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In vitro establishment and multiplication of *Aechmea fasciata* (Lindl.) Baker, a bromeliad of commercial interest

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ABSTRACT

Objective: To develop a protocol for the aseptic in vitro establishment and multiplication of *Aechmea fasciata* (Lindl.) Baker.

Design/Methodology/Approach: Different concentrations of NaClO (2, 3, 4, and 5%) were assessed for the aseptic establishment of *A. fasciata*. During the shoot induction stage, the three following treatments were tested: T_1=6-benzyladenine+naphthalene-1-acetic acid (BA+ANA; 5+2 mg L^{-1}); T_2=zeatin+2,4-dichlorophenoxyacetic acid (Zea+2,4-D; 5+2 mg L^{-1}); and T_3=control with no plant growth regulators. During the shoot multiplication stage, three more treatments were assessed: T_1=Zea+2,4-D (2.5+1 mg L^{-1}); T_2=Zea+2,4-D (5+2 mg L^{-1}); and T_3=Zea+2,4-D+gibberellic acid (AG_3) (2.5+1+3.5 mg L^{-1}). To assess the number of cellular events, different nitrate concentrations were tested in the medium (18.8, 39.4, and 60 mM NO_3^-). Finally, during the shoot regeneration stage, nine treatments derived from the combination of three concentrations of kinetin (KIN: 0, 0.1, and 0.5 mg L^{-1}) and of indole-3-acetic acid (AIA: 0, 0.3, and 0.4) were assessed. Completely randomized designs were used in each stage. Duncan's test ($p \leq 0.05$) was used to compare the means during the shoot induction and multiplication stages. A regression analysis was carried out to study the aseptic establishment and a non-parametric test (Kruskal-Wallis) was made to assess the "amount of microshoots during regeneration" variable.

Results: *A. fasciata* aseptic explants with 4% NaClO were established. Shoot induction was most effective with the BA+ANA (5+2 mg L^{-1}) treatment. The highest callus production was reported with the Zea+2,4-D (5+2 mg L^{-1}) treatment. The largest number of microshoots was obtained with high nitrate doses. Meanwhile, the most successful regeneration was achieved with the 0.1 mg KIN L^{-1} and 0.4 mg AIA L^{-1} treatment.

Study Limitations/Implications: The application of Zea and 2,4-D during multiplication induced callus formation.

Findings/Conclusions: Apical bud explants in an MS medium with BA and ANA present organogenesis. The use of Zea and 2,4-D forms calluses in the already established *in vitro* shoots, which regenerate with the use of KIN and AIA. Better microshoot coloring and development were achieved with MS salts, which have a medium nitrate content.

Keywords: Plant growth regulators (RCV), Microshoots, Bromeliads, Tropical forest, Endangered species.

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INTRODUCTION

Bromeliaceae are one of the main components of tropical forests. Their tank-shaped anatomy enables the development of microhabitats (Martinelli *et al.*, 2008). Microhabitats created by bromeliads establish interactions among different species and consequently contribute to the stability of forest ecosystems (Dal-Vesco *et al.*, 2014). However, a number of bromeliad species are endangered due to soil loss, forest fragmentation, and illegal exploitation of plants with high ornamental and commercial value (Balke *et al.*, 2008; Negrelle *et al.*, 2012). As a consequence of the severe habitat loss of bromeliads and their high commercial demand, a need to develop protocols or techniques for the propagation of species in vulnerable conditions has arisen. One of them is the Brazilian bromeliad *Aechmea fasciata*, one of the most valued and commercialized ornamental species due to its shape and inflorescence (Ping-Lung *et al.*, 2010). The most common multiplication methods used for this species include seed propagation and vegetative bud propagation —which usually produces 4 to 8 shoots. However, these traditional methods are not adequate for the massive propagation that is required to commercialize this plant and satisfy the market's demands, since both techniques lead to low production levels and a slow growth —approximately 2 to 3 years (Martins *et al.*, 2013). One of the main alternatives for massive plant production is micropropagation. This method has several advantages over conventional propagation: a large number of plants can be produced from a small tissue segment, in a short period of time, and in a significantly reduced space, free of pests and disease (Guerra and Dal-Vesco, 2010; Pickens *et al.*, 2006). Micropropagation is considered an important technique to optimize bromeliad production and thus satisfy the ornamental plant market. It is currently necessary to develop in vitro cultivation protocols to propagate bromeliads. These protocols should allow for a sustainable exploitation of wild species that will satisfy the local market's demands, while reducing overexploitation and recovering endangered species. Therefore, this research seeks to develop a protocol for the aseptic in vitro establishment and multiplication of *Aechmea fasciata* (Lindl.) Baker, a species with ornamental potential.

MATERIALS AND METHODS

Vegetable matter

A. fasciata plants were kept in greenhouse conditions. Apical bud explants taken from the shoots were used for the aseptic establishment and shoot induction. To reduce and avoid the proliferation of pathogenic microorganisms, donor plants were sprinkled every other day with a solution made of 5 g L^{-1} of Agri-mycin® 500 (*a. i.* tribasic copper sulfate, streptomycin sulfate, and oxytetracycline hydrochloride) and 5 g L^{-1} of Amistar® (a. i. azoxystrobin and difenoconazole).

Aseptic establishment

A pre-disinfecting measure was carried out in the lab: the explants were washed with commercial detergent for 5 minutes and then rinsed. Afterwards, the explants were submerged in 100% acetic acid for 20 minutes and then placed in 100% hydrogen peroxide for 10 minutes. Finally, they were soaked in a 5 g L^{-1} solution of Agri-mycin® 500 for 30

minutes, after which they were rinsed with distilled water. Once under aseptic conditions, the first superficial disinfection was carried out with 70% ethanol for 1 minute. The explants were subsequently immersed in four concentrations of NaClO (2, 3, 4, and 5% a. i.) for 20 minutes, adding a drop of Tween™ 20. All explants were rinsed thrice with sterile distilled water mixed with 100 mg of citric acid L^{-1}. The culture medium consisted of MS inorganic salts, supplemented with Morel and Wetmore vitamins (1951) and 2 g L^{-1} of activated charcoal. Incubation conditions were a 16/8 h light/dark photoperiod. The number of contaminated explants was assessed 15 days after sowing.

Culture media

The formula proposed by Murashige and Skoog (1962) was used as culture media, adding Morel and Wetmore vitamins (1951), enriching it with 3% sucrose, and using 2.25 g L^{-1} of Phytagel (Sigma Aldrich) as gelling agent. In each experiment, the appropriate plant growth regulators (RCV) were added to the medium. The media pH was adjusted to 6.0 with KOH or HCl 1 N. Finally, the media were sterilized in an autoclave at a temperature of 120 °C and a pressure of 1.05 kg cm^{-2} for 15 minutes.

Shoot induction and multiplication

Three treatments were assessed in the shoot induction stage: T_1=6-benzyladenine+naphtalene-1-acetic acid (BA+ANA; 5.0+2.0 mg L^{-1}); T_2=zeatin+2,4-dichlorophenoxyacetic acid (Zea+2,4-D; 5.0+2.0 mg L^{-1}); and T_3=control with no RCV in apical bud explants. The following variables were assessed: number of shoots per explant (the shoots resulting from individuation); shoot length (the length of shoots resulting from individuation, measured in mm); and number of leaves.

Considering the presence of calluses as response variable, three treatments were assessed in the multiplication stage: T_1=Zea+2,4-D (2.5+1.0 mg L^{-1}); T_2=Zea+2,4-D (5.0+2.0 mg L^{-1}); T_3=Zea+2,4-D+gibberellic acid (AG_3), (2.5+1.0+3.5 mg L^{-1}).

Modification of nitrate concentration in MS medium

An experiment was conducted where the nitrate concentration $\left(NO_3^-\right)$ in the MS medium salts was modified. Three treatments were assessed (T_1=18.8 mM, T_2=39.4 mM, T_3=60 mM), in order to determine which one had the largest number of cellular events.

Shoot regeneration based on *A. Fasciata* calluses

In the shoot regeneration stage, the effects of nine treatments —resulting from the combination of three kinetin concentrations (KIN: 0, 0.1, 0.5 mg L^{-1}) with three indole-3-acetic acid concentrations (AIA: 0, 0.3, 0.4 mg L^{-1})— were assessed, in order to identify the concentration required to achieve shoot formation.

Statistical model

A completely randomized design was chosen. Four repetitions per treatment were randomly sown for shoot induction; eight repetitions per treatment, for multiplication;

five repetitions per treatment, for callus-based shoot regeneration; and six repetitions per treatment, for the assessment of nitrate concentration. Each jar with an explant was considered an experimental unit. Data were analyzed separately for each test with the Infostat software v. 2016, while the means were compared using Duncan's test (p=0.05). To analyze the "contamination" variable, the presence or absence of bacterial or fungal colonies on the explant surface was assessed. A regression analysis was carried out based on the data obtained. A non-parametric Kruskal-Wallis test was used to analyze callus-based shoot regeneration.

RESULTS AND DISCUSSION

The apical bud contamination decreased when the concentration of NaClO in the disinfecting solution increased. The lower number of contaminated explants was obtained with treatments T_3 and T_4 (4 and 5% NaClO, respectively), with a value of p=0.034 (Figure 1). Although less contaminated explants were obtained with the 5% NaClO treatment, necrosis was observed, probably due to NaClO-induced cytotoxicity. Rodríguez *et al.* (2001) suggest that high contamination levels in bromeliad *in vitro* cultures are caused by leaf shape and arrangement, since the union between leaves and stalks is a suitable reservoir for the development of microorganisms —particularly bacteria and fungi. However, the disinfection method used in this work enabled the elimination of contamination and the successful establishment of the *A. fasciata* apical bud explants.

Shoots were produced after a 20-day development, as a consequence of the disinfection process applied to the explants and their *in vitro* establishment. Shoot production had a better response with BA+ANA (5.0+2.0 mg L^{-1}) than with the Zea+2,4-D (5.0+2.0 mg L^{-1}) treatment and the control with no RCV (Table 1). The high redifferentiation potential of the apical bud explants accounts for their organogenic response, since they contain the vegetative apical meristem which, owing to its characteristics and cell types, allows them to achieve a higher regeneration rate (Firoozabady and Moy, 2004). The results of this study agree with Skoog and Miller's model of organogenesis (1957), according to which vegetative bud differentiation is fostered by the auxin/cytokinin balance, favorable to cytokinin (Segura, 2008). Saucedo *et al.* (2008) suggest that adding auxin to the culture

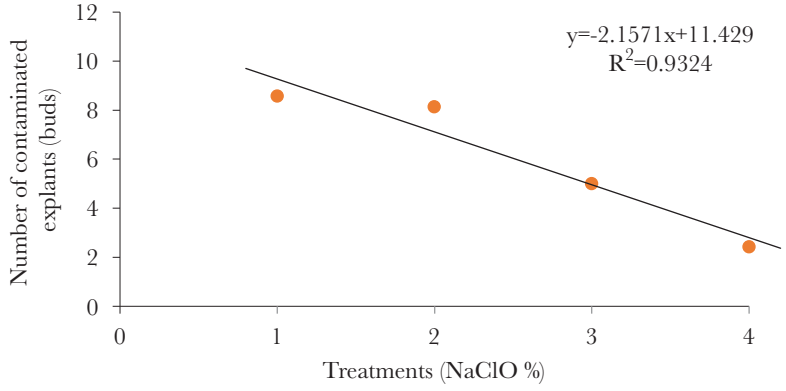

Figure 1. Effect of four different NaClO concentrations (T_1=2%, T_2=3%, T_3=4%, and T_4=5% with a 20-minute immersion) on the aseptic establishment of *A. fasciata* apical buds.

Table 1. Response of plant growth regulators (RCV) in apical buds during *A. fasciata* shoot induction.

Treatments	Number of leaves	Number of shoots	Length of shoots (cm)
Control (No RCV)	Unanswered	Unanswered	Unanswered
T_1 (BA+ANA; 5.0+2.0 mg L^{-1})	7.50 a	6.75 b	1.75 a
T_2 (Zea+2,4-D; 5.0+2.0 mg L^{-1})	6.25 a	2.25 a	1.58 a

Values with a different letter in each column indicate statistical differences (Duncan, $p \leq 0.05$). BA=6-benzyladenine; ANA=naphtalene-1-acetic acid; Zea=zeatin; 2,4-D=2,4-dichlorophenoxyacetic acid.

medium could directly influence the number of shoots, because an adequate cytokinin/auxin balance hinders the elongation of shoots, inhibits rooting, and stimulates the emission of shoots.

During the multiplication stage, the assessed treatments resulted in the formation of calluses instead of shoots. No significant differences were found in the variance analysis regarding the "callus presence" variable; however, the Zea+2,4-D (5.0+2.0 mg L^{-1}) treatment showed a higher callus development (Figure 2). These results match the findings of Obukosia *et al.* (2005), who found that a high 2,4-D concentration leads to callus formation in the chrysanthemum. Similarly, Ping-Lung *et al.* (2010) report callus formation when a combination of 2.0 mg 2,4-D L^{-1} and 1.0 mg ANA L^{-1} is used in *A. fasciata* ovary explants. The formation of calluses instead of shoots could be explained by an inadequate balance between endogenous and exogenous regulators or by the influence of the *A. fasciata* genotype on the response to *in vitro* morphogenesis.

The largest amount of microshoots was obtained with 60 mM NO_3^-, which produced an average of 213.43 microshoots, while 18.8 mM NO_3^- yielded an average of 28 shoots. Although there was no statistical difference in the amount of microshoots produced by treatments with 60 and 39.4 mM NO_3^- (the latter producing an average of 166.67 microshoots), better development and coloring (bright green) were observed in the microshoots produced with a concentration of 39.4 mM NO_3^- (Figure 3). These results match the findings of Droste *et al.* (2005), who found higher survival rates among

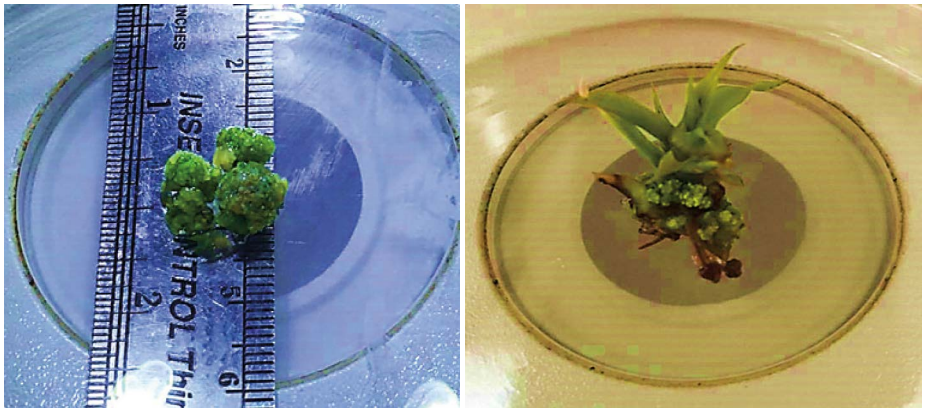

Figure 2. Cellular response during *A. fasciata* multiplication with Zea+2,4-D (5.0+2.0 mg L^{-1}).

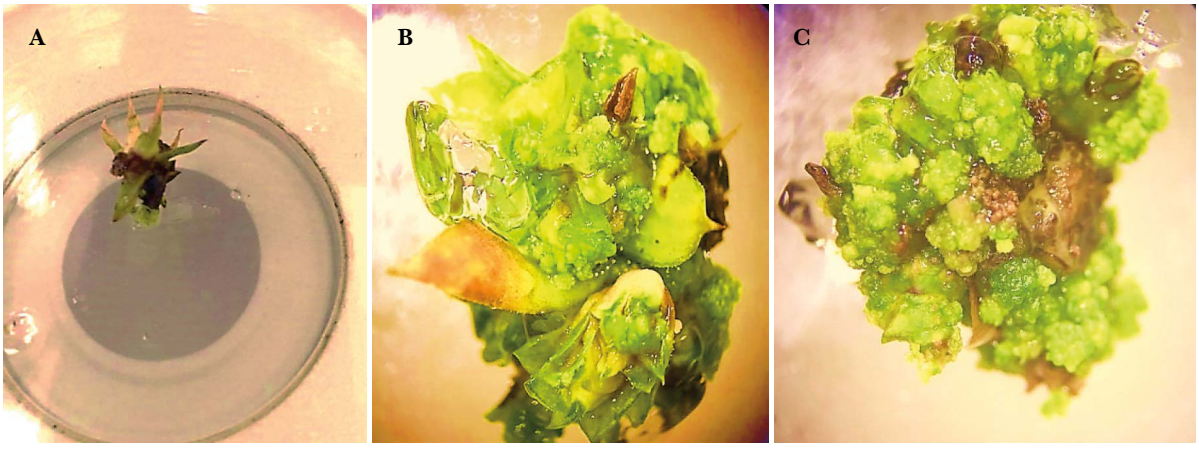

Figure 3. Cellular response of *A. fasciata* calluses to nitrate concentrations in the MS medium. A) 18.8 mM NO_3^-; B) 39.4 mM NO_3^-; and C) 60 mM NO_3^-.

Tillandsioideae seedlings, a subfamily of the Bromeliaceae, using the MS medium with 50 and 25% macronutrient concentrations. One morphological characteristic of this species are its epidermal-trichome-covered leaves, which are important for the absorption of water, amino acids, and moisture. For this reason, the roots no longer fulfill that function and they are apparently used exclusively for anchorage purposes. Therefore, they have become independent from their substrata and developed the ability to colonize nutrient-poor habitats (Estrada-Méndez, 1995). Based on this information and on the results of this work, these *in vitro* cultured species might require, instead of low or high nitrate concentrations, intermediate quantities of the total content of the MS medium.

A non-parametric analysis was necessary to examine the behavior of the values provided by the "number of shoots" variable in relation to the different treatments in the shoot regeneration based on *A. fasciata* calluses. The Kruskal-Wallis test was used to determine that there is no significant difference with a value of $x^2=12.50$ and $p=0.13$. However, the 0.1 mg KIN L^{-1} and 0.4 mg AIA L^{-1} treatment produced more microshoots than the other treatments. Similarly, Dal-Vesco *et al.* (2014) report a high shoot proliferation rate regarding *Vriesea reitzii* callus regeneration in an MS medium supplemented with 0.7 mg of AIA L^{-1}. Meanwhile, Alves *et al.* (2006) report a regeneration rate of 60 *V. reitzii* shoots, obtained by cultivating the leaf's basal region in a MS medium supplemented with KIN and two cytokinins (BAP and 2-iP).

CONCLUSIONS

The aseptic establishment of *A. fasciata* apical buds succeeded when they were immersed in 4% a. i. NaClO for 20 minutes. The highest shoot average was produced by the BA+ANA (5.0+2.0 mg L^{-1}) treatment. The application of Zea+2,4-D (5.0+2.0 mg L^{-1}) to the MS medium during the multiplication stage produces calluses. The MS medium with the 60 mM NO_3^- treatment produces more *A. fasciata* microshoots, with no statistical difference, with the 39.4 mM NO_3^- treatment. However, better coloring and development were observed with 39.4 mM NO_3^-. Callus-based shoot regeneration was

achieved in an MS medium with 0.1 mg KIN L^{-1} and with 0.4 mg AIA L^{-1}. This proves that mass production is feasible, if alternative sources for *in vitro* culture are used.

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