities such as antiviral and antitumor (KAUR *et al*., 2009) and anti-inflammatory (KIM *et al*., 2006). Benzoic acids are capable of inhibiting bacterial growth and are widely used to acidify food products (DONG; WANG, 2006).

CONCLUSIONS

The results demonstrate the potential of callus cultures of *B. verbascifolia* as a new source of bioactive phenolic compounds *in vitro*; moreover, good yield of callus in relation to the initial explant encourages further studies aimed at improving protocols for *in vitro* cultivation and production of phenolic compounds of interest in commercial scale.

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REFERENCES

ABAD-GARCÍA, B. *et al.* A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. **Journal of Chromatography A**, v. 1216, n. 28, p. 5398-5415, 2009.

ALMEIDA, S. P. de *et al*. **Cerrado**: espécies vegetais úteis. Planaltina: EMBRAPA-CPAC, 1998. 464 p.

AN, S. M.; KOH, J. S.; BOO, Y. C. p-coumaric acid not only inhibits human tyrosinase activity in vitro but also melanogenesis in cells exposed to UVB. **Phytotherapy Research**, v. 24, n. 8, p. 1175-1180, 2010.

ARUNYANART, S.; CHAITRAYAGUN, M. Induction of somatic embryogenesis in lotus (*Nelumbo nucifera* Geartn.). **Scientia Horticulturae**, v. 105, n. 3, p. 411-420, 2005.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. **Official methods of the association of official analytical chemists**. 11. ed. Washington: AOAC International, 1995. 1015 p.

CASTRO, A. H. F. *et al.* Calogênese e teores de fenóis e taninos totais em barbatimão [*Stryphnodendron adstringens* (Mart.) Coville]. **Ciência e Agrotecnologia**, v. 33, n. 2, p. 385-390, 2009.

CECÍLIO, A. B. *et al*. Screening of Brazilian medicinal plants for antiviral activity against rotavirus. **Journal of Ethnopharmacology**, v. 141, n. 3, p. 975-981, 2012.

CHERDSHEWASART, W.; SUBTANG, S.; DAGKAB, W. Major isoflavonoid contents of the phytoestrogen rich-herb *Pueraria mirifica* in comparison to *Pueraria lobata*. **Journal of Pharmaceutical and Biomedical Analysis,** v. 43, n. 2, p. 428-434, 2007.

COENEN, C.; LOMAX, T. L. Auxin-cytokinin interactions in higher plants: old problems and new tools. **Trends in Plant Science**, v. 2, n. 9, p. 351-356, 1997.

DONG, C.; WANG, W. Headspace solid-phase microextraction applied to the simultaneous determination of sorbic and benzoic acids in beverages. **Analytica Chimica Acta**, v. 562, n. 1, p. 23-29, 2006.

FERREIRA, D. F. Sisvar: a computer statistical analysis system. **Ciência e Agrotecnologia**, v. 35, n. 6, p. 1039-1042, 2011.

FUKUDA, N. *et al*. Directional blue light irradiation triggers epidermal cell elongation of abaxial side resulting in inhibition of leaf epinasty in geranium under red light condition. **Scientia Horticulturae**, v. 115, n. 2, p. 176-182, 2008.

GONÇALVES, C. A. *et al.* Gestational exposure to *Byrsonima verbascifolia*: teratogenicity, mutagenicity and immunomodulation evaluation in female Swiss mice. **Journal of Ethnopharmacology**, v. 150, n. 3, p. 843-850, 2013.

GUILHON-SIMPLICIO, F.; PEREIRA, M. de M. Aspectos químicos e farmacológicos de *Byrsonima* (Malpighiaceae). **Química Nova**, v. 34, n. 6, p. 1032-1041, 2011.

HAGERMAN, A. E. Radial diffusion method for determining tannin in plant extract. **Journal of Chemical Ecology**, v. 13, n. 3, p. 437-449, 1987.

KAKANI, A.; PENG, Z. ARR5 and ARR6 mediate tissue specific cross-talk between auxin and cytokinin in *Arabidopsis.* **American Journal of Plant Sciences**, v. 2, n. 4, p. 549-553, 2011.

KAUR, M. *et al.* Gallic acid, an active constituent of grape seed extract, exhibits anti-proliferative, pro-apoptotic and anti-tumorigenic effects against prostate carcinoma xenograft growth in nude mice. **Pharmaceutical Research**, v. 26, n. 9, p. 2133-2140, 2009.

KIM, S. H. *et al.* Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. **Toxicological Sciences**, v. 91, n. 1, p. 123-131, 2006.

LOREDO-CARRILLO, S. E. *et al.* Establishment of callus from *Pyrostegia venusta* (Ker Gawl.) Miers and effect of abiotic stress on flavonoids and sterols accumulation. **Journal of Plant Biochemistry and Biotechnology**, v. 22, n. 3, p. 312-318, 2013.

MÄÄTTÄ, K. R.; KAMAL-ELDIN, A.; TÖRRÖNEN, A. R. High-performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: ribes species. **Journal of Agricultural and Food Chemistry**, v. 51, n. 23, p. 6736-6744, 2003.

MANEECHAI, S. *et al.* Flavonoid and stilbenoid production in callus cultures of *Artocarpus lakoocha*. **Phytochemistry**, v. 81, p. 42-49, 2012.

MEYER, J. E.; PE´PIN, M. F.; SMITH, M. A. L. Anthocyanin production from *Vaccinium pahalae*: limitations of the physical microenvironment. **Journal of Biotechnology**, v. 93, n. 1, p. 45-57, 2002.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and biossays with tabacco tissue cultures. **Physiologia Plantarum**, v. 15, n. 3, p. 473-497, 1962.

OKSMAN-CALDENTEY, K.; INZÉ, D. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. **Trends in Plant Science**, v. 9, n. 9, p. 433-40, 2004.

PALACIO, L. *et al.* Phenolic compound production in relation to differentiation in cell and tissue cultures of *Larrea divaricata* (Cav.). **Plant Science**, v. 193-194, p. 1-7, 2012.

SAHIN, G.; VERMA, S. K.; GUREL, E. Calcium and magnesium elimination enhances accumulation of cardenolides in callus cultures of endemic *Digitalis* species of Turkey. **Plant Physiology and Biochemistry**, v. 73, p. 139-143, 2013.

SAKAKIBARA, H. *et al.* Simultaneous determination of all polyphenols in vegetables, fruits, and teas. **Journal of Agricultural and Food Chemistry**, v. 51, n. 3, p. 571-581, 2003.

SILVA, L. C. *et al.* Cell viability of *Byrsonima intermedia* A. Juss Calli. **Journal of Agricultural Science and Technology**, v. 2, n. 8, p. 713-720, 2012.

SZOPA, A.; EKIERT, H. Production of biologically active phenolic acids in *Aronia melanocarpa* (Michx.) Elliott *in vitro* cultures cultivated on different variants of the Murashige and Skoog medium. **Plant Growth Regulators**, v. 72, n. 1, p. 51-58, 2014.

WOISKY, R. G.; SALATINO, A. Analysis of propolis: some parameters and procedures for chemical quality control. **Journal of Apicultural Research**, v. 37, n. 2, p. 99-105, 1998.