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Effect of auxin and cytokinin on phenolic content of *Baccharis myriocephala* DC. (Asteraceae) produced *in vitro*

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A protocol for large-scale *in vitro* production of *Baccharis myriocephala* was developed. Total phenolic content was analyzed in plants cultured on MS + 1.0 mg.L^{-1} of indoleacetic acid (IAA), benzyladenine (BA) or kinetin (KIN). Nodal segments obtained from seeds germinated *in vitro* on Murashige and Skoog basal medium (MS) were subcultured on MS basal medium, hormone-free or with different growth regulators added: IAA, BA or KIN. The total phenolic content was determined using the Folin-Denis spectrophotometric method, and the content of phenolics of extracts was expressed in terms of gallic acid-equivalent (µg GA/mg dry weight). The best growth and development of the plants was induced by MS + 1.0 mg.L^{-1} IAA, which stimulated the production of 23 new nodal segments from a single phytomer after 60 days of *in vitro* culture. However, MS + 1.0 mg.L^{-1} IAA accumulated the lowest phenolic content, and only 18.46 µg/mg dry weight. A significant negative correlation was noted, since the phenolic content increased when the dry weight decreased. This culture system is capable of supplying good-quality raw material in adequate quantity to meet the growing demand for medicinal plants. However, the accumulation of polyphenols is lower in cultures with higher dry weight.

Key words: Carqueja, micropropagation, growth regulators, phenolics accumulation.

INTRODUCTION

Baccharis myriocephala DC. (Asteraceae) is a three winged branched shrub; in Brazil, this and other species of the genus are known as *carqueja* (Simões-Pires et al., 2005; Abad, 2007). The use of medicinal plants such as *carqueja* generates an economic value that sometimes leads to cultivation of the species (Castro et al., 1999a) to meet the demands of industry and traditional users (Gupta and Birdi, 2017; Park et al., 2017). In natural

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populations, unregulated extraction contributes to a decline in numbers, consequent loss of genetic variability, and even extinction (Martinelli and Moraes, 2013; Leitão et al., 2014).

The allogamy of *Baccharis* species is the source of considerable genetic variability (Castro et al., 1999a, b). This causes difficulties in seedling production, generates plants with non-standard agronomic and phytochemical attributes, and consequently reduces the potential of a plant as raw material for medicine preparation.

In vitro culture techniques such as micropropagation have been widely used as an alternative to produce good-quality raw materials from the phenotype of interest (Cardoso and Teixeira da Silva, 2013; Ozarowski and Thiem, 2013; Ahmad et al., 2015). The micropropagation technique enables a plant to be produced when there are difficulties in the natural mechanisms of reproduction, preservation of endangered species, and maintenance of plant germplasm collections, and is the only tissue-culture technique that has been effective for large-scale production of plants of economic interest (Cardoso and Teixeira da Silva, 2013; Chen et al., 2016). Phenolic compounds, widespread in the plant kingdom, have beneficial effects on human health, such as reduction of blood glucose levels (Oliveira et al., 2014), antioxidant effects (Pádua et al., 2010) and many others (Hossen et al., 2017; Hu et al., 2017). Biotic and abiotic factors produce changes in the phytochemical profile of plants and may increase the production of chemicals of interest (Sartor et al., 2013; Victório et al., 2015).

The potential use of *carqueja* as raw material for a plant drug motivated the authors to develop a protocol for *in vitro* propagation of *B. myriocephala,* in order to provide an optimized alternative for large-scale production. The effects of different growth regulators on the development of *in vitro* plants and on the production of total phenolics were investigated.

MATERIALS AND METHODS

Plant material

Achenes of *B*. *myriocephala* were collected in Santa Rita de Jacutinga, Minas Gerais State, Brazil, and were identified by Gustavo Heiden from the Rio de Janeiro Botanical Garden Research Institute. A voucher specimen of this plant material was deposited in the Herbarium (RB 439.238) at the Institute.

Plant tissue culture

Achenes were pre treated by immersion in 2% commercial bleach solution for 20 min, and then rinsed thoroughly in running tap water inside the laminar air-flow chamber, the achenes' surfaces were sterilized in 70% ethanol for 20 min, followed by 15 min in a 20% commercial bleach solution containing 2 to 3 drops of Tween 20, and rinsed three times with sterile distilled water. The disinfected achenes were inoculated on MS (Murashige and Skoog, 1962) solid medium. Nodal segments were excised from plants emerged from achenes held in *vitro* for 10 days. Nodal segments were grown in sterile flasks with MS basal medium, hormone-free or supplemented with kinetin (KIN; 0.01, 0.1, 0.5 and 1.0 $mg.L^{-1}$), benzyladenine (BA; 0.01, 0.1, 0.5 and 1.0 $mg.L^{-1}$) or indoleacetic acid (IAA; 0.01, 0.1, 0.5 and 1.0 $mg.L^{-1}$). The effects of growth regulators on the organogenesis of shoots and roots were recorded after 60 days of culture.

In all media, the pH was adjusted to 5.8 ± 0.1 before addition of 7.5 $g.L^{-1}$ agar-agar. *In vitro* cultures were maintained in a growth room at 25 ± 2°C under a 16/8 h light/dark photoperiod (Silvania daylight fluorescent lamps, 23 mmol $m^{-2}.s^{-1}$).

Acclimatization

Seedlings cultured for 60 days on hormone-free MS basal medium were removed and their roots rinsed in running tap water to completely remove the culture medium, and then kept in bottles with water for 24 h. Then, the seedlings were transferred to plastic containers, 6 cm in diameter, containing soil conditioner enriched with humus. These plants were initially kept in an aquarium covered with transparent plastic, and generously irrigated to create a high-humidity environment. The plastic cover was removed gradually until day 30 when it was completely removed to transfer the plants to outdoor conditions.

Determination of total phenolic content

The total phenolic content was determined using the spectrophotometric method of Folin-Denis reagent, as described by Waterman and Mole (1994). Samples with 200 mg of shoots from the culture medium containing 1.0 $mg.L^{-1}$ IAA, BA or KIN were lyophilized and milled, and then extracted four times with 5 mL of acetone : water (7:3, v/v) solution. The entire extract volume was combined with distilled water to make it up to 25 mL. A volume of 0.5 mL of Folin-Denis reagent was added to 0.5 mL plant extract plus 3 mL water. One milliliter of Na_2CO_3 saturated solution was added after 1 h of reaction, and then the absorbance of the extract was read in a spectrophotometer at 760 nm. The reference cuvette contained distilled water only. Samples were prepared in triplicate for each analysis, and the mean absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid to generate a calibration curve. Based on the measured absorbance, the concentration of phenolics was read (µg/mL) from the calibration curve, then the content of phenolics in the extracts was expressed in terms of gallic acid-equivalent (µg GA/mg dry weight).

Statistical analysis

The layout was totally randomized in all experiments. Tests with growth regulators were done in duplicate (n = 30 per treatment), and the rooting percentage was analyzed by the difference in the percentage (p_1 and p_2), at 5% significance. Total phenolic contents were analyzed in triplicate. The data were analyzed statistically with one-way ANOVA, using GraphPad Prism version 7.02. The significance of differences among mean values was assessed with Tukey's multiple comparison test at $p \leq 0.05$ or $p \leq 0.001$. The Pearson correlation coefficient was calculated using Microsoft Excel 2010.

RESULTS AND DISCUSSION

Effect of growth regulators on culture

Some easily measured characteristics were observed to establish the best combination of the basic salt medium

Table 1. Effects of plant growth regulators on *B. myriocephala* after 60 days of *in vitro* culture.

Culture medium ($mg.L^{-1}$) *	Number of buds x ± SD**	Number of nodal segments per bud	Multiplication rate	Plant elongation (cm) x ± SD**	Rooting (%)
MS	1.4±0.6^{f}	7.0^{a}	9.9^{b}	2.9±2.4^{d}	82.0^{c}
0.01 IAA	1.6±0.9^{f}	6.0^{b}	4.5^{c}	4.8±2.6^{b}	82.6^{c}
0.1 IAA	1.6±0.6^{f}	5.7^{b}	8.4^{b}	3.6±1.5^{c}	100.0^{a}
0.5 IAA	3.9±1.9^{d}	5.3^{c}	18.7^{a}	6.0±1.8^{a}	100.0^{a}
1.0 IAA	6.7±3.4^{b}	3.7^{d}	23.0^{a}	6.0±2.4^{a}	96.7^{b}
0.01 BA	1.6±0.7^{f}	5.9^{b}	8.6^{b}	2.0±1.0^{e}	66.0^{d}
0.1 BA	1.2±0.4^{f}	1.8^{e}	2.1^{d}	1.0±0.3^{f}	0
0.5 BA	***	***	***	***	0
1.0 BA	***	***	***	***	0
0.01 KIN	1.4±0.7^{f}	4.8^{c}	6.1^{c}	2.3±1.6^{e}	34.5^{e}
0.1 KIN	2.3±1.4^{e}	5.0^{c}	9.3^{b}	1.7±0.7^{e}	18.2^{f}
0.5 KIN	4.8±2.5^{c}	3.6^{d}	15.0^{a}	1.3±0.4^{f}	6.0^{g}
1.0 KIN	7.2±3.0^{a}	1.1^{e}	6.8^{c}	1.6±0.4^{e}	0

*Murashige and Skoog (1962) basal medium, hormone-free or supplemented with kinetin (KIN); benzyladenine (BA) or indoleacetic acid (IAA). **Mean (x) ± standard deviation (SD), n = 2 × 30 per treatment. Values in the same column followed by the same letter do not differ statistically at P ≤ 0.05. *** High proliferation of non-elongated shoots.

with the different growth regulators added to the medium, including the number of buds, nodal segments per bud, shoot length, and rooting produced in each culture medium. The ideal culture medium increased the multiplication rate in addition to producing elongated and rooted plants, to avoid the need for additional steps in the production of *in vitro* plants.

While increasing the IAA concentration favored organogenesis of shoots and rooting, making the culture more efficient, BA and KIN had the opposite effect because the increased concentration of these hormones reduced the multiplication rate and rooting or produced very short shoots, making it more difficult to isolate nodal segments for subculturing. The culture medium with 1.0 $mg.L^{-1}$ IAA allowed the production of 23 new nodal segments from a single phytomer inoculated. This result is statistically equivalent to that from 0.5 $mg.L^{-1}$ IAA which produced about 18 new nodal segments from a single phytomer inoculated. Both 0.5 and 1.0 $mg.L^{-1}$ IAA provided satisfactory rooting and elongation of plants (Figure 1 and Table 1).

Cytokinins, BA and KIN have been used in the induction of *in vitro* shoots of *Eclipta alba*, a member of Asteraceae of recognized medical value. Cytokinins promote shooting but often unsatisfactory shoot elongation or rooting, which for *E. alba* necessitated an additional step in the tissue culture (Dhaka and Kothari, 2005; Baskaran and Jayabalan, 2005; Ray and Bhattacharya, 2008).

The composition of the basic saline growth medium also affected the regeneration of new shoots in *E. alba* (Baskaran and Jayabalan, 2005) and *Baccharis tridentata* (Kajiki and Shepherd, 2006), where the MS medium gave the best results for shoot regeneration in both plants. Because many plants develop satisfactorily in MS medium, this was chosen for the *B*. *myriocephala in vitro* culture (Figure 1).

The results obtained in the tissue culture of *B. myriocephala* concord with the effects of auxins on rooting in *E. alba* (Dhaka and Kothari, 2005). Shoot elongation and multiplication combined with good rooting saves time and culture medium, by shortening the micropropagation process.

Acclimatization

A complete micropropagation protocol must include a well-established acclimatization process. Therefore, the transfer to outdoor conditions is still the greatest problem for the micropropagation of many plant species (Chandra et al., 2010). Because *B. myriocephala* cultured in hormone-free MS basal medium is highly sensitive to dehydration, it was necessary to provide a high-humidity environment for the first stage of acclimatization. With this strategy, the plants were well adapted to their new environment, with 100% survival. The plants did not show any variation in morphology. Previous rooting was not necessary for *B. myriocephala,* making the protocol more efficient and lower-cost. A prior rooting step was required for *Stevia rebaudiana* (Singh and Dwivedi, 2014; Ramírez-Mosqueda et al., 2016), *E. alba* (Singh et al., 2012) and *Gerbera jamesonii* (Cardoso and Teixeira da Silva, 2013).

Total phenolic content

Environmental factors lead to variations in the phenolic

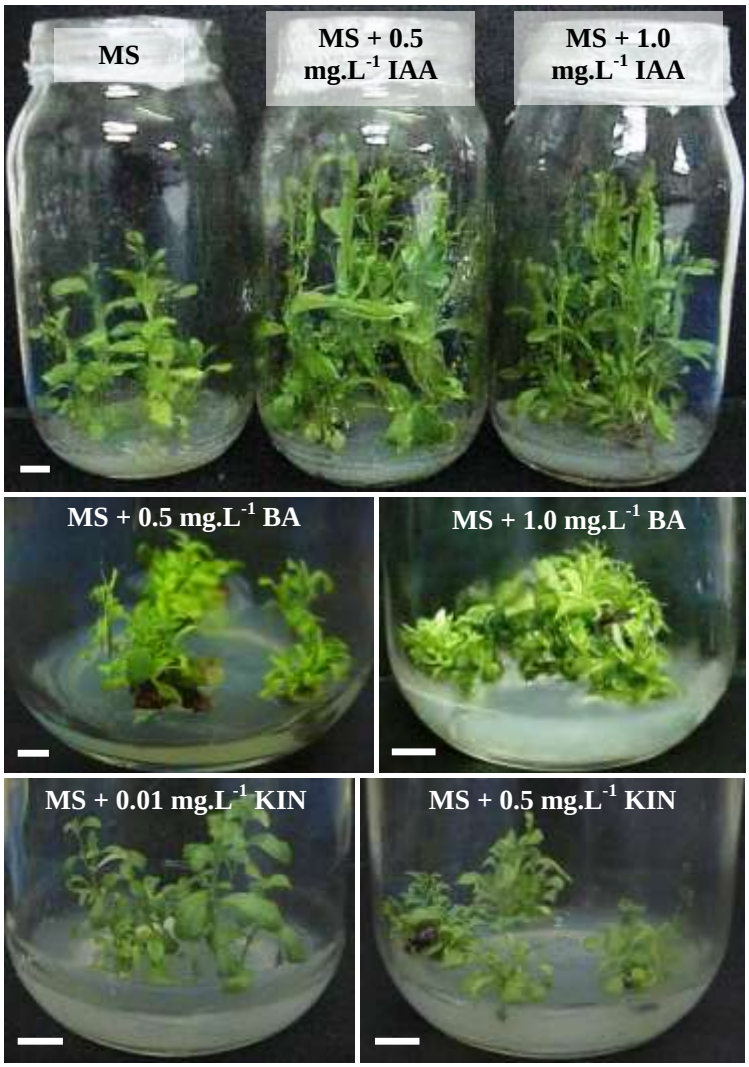

Figure 1. Effects of growth regulators on *B. myriocephala* after 60 days of culture. MS, Murashige and Skoog basal medium; IAA, Indoleacetic acid; BA, benzyladenine; KIN, kinetin. Bar = 1.0 cm.

content. The carbon fixed by the plant is managed between the primary and secondary metabolisms, and therefore in situations or seasons of lower growth of the plant body, the production of phenolics is higher (Sartor et al., 2013).

The environmental conditions of the *in vitro* culture are controlled, stable and predictable, and it is possible to relate the amount of polyphenol produced to the effect of the culture-medium composition on the development of the plant. The polyphenol versus biomass accumulation profile presented corroborates the observation in other studies, that plants with smaller growth and development accumulate more polyphenols (Grzegorczyk-Karolak et al., 2015; Ahmad et al., 2016; Kousalya and Bai, 2016). Nevertheless, the polyphenol accumulation can be influenced by the plant growth regulator utilized, regardless of biomass accumulation, or the two may show a positive relationship (Szopa et al., 2013; Kousalya and Bai, 2016).

Thus, the culture medium MS + 1.0 mg.L^{-1} IAA induced the best growth and development of the plants but led to the lowest accumulation of polyphenols; only 18.46 µg/mg dry weight (Table 2). The hormone-free MS and the MS + 1.0 mg.L^{-1} BA culture media produced similar amounts of polyphenols (Table 2), and these plants grew reasonably well, although plants cultured on the BA-containing medium showed high shoot proliferation, so that *in vitro* development parameters could not be analyzed (Table 1). Budding with low growth and a lower rate of multiplication were observed in the medium with MS + 1.0 mg.L^{-1} KIN, but the plants cultured on this medium had the highest polyphenol content; 27.16 µg/mg dry weight (Table 2). The Pearson correlation coefficient between the variables: phenolic accumulation (x) and dry

Table 2. Total phenolics content of *Baccharis myriocephala* cultured on MS basal medium, hormone-free or supplemented with 1 mg L IAA, BA or KIN (mean ± SD).

Culture medium (mg.L^{-1})*	Total phenolics (µg/mg dry weight) **
MS	23.81 ± 0.029^{b}
1.0 IAA	18.46 ± 0.026^{d}
1.0 BA	23.14 ± 0.036^{c}
1.0 KIN	27.16 ± 0.105^{a}

*Murashige and Skoog (1962) basal medium, hormone-free or supplemented with with kinetin (KIN); benzyladenine (BA) or indoleacetic acid (IAA). ** Different letters in a column indicate a significant difference (P ≤ 0.001, Tukey's multiple comparisons test).

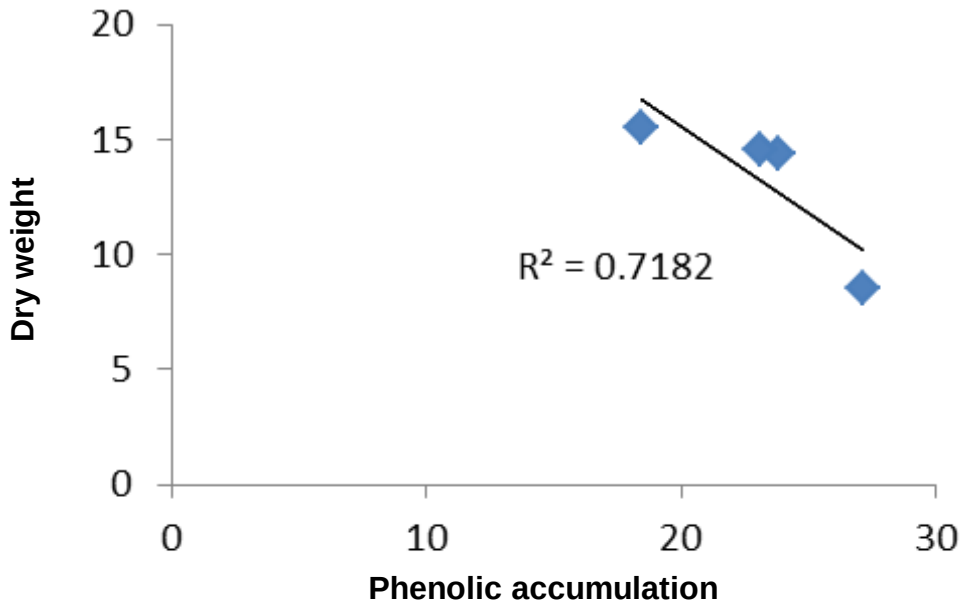

Figure 2. Correlation between phenolic accumulation and dry weight for plants cultured on MS basal medium, hormone-free, MS + 1 mg.L^{-1} IAA, MS + 1 mg.L^{-1} BA or MS + 1 mg.L^{-1} KIN, at 60 days of culture.

weight (y) was r = - 0.847447, showing a strong negative correlation between variables, since the phenolics accumulate when the dry weight decreases. In addition, 71% of the dry weight variation was due to variation in the accumulation of polyphenols (r^2 = 0.7182) (Figure 2).

Conclusion

The present results demonstrated that the MS medium is suitable for *in vitro* culture of *B. myriocephala.* Supplementation of MS medium with 1.0 mg.L^{-1} IAA maximized the multiplication rate to 23 new nodal segments from a single phytomer inoculated. In addition, IAA provided satisfactory rooting and elongation of plants, making the micropropagation process faster and more cost-effective. The success of acclimatization depends on an environment with initially high humidity that is gradually lost. Plants cultured on MS + 1.0 mg.L^{-1} KIN accumulated the highest content of polyphenols.

The efficient *in vitro* production system developed in this study provided sterile and consistent tissues for large-scale production of *B. myriocephala*, or for investigation of phytochemicals and germplasm conservation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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