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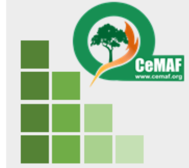

Physiological responses of *Aechmea setigera* Mart. ex Schult. & Schult. f. (Bromeliaceae) in vitro cultured

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INFO

Keywords
micropropagation
biotechnology
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ABSTRACT

In vitro propagation of bromeliads is widely used to produce plantlets intended for conservation. This study aims at evaluating physiological responses of *Aechmea setigera in vitro* cultured under different concentrations of 6-benzylaminopurine (BAP). The experiment was conducted in completely randomized design with five treatments (0, 1.0, 2.0, 4.0 and 8.0 mg L^{-1} of BAP). After 90 days of *in vitro* cultured in stationary liquid MS culture medium it were assessed morphological and biochemical parameters. The BAP increment increased stomatal density, reduced plant size and root number, but increased the number of shoots. There was significant effect of BAP dose on fresh and dry mass. High BAP doses (4.0 and 8.0 mg L^{-1}) promoted the reduction of chlorophyll *a* and total chlorophyll content, but there was no change of chlorophyll *b* and carotenoids. The concentration of starch and total soluble carbohydrates decreased above 2.0 and 4.0 mg L^{-1} of BAP, respectively. The total phenolic content decreased with increasing BAP concentrations. Evaluated changes in metabolism elucidate the phenotypic plasticity for *in vitro* multiplication process in *A. setigera*.

RESUMO

Palavras-chaves
micropropagação
biotecnologia
clorofilas
carboidratos
compostos fenólicos

Respostas fisiológicas de Aechmea setigera *Mart. ex Schult. & Schult. f. (Bromeliaceae) cultivada in vitro.* A propagação *in vitro* de bromélias é amplamente utilizada para produzir plantas visando a conservação. O objetivo desse trabalho foi avaliar as respostas fisiológicas de *Aechmea setigera* cultivada *in vitro* sob diferentes concentrações de 6-benzilaminopurina (BAP). O experimento foi conduzido em delineamento completamente casualizados com cinco tratamentos (0; 1,0; 2,0; 4,0 e 8,0 mg L^{-1} de BAP). Após 90 dias de cultivo *in vitro* em meio de cultura MS líquido estacionário, foram avaliados parâmetros morfológicos e bioquímicos. O aumento das concentrações de BAP promoveu aumento da densidade estomática, redução do tamanho da planta e do número de raízes, mas aumentou o número de brotos. Existiu efeito significativo entre as doses de BAP e as massas fresca e seca. Elevadas doses de BAP (4,0 e 8,0 mg L^{-1}) promoveu a redução dos teores de clorofila a e total, mas não alterou os teores de clorofila b e carotenóides. As concentrações de amido e carboidratos solúveis diminuíram com o uso de concentrações acima de 2,0 e 4,0 mg L^{-1} de BAP, respectivamente. O teor de compostos fenólicos totais diminui com o aumento da concentração de BAP. As modificações avaliadas no metabolismo elucidam a plasticidade fenotípica nos processos de multiplicação *in vitro* de *A. setigera*.

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INTRODUCTION

The *Aechmea setigera* Mart. ex Schult.. & Schult. f. (Figure 1A), epiphytic plant of Bromeliaceae family, is found in Panama, Colombia, Venezuela, Guyana and Brazil between 70 and 550 meters of altitude (Missouri Botanical Garden, 2021). In Brazil, this plant occurrence has been recorded in Acre, Amazonas, Pará, Mato Grosso and in the State of São Paulo (Brito et al., 2019). However, the massive deforestation dramatically damaged this species population and currently, *A. setigera* is considered unique in the Amazonian region. This species is epiphytic, with leaves up to 1 meter in length, peduncular bracts with a spiny margin and curved inflorescence (Koch et al., 2015). *A. setigera* has large, intense green and succulent leaves, and because of its beauty, it has ornamental potential (Leão et al., 2014).

In general, bromeliad species in the Amazon are poorly known (Quaresma and Jardim, 2012), and can be considered threatened due to deforestation. In view of existing threats to populations of plant species, biotechnological techniques have been surveyed aiming at conservation and clonal propagation, such as in vitro culture (Guerra and Dal Vesco, 2010). *In vitro* techniques have been widely used for propagation and as alternative to minimize the extraction of a high number of bromeliads, which are rare or endangered (Guerra and Dal Vesco, 2010). According Silva et al. (2020) the germplasm conservation of bromeliads needs to consider the genetic diversity of the species' natural conditions, therefore, the use of *in vitro* germinated seeds is essential for the maintenance of diversity aiming at *in vitro* conservation.

The combination of growth regulators and explant type is defined according to the desired morphogenetic responses (Faria et al., 2018), which are activated by complex metabolic pathways. Thus, plant growth regulators can lead to various responses, generate metabolic pathway activation and promote the accumulation or reduction of compounds essential for plant development (Rosa et al., 2018). Guerra and Dal Vesco (2010) suggest the use of growth regulators such as naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) in the *in vitro* multiplication phase of bromeliads in general. Biochemical responses, assessing the content of soluble carbohydrates, starch and soluble proteins were performed at *A. blanchetiana in vitro* cultured under the effect of different auxins (Chuet al., 2010).

Cytokinins especially BAP are used in different concentrations and combinations in order to facilitate the development of plant micropropagation techniques for inducing organogenesis (Maláet al., 2013), especially in bromeliads (Silva et al., 2020). Micropropagation studies using BAP of *A. setigera* are still recent (Leão et al., 2014; Vasconcelos et al., 2015).

This study aims at evaluating the physiological responses of *A. setigerain vitro* cultured under different BAP concentrations.

MATERIAL AND METHODS

The fruits of *Aechmea setigera* Mart.Ex Schult. & Schult. f. (Figure 1) at physiological maturity stage were collected from adult plants in natural population on the AC-90 Road, km 10, at 01'16.9"S and 67 55'26.6"W in Rio Branco (AC). The collections took place in November (the Amazon rainy season), with the manual removal of the fruits and stored in a refrigerator (4 °C) for 30 days. For species identification, exsiccates in duplicates were sent to the deposit in the Herbarium of the Rio de Janeiro Botanical Garden (Herbarium RB) under the number Rb550638. Seeds were manually removed and disinfected for 2 min in 70% ethanol, followed by 25 min immersion in commercial bleach solution (2.0-2.5% active chlorine) and added a drop of Tween 20 in each 100 mL of solution, according to Leão et al. (2014). Then, seeds were rinsed three times in sterile and distilled water and inoculated into canned-type glass (340 mL) containing 30 ml of MS culture medium (Murashige and Skoog, 1962) supplemented with vitamins of Morel (Morel and Wetmore, 1951), 30 g L^{-1} of sucrose and gelled with 6 g L^{-1} of agar. The pH of culture medium was adjusted to 5.8 prior to sterilization for 15 min at 1.3 kgf cm^{-2}. From *in vitro* introduction, plants multiplication was preceded.

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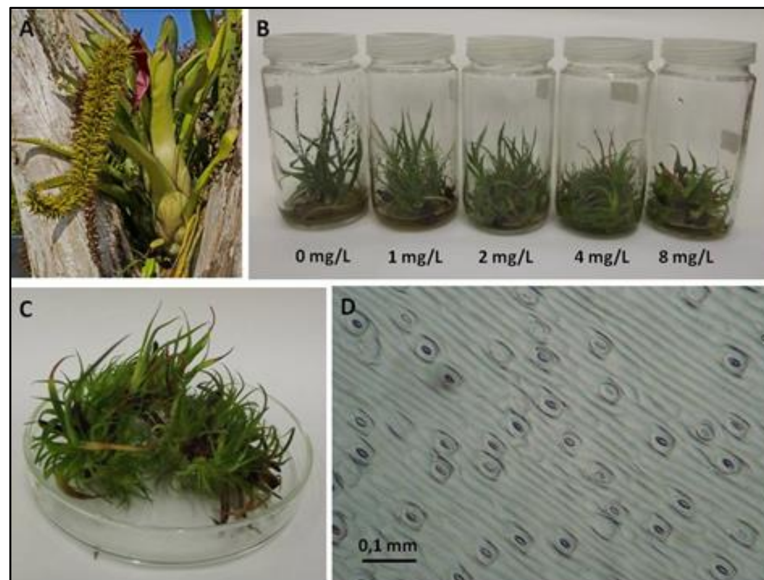

Figure 1 - *Aechmea setigera* Mart. ex Schult. & Schult. f. (Bromeliaceae) under natural conditions - image by Andrés Hernández with Smithsonian permission - www.si.edu (A), *in vitro* plants treated with different BAP concentrations (B), 4.0 mg L^{-1} treatment explant detail (C) and leaf abaxial surface image with stomata distribution (D).

Plants with 60 days of culture containing young leaves of about 1 cm inlength were excised and inoculated into glass bottles containing MS culture medium (Murashige and Skoog, 1962) in a stationary liquid phase with vitamins of Morel (Morel and Wetmore, 1951) sucrose (30 g L^{-1}) and 6-benzylaminopurine (BAP) at 0; 1.0;2.0;4.0 and 8.0 mg L^{-1}concentrations. After 90 days of culture (Figure 1B and 1C), assessments for biomass accumulation, stomatal density, leaf pigment contents and chemical compounds were performed.

Biomass accumulation was estimated from the number of shoots formed in each of the plants contained in the bottles. The length of the longest leaf and the largest root was measured using a caliper. The numbers of leaves in the largest shoot and the number of leaves per shoot were obtained from counting in each shoot. The fresh biomass of shoots and roots was determined in analytical precision balance (Kern 430-21, Kern, Balingen, Germany). The dry biomass of shoot and roots was performed after drying in greenhouse under air circulation at 65 °C for 72 hours. The total fresh and total dry biomasses were estimated by summing those in the shoot and roots.

The stomatal density was determined using the modeling technique with colorless enamel. The dry enamel was subsequently removed with clear adhesive tape and fixed on slides. Three leaves of different plants were randomly collected in each bottle, totaling 15 counts per treatment. Abaxial (Figure 1D) and adaxial photomicrographs of surfaces of leaves were performed under optical microscope (Feldmann Wild Leitz 1500 FWL T) with attached camera. With ANATI QUANTI software (version 2.0, Plant Anatomy Laboratory/UFV, Viçosa, Brazil), the number of stomata was determined (Aguiar et al., 2007).

The leaf pigment content was estimated using the SPAD index, measured by a chlorophyll portable meter (SPAD 502 Plus Chlorophyll Meter, Spectrum technologies, Bridgend, United Kingdom). Readings were performed on three leaves randomly selected of different plants in each replication, totaling 15 counts per treatment. The assessmentof chlorophyll *a* and *b*, total chlorophyll and total carotenoids content was performed according to the methodology described by Hiscox and Israelstam (1979). Samples of 100 mg fresh, healthy, full leaves were incubated in water bath with 7 mL of dimethylsulfoxide (DMSO) for two hours at 65 °C, with no maceration. After filtering, the total volume was adjusted to 10 mL. The values were obtained by visible-UV spectrophotometry in microplate (SpectraMax Paradigm Multi-mode Detection Platform Microplate Reader, Molecular Devices, Sunnyvale, USA) considering the optical density measured at 480 nm, 649 nm and 665 nm. The estimate of chlorophyll and carotenoid contents was performed using the equations (Wellburn, 1994): Chlorophyll *a* (Chla) = [(12.19 x A665) - (3.45 x A649)]; Chlorophyll *b* (Chlb) = [(21.99 x A649) - (5.32 X A665)]; Total chlorophyll = (Chla + Chlb) and Total carotenoids = [(1,000 x A480) - (2.14 x Chla) - (70.16 x Chlb)] / 220.

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The total soluble carbohydrate content was determined by phenol-sulfuric method (Dubois et al., 1956). Samples containing about 1 g of fresh mass were macerated with liquid nitrogen (-196 °C) using mortar and pestle and then subjected to a triple extraction by boiling in water bath (100°C) with 80% ethanol for 5 minutes. Extracts were centrifuged at 3000 rpm at 20 °C for 10 minutes and filtered through glass wool. From the combination of the three alcoholic extracts was obtained a corresponding portion to soluble sugar fraction, adjusting final volume to 10 mL with 80% ethanol. In 10 mL testing tubes was pipetted 50µl extract and added 450 µl distilled water, 0.5 mL phenol (5%) and 2.5 mL concentrated sulfuric acid (98%). The reading was carried out in visible-UV spectrophotometer in microplate (SpectraMax Paradigm Multi-mode Detection Platform Microplate Reader, Molecular Devices, Sunnyvale, USA) at 490 nm absorbance. The total carbohydrate content was estimated from a standard curve determined based on a standard carbohydrate (glucose), and the analysis performed in triplicate.

The starch extraction was carried out according to the methodology of McCready et al. (1950). To the precipitated residue from total soluble carbohydrates extraction was added 2 mL ice-cold distilled water (4 °C) and 2.6 mL perchloric acid (52%) iced, being stirred with a glass rod for 15 minutes. A total of 4 mL ice-cold distilled water was added and centrifuged at 3000 rpm for 15 minutes. The supernatant was filtered through glass wool and to precipitate was added 1 mL of ice-cold distilled water, followed by adding 1.3 mL of 52% cold perchloric acid, and being stirred with a glass rod for 15 minutes. It was centrifuged again at 3000 rpm for 15 minutes and the supernatant filtered through glass wool, joining all extract fractions, volume was adjusted to 20 mL with distilled water, and then homogenized. A fraction of 50µl was removed from the extract and added 450µl distilled water, 0.5 mL of phenol (5%) and 2.5 mL of concentrated sulfuric acid (98%). The total carbohydrate content was estimated from a standard curve determined based on a standard carbohydrate (glucose), and the analysis performed in triplicate. Quantification of starch content was according to the methodology of Dubois et al. (1956) in visible-UV spectrophotometer in microplate (SpectraMax Paradigm Multi-mode Detection Platform Microplate Reader, Molecular Devices, Sunnyvale, USA) at 490 nm absorbance.

The total polyphenol content was determined using the Folin-Ciocalteu method described by Singleton and Rossi (1965). The mass of 1 gram of fresh plant material was macerated in liquid nitrogen (-196°C) with mortar and pestle. The sample was placed in a test tube and added 10 mL of 80% methanol, followed by stirring for 2 minutes. Samples remained for 24 hours in the dark at 4 °C. The extract was filtered on vacuum pump. It was added 200 µL of sample extract and 1 mL of Folin-Ciocalteu reagent (diluted 1:9 v/v) intest tubes, followed by 800 µL of sodium carbonate (7.5%). Test tubes were covered with PVC film and kept away from light for 30 minutes. The test tube corresponding to blank received 200 µL of 80% methanol instead of sample. After incubation, absorbance was determined in visible-UV spectrophotometer in microplate (SpectraMax Paradigm Multi-mode Detection Platform Microplate Reader, Molecular Devices, Sunnyvale, USA) at 765 nm and analysis performed in triplicate. The total polyphenol content was estimated from a standard curve determined based on a standard phenolic compound (Gallic acid).

The experiment was conducted in completely randomized design with 5 treatments and 5 replications, and one glass bottle with 4 plants was the experimental unit. The results were subjected to analysis of variance and regression, using the statistical software Sisvar® 4.3 (5% error probability).

RESULTS

The length of the longest leaf of *Aechmea setigera* decreased linearly (R^2=0.80) with increased BAP concentration. Plant leaves in medium without growth regulator and highest BAP concentration showed 10.0 cm and 4.2 cm length, respectively. The length of the longest root has followed a similar trend to the leaf size, with exponential decrease (R^2=0.89) (Figure 2A). The number of leaves with higher shoot was not significantly changed by cytokinin addition to the culture medium. However, the total number of roots showed linear decrease with increased BAP concentration (Figure 2B). Culture medium supplementation with growth regulator favored shoot induction during *in vitro* culture of *A. setigera* (Figure 2C). The cultivation of plants in the absence of BAP resulted in low multiplication rate (2.2 shoots per explant). The increased concentration of growth regulator promoted linear increase in the number of shoots per explant (R^2 = 0.90) with 20.6 shoots per explant at 8.0 mg L^{-1}. The average number of leaves per shoot suffered no significant effect of BAP addition, being close to four in all treatments.

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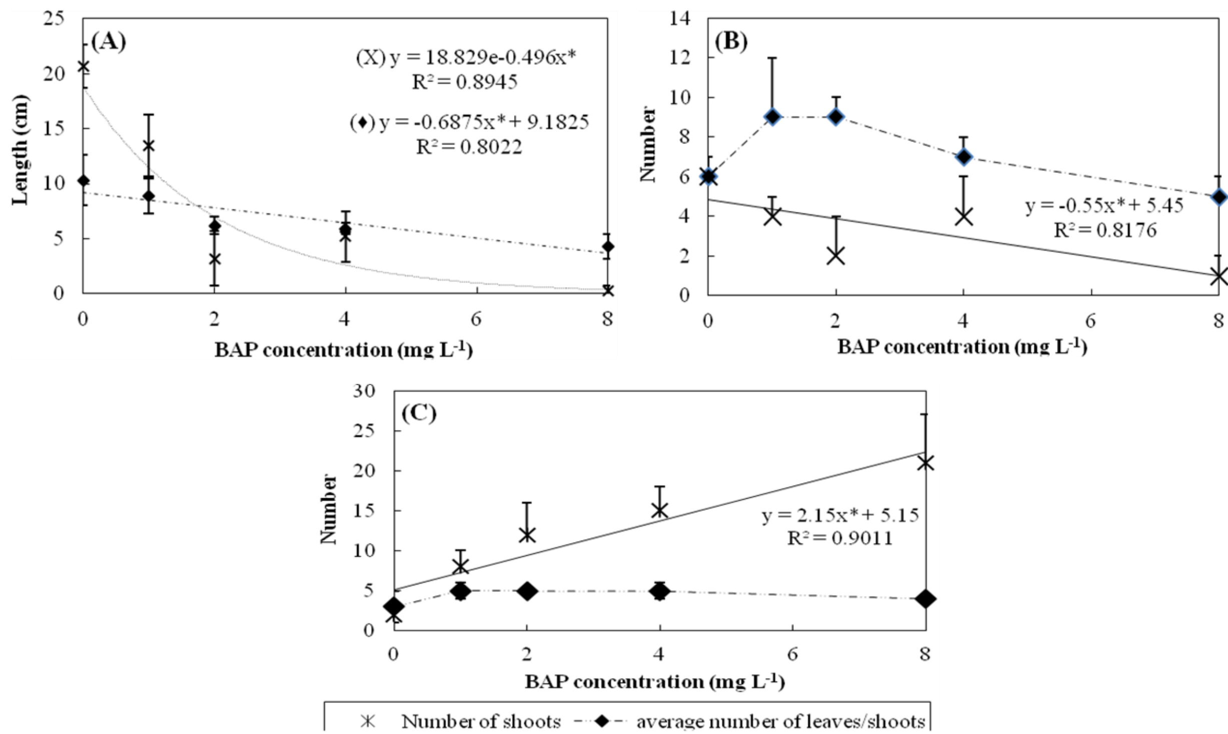

Figure 2 - Morphological parameters of *Aechmea setigera* Mart. ex Schult. & Schult. f.*in vitro* cultured under different concentrations of 6-benzylaminopurine (BAP). (A) Leaf and root length of the largest shoot. (B) Number of leaves and roots of the largest shoot. (C) Number of shoots and average number of leaves per shoot. Error bars indicate standard deviation. *Significant at 5% error probability (p <0.05)

BAP influenced the fresh and dry biomass accumulation besides modifying the number and length of shoots, (Figure 3). The shoot fresh matter was significantly increased (R^2=0.88), following a polynomial modeland increasing cytokinin concentration to the culture medium, while root system fresh matter was exponentially reduced (R^2=0.92) (Figure 3A). The accumulation of shoot fresh matter resulted from the expressive increased number of shoot (Figure 2C). In the dry biomass the same trend was observed (Figure 3B). However, there was no effect on the total biomass accumulation of *A. setigerain vitro*cultured, which showed average of 2.28 g fresh matter per explant and 0.18 g of dry matter per explant. The average of 7.7% dry matter was observed on the total biomass of explants.

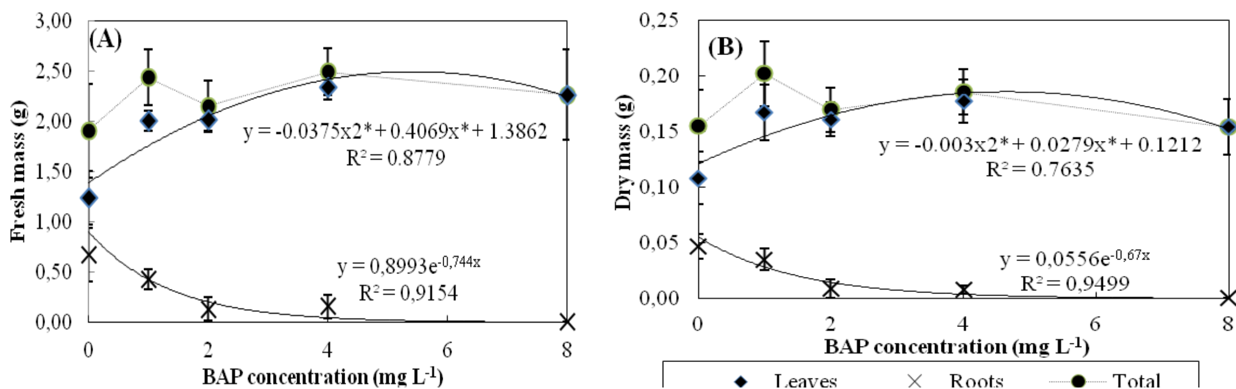

Figure 3 - Fresh matter of shoots, roots and total fresh matter (A) and dry matter of shoots, roots and total dry matter (B) of *Aechmea setigera* Mart. ex Schult. & Schult. f.*in vitro* cultured under different concentrations of 6-benzylaminopurine (BAP). Error bars indicate standard deviation. * Significant at 5% error probability (p <0.05)

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The increased cytokinin concentration in the culture medium resulted in linear increase (R^2=0.89) in stomatal density on the leaves abaxial surface of *A. setigera* (Figure 4). Plants cultivated with 8.0 mgL^{-1} BAP addition presented on average of 212 stomata mm^{-2}, three times over the density observed in plants kept without this growth regulator (71 stomata mm^{-2}).

The SPAD index is a direct and non-destructive measurement that represents an estimate of green pigments present in leaves. As the total chlorophyll content, SPAD index was influenced by the BAP addition to the culture medium, showing significant reduction with increased cytokinin concentration (Figure 4). The reduction followed a polynomial mathematical model (R^2=0.84). These results indicate that high concentrations of cytokinin in the culture medium can be harmful to the photosynthetic apparatus. However, these losses are compensated by the addition of sugars to the culture medium, allowing the multiplication of plants.

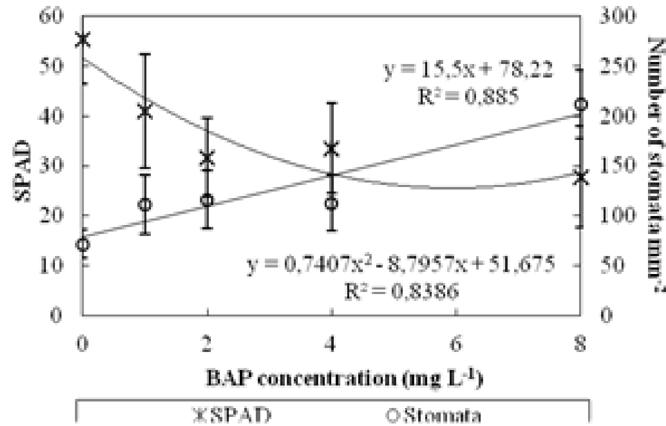

Figure 4 - Stomatal density (stomata mm^{-2}) in the leaf abaxial surface and SPAD index of *Aechmea setigera* Mart. ex Schult. & Schult. f.*in vitro* cultured under different concentrations of 6-benzylaminopurine (BAP). Error bars indicate the standard deviation. *Significant at 5% error probability ($p < 0.05$)

Total chlorophyll content (R^2=0.87) and chlorophyll *a* (R^2=0.89) in leaves of *A. setigera* were significantly reduced with increasing BAP concentrations. The reduction of leaf pigments followed linear pattern and the chlorophyll *b* and total carotenoids content have no differences in plants cultivated in culture medium with varied BAP concentrations (Figure 5). The total chlorophyll content ranged from 0.65 to 1.0 mg g^{-1} of fresh matter. Chlorophylls represented on average 87% of the pigments present in leaves, on average ratio of 7.0 between total chlorophyll and carotenoids. The content of chlorophyll *a* was equivalent to 73% of the total chlorophyll and average ratio of 2.8 chlorophyll *a*/*b*.

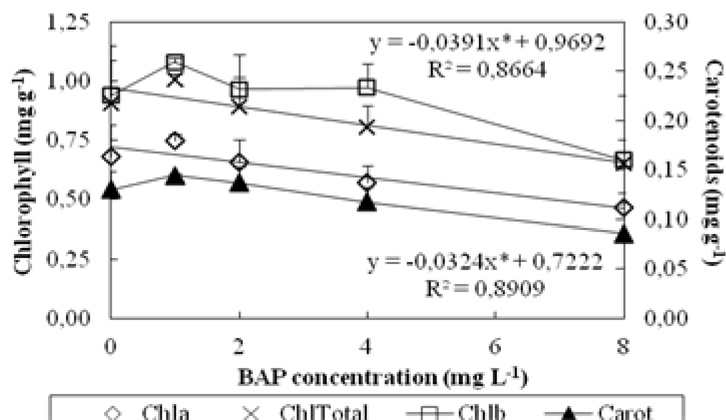

Figure 5 - Chlorophyll contents (*a*, *b*, total) and carotenoids in leaves of *Aechmea setigera* Mart. ex Schult. & Schult. f.*in vitro* cultured under different concentrations of 6-benzylaminopurine (BAP). Error bars indicate the standard deviation. *Significant at 5% error probability ($p < 0.05$)

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The analysis of starch content showed that BAP addition changed this polysaccharide amount in *A. setigera* leaves (Figure 6), with marked reduction above the use of 2.0 mg L^{-1} BAP. The BAP addition altered significantly the total content of soluble carbohydrates in leaves of *A. setigera* (Figure 6). There was increase in the carbohydrate content with increasing concentration up to 4.0 mg L^{-1} BAP. Plants cultivated in the presence of BAP had average carbohydrate content 3.2 times higher than those maintained in medium without regulator addiction. Concentrations above 4.0 mg L^{-1} BAP resulted in decreased soluble sugar content.

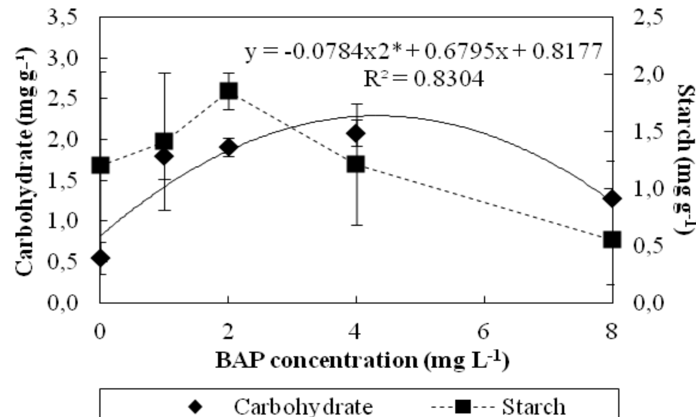

Figure 6 - Soluble carbohydrates and starch content in leaves of *Aechmea setigera* Mart. ex Schult. & Schult. f.*in vitro* cultured under different concentrations of 6-benzylaminopurine (BAP). Error bars indicate the standard deviation. *Significant at 5% error probability ($p < 0.05$)

The total phenolic compounds content was significantly reduced (R^2=0.91) with increase of BAP concentration to the culture medium (Figure 7). *A. setigera* plants maintained without growth regulator showed about 30% more phenolic compounds compared to plants under higher BAP concentrations (8.0 mg L^{-1}).

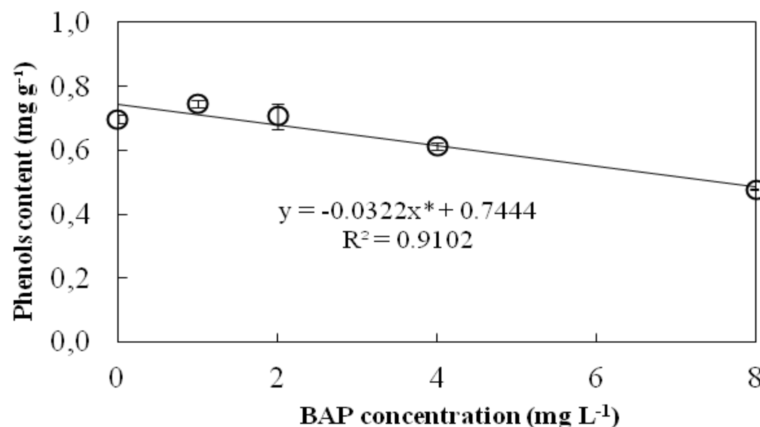

Figure 7 - Total phenolic compounds content in leaves of *Aechmea setigera* Mart. ex Schult. & Schult. f.*in vitro* cultured under different concentrations of 6-benzylaminopurine (BAP). Error bars indicate the standard deviation. *Significant at 5% error probability ($p < 0.05$)

DISCUSSION

The increased BAP concentration promoted increase in shoot number and lower growth of them. This observation was also verified in other studies on *in vitro* plants with the activation of lateral meristems and beginning of new shoot formation (Galvanese et al., 2007; Faria et al., 2018). In studies on micropropagation using *Nidularium innocentii* and *N. procerum* bromeliads, the increased BAP concentration also promoted increase in the number of regenerated shoots (Silva et al., 2012). Santa-Rosa et al. (2013) studying bromeliads of *Aechmea* genera, observed that the BAP addition to the culture medium

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resulted in shoot formation without roots or with poorly developed roots. This observation is in accordance to the trend observed in this study. The addition of BAP growth regulator to the culture medium changed the hormonal balance of *A. setigera* explants by modifying the investment in plant development, with more expressiveness in the formation of shoots as increased BAP concentration. Consequently, growth of leaves and roots reduced in the highest concentrations of phytoregulator.

The results observed for the number and length of leaves or roots and the biomass accumulation confirmed that the increased BAP concentration favored *A. setigera* multiplication rather than the growth of plant organs. Similar results with the use of BAP and ANA were obtained with bromeliad *Alcantarea nahoumii* (Silva et al., 2020), indicating that the use of this cytokinin in higher concentrations increases the multiplication rate, an important characteristic for the propagation and conservation of the species.

Stomata in plants *in vitro* cultured are significantly related to the photosynthetic capacity and to the acclimatization process (Borghezan et al., 2003; Tavares et al., 2015; Aliniaeifard et al., 2020). The cytokinins act in many morphogenic processes such as the vascular system development (Aloni, 2001), formation and maintenance of the photosynthetic apparatus (Rosa et al., 2018), and stomata functionality (Pospíšilová et al., 1999; Veselova et al., 2005). Plants maintained *in vitro* environment typically have unique characteristics, such as increase in size and stomatal density and reduced control on the opening and closing mechanism of stomatal pores (Hazarika, 2006). However, it has not been reported cytokinins action on the stomata formation, although it is widely known the role of these regulators on the control of stomatal conductance (Pospíšilová et al., 1999; Veselova et al., 2005). In this study, the increase in stomatal density by increasing BAP concentration may be related to the probable increase of gas exchange for the most metabolic activity. Such relation is supported by the increased formation, also with linear pattern, of the number of shoots regenerated with increasing BAP concentrations (Figure 2C).

Leaf pigments contents and ratios between pigments are in agreement with the values observed for other bromeliad species (Kurita and Tamaki, 2014; Martins et al., 2015). Although growth regulator addition has resulted in significant reduction of chlorophyll content, the highest BAP concentrations were not limiting for the multiplication of *in vitro* plants, considering that a carbon source (sucrose) was added to the culture medium. Cytokinins are growth regulators widely used in micropropagation of axillary and adventitious shoot (Dobránszki and Silva, 2010), which plays important role in the formation and development of chloroplasts (Rosa et al., 2018).

In order to minimize plant mortality during the acclimatization phase, the use of an intermediate culture medium with lower concentrations of both growth regulators and sugars stimulating root development is an important step for the propagation of bromeliads as established by Guerra and Dal Vesco (2010) and Leão et al. (2014).

Total content of soluble carbohydrates results reflect the balance between the source (photosynthesis and added source) and sinks (growth, multiplication and maintenance) of carbon at *in vitro* conditions. These sprouting were smaller and in higher number, using sugars rapidly, when compared with plants in medium without BAP. Similar pattern of response was observed by Leão et al. (2014), who suggest that intense metabolic activity of shoots justifies the most direct use of carbohydrates and lower stored content (starch).

The results disagree with other authors, who observed that culture medium with highest concentrations of growth regulators induced higher production of phenolic compounds (Dias et al., 2016). These authors report that *in vitro* cultured, produced phenolic compounds can be oxidized, causing blackening of the culture medium and later explants. These oxidized compounds become toxic to cells, negatively affecting plant development, compromising the action of enzymes and can lead to death and loss of cultures. However, for the *in vitro* culture of *A. setigera*, the content of phenolic compounds caused no change in coloring of the culture medium and did not reach toxic levels, without affecting the growth of plants.

The *in vitro* propagation system of bromeliads of *Aechmea* genera performed from shoots presented viability and can be used as a biotechnology strategy for the conservation of bromeliads, minimizing the extractive activity, as suggested by other authors (Santa-Rosa et al., 2013; Leão et al., 2014, Silva et al., 2020). This system can also be used for clonal propagation of plants with ornamental potential (Guerra and Dal Vesco, 2010; Faria et al., 2018). In addition to use in propagation and conservation systems, the results of this study contributed to extend the understanding of the BAP effect on the physiological behavior and metabolism of *A. setigera* plants.

CONCLUSIONS

The addition of cytokinin 6-benzylaminopurine (BAP) to MS culture medium promotes increased

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multiplication of *A. setigera* and the greater investment in the shoots. The stomatal density in leaves increases with BAP concentration. Starch and total soluble carbohydrates concentration decrease above 2.0 and 4.0 mg L^{-1} BAP, respectively. The content of total phenolic compounds decreases with increased BAP concentrations. *A. setigera* has facility at *in vitro* propagation and the addition of 8 mg L^{-1} BAP favored the multiplication rate without affecting the explants development, therefore, the best concentration for micropropagation and conservation.

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