




Spontaneous vegetative propagules differentiation in *Bowdichia virgilioides* seedlings maintained at MS basal medium

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ABSTRACT

Bowdichia virgilioides Kunth is a medium size tree used in folk medicine and in furniture manufacturing which makes it a target of extractive processes. The aim of this work was to evaluate the endogenous content of polyamines, amino acids, and sugars in seedlings of *Bowdichia virgilioides* that presented or not the development of spontaneously differentiated vegetative propagules in their roots. An average of thirty percent *Bowdichia virgilioides* in vitro growing plants cultivated in MS basal medium without growth regulators presented the differentiation of vegetative propagules. It was noted the propagules units presents embryo-like structures (globular - to leaf-shaped features) and these plants showed a compromised development when compared to the plants that do not presented these morphogenetic structures. Total sugars and amino acids content was significantly higher in the roots of plants with the vegetative propagules. With regard to polyamines content, there was no statistical difference, for spermine and spermidine. However, higher levels of putrescine were found in plants developing vegetative propagules. In this way, these results showed that this event are related to the amino acids, sugars and polyamines, mainly putrescine content.

Keywords: sucupira preta, putrescine, plant tissue culture.

INTRODUCTION

Bowdichia virgilioides Kunth is a tree species that belongs to the Fabaceae Family. It is commonly known by sucupira-preta, sucupira-do-cerrado, sucupira-do-campo, angelim-amargoso e coração-de-negro. It's wood has naturally high density and long durability and is widely used in edifications and furniture manufacturing. Besides it is a pioneer plant adapted to poor and dry soils, which makes it an interesting target for reforestation processes (Arantes *et al.*, 2015). Furthermore, *Bowdichia virgilioides* also has medicinal properties having been included in the first edition of the Brazilian pharmacopoeia (Albuquerque *et al.*, 2007). So it is important to implement conservation strategies for this species. In this sense, vegetative propagation is one of the most important tools

for plant conservation. It can be used to produce plants commercially, or to carry out basic studies of genetics, biochemistry and many others (Fehér, 2015). Briefly, differentiated plant cells under certain circumstances can revert to an earlier developmental state (dedifferentiate). However, is not a simple event and understanding different factors involved in this process has been the focus of many scientific works all over world (Mahendran & Bai, 2016; Mahdavi-Darvari *et al.*, 2015; Salvo *et al.*, 2014).

One of these factors is the polyamines (PAs) content, that are small aliphatic amines implicated in several physiological processes in plants such as, organogenesis, embryogenesis, leaf senescence, fruit development and ripening and response to abiotic and biotic stresses (Ashraf *et al.*, 2011; Alcázar & Tiburcio, 2014). The PAs

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metabolism is intrinsically connected with the nitrogen metabolism especially those related to the biosynthesis of amino acids. Therefore, many scientific studies have demonstrated the role of polyamines in the initiation and/or development of plants morphogenetic structures (Cheng *et al.*, 2015; Reis *et al.*, 2015; Vondráková *et al.*, 2014). The aim of this work was to evaluate the endogenous content of polyamines, amino acids, and sugars in seedlings of *Bowdichia virgilioides* growing in MS basal medium without growth regulators that presented or not the development of spontaneous differentiated vegetative propagules in their roots.

To our knowledge, this is the first study to demonstrate the occurrence of spontaneous vegetative propagules differentiation in *Bowdichia virgilioides* and its relation with biochemical parameters.

MATERIAL AND METHODS

Plant material, growth conditions and experimental design

Seeds of *Bowdichia virgilioides* were collected between the months of november and february in four different locations as follows: First location, 19°46'55" S and 45°15'38" W; second location, 19°45'02" S and 45°14'09"; third location, 21°06'42" S and 45°34'18" W; fourth location 26°46'40" S and 45°18'32".

To break the dormancy, in the moment of use, the seeds were submerged in concentrated sulfuric acid for 10 min. Subsequently they were washed three times with distilled and autoclaved water, disinfested with sodium hypochlorite 2.5% for 5 min and washed again. Then, the seeds were inoculated in culture flasks containing 20 mL of MS medium (Murashige & Skoog, 1962). The pH was adjusted to 5.8 ± 0.1 before autoclaving, which was carried out at 121 °C, pressure of 1.0 atm for a period of 20 min. The flasks were kept at 25 ± 1 °C, photoperiod of 12 h and irradiance of $36 \mu\text{mol.m}^{-2}.\text{s}^{-1}$. There were inoculated 800 seeds, being 200 seeds for each collection point as mentioned above. The plants were randomly distributed in the growth chamber and remained in this condition for 11 months (pre-established period for the development of vegetative propagules) without changes in the culture medium. At the end of this period, it was evaluated the number of plants with spontaneous vegetative propagules differentiation. For biochemical analysis were collected four roots of each location point of plants with vegetative propagules differentiation and four roots of plants without vegetative propagules differentiation.

Extraction and analysis of amino acids and sugars

Frozen tissue samples (200 mg) were ground in 2 mL of NaCl solution (methanol:chloroform:water, 12:5:3

v:v:v) and incubated at room temperature for 24 h. After this period, the samples were centrifuged for 30 min at 1500 g, and the supernatant was mixed with chloroform and water (4:1:1.5 v:v:v). The aqueous phase was collected after 24 h and used for the analysis of amino acids and sugars.

Total soluble sugars were determined colorimetrically after reaction with anthrone (Yemm & Willis, 1954). Briefly, samples were mixed with water in a final volume of 1 mL and mixed with 2 mL of anthrone reagent (20 mg anthrone, 500 μL water and 10 mL concentrated H_2SO_4). The samples were shaken and subsequently heated at 100 °C for 5 min. Absorbance was determined at 620 nm and quantification based on standard curve of glucose.

Total amino acid content was determined according to Yemm & Cocking (1955). Aliquots of the extracts were added to distilled water in a final volume of 1 mL. Subsequently, 1.7 mL of the following reagents were added: 0.2 M sodium citrate buffer pH 5 (0.5 mL), 5% methanol solubility in water (0.2 mL) and 2% KCN in methylcellulose (1 mL). This mixture was stirred and heated at 100 °C for 20 min. After cooling at room temperature, 1.3 mL of 60% ethanol was added to the samples and the absorbance at 570 nm was determined. Quantification was performed based on standard curve of glutamine.

Free polyamines determination

Frozen samples (500 mg) were macerated in 3 mL of 5% perchloric acid (v/v) and kept at 4 °C for 1 h. After that, the samples were centrifuged at 15000 g for 20 min at 4 °C. The supernatant containing the free polyamines was removed and the pellets were re-extracted. The supernatants were combined and the pellets were eliminated. Free polyamines were derivatized by dansyl chloride diluted in acetone at the concentration of 5 mg.mL^{-1} . An aliquot of 40 μL of the samples were added to 100 μL of dansyl chloride and 50 μL of saturated sodium carbonate. This mixture was incubated in the dark for 50 min at 70 °C.

After that, the excess of dansyl chloride was converted to dansyl proline by the addition of 25 μL of proline (100 mg mL^{-1}). After 30 min incubation the dansylated polyamines were extracted by the addition of 200 μL of toluene. The toluene phase was collected and dried in nitrogen and the dansylated polyamines were solubilized in 200 μL of acetonitrile. The polyamines were separated and analyzed by reverse phase HPLC using a 5 μm , 250 mm x 4.60 mm C-18 column. The mobile phase was composed of absolute acetonitrile (solvent A) and 10% acetonitrile in water (pH 3.5; solvent B) in a flow rate of 1 mL/min at 40 °C. The gradient of solvent A was as follows: 65% over the first 10 min, 65 to 100% between 10 and 13 min and 100% between 13 and 21 min. (Silveira *et al.*, 2004).

The concentration of the polyamines was monitored with a fluorescence detector operating at 340 and 510 nm of excitation and emission wavelength respectively. Free polyamines were derivatized by dansyl chloride diluted in acetone at the concentration of 5mg ml⁻¹. An aliquot of 40 µl of the sample was added to 100 µl of dansyl chloride, 50 µl of saturated sodium carbonate. As standards were used the commercial compounds putrescine, spermidine and spermine.

STATISTICAL ANALYSIS

The data were submitted to analysis of variance and the means were compared by the Tukey test at 5% of significance.

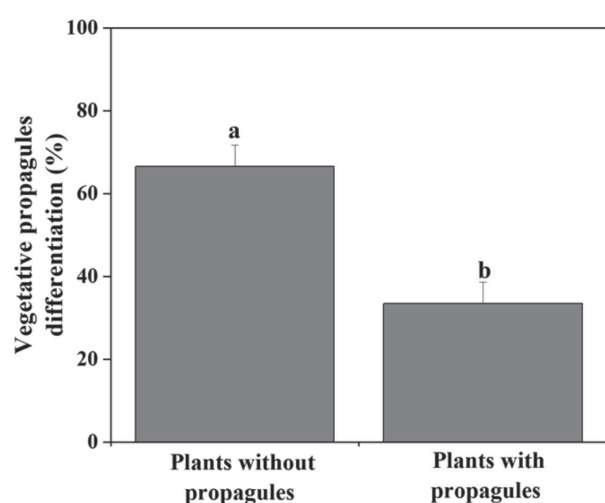


Figure 1: Percentage of *in vitro* *Bowdichia virgilioides* seedlings with or without spontaneous vegetative propagules differentiation after eleven months of *in vitro* cultivation. The bar represent the mean \pm SD. Different letters indicate that the data are statistically different by the Tukey test at 5% significance level.

RESULTS AND DISCUSSION

It was observed that some plants of *Bowdichia virgilioides* cultivated *in vitro* in MS medium without supplementation of growth regulators presented after a few months the development of vegetative propagules in their roots. With this in mind, *Bowdichia virgilioides* seeds were inoculated and the plants were cultivated for eleven months in order to analyse the vegetative propagules formation. It is worth mentioning that the plants were randomly allocated at the growth chamber and maintained at the same conditions throughout the cultivation period. This period of cultivation was chosen because we observe in previous experiments that the propagules formation begins around the sixth month and after the eleventh month there were no emergence of new propagules units. As can be seen in the Figure 1 an average of thirty percent of the obtained plants presented the development of vegetative propagules. Besides that, was noted that these plants showed a compromised development when compared to the plants that did not present vegetative propagules (Figure 2 A and B). According to Vila *et al.* (2005), the use of roots as a source of explants for *in vitro* regeneration is limited to a few number of plant species. Besides that, there are several reports about the development of organogenic structures in regeneration systems from roots (Choffe *et al.*, 2000; Da Cruz *et al.*, 2014; Da Silva *et al.*, 2011). However, to a better knowledge of these structures histological examinations are necessary. From this point of view Haensch (2004) presents an interesting argumentation about embryo-like structures in *Pelargonium hortorum*. He reports globular - to leaf-shaped structures or similar to shoots, but with vascular connections with the parent explant and the lack of bipolarity. As can be seen in

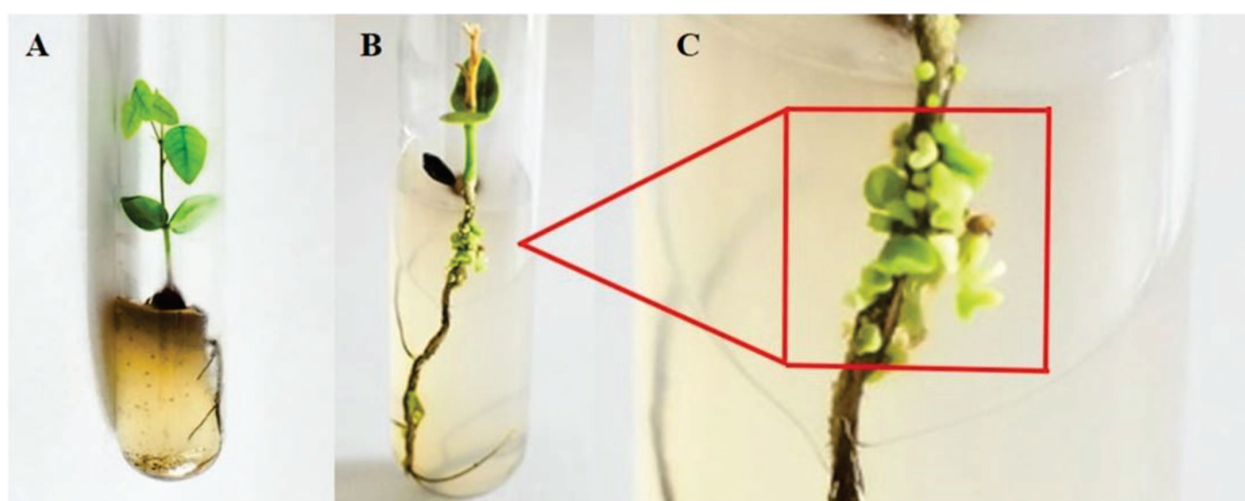


Figure 2: Samples of seedlings without (A) and with (B) spontaneous vegetative propagules differentiation and approximate detail (C) of roots developing propagules units.

more detail in the figure 2C, the propagules units presents globular - to leaf-shaped features. However, ultrastructural characteristics of the cells were not elucidate.

It is well known that the addition of growth regulators is an extremely relevant factor for the induction of organogenetic process in *in vitro* growing plants (Asthana *et al.*, 2017; Mose *et al.*, 2017; Roshanfekrrad *et al.*, 2017; Shen *et al.*, 2018, Da Cruz *et al.*, 2014; Da Silva *et al.*, 2011). For this, it is unusual that the propagules differentiation ocured without the addition of growth regulators, However, the *in vitro* tissue culture conditions *per se* expose the plants to a significant stresses (Feher, 2003). Under these condtions, some plant cells presents a remarkable feature of change their fate as a stress response (Kaur *et al.*, 2018) and the adaptations include several changes in the physiology and metabolism of the cells.

With this in mind, was carried out biochemical tests in the roots in order to point out possible biochemical diferences which could be related to the embryo emergence of the propagules units. This was thought because there is some evidences that changes in the levels of sugars, amino acids and polyamines are associated with development of morphogenetic responses in plants.

As can be seen in the figure 3A, the total sugars content was significantly higher ($62.67 \pm 6.57 \mu\text{g.g}^{-1}$) in the roots of the plants presenting propagules units when compared to the plants without these structures ($29.94 \pm 5.81 \mu\text{g.g}^{-1}$). Grzyb *et al.* (2017) and Aragão *et al.* (2016) argue that the relationship between the endogenous content of sugars and the development of morphogenetic responses is not well known, but they suggest that these biomolecules also play a relevant role in cell signaling, acting as promoting agentes. In the same way, total amino

acid content (Figure 3B) also presented higher content (19.2326 ± 5.3621) in the plants with progules units differentiation when compared with to the plants without these strucutres (11.2709 ± 3.59512).

With regard to free polyamine content (figure 4), there was no statistical difference between the plants, for spermine (Spm) and spermidine (Spd). However, higher levels of putrescine were found in the plants with progules units differentiation. Such higher content of putrescine content suggest a relationship with the the amino acid content, since the ornithine and arginine are the polyamines precursors. (Martin-Tanguy, 2001). Thus, we can hypothesize that the accumulation of amino acids supports the putrescine biosynthesis and these events,

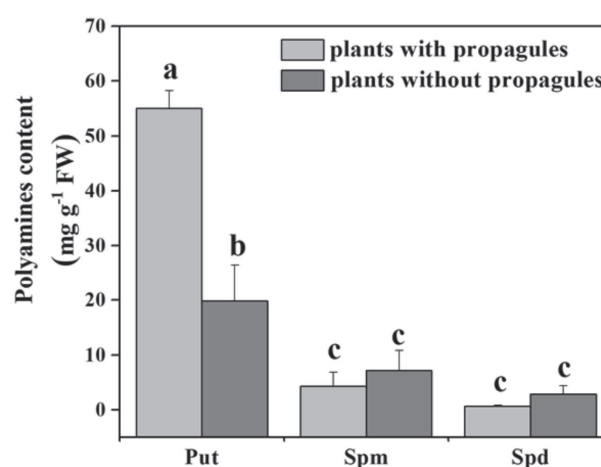


Figure 4: Free Polyamines in roots of *Bowdichia virgilioides* plants with and without spontaneous vegetative propagules differentiation. Put, putrescine; Spm, spermine; Spd, spermidine. The bar represent the mean \pm SD. Different letters indicate that the data are statistically different by the Tukey test at 5% significance level.

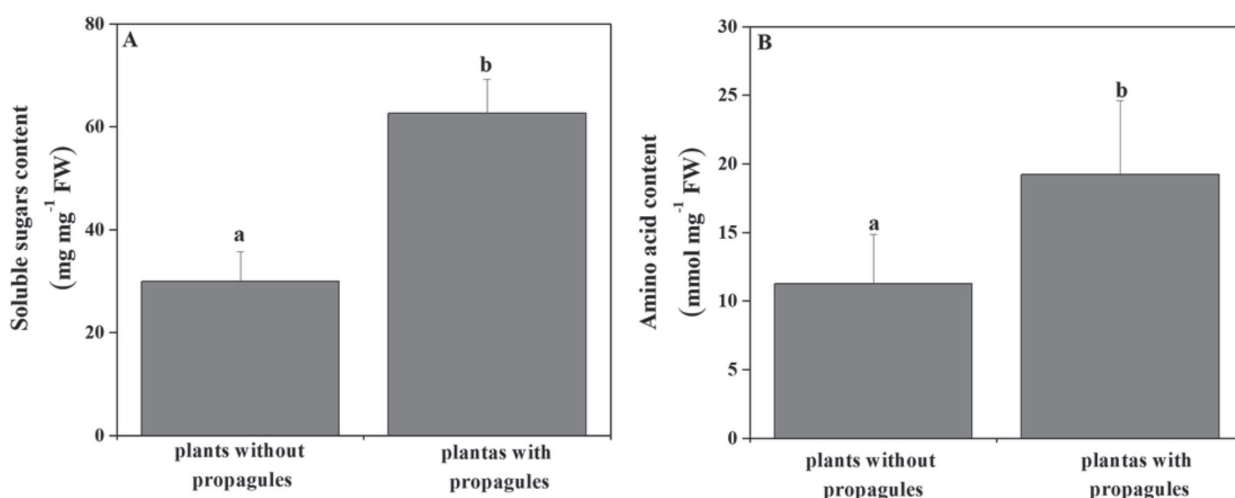


Figure 3: Total concentration of soluble sugars (A) and amino acids (B) in roots of *Bowdichia virgilioides* plants without and with spontaneous vegetative propagules differentiation. Bar represent the mean \pm SD. Different letters indicate that the data are statistically different by the Tukey test at 5% significance level.

together, may be associated with the emergence of the vegetative propagules. The polyamines are, intrinsically associated with the morphogenetic responses as demonstrated by Reis *et al.* (2015) in sugarcane, which obtained the largest quantity of embryos with putrescine supplementation. Other works also supports the role of polyamines, mainly putrescine, on the induction of morphogenetic responses like *in vitro* rooting (Matam & Parvatam, 2017), conversion of protocorms like-bodies to shoots (Wei *et al.*, 2010) and shoot organogenesis (Parimalan *et al.*, 2011).

CONCLUSION

Bowdichia virgilioides in vitro growing plants cultivated in MS basal medium without growth regulators presented the differentiation of vegetative propagules in their roots;

This event are related to the amino acids, sugars and polyamines content;

The propagules units presents embryo-like structures (globular - to leaf-shaped features), however, the ultrastructural characteristics will be determined in future works.

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