Short Communication

6-Benzylaminopurine and 3-Indolebutyric acid on the *in vitro* multiplication of *Eugenia involucrata*[1]

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ABSTRACT

Eugenia involucrata is an important Brazilian forest species, which it can be used for timber, such as firewood and charcoal, and non-timber purposes, for culinary and medicinal use. Considering the limitations of its seminal reproduction, vegetative propagation, particularly by micropropagation, becomes an alternative for plantlets production. In this study we evaluated the effect of the phytoregulators 6-benzylaminopurine (BAP) and 3-indolebutyric acid (IBA) on the *in vitro* multiplication stage of nodal segments. The experiment was carried out in a completely randomized design, in a 3 x 4 factorial array, the main factors were BAP (0; 0.1 or 0.2 µM) and IBA (0; 10; 20 or 30 µM). After 60 days of *in vitro* inoculation, evaluations on the the number of buds, number of shoots, number of leaves, callus formation (%) and number of roots per explant were carried out. BAP had not significant effect, however, the use of IBA alone favours the callogenesis and negatively affected leaves, shoots and roots formation. A moderate multiplication rate was obtained when considering *in vitro* bud formation. BAP and IBA are dispensable in *in vitro* multiplication of *E. involucrata*.

Keywords: micropropagation; phytoregulators; multiplication rate.

INTRODUCTION

Eugenia involucrata DC. (Myrtaceae) is a forest tree species native from Brazil and it has natural occurrence in the States of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo and Minas Gerais. Its dispersion was recorded in other Southern American countries such as Argentina, Uruguay and Paraguay (Carvalho, 2009). The timber is used to make small agricultural tools, for firewood and charcoal (Carvalho, 2009; Lorenzi, 2016), as well as for landscape, environmental, culinary and medicinal purposes. Despite its great economic and environmental potential, the species still remains underutilized, it being grown only in domestic orchards in the South and Southeast regions of Brazil (Prado, 2009). In addition, the species is under intense human pressure, due to the fragmentation of its natural habitat. Furthermore, the seeds are recalcitrant, a fact that they can compromise the reproduction, considering that they need to maintain a relatively high water content so that germination is not jeopardized (Carvalho, 2009).

In view of the limitations of seminal reproduction of certain native forest species, such as *E. involucrata*, vegetative propagation is an alternative for plantlets production for both purposes commercial and conservation of genetic resources (Oliveira *et al*., 2013). Among the techniques of vegetative propagation, micropropagation stands out as a tool of great impact, providing alternatives and even unique solutions in certain cases, like the production of seedlings from recalcitrant seeds. In addition, this technique enables to obtain plantlets with better phytosanitary pattern (Paiva *et al*., 2002), it being extremely relevant for the propagation of native forest tree species.

In this way and once for the complete plant development through *in vitro* propagation, hormonal balances between cytokinins and auxins influence many aspects of plant cell growth and differentiation. It is important to adjust the appropriate concentrations of plant hormones to propagate a particular species (Victório *et al*., 2012).

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Two studies on *in vitro* establishment of *E. involucrata* were performed by our research group, in which promising results were obtained by using nodal segments of this species inoculated in ½MS nutrient medium (Murashige & Skoog, 1962) (Golle *et al*., 2012) as well as adjusting the pH to 6.0 and adding 10 gL^{-1} sucrose and 4 gL^{-1} agar to the medium (Stefanel, 2016).

However, studies on the effects of phytoregulators on the *in vitro* multiplication of *E. involucrata* are still incipient. In the only study to date in this respect, Golle *et al*. (2017) obtained two shoots per explant when combined 0,5μM NAA and a very high concentration (32μM) of Thidiazuron (TDZ). Such result, however, it is economically inefficient and with a low multiplication rate, the reason that motivated this study. The use of other phytoregulators, such as 6-benzylaminopurine (BAP) and/or 3-indolebutyric acid (IBA), showed promising results on the *in vitro* multiplication of other species, such as *Eugenia uniflora* (Souza *et al*., 2008), *Eremanthus elythropappus* (Prudente *et al*., 2016a), *Campomanesia adamantium* (Goelzer *et al*., 2019), *Angelonia integerrima* (Winhelmann *et al*., 2019), among others. In this work, we aimed to evaluate the effect of BAP and IBA on the *in vitro* multiplication stage of *E. involucrata* micropropagation.

MATERIAL AND METHODS

The experiment was carried out at Universidade Federal de Santa Maria (UFSM), in a completely randomized design, in a 3 x 4 factorial array, with six replicates, each one constituted of six 150 mL capacity glass bottles sealed with aluminum foil, containing 30 mL of nutritive medium and two explants, totaling 72 experimental units. The main factors were BAP (0; 0.1 or 0.2 μM) and IBA (0; 10; 20 or 30 μM).

The nutritive medium used was ½MS (Murashige & Skoog, 1962) composed by the dilution of MS medium in half of the normal concentration of its salts and vitamins, plus sucrose (30 g L^{-1}), myo-inositol (50 mg L^{-1}) and agar (7 g L^{-1}), with pH adjusted to 5.8. The *in vitro* cultures were maintained in a growth room at 25 ± 2°C, with 12 h photoperiod and 20 μm m^{-2} s^{-1} irradiance obtained from cold white daylight fluorescent lamps (Golle *et al*., 2012).

The explants used were isolated from *in vitro* germinated *Eugenia involucrata* seeds, which were superficially disinfested prior to their inoculation in a nutrient medium. For superficial disinfestation, performed on the laminar flow table, the seeds were submerged in ethanol at 70% (v/v) for 1 min, followed by immersion in calcium hypochlorite at 3% (v/v) for 15 min, and in sodium hypochlorite at 3% (v/v) for 15 min. Finally, the seeds were washed three times in distilled and autoclaved water.

Subsequently, the seeds were inoculated with the aid of sterilized tweezers in agar-water medium, previously autoclaved at 121 ºC and 1 atm for 15 min. After inoculation, the flasks were closed with aluminum foil and placed in the growth room at 25±2 °C, 16 h photoperiod, under 20 μmol m^{-2} s^{-1} white fluorescent lamps, where they remained for 60 days. After *in vitro* germination, the apical stem segments, approximately 1 cm long apical buds, were isolated, which were used as explants in the experiment.

Evaluations were carried out 60 days after *in vitro* inoculation. The number of buds, number of shoots, number of leaves, callus formation (%) and number of roots per explant were evaluated. The average multiplication rate was estimated by counting the number of buds formed, except the apical bud (Pereira & Fortes, 2004).

After testing the normality of the errors by the Kolmogorov-Smirnov test and the homogeneity of variances by the Bartlett test, the variables were transformed, when necessary, by the function $\sqrt{x + 0.5}$, where *x* is the observed value. Analysis of variance (ANOVA) and polynomial regression analysis were performed using Sisvar software (Ferreira, 2014) version 5.6 at 0.05 significance level. The precision of the tests was estimated by the variation index (IV), calculated by $\frac{CV}{\sqrt{N}}$, where IV equals to coefficient of variation (CV) divided by the square root of the number of repetitions (N) (Pimentel-Gomes, 2009). Charts were plotted in Microsoft Office Excel.

RESULTS AND DISCUSSION

For the number of buds per explant no significant effect of any main factor was observed, nor with its interaction. In turn, for the number of shoots (p = 0.0447) (Figure 1A), number of leaves (p = 0.0166) (Figure 1B), callus formation (p = 0.0470) (Figure 1C) and number of roots (p = 0.0284) (Figure 1D), there was significant effect for IBA concentrations, but no significant effect for BAP or the interaction between the factors was observed.

Regarding the number of buds, a satisfactory mean of 1.04 was obtained, which it can be explained by the endogenous balance of phytoregulators favorable to the increase of this variable in *E. involucrata* cultures. This result is considered satisfactory since it was necessary to supplement 16 μM of Thidiazuron (TDZ) to obtain a similar number of buds (1.57) in previous studies carried out with the same species (Golle *et al*., 2017). However, these means are lower than that recorded in *Bowdichia virgilioides*, in which there was the formation of 7.29 buds per explant in nodal segments and 2.50 cotyledonary segments with the use of 1.32 μM BAP added to the WPM nutritive medium (Moura *et al*., 2012).

BAP is the most used cytokinin in the micropropagation of forest species due to its effectiveness on promoting cell multiplication and, thus, the induction of adventitious buds

in vitro (Botin & Carvalho, 2015). However, in the present study, there was no effect of this phytoregulator for bud proliferation in *E. involucrata*, demonstrating that the effect of phytoregulators is not the same in all species.

For the number of shoots, the model with the best fit was square (Figure 1A) and, according the technical efficiency calculation, the highest average would be obtained at 5,4 µM IBA. Auxin supplementation with low concentrations caused an increase in the number of shoots until 9 µM from which was observed a decrease. Shoot formation in the absence of phytoregulators may indicate the presence of endogenous cytokinins in *E. involucrata*

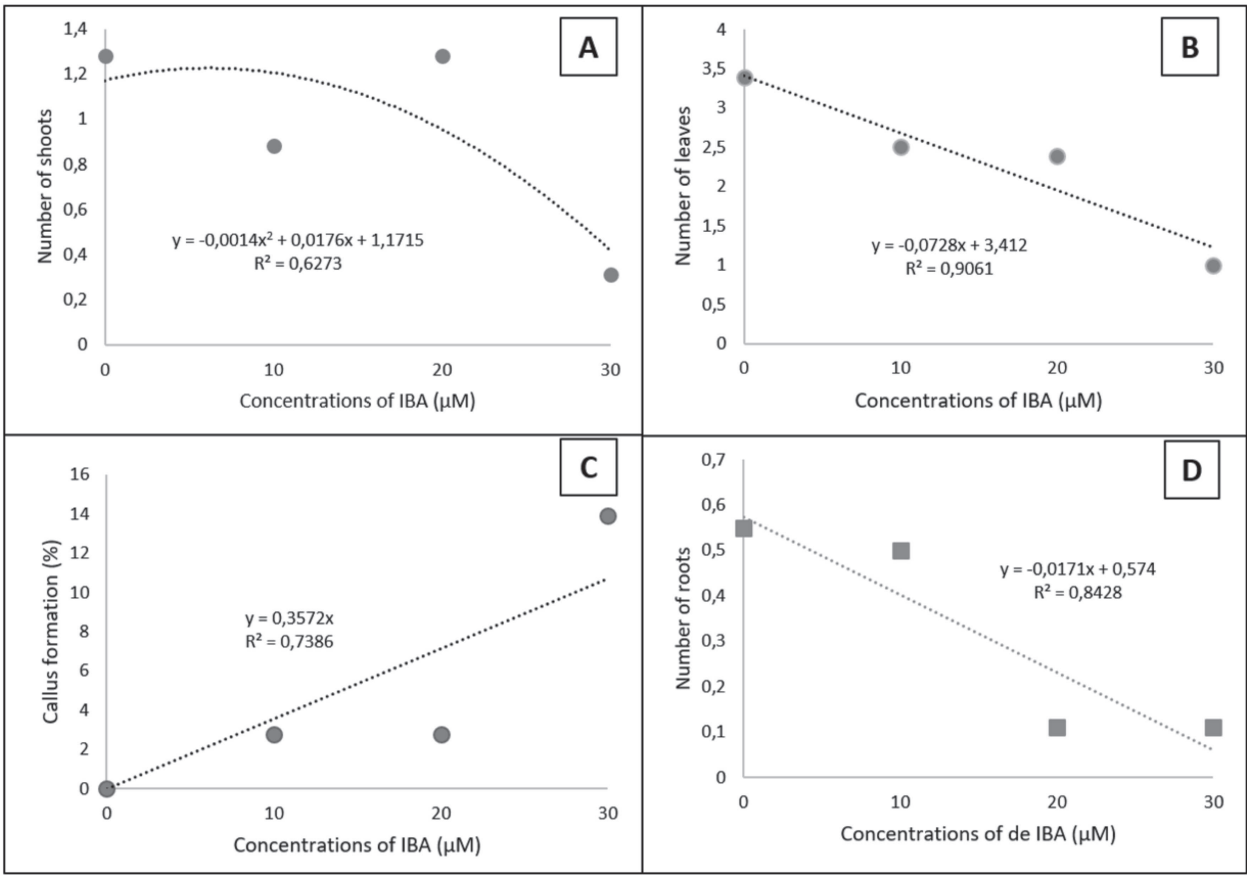

Figure 1: Number of shoots (A), number of leaves (B), callus formation (%) (C) and number of roots (D) in *E. involucrata* nodal segments on different indolebutyric acid (IBA) concentrations, regardless benzylaminopurine (BAP) concentrations, after 60 days of *in vitro* culture on ½MS nutritive medium.

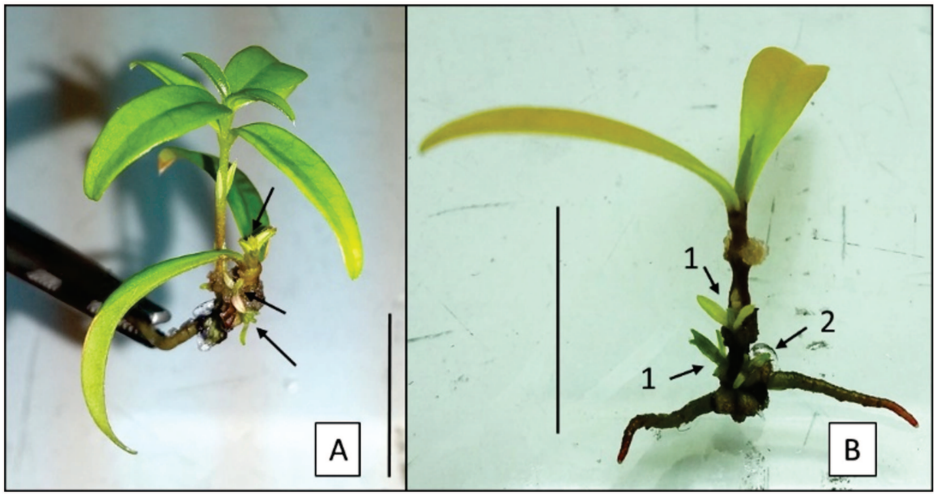

Figure 2: Aspects of *E. involucrata* explants in the MS nutritive medium, in the absence of phytoregulators, after 60 days of *in vitro* culture. It is observed, in 'A', the emission of several shoots (highlighted by the arrows) and, in 'B', the aspect of bud formation (arrow 1) and the development of adventitious bud (arrow 2). Bar = 1 cm.

(Figures 2A and 2B). It is a good finding as plantlets of this species can be micropropagated at reduced costs. However, this value (1.28 shoots per explant) is still low and further studies to optimize this finding are required. Similar results were found in other study with *E. involucrata* in which 1.96 shoots per explant were obtained in the absence of phytoregulators and, when combined with 0.5 µM α-naphthaleneacetic acid (NAA) and 32 µM Thidiazuron (TDZ), no significant alteration was obtained (Golle *et al*., 2017). An opposite result was observed in *Cyrtopodium saintlegerianum*, where the concentration of approximately 1 µM IBA or 1 µM NAA increased the number of shoots (Silva *et al*., 2013).

Regarding the number of leaves, the model with the best fit was linear (Figure 1B). The highest mean value (3.39 leaves per explant) was observed in the absence of IBA, decreasing with the increase of auxin concentration in the nutritive medium (Figures 3A and 3B). The observed response is probably due to the endogenous hormonal balance adequate for morphogenesis, such that the auxin supplementation has impaired leaf formation. This finding supports the recognized action of auxins on plant

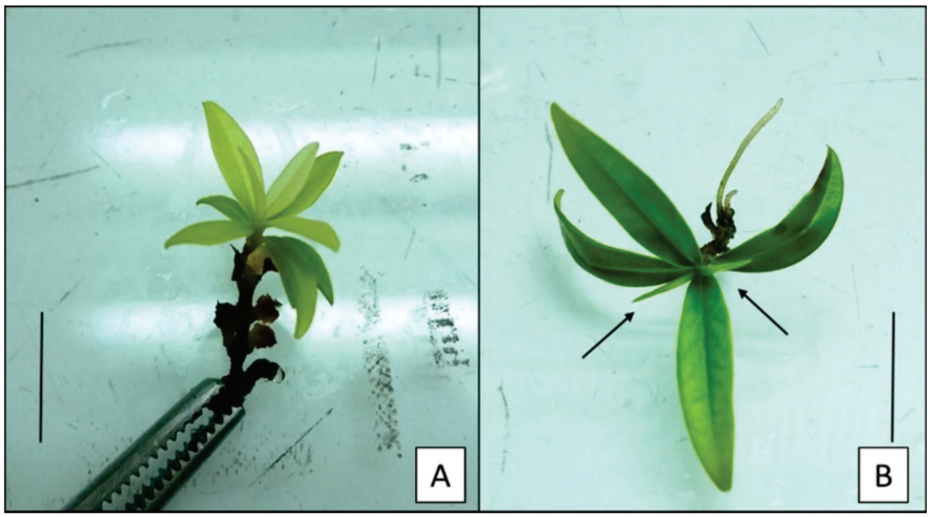

Figure 3: Leaf emission in *E. involucrata* explants after 60 days of *in vitro* culture on the ½MS nutritive medium as a function of different 3-indolebutyric acid (IBA) concentrations, regardless 6-benzylaminopurine (BAP) concentrations. A. Several leaves emitted in the absence of phytoregulators. B. The aspect of leaf formation in the presence of IBA (30 µM) (highlighted by the arrows). Bar = 1 cm.

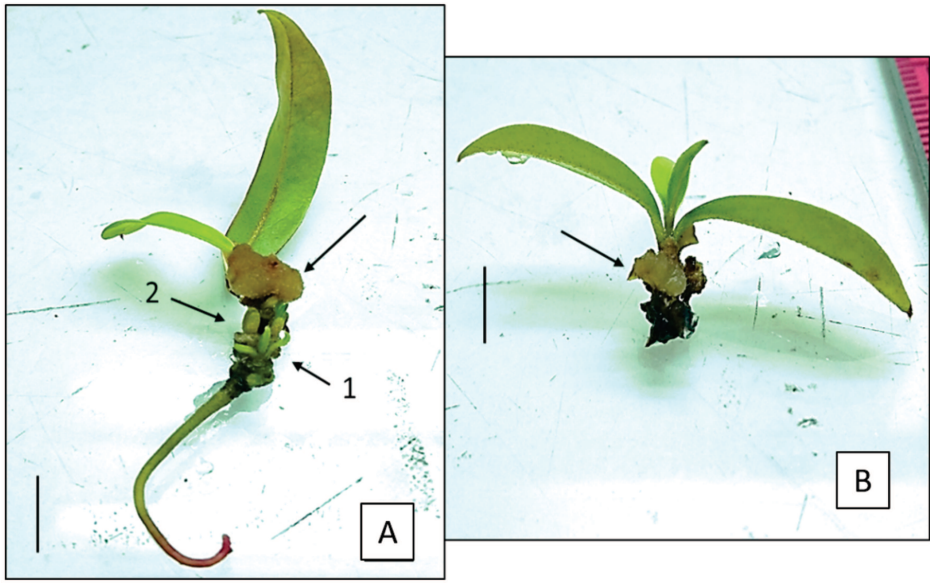

Figure 4: Callus formation in *E. involucrata* after 60 days of *in vitro* culture on the ½MS nutritive medium as a function of different 3-indolebutyric acid (IBA) concentrations, regardless 6-benzylaminopurine (BAP) concentrations. A. Callus formation in the presence of IBA (20 µM) (highlighted by the arrow), shoot emission (arrow 1) and adventitious bud formation (arrow 2). B. The aspect of callus formation in the presence of IBA (30 µM) (highlighted by the arrow). Bar = 1 cm.

rhizogenesis as well as on stimulating the formation of leaves, axillary or apical buds, embryos and calluses (Cid & Teixeira, 2010). Leaf production at the multiplication stage is crucial for shoot production since new shoots rise from buds formed at the insertion point of leaves on the stem, consequently, increasing seedling production (Costa *et al*., 2010). Similar results were obtained with *Rubus* sp. in which the highest number of leaves was obtained in the absence of BAP phytoregulators (Toledo & Biasi, 2018) and in *Cyrtopodium saintlegerianum*, in which the use of IBA in the nutritive medium promoted a decrease in number of leaves (Silva *et al*., 2013).

For callus formation, the model with the best fit was linear (Figure 1C), it being observed an increase in callogenesis when IBA concentration increased. It should be clarified that callus formation did not hinder explant development, instead, allowed simultaneous emission of shoots and adventitious buds (Figures 4A and 4B). Callus formation may be promoted by the balance of endogenous cytokinins and the supplemented IBA auxin in the nutrient medium. Auxins can stimulate various physiological responses in plants, such as callus induction (Cid & Teixeira, 2010), as it was observed in the present study, where the higher concentrations of auxin combined with low concentrations of cytokine resulted in a balance favoring callogenesis. Similar results were observed by Fermino-Júnior & Scherwinski-Pereira (2012) in *Amburana acreana* explants, in which the use of 1 and 2 mg L^{-1} of IBA promoted callus formation whereas its absence and 0.5 mg L^{-1} did not promote.

Regarding the multiplication rate, 65 new buds were formed at the end of the cultivation period, that it can be considered a favorable rate for the *in vitro* multiplication of *E. involucrata*. Micropropagated woody species generally have a low multiplication rate as observed in *Libidibia ferrea*, which the highest rate (27.75) was obtained when the explants were grown in the presence of 3.96 µM of BAP (Silva, 2019). Although BAP is one of the most widely used phytoregulators to increase the *in vitro* multiplication rate, this cytokinin does not always have a positive effect for all species (Vidal *et al*., 2013), a statement that corroborates with the data obtained in the present study with *E. involucrata*. This result confirms therefore the importance of adjusting the propagation protocols in order to increase the mass production of plants (Silva, 2019). Multiplication rate estimates the growth and development capacity of plant material, being it an important variable to evaluate the performance of

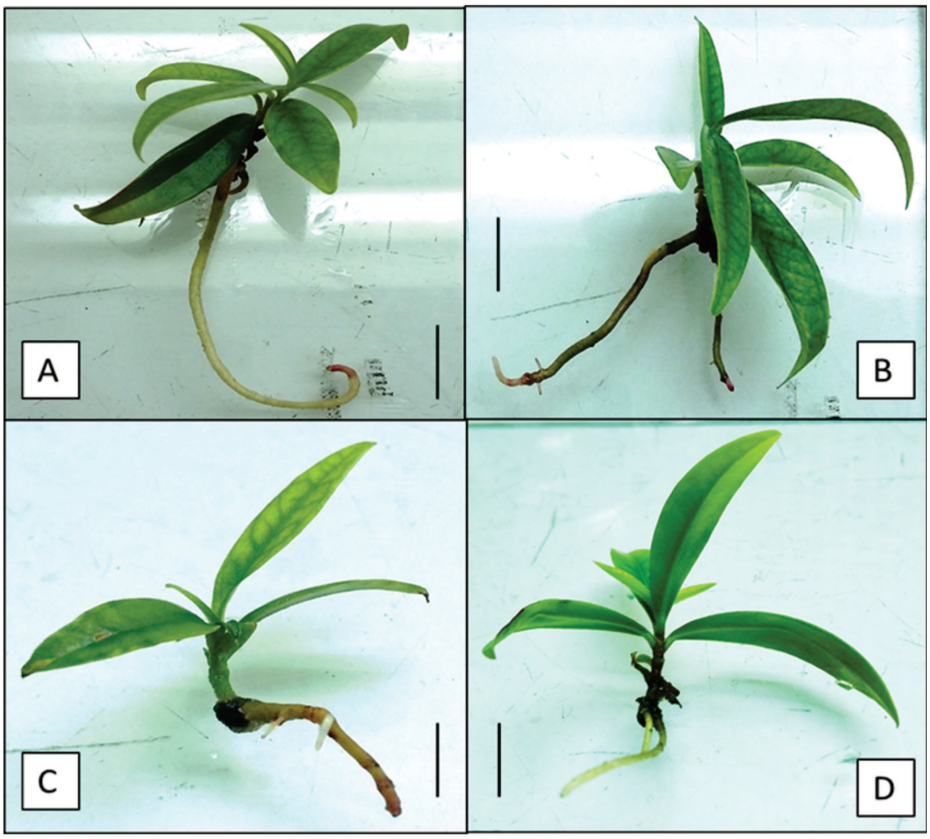

Figure 5: Number of roots in *E. involucrata* after 60 days of *in vitro* culture on the ½MS nutritive medium as a function of different 3-indolebutyric acid (IBA) concentrations, regardless 6-benzylaminopurine (BAP) concentrations. A. Root emission in the absence of IBA. B. Aspect of root formation in the presence of 10µM of IBA. C. Aspect of root formation in the presence of 20µM of IBA. D. Aspect of root formation in the presence of 30µM of IBA. Bar = 1 cm.

micropropagation (Santiago, 2011), especially when it comes to native forest species which most studies do not evaluate this variable.

With regard to the number of roots, the model with the best fit was linear (Figure 1D). There was observed a decrease in the number of roots when IBA concentration increased, indicating a possible inhibitory effect of this fitoregulator (Figure 5). Probably, the supplementation with higher IBA concentrations alters the hormonal balance, disfavoring the auxins and, consequently, the rhizogenesis.

Similar result was obtained in *Miconia ligustroides* (Prudente *et al*., 2016b), where *in vitro* rhizogenesis induction occurred without exogenous auxin supplementation to the medium. Auxins induce rhizogenesis, but do not yield universal responses and some species do not root, even in the presence of this fitoregulator (Hartmann *et al*., 2011), as observed in our study. Despite we obtained the highest averages of rhizogenesis without adding auxin, these averages are very low (0.55) (Figure 1D), confirming the existence of rooting recalcitrance in these species.

CONCLUSIONS

In view of the above, we conclude that BAP and IBA are dispensable for *in vitro* multiplication of *E. involucrata*, and IBA alone, at concentrations 10, 20 or 30 μM, favours the callogenesis, negatively affects leaves, shoots and roots formation.

A high multiplication rate was obtained when considering *in vitro* bud formation.

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