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MICROPROPAGATION OF *Buddleja cordata* AND THE CONTENT OF VERBASCOSIDE AND TOTAL PHENOLS WITH ANTIOXIDANT ACTIVITY OF THE REGENERATED PLANTLETS

MICROPROPAGACIÓN DE *Buddleja cordata* Y EL CONTENIDO DE VERBASCÓSIDO Y FENOLES TOTALES CON ACTIVIDAD ANTIOXIDANTE DE LAS PLÁNTULAS REGENERADAS

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Abstract

Buddleja cordata is a medicinal plant distributed in Mexican territory that is characterized for producing phenolic compounds possessing antioxidant activity. It was evaluated the type of morphogenetic responses induced by plant growth regulators. Furthermore, the content of verbascoside and total phenols was determined, as well as the antioxidant activity in regenerated plantlets. The greatest shoot proliferation (29.2 shoots per explant) was achieved in the stem-nodes that were grown in a half-strength Murashige and Skoog medium containing 4.44 μM N^6-benzyladenine. The greatest shoot heights of 4.3 and 4.7 cm (statistically not different) were obtained with gibberellic acid at 4.34 and 8.67 μM, respectively. The highest percentage of rooting (89.4%) occurred with 2.45-μM indole-3-butyric acid with 20.3 roots per shoot and an average root length of 4.4 cm. Of the rooted shoots, 91.7% were able to survive after 30 days of acclimatization. The verbascoside (1.0 mg g^{-1}) and total phenols (24.8 mg of gallic acid equivalents g^{-1}) content was related to antioxidant activity of the regenerated plantlets. The micropropagation of *B. cordata* might represent an alternative about massive production and depict the basis of the establishment of commercial crops and genetic studies.

Keywords: *Buddleja cordata*, *in vitro* propagation, plant growth regulators, secondary metabolites, radical scavenging.

Resumen

Buddleja cordata es una planta medicinal distribuida en territorio Mexicano caracterizada por producir compuestos fenólicos poseedores de actividad antioxidante. Se evaluó el tipo de respuesta morfogenética inducida en *B. cordata* por reguladores de crecimiento vegetal y se determinó el contenido de verbascósido y fenoles totales así como la actividad antioxidante de las plántulas regeneradas. La mayor proliferación de brotes (29.2 brotes por explante) se obtuvo con nodos de tallo crecidos en medio de cultivo Murashige y Skoog (50%) adicionado con N^6-benciladenina a 4.44 μM. La mayor longitud de los brotes de 4.3 y 4.7 cm se obtuvó con ácido giberélico a 4.34 y 8.67 μM, respectivamente. El máximo porcentaje de rizogénesis (89.4%) se encontró con ácido indol-3-butírico a 2.45μM con una producción de 20.3 raíces por brote y 4.4 cm de longitud. El 91.7% de los brotes enraizados sobrevivieron después de 30 días de aclimatización. El contenido de verbascósido (1.0 mg g^{-1}) y fenoles totales (24.8 mg equivalentes de ácido gálico g^{-1}) se relacionó con la actividad antioxidante de las plántulas regeneradas. La micropropagación de *B. cordata* podría ser una alternativa de propagación masiva y constituir la base del establecimiento de cultivos comerciales y estudios genéticos.

Palabras clave: *Buddleja cordata*, propagación *in vitro*, reguladores de crecimiento vegetal, metabolitos secundarios, secuestro de radicales.

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1 Introduction

Buddleja cordata is a species of dioecious shrub or tree that is distributed across a variety of habitats in the Mexican territory (Aguilar-Rodríguez *et al*., 2006). This species is highly important due to its diversity of uses that include ornamentation, foraging, ecological restoration, as a pesticide and medicinal purposes (Mendoza-Hernández 2003; Ramos-Palacios *et al*., 2012; Rodríguez-Zaragoza *et al*., 1999). The woody parts of this plant have been used for decorative purposes, as fodder and to produce high-quality paper (Romero *et al*., 2003). Phytochemical studies have highlighted the use of this plant in folk medicine to counteract several diseases that are primarily related to inflammation and microbial infection (Martínez 1989). Some secondary metabolites type phenolic compounds have been attributed to medicinal properties such as the flavonoid linarin, hydroxycinnamic acids and the phenyletanoid verbascoside (Rodríguez-Zaragoza *et al*., 1999). Phenolic compounds are a proven source of significant health benefits due to their antioxidant activity which reduce the level of damage caused to biomolecules by oxygen free radicals, hence it has played a crucial role in the development of cures for a large number of diseases (Kennedy and Wightman 2011; Molavi and Mehta 2004; Soobrattee *et al*., 2005; Valko *et al*., 2006). In the last decades, verbascoside has attracted great interest due to its clinical potential. It has exhibited diverse biological activities (Alipieva *et al*., 2014) such as antiviral (Bermejo *et al*., 2002; Kernan *et al*., 1998), photoprotective (Avila *et al*., 2005), *in vitro* DNA protecting (Zhao *et al*., 2005), anti-inflammatory (Korkina *et al*., 2007; Paola *et al*., 2011), anti-tumor (Zhang *et al*., 2002), anti-fungal (Furani *et al*., 2012), anti-obesity (Wu *et al*., 2014), protection against Alzheimer's disease (Bai *et al*., 2013; Kurisi *et al*., 2013), chemopreventive against skin cancer (Kostyuk *et al*., 2013), ocular tissue protective (Mosca *et al*., 2014), intracellular radical scavenging (Mazzon *et al*., 2009) and antioxidant (Si *et al*., 2013).

Plant tissue culture is a biotechnological tool that has been shown to be an alternative and feasible source of important bioactive secondary metabolites. In this regard, the cell suspension cultures from *B. cordata* produced phenolic secondary metabolites that have been credited with the therapeutic properties of this species. In fact, the content of verbascoside in cell suspension cultures was significantly higher than that in wild plants (Estrada-Zúñiga *et al*., 2009). Another important plant tissue culture technique that is related to secondary metabolites is micropropagation, also known as *in vitro* propagation. It can be used to propagate species that possess desirable attributes (Mustafa 2012). In many medicinally important plant species, the secondary metabolite contents of plantlets obtained through micropropagation have exhibited less variation than that exhibited by cultivated or wild counterparts (Sagare *et al*., 2001). Furthermore, this technique can represent the first step toward the optimization of genetic transformation procedures (Oksman-Caldentery and Inze 2004). However, to our knowledge, the micropropagation of *B. cordata* has not been reported. In Mexico, natural populations of *B. cordata* have decreased due to its use and other anthropogenic activities (personal communications). Moreover, the natural regeneration of *B. cordata* is limited, and it is difficult to propagate this plant from seeds (Ramos-Palacios *et al*., 2012). The micropropagation of this species might be an alternative for multiplying its numbers without undermining natural populations and even for establishing commercial crops as a source of bioactive compounds. The aim of this study was to develop a micropropagation protocol from *B. cordata* and evaluate the content of verbascoside and total phenols, as well as the antioxidant activity of propagated plantlets.

2 Material and methods

2.1 Plant material

Five-year-old *B. cordata* plants used by Estrada-Zúñiga *et al*., (2009), previously identified and registered with the number 61170 at Herbario Metropolitano Ramón Riba y Nava Esparza, Universidad Autónoma Metropolitana, Iztapalapa Campus (UAM-I), were provided by the UAM-I. These plants were maintained in a greenhouse and fertilized once a week (Triple 17®, Royal Garden, Guadalajara, Mexico) to obtain healthy explants. The stem-node explants (0.5-1-cm length) of the five-year-old plants were removed from the apical parts of the plants, and a disinfestation procedure was conducted in order to obtain aseptic explants to be used in subsequent experiments of micropropagation. This procedure consisted of sequential immersions with low-level continuous shaking in 5% (w/v) soap (Roma® biodegradable detergent, La Corona, Mexico City, Mexico) for 15 min; 1% (v/v) fungicide (Bravo®

720, Syngenta, Mexico City, Mexico) for 30 min; immersion in a mixture of ampicillin (100 mg L^{-1}), tetracycline (10 mg L^{-1}) and cefotaxime (10 mg L^{-1}, Sigma-Aldrich Co LLC, MO, USA) for 30 min; 70% (v/v) ethanol for 30 s and 0.9% (v/v) sodium hypochlorite with added Tween-20 (three drops per 100 mL of solution) for 15 min. The stem-node explants were rinsed four times with sterile distilled water and placed in a sterile solution consisting of 150 mg L^{-1} ascorbic acid for 20 min to control the damage caused by mechanical injuries because ascorbic acid is a natural antioxidant that occurs in plants. On the other hand, the leaves of 6-month-old wild plants, 6-month-old micropropagated plantlets and five-year-old plants (source of explants) were used to carry out the phytochemical studies. The wild plants were collected in the vicinity of the UAM-I because the length of their leaves and stem were similar to that of micropropagated plantlets. All the leaves used for phytochemical analysis were collected on july 5th, 2012^{th} in the morning. The wild plants were grown under uncontrolled environmental conditions, while the rest of plants were grown in the greenhouse at 25±5°C.

2.2 Culture medium and incubation conditions

Half-strength Murashige and Skoog (MS, Murashige and Skoog 1962) medium with 3% (w/v) sucrose was used as the basal medium for all treatments. The plant growth regulators N^6-benzyladenine (BA), gibberellic acid (GA_3) or indole-3-butyric acid (IBA) were added at different concentration to achieve micropropagation. The pH value of the medium was adjusted to 5.8, gelled with 0.2% (w/v) Phytagel and sterilized by autoclaving at 121°C for 18 min. The culture medium (15 mL) was placed in culture tubes (25 x 150 mm). All *in vitro* cultures and rooted shoots were acclimatized and incubated at 25±2°C under warm-white fluorescent light (50 μmol m^{-2} s^{-1}) with 16-h (light)/8-h (dark) photoperiod for 30 days of culture.

2.3 Proliferation and elongation of shoots

The micropropagation of *B. cordata* was performed in four successive stages: 1) proliferation, 2) elongation, 3) rooting and 4) acclimatization. Aseptic stem-node explants were vertically placed in culture tubes containing medium with 0.00, 0.44, 2.22, 4.44 or 13.32 μM BA to achieve proliferation. The shoot formation frequencies and numbers of induced shoots per explant were measured after 30 days of culture. All explants that received the best treatment for inducing shoots (4.44 μM BA) were subcultured in fresh induction medium to increase the numbers of shoots. The shoot proliferation rate was assessed at 30 days of culture, and the resulting shoots were tested to achieve elongation. The shoots were transferred to a medium containing 0.00, 4.34 or 8.67 μM GA_3. The shoot lengths and the frequencies of shoots that were elongation were determined after 30 days. All frequency values are expressed as percentages of the explants that exhibited suitable developmental responses compared with the total of the tested explants.

2.4 Shoot rooting and acclimatization

The elongated shoots were individually separated and treated with 0.00 or 2.45 μM IBA for rooting. After 30 days of culture, the frequencies of root formation, the numbers of induced roots per shoot and the corresponding root lengths were measured. Afterwards, for acclimatization, the rooted shoots were transferred to pots containing a sterile mixture of peat moss and agrolite (Agrolita Peat Moss®, Agrolita Perlita®, respectively, Agrolita de México, México, in a 1:1 ratio). The plantlets were covered with clear bags, and the cover was gradually perforated until it was completely removed after 30 days of incubation. The substrates of the pots were irrigated twice per week with 5 mL of a 1% (v/v) antifungal solution (Bravo® 720). The frequency of rooted shoot survival was measured after 30 days. All frequency values are expressed as percentages of the explants that exhibited suitable developmental responses compared with the total of the tested explants. The surviving plantlets were transferred to a greenhouse at UAM-I, maintained for 6 months with weekly fertilization (Triple 17®) and used for the phytochemical studies.

2.5 Extraction of phenolic compounds

The leaves of the micropropagated plantlets (6-months-old), the greenhouse plants (the source of the stem-node explants), and the wild plants (approximately 6-months-old) were lyophilized, ground, weighed (500 mg), extracted by boiling in MeOH (20 mL) over 1 h and filtered under vacuum. Quercetin (Sigma-Aldrich Co LLC, MO, USA) was used as an internal standard in order to know the effect of the extraction procedure on the content of

phenolic compounds that were extracted. So, 100 μg mL^{-1} was added before starting the extraction procedure; a control consisting of just quercetin was simultaneously performed. The resulting extracts were used to determine the total phenol and verbascoside content and antioxidant activity. Moreover, if the resulting values of that analysis were lower or higher to the limit values established by the calibration curve itself, the extracts would be diluted in MeOH (10^{-1} to 10^{-2}) or concentrated (1 to 10 mL) under reduced pressure in a rotator evaporator (Büchi Labortechnik AG, Switzerland) .

2.6 *Determination of total phenol and antioxidant activity*

The determination of the total phenol content (TPC) consisted in mixing 1 mL of diluted extract with 1 mL of Folín-Ciocalteu reagent (diluted in water in ratio 1:10). The mixture was incubated during 1 min at room temperature. Then, 0.8 mL of 7.5% (w/v) sodium carbonate was added, mixed, incubated during 1 h at room temperature and used to record the absorbance at 765 nm. Gallic acid was used as reference to build the calibration curve (1-25 mg mL^{-1}; r^2 = 0.9899). The results are expressed as milligrams of gallic acid equivalents (GAE) per gram of biomass extracted (mg GAE g^{-1}). The antioxidant capabilities were determined based on the abilities of the extracts to scavenge DPPH$^+$ or ABTS$^+$ radicals. The DPPH assay consisted of mixing 50 μL extract with 950 μL 0.1 mM DPPH$^+$ (Sigma-Aldrich Co LLC, MO, USA). After 15 min in darkness and incubation at a room temperature of 25±2°C, the absorbance was recorded at 515 nm. The ABTS assay consisted of radical cation generation (ABTS$^+$) via a reaction of 7 mM ABTS (Sigma-Aldrich Co LLC, MO, USA) with 2.45 mM potassium persulfate (Sigma-Aldrich Co LLC, MO, USA) for 16 h in darkness and at a room temperature of 25±2°C. The solution thus obtained was diluted with MeOH to achieve an absorbance value of 0.7±0.05 at a wavelength of 734 nm. Nine hundred fifty milliliters of diluted solution was mixed with 50 μL of extract and incubated in darkness at a room temperature of 25±2°C for 15 min. The absorbance was recorded at 734 nm. In both assays, blanks were simultaneously prepared using MeOH instead of the extract. The results are expressed as millimoles of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Sigma-Aldrich Co LLC, MO, USA) equivalent antioxidant activity (TEAC) per gram of extracted biomass (mmoles TEAC g^{-1}); thus, a calibration curve (100, 200, 300, 400 and 500 μM; r^2 = 0.9910 to 0.9960) was developed for every assay.

2.7 *Determination of verbascoside by HPLC*

The verbascoside content was determined using an Agilent Technologies 1100 series HPLC system with a G1311A Quatpump that was equipped with a Platinum EPS-C18 column (7 mm x 53 mm, 3 μm) and a G1315B photodiode array detector (DAD) (Agilent Technologies, CA, USA). The samples were filtered through a 0.45-μm nylon filter. The working conditions consisted of a 20 μL volume injection, a 1.0 mL min^{-1} flow rate, a 30°C column temperature, a 334-nm wavelength for the DAD and a mobile phase of 0.3% (v/v) phosphoric acid solution (solvent A) with acetonitrile (solvent B). The system was run with the following gradient program: 5 min, 100% to 87% A; 33 min, 87% to 78.2% A; and finally, 33 min, 78.2% to 65% A. A verbascoside standard (Extrasynthese, Genay, France) was used to prepare the solutions to build a calibration curve (40, 80, 120, and 240 μg mL^{-1}). The verbascoside standard and the extracts samples were run in the same manner. The CHEMSTATION chromatography software (Rev. A.08.03; Agilent Technologies, CA, USA) was used to acquire all data from the detector. The data corresponding to the verbascoside standard in the chromatograms (retention time and peak area) were used to detect, identify and quantify this compound in the extract samples.

2.8 *Statistical analysis*

All experiments involved in the *in vitro* propagation and phytochemical analyses were repeated three times, and 10 replicates were used per treatment (n=30). The corresponding results were statistically analyzed and are presented as the means ± the standard deviations (SDs). An analysis of variance (ANOVA) and Tukey-Kramer's multiple range tests were performed using NCSS version 5 statistical software (Wireframe Graphics, Kaysville, UT, NCSS 2001) to determine the significant differences which were assumed to be below $p < 0.05$.

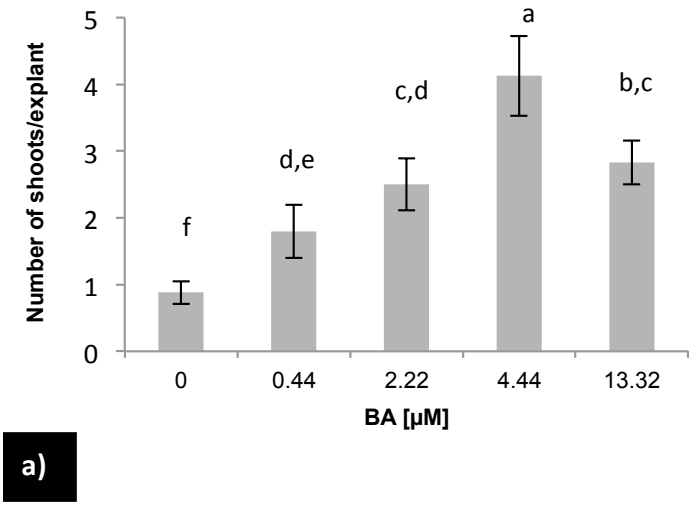
3 Results and discussion

3.1 Proliferation and elongation of shoots

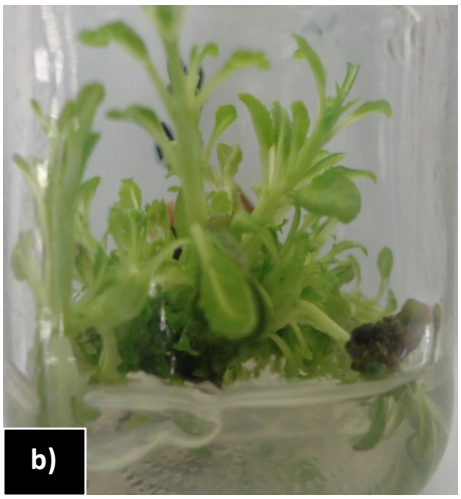
The PGR treatments significantly affected the morphogenetic responses of *B. cordata*. The BA significantly enhanced the shoot proliferation rate in stem-node explants compared with the control (without BA; Figure 1a). The shoot induction percentages ranged from 61 to 75% among the BA treatments and were not significantly different (data not shown). The concentration of 4.44 μM BA promoted the highest shoot proliferation rate (4.1 shoots per explant), whereas the control produced 0.9 shoots per explants (Figure 1a). A similar concentration of BA (5 μM) promoted the highest proliferation shoot rate (12 shoots per explant) in 100% of the explants of *Buddleja davidii* (Phelan *et al*., 2009). The use of 4.44 μM BA combined with 1.07 μM NAA has been shown to induce 3.43 shoots per stem-node explant in *Scrophularia yoshimurae*, which is a species that is taxonomically near *B. cordata* (Sagare *et al*., 2001). A succession of subcultures can maximize the number of induced shoots during the shoot proliferation stage (Iliev *et al*., 2010). Subculture in the same shoot induction medium significantly increases the numbers of induced shoots in *Scrophularia yoshimurae* (10.3 shoots per stem-node) (Sagare *et al*., 2001). The present study showed that subculture in the best treatment for shoot proliferation (4.44 μM BA) increased the number of induced shoots per explant by nearly sevenfold (29.2±4.2), whereas the control remained unchanged (0.9 shoots per explant). Subculture with 4.44 μM BA was also tested for the other initial BA treatments, but the numbers of induced shoots were lower than 29.2 (data not shown); therefore, the shoots were discarded for the subsequent experiments. Various factors are known to affect the failure or success of any *in vitro* propagation protocol. Plant growth regulators have been highlighted because they can exert relative control over morphogenesis *in vitro* (Hill and Schaller 2013). Cytokinins are frequently used to promote the induction of adventitious shoots. However, the application of a cytokinin to explants can induce shoots without elongation that tend to lose their regeneration ability (Iliev *et al*., 2010). The addition of gibberellic acid promotes elongation responses (Santner *et al*., 2009; Wang and Irving 2011). After the proliferation stage, the *B. cordata* shoots had an average length of 1.2±0.4 cm, which was subsequently enhanced with GA_3 (Figure 1b). The addition of 4.34 or 8.67 μM GA_3 to the shoots produced increases in their lengths (4.3 and 4.7 cm, respectively) compared with the control (3.1 cm). The frequencies of the shoots that were elongated were not statistically different between the 4.34 and 8.67 μM GA_3 treatments (58.3% and 57.8%, respectively, Figure 1c). The remaining shoots (41.7% and 42.2%) were not significantly different in length (2.9 and 3.0 cm for the 4.34 and 8.67 μM GA_3 treatments, respectively) compared with the control (3.1 cm). Visually, these shoots exhibited darkening and chlorosis (Figure 1d) and were therefore unable to achieve regeneration. In related studies of *Zeyheria Montana*, a species that is taxonomically similar to *B. cordata*, the use of 1.44 μM GA_3 combined with 0.44 μM BA resulted in the regeneration of 7.3 shoots per nodal explant, and these shoots were capable of rooting (Cardoso and da Silva 2013). In the present work, 4.34 and 8.67 μM GA_3 promoted regenerations of 17 and 16.8 shoots (these values were not statistically different), respectively, from a total of 29.2 treated shoots.

3.2 Rooting and acclimatization

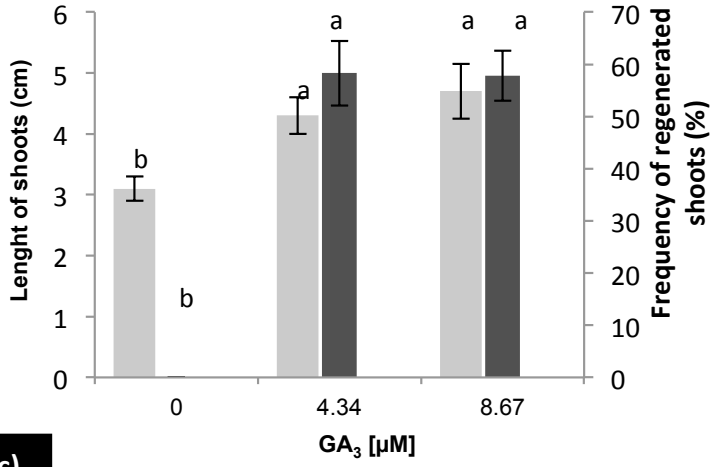
The rooting response of the regenerated shoots of *B. cordata* significantly increased with 2.45 μM IBA (Figure 1e). Rooting occurred from the base of the shoots after 11-15 days of culture, and the rooted shoots were visually observed to be slightly hyperhydric (Figure 1f). Those shoots that were unable to induce roots exhibited morphological abnormalities, such as darkening (Figure 1g), and then died. The frequency of rooted shoots (89.4%), root number (20.3) and root length (4.4 cm) were significantly higher with the 2.45 μM IBA treatment than in the control (28.9%, 13.3 and 3 cm, respectively, Figure 1e). No significant differences were observed regarding the rooting of the shoots treated with 5 μM IBA and 2.45 μM IBA (data not shown). In studies of *Buddleia davidii*, IBA at concentrations from 0.1 to 10 μM significantly increase the frequency of rooted shoots from 83.4 to 100% depending on the cultivar (Phelan *et al*., 2009). Higher IBA concentrations, such as 490 μM IBA, promotes shoot rooting in *B. cordata* for propagation purposes (with a probability of rooting less than 0.2) (Ramos-Palacios *et al*., 2012), and 30 to 100 μM IBA is effective in *Buddleia globosa* (Rose *et al*., 2000). These reports and the present study highlight the effects of IBA on rooting in species that are taxonomically similar to *B. cordata* and corroborate the finding that the effects of IBA depend on the cultivar, the species and genetics.


Number of shoots/explant
0
1
2
3
4
5
f
d,e
c,d
a
b,c
0
0.44
2.22
4.44
13.32
BA [µM]

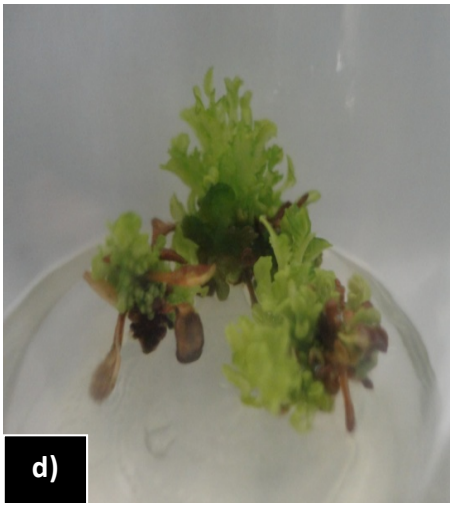
a)



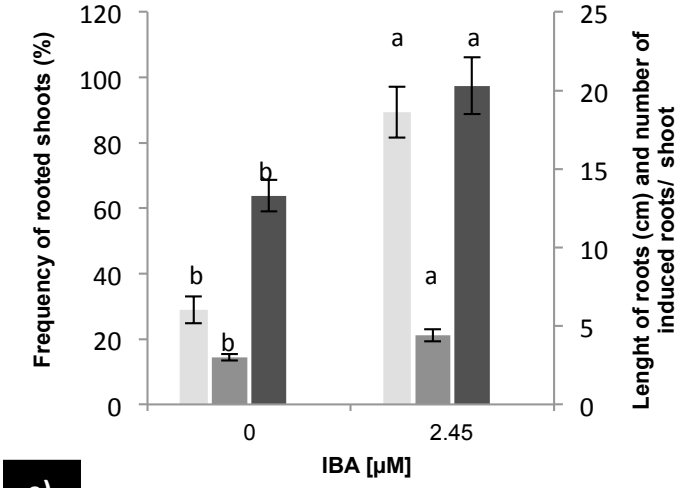
b)


Lenght of shoots (cm)
Frequency of regenerated shoots (%)
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
b
b
a
a
a
a
0
4.34
8.67
GA3 [µM]

c)



d)


Frequency of rooted shoots (%)
Lenght of roots (cm) and number of induced roots/ shoot
0
20
40
60
80
100
120
0
5
10
15
20
25
b
b
b
a
a
a
0
2.45
IBA [µM]

e)

f)

g)

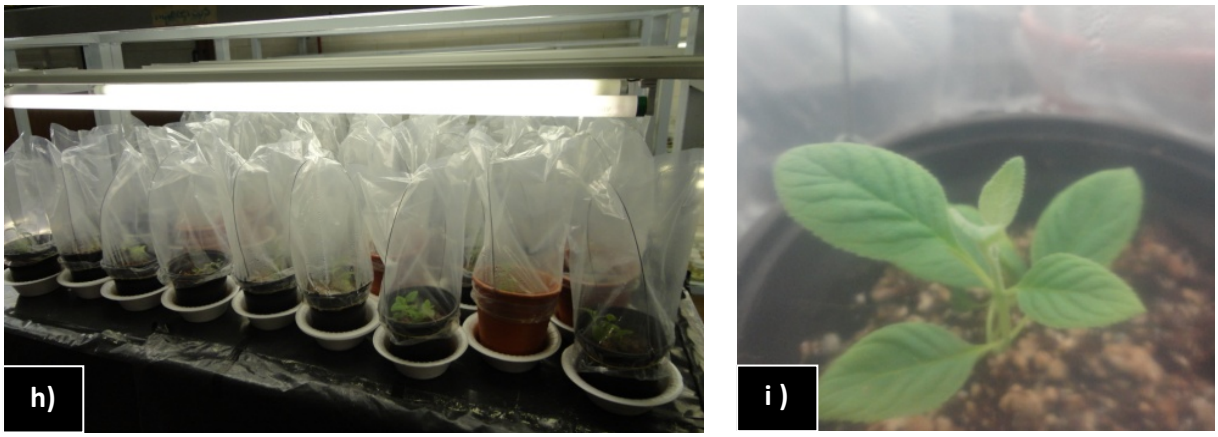


Fig. 1. Propagation *in vitro* of *Buddleja cordata*. a) ■ Effect of BA (0.00 to 13.32 μM) on the number of induced shoots in stem-node explants. b) Regenerated shoots under GA_3 (4.34 or 8.67 μM); c) Effect of GA_3 (0.00 to 8.67 μM) on, ■ the shoot length and ■ regeneration frequency. d) Phenotypic characteristics of the shoots that lost their regeneration ability; e) Effect of IBA (0.00 and 2.45 μM) on the rooting (■ frequency of rooted shoots, ■ length and ■ number of roots) of the elongated shoots. f) Rooted shoot after 30 days in culture. g) Phenotypic characteristics of the shoots that were unable to develop roots. h) Rooted shoots during acclimatization.; i) Phenotypic characteristics of the acclimatized plantlets that exhibited the disappearance of vitrification in the leaves. The results in a), c) and e) are presented as the means ± the SDs (indicated by vertical bars) of triplicate measurements by treatment. The means with bars followed by the same letter within the columns of the same color were not significantly different ($p < 0.05$).

The success of a micropropagation procedure strongly depends on shoot rooting, which is typically triggered by auxins (Iliev *et al*., 2010). When the *B. cordata* rooted shoots were acclimatized (Figure 1h), the hyperhydric appearance of plantlets gradually disappeared (Figure 1i) until they were phenotypically equal to their wild relatives. Thirty days after the transplantation, those shoots that developed roots in IBA exhibited a higher survival rate (91.7±9.8) than the controls (67.5±5.0). The results from the *Buddleia davidii* cultivars are comparable to those from *B. cordata* because the regenerated plantlets that were developed with IBA exhibited a survival capability above 95% (Phelan *et al*., 2009). Rooting with auxins can allow increased shoot survival during the transplantation process from *in vitro* to *ex vitro* conditions (Iliev *et al*., 2010).

3.3 Content of verbascoside and total phenols with antioxidant activity

The phytochemical analysis indicated that the micropropagated plantlets (PP) obtained through *in vitro* culture produced 24.8 mg GAE g^{-1} and 1.0 mg g^{-1} verbascoside that were related to the antioxidant activities of 0.9 and 0.8 mmol TEAC g^{-1} in the $DPPH^+$ and $ABTS^+$ assays, respectively (Figure 2a, b, c). The total phenol content (TPC) and antioxidant activities of the leaves of *in vitro*-regenerated plants of the *Rehmannia glutinosa* species have been reported to produce verbascoside at 60 mg GAE g^{-1} with 0.2649 mmol Trolox g^{-1} in the $ABTS^+$ assay (Piatczak *et al*., 2014). Statistical differences regarding the corresponding values of the total phenol and verbascoside contents and the antioxidant activity were determined among the leaves of PP, wild plants (WP) and greenhouse plants (GP) of *B. cordata*. In the leaves of GP, the higher values were determined whilst the lower values corresponded to PP (GP>WP>PP: 84.2>36.6>24.8 mg EGA g^{-1} for TPC; 8.1>1.5>1.0 mg of verbascoside g^{-1}; 1.9>1.4>0.9 mmol TEAC g^{-1} in the DPPH assay; 2.2>1.2>0.8 mmol TEAC g^{-1} in the ABTS assay) (Figure 2a, b, c). Also, chromatograms from the leaves of PP, WP and GP showed a different pattern for the peaks. This indicates that, in addition to verbascoside, other different compounds were produced (Figure 2c).

In the case of *Thymus moroderi*, the profile of the contents of the secondary terpene metabolites of the *in vitro* propagated plantlets was different from that of their wild relatives (Marco-Medina and Casas 2014).

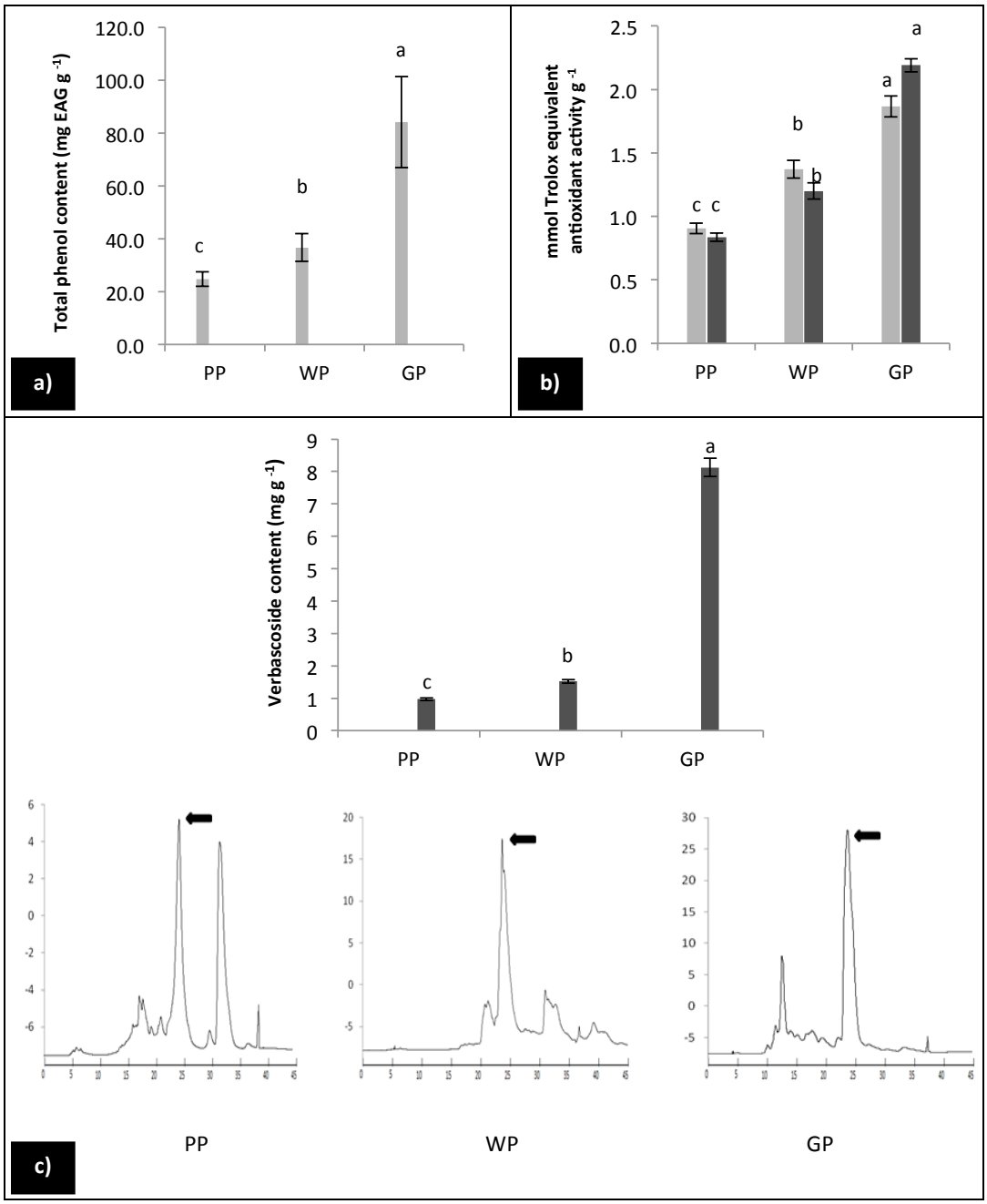


Fig. 2. Total phenol and verbascoside contents with Trolox equivalent antioxidant activities based on the $DPPH^+$ and $ABTS^+$ assays of the extracts derived from the leaves of the micropropagated (PP), wild (WP) and greenhouse (GP) *B. cordata* plants. a) ■ Contents of total phenols. b) Trolox equivalent antioxidant activity based on the ■ $DPPH^+$ and ■ $ABTS^+$ assays. c) ■ Content of verbascoside and chromatograms. The results are presented as the means ± the standard deviations (indicated by the vertical bars) of triplicate measurements by treatment. The means with bars followed by the same letter within the columns of the same color were not significantly different ($p < 0.05$). In the chromatograms, the arrows indicate the peak corresponding to verbascoside (retention time of 24.27 min).

Those differences in gene expression have been attributed to genetic and epigenetic factors. Epigenetic changes are often temporary, and the plants might revert to the normal phenotype (Smulders and Klerk 2011). Moreover, phenolic compounds are secondary metabolites that are often related to ecological issues because they play an important role in plant-environment interactions (Gouvea *et al*., 2012). The presence and the quantities of these compounds are significantly affected by several factors, such as genetic, biochemical and physiological processes, attack of microorganisms, environmental fluctuations and plant age, species and interspecies (Bowers and Stamp 1993; Gobbo-Neto and Lopes 2007). Among the plant species of the genus *Verbascum*, verbascoside contents are variable and range from 0.2% in *Verbascum phoeniceum* to 3% in *V. nigrum* (Georgiev *et al*., 2011). Even the GP analyzed in the present work, which were the vegetal material used by Estrada-Zúñiga at 2009, showed a verbascoside content (8.1 mg g^{-1}) lower in comparison with the value determined three years ago (10.10 mg g^{-1}) (Estrada-Zúñiga *et al*., 2009). The different conditions that took place on the age and environmental conditions of the GP, WP and PP of *B. cordata* might all have contributed to the differences found in the total phenols and verbascoside contents, antioxidant activity, as well as pattern of peaks of chromatograms.

The results for *B. cordata* in the present study, which allowed for the determination of the relationships of total phenol contents and verbascoside with antioxidant activity, are similar to those that have been reported in studies of species that are taxonomically related to *B. cordata*. Across several species of Acanthaceae, the total phenol contents range from 28.2 to 236.7 mg GAE g^{-1}, and antioxidant activity values range from 785.67 to 16.33 μg mL^{-1} (lower amounts of extract in terms of μg mL^{-1} indicate greater antioxidant activity) (Sawadogo *et al*., 2006; Sepúlveda-Jiménez *et al*., 2009). *Wendita calysina* has 87.5 mg GAE g^{-1} and 2.4 TEAC mmol g^{-1}, and both of these values are slightly lower than those of black tea (a potential source of phenols with health promoting benefits (Piccinelli *et al*., 2004), similar to those of the GPs of *B. cordata* (84.2 mg GAE g^{-1} and 1.9 and 2.2 mmol TEAC g^{-1}) and lower than those of the PPs. Moreover, although *in vitro* propagation has been achieved in species that are closely related to *B. cordata*, such as *Zeyheria montana* (Cardoso and da Silva 2013) and *Justicia gendarussa* (Thomas and Yoichiro 2010), only a few studies have reported on the production of bioactive secondary metabolite compounds with potential therapeutic uses, e.g., in *Scrophularia yoshimurae* (Sagare *et al*., 2001). Instead, cell or organ cultures are commonly conducted to produce bioactive secondary metabolites, e.g., the callus of *Iris pseudacorus* exhibits 0.4% w/w ($\sim$ 4 mg g^{-1}) polyphenolic compounds (Tarbeeva *et al*., 2013); the callus and cell suspension cultures of *Stephania venosa* are able to produce 19.6 and 26 mg g^{-1} of dicentrine, respectively (Kitisripanya *et al*., 2013); shoot cultures of *Castilleja tenuiflora* (Orobanchaceae) produced verbascoside at 19.19 mg g^{-1} and the total phenol content ranged from 21.6 to 49.5 mg GAE g^{-1} and those values were significantly triggered by elicitation procedure (Cardenas-Sandoval *et al*., 2015). However, one outstanding case is *Olea europea*, in which the biotechnological advances have significantly contributed to its genetic improvement and the *in vitro* propagation has played a crucial role in the regeneration of species that possess desirable features (Pollastri 2008). Additionally, *O. europea* leaves have recently received growing interest because they exhibit great benefits to health and contain verbascoside in amounts that range from 0.0002 to 0.016 mg g^{-1} (Quirantes-Piné *et al*., 2013; Ramos *et al*., 2013). These values are significantly lower than those reported here for the leaves of *in vitro* propagated *B. cordata* (1.0 mg g^{-1}). Moreover, important applications of *in vitro* propagation have begun to appear in the marketplaces of America, Japan and some European countries as viable methods to commercially exploit plants (Onay *et al*., 2011). The present study has contributed to the development of biotechnological advances for *B. cordata*. The findings highlight the viability of *in vitro* culture techniques developed for *B. cordata* that seek to propagate this species and to produce verbascoside, which is a secondary metabolite that is capable of promoting health benefits. *B. cordata* is an option for obtaining verbascoside and other phenols in a sustainable manner. *Wendita calysina*, is a species that produces verbascoside (2.9% w/w) and is used on an industrial scale as a food supplement, in cosmetics, in phytomedicines and as an ingredient in different formulations (Marzocco *et al*., 2007; Piccinelli *et al*., 2004; Vertuani *et al*., 2011). Another potentially valuable source of verbascoside (recovery rate of 2.6 g/kg) in addition to other phenolic compounds that possess antioxidant activity is the industrial byproduct of the processing of the fruit and leaves of the olive (De Marco *et al*., 2007; Obied *et al*., 2007; Quirantes-Piné *et al*., 2013; Ramos *et al*., 2013). There are diverse industrial applications of the phenolic

compounds produced by plants; hence a design of the bioprocess includes applying abiotic stress to increase the production of phenolic compounds (Sánchez-Rangel *et al*., 2014). Further research must be performed to determine the environmental factors that trigger verbascoside production.

Conclusions

A procedure for *in vitro* propagation from stem-node explants of *B. cordata* treated with BA (4.44 μM), GA_3 (4.34 and 8.67 μM) and IBA (2.45 μM) promoted strong shoot regeneration responses (16.8-17 shoots per explant), rooting (89.4%) and survival (91.7%). The regenerated plantlets produced phenolic compounds (24.8 mg GAE g^{-1}) that included verbascoside (1.0 mg g^{-1}) and displayed antioxidant activity (0.9 and 0.8 mmol TEAC g^{-1} in the $DPPH^+$ and $ABTS^+$ assays, respectively). The present research highlights the possibility of propagation for the prevention of decreases in the natural populations of the *B. cordata*. Furthermore, this work lays the foundation for the establishment of commercial crops and the continuation of biotechnological research with *B. cordata*, e.g., genetic studies, to improve the desirable characteristics of this species.

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Nomenclature

ABTS	2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt
ANOVA	analysis of variance
BA	N^6-benzyladenine
DAD	photodiode array detection
DPPH	2,2-dipheny-1-picrylhydrazyl
GA_3	gibberellic acid
GAE	gallic acid equivalents
HPLC	high performance liquid chromatography
IBA	indole-3-butyric acid
MS	Murashige and Skoog culture medium
NAA	α-naphthaleneacetic acid
PGR(s)	plant growth regulator(s)
TEAC TPC	trolox equivalent antioxidant activity total phenol content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid tiempo

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