

COMBINATION OF NAA AND TDZ FOR *In Vitro* MULTIPLICATION OF *Eugenia involucrata* DC¹

Diego Pascoal Golle², Lia Rejane Silveira Reiniger^{3*}, Charlene Moro Stefanel⁴, Marlove Fátima Brião Muniz⁵ and Karol Buuron da Silva⁴

¹ Received on 20.04.2015 accepted for publication on 04.10.2017.

² Universidade de Cruz Alta, Pró-Reitoria de Pós-Graduação, Cruz Alta, Rio Grande do Sul - Brasil. E-mail: <diego.golle@gmail.com>.

³ Universidade Federal de Santa Maria, Departamento de Fitotecnia, Santa Maria, Rio Grande do Sul - Brasil. E-mail: <liarsr@ufsm.br>.

⁴ Universidade Federal de Santa Maria, Programa de Pós-Graduação em Engenharia Florestal, Santa Maria, Rio Grande do Sul - Brasil. E-mail: <charlenestefanel@yahoo.com.br> and <karolbuuron@hotmail.com>.

⁵ Universidade Federal de Santa Maria, Departamento de Defesa Fitossanitária, Santa Maria, Rio Grande do Sul - Brasil. E-mail: <marlovenuniz@yahoo.com.br>.

*Corresponding author.

ABSTRACT – *Eugenia involucrata* DC. (Myrtaceae), an economically important forest tree species, is prized for its timber and fruits, and is also an important ornamental plant. This study aimed to evaluate the effect of Thidiazuron (TDZ) and α -Naphthaleneacetic acid (NAA) on *in vitro* multiplication of nodal segments of *E. involucrata*. We tested the effect of the absence and presence of NAA (0.5 μ M), combined with TDZ at concentrations of 0, 2, 4, 8, 16, or 32 μ M, on the *in vitro* multiplication of *E. involucrata*. The use of TDZ combined with NAA (0.5 μ M) favored the formation of shoots and buds in the explants, especially at 32 μ M TDZ concentration. Intermediate concentrations of TDZ also promoted shoot formation but induced hyperhydricity in the explants. It is possible to induce organogenesis leading to the multiplication of *E. involucrata* nodal segments using TDZ, preferably combined with NAA.

Keywords: *Micropropagation; Adventitious buds; Organogenesis.*

COMBINAÇÕES DE ANA E TDZ NA MULTIPLICAÇÃO *In Vitro* DE *Eugenia involucrata* DC

RESUMO – *Eugenia involucrata* DC. (Myrtaceae) é uma espécie florestal com potencialidade econômica, para uso madeireiro, frutícola e ornamental. Este trabalho objetivou avaliar o efeito de Thidiazuron (TDZ) e Ácido α -Nafialeno Acético (ANA) sobre a multiplicação *in vitro* de segmentos nodais de *E. involucrata*. Foi testada a ausência e a presença de ANA (a 0,5 μ M) combinadas ao TDZ nas concentrações 0, 2, 4, 8, 16 ou 32 μ M sobre algumas variáveis da multiplicação *in vitro* de *E. involucrata*. O uso de TDZ combinado a ANA (a 0,5 μ M) favoreceu a emissão de brotações e de gemas nos explantes, especialmente a 32 μ M. Concentrações intermediárias de TDZ também promoveram brotações, mas induziram à ocorrência de hiperhidricidade nos explantes. É possível multiplicar e induzir segmentos nodais de *E. involucrata* à organogênese na presença de TDZ, preferencialmente combinado com ANA.

Palavras-Chave: *Micropropagação; Gemas adventícias; Organogênese.*



1. INTRODUCTION

Eugenia involucrata DC. belongs to the family Myrtaceae, and naturally occurs in several Brazilian states, ranging from Rio Grande do Sul to Minas Gerais. It has medicinal properties and economic potential as a source of timber and fruits, and has substantial ornamental use as well. However, *E. involucrata* propagation is hindered because its seeds are recalcitrant, that is, they lose viability when stored (Backes and Irgang, 2002; Carvalho, 2008; Lorenzi, 2008).

Similar to other forest tree species, *E. involucrata* requires a long time to reach reproductive and phenotypic maturity. Crossing processes are also arduous, limiting eventual plant breeding programs (Tzfira et al., 1998; Resende, 2001). A possible alternative to speed up these programs consists of the use of tissue culture techniques that have the advantages of possible *in vitro* conservation of the germplasm, propagation of superior clones, production of pathogen-free cultures, and patenting of biotechnological processes and materials. Furthermore, *in vitro* cultures may be the source material for other procedures, such as genetic transformation (Xavier et al., 2007).

Optimization of *in vitro* propagation from shoots may be applied to fast clonal propagation of *E. involucrata* seedlings, thereby hastening the multiplication of individuals with essential agronomic characteristics for use in plant breeding programs. Micropropagation of woody species has been studied through the evaluation of physiological responses in the presence of several types of exogenous cytokines. The most frequently used micropropagation protocols include 6-benzylaminopurine (BAP). However, kinetin (KIN) and Thidiazuron (TDZ) have shown promising results as well (Oliveira et al., 2013). Cytokines are often combined with auxins, and have been used to produce propagules by means of *in vitro* multiplication (Hinojosa, 2000).

Auxins induce cell division and control cell growth and elongation (Li et al., 2010), while cytokines promote cell division and adventitious bud formation (Barrueto, 2000). TDZ is a cytokine frequently used in the tissue culture of woody species. TDZ is usually required at higher concentrations than other cytokines, such as aminopurines (Sujatha and Hazra, 2007).

The objective of the present work was to evaluate the *in vitro* multiplication of *E. involucrata* using

different phytohormone combinations in the presence or absence of α -Naphthaleneacetic acid (NAA) and distinct TDZ concentrations.

2. MATERIAL AND METHODS

The experiment was carried out in the Laboratory of Plant Tissue Culture at the Nucleus of Biotechnology and Plant Breeding, Department of Crop Plants, Federal University of Santa Maria (UFSM), Rio Grande do Sul. Parent plants were grown in a greenhouse, and at 36-months old they were used for explant isolation. These plants were trimmed approximately four weeks before the explants were collected for the production of young shoots and kept in vases (depth – 22 cm, diameter – 24 cm, volume approximately 8 L) filled with the Plantmax[®] substrate. Plants were irrigated daily, and every 15 days, 400 mL of a 1 g L⁻¹ solution containing nitrogen, phosphorus, and potassium (N-P-K: 5-20-20) were added to the substrate, which was supplemented with 400 mL at 1 g L⁻¹ N (urea) once a week. One day before the weekly collection of the explants, the plants were sprayed until saturation with a solution containing 1 g L⁻¹ Cercobin700PM[®] (Thiophanate-methyl) and 0.1 g L⁻¹ streptomycin sulfate. This procedure was a pre-treatment that facilitated surface disinfection of the explants in the laboratory.

Young branches with a green color and no woody areas present between the third and fourth nodal segments were selected for the experiment. The most apical segment was discarded, and the most basal segment was also discarded if it appeared woody or more developed than the other parts. Some of the leaves were removed upon collection, and the segments were placed in distilled water with 1 g L⁻¹ Benlate 500[®] (Benomyl) and 0.1 g L⁻¹ streptomycin sulfate for approximately 30 min. In the laboratory, the branches were washed for 5 min under running water with a commercial detergent using a sponge. The nodal segments were cut in a laminar flow chamber and subsequently subjected to surface disinfection as follows: immersion in 70% (v/v) ethanol for 30 s, a wash with distilled water, agitation in a 1.5% (v/v) sodium hypochlorite solution (NaOCl) for 15 min, immersion in a 0.05% (w/v) mercury bichloride (HgCl₂) solution for 10 min, a wash with sterile water, and immersion for 10 min in a 1.5% (v/v) NaOCl solution with three drops of commercial detergent added to break the surface tension. Finally, the material was rinsed three times with sterile water.

After the last rinse, the explants remained in a sterile water solution containing 100 mg L⁻¹ ascorbic acid. Leaf remains were removed from the nodal segments, which were then cut 0.3 cm above and 0.8 cm below the node. Bevel cuts were performed in the basal region to increase the surface area in contact with the nutritive medium.

The experiment was conducted with a completely randomized design in a 2 × 6 factorial array. The levels of the “A” factor were defined by the absence or presence (at 0.5 μM) of α-NAA, while the levels of the factor “B” referred to different TDZ concentrations (0, 2, 4, 8, 16, or 32 μM). Eight replicates were performed with two explants each, for a total of 96 experimental units and 192 inoculated nodal segments. Each experimental unit consisted of a 150-mL glass flask containing 30 mL MS nutritive medium (Murashige and Skoog, 1962) with half the normal salt concentration (½ MS), 30 g L⁻¹ sucrose, 50 mg L⁻¹ myoinositol, and 7 g L⁻¹ agar. The pH of the medium was adjusted to 5.8 after supplementation with phytohormones but before the addition of agar. Flasks were covered with aluminum foil and autoclaved at 121 °C and 1.5 atm for 20 min.

After 60 days of culture, the following variables were evaluated: average number of shoots per explant, average number of buds produced per explant (counting only the adventitious buds generated by tissue organogenesis and not for the pre-existing buds frequently found in nodal segments), average number of leaves per explant, the percentage (%) of nodal segments forming calli at the base, and hyperhydricity (%).

The data were tested for normality using the Kolmogorov-Smirnov test and for homogeneity using Bartlett’s test, and, when required, transformed using the following equation: $\sqrt{x + 0,5}$. The data were then subjected to an analysis of variance. The qualitative analyses were carried out using Tukey’s test at a 5% significance level, while the quantitative analyses were carried out by polynomial regression. All statistical analyses were performed using the software SISVAR (Ferreira, 2014).

3. RESULTS

There was a significant effect of the interaction ($p = 0.0021$) between the phytohormones tested on the variable number of shoots produced per explant (Figure 1). In the absence of NAA, it was not possible to adjust

the behavior of the explants to a polynomial equation. The highest average number of shoots produced per explant (1.95) occurred in the absence of TDZ. Nevertheless, the combination of different TDZ concentrations with 0.5 μM NAA allowed for the fit at a quadratic equation. In the absence of TDZ, the number of shoots was higher than that observed for most of the concentrations tested, with the lowest value, according to calculations, associated with 11.62 μM TDZ. However, above that value, the number of shoots increased with increasing TDZ concentration, reaching a maximum at 32 μM TDZ when an average of 2.0 shoots were produced per explant.

Regarding the formation of adventitious buds (Figure 2), there was again a significant effect of interaction ($p = 0.0047$) between the phytohormones tested. In the absence of NAA and at TDZ concentrations of 0, 2, 4, or 8 μM, no buds were produced. Buds were observed only at 16 (0.28) and 32 μM (2.83) TDZ. Bud

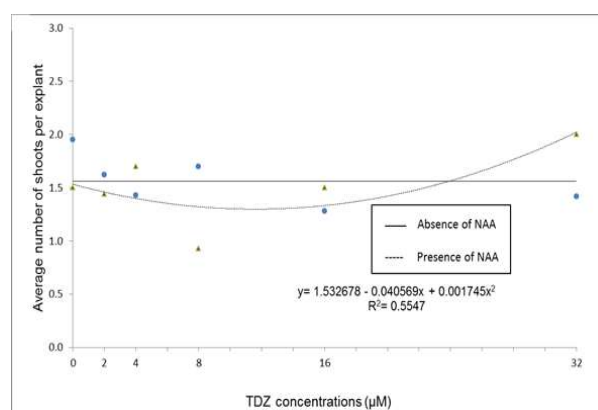


Figure 1 – Average number of shoots produced in nodal segments of *Eugenia involucrata* DC. as a function of the interaction between the absence or presence of α-Naphthaleneacetic acid (NAA; at 0.5 μM) and Thidiazuron (TDZ) at 0, 2, 4, 8, 16, or 32 μM after 60 days of *in vitro* culture on ½MS nutritive medium. Santa Maria, RS, UFSM, 2010.

Figura 1 – Média de brotações emitidas em segmentos nodais de *Eugenia involucrata* DC. em função da interação entre a ausência ou presença (a 0,5 μM) de Ácido α-Naftalenoacético (ANA) e concentrações 0, 2, 4, 8, 16 ou 32 μM de Thidiazuron (TDZ), aos 60 dias de cultivo *in vitro* em meio nutritivo MS, cuja concentração de sais foi reduzida à metade (½MS). Santa Maria, RS, UFSM, 2010.

formation in the absence of NAA fit a linear model, with the production of buds increasing with increasing TDZ concentration. The use of 0.5 μM NAA resulted in a positive linear relationship between variable bud formation and the increase in the number of buds per explant following increase in TDZ concentrations. No buds were produced at TDZ concentrations of 0, 2, and 4 μM , but were produced only at 8 (0.67), 16 (1.57), and 32 μM (2.50) of TDZ.

Similarly, there was a significant interaction ($p=0.0259$) between phytohormones tested and the number of leaves produced per explant (Figure 3). In the absence of NAA, the variable fitted a quadratic behavior, with a reduction in the number of leaves per explant at lower TDZ concentrations, reaching the lowest value at 16 μM (1.66). According to the calculated value for the vertex of the equation, the fewest number of leaves would have been produced at a TDZ concentration of 16.33 μM . In the presence of 0.5 μM NAA it was not possible to adjust the values observed to any mathematical model. The highest values for the number of leaves per explant occurred at TDZ concentrations of 0 (2.22), 16 (2.08), and 32 (2.08) μM ,

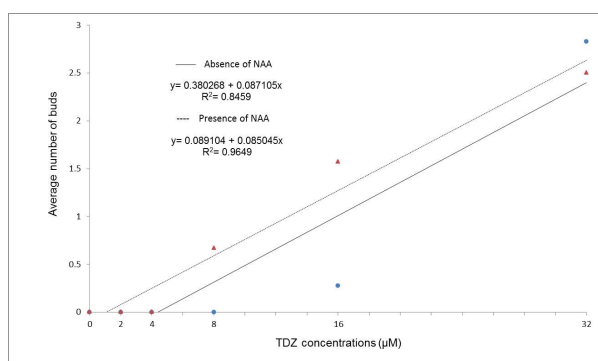


Figure 2 – Average number of adventitious buds produced in the nodal segments of *Eugenia involucrata* DC. as a function of the interaction between the absence or presence of α -Naphthaleneacetic acid (NAA; at 0.5 μM) and Thidiazuron (TDZ) at 0, 2, 4, 8, 16, or 32 μM after 60 days of *in vitro* culture on $\frac{1}{2}$ MS nutritive medium. Santa Maria, RS, UFSM, 2010.

Figura 2 – Média de gemas adventícias emitidas em segmentos nodais de *Eugenia involucrata* DC. em função da interação entre a ausência ou presença (a 0,5 μM) de Ácido α -Naftalenoacético (ANA) e concentrações 0, 2, 4, 8, 16 ou 32 μM de Thidiazuron (TDZ), após 60 dias de cultivo *in vitro* em meio nutritivo MS, cuja concentração de sais foi reduzida à metade ($\frac{1}{2}$ MS). Santa Maria, RS, UFSM, 2010.

whereas the lowest values were observed at 2 (1.59), 4 (1.85) and 8 (1.32) μM of TDZ.

Regarding the formation of calli, which were particularly evident at the base of the explants, there was no significant interaction between the phytohormones tested and calli formation. Nevertheless, changes occurred ($p=0.0004$) in response to different TDZ concentrations in a quadratic model (Figure 4). In the absence of TDZ, a lower percentage of explants (34.37%) formed calli. This value was higher at TDZ concentrations of 2 (54.12%), 4 (79.28%), and 8 μM (81.25%). According to the calculated vertex of the equation, the TDZ concentration that resulted in the highest percentage of callus formation was 16.21 μM . However, reduced values for calli formation were observed at 16 μM (63.37%) and 32 μM (53.12%) of TDZ.

A significant interaction ($p=0.0000$) between the phytohormones tested and explant hyperhydricity was found (Figure 5). In the absence of NAA, hyperhydricity displayed quadratic behavior as a function of the TDZ concentration. The highest percentage of hyperhydricity (40%) occurred at 8 μM TDZ. According to the calculated value for the vertex of the polynomial equation, that percentage would have reached a maximum at 15.70

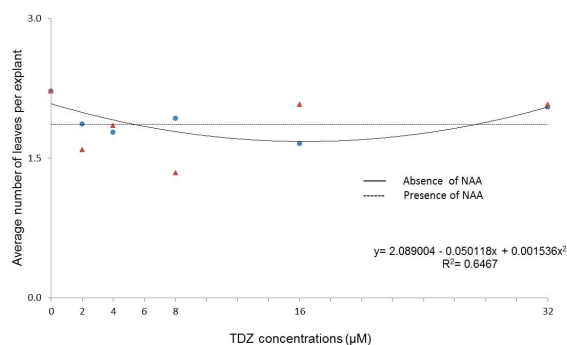


Figure 3 – Average number of leaves produced on nodal segments of *Eugenia involucrata* DC. as a function of the interaction between the absence or presence of α -Naphthaleneacetic acid (NAA; at 0.5 μM) and Thidiazuron (TDZ) at 0, 2, 4, 8, 16, or 32 μM after 60 days of *in vitro* culture on $\frac{1}{2}$ MS nutritive medium. Santa Maria, RS, UFSM, 2010.

Figura 3 – Média de folhas emitidas em segmentos nodais de *Eugenia involucrata* DC. em função da interação entre a ausência ou presença (a 0,5 μM) de Ácido α -Naftalenoacético (ANA) e concentrações 0, 2, 4, 8, 16 ou 32 μM de Thidiazuron (TDZ), após 60 dias de cultivo *in vitro*, em meio nutritivo MS, cuja concentração de sais foi reduzida à metade ($\frac{1}{2}$ MS). Santa Maria, RS, UFSM, 2010.

μM . Above this concentration, callus formation tends to decrease. In the presence of NAA, the resulting hyperhydricity was 9.05%, and it was not possible to adjust the values to any mathematical model.

4. DISCUSSION

Regarding the number of variable shoots produced by explants, high TDZ concentrations combined with NAA promoted bud formation. The initial reduction in the number of buds produced is potentially associated with the swelling of the budding regions of the explants—the location of the dormant buds—and increasing TDZ concentrations. That decrease occurred mostly in the interval between the TDZ concentrations of 2 and 16 μM . However, the highest concentration provided the required stimulus to increase the number of buds produced per explant.

We can infer that at the lowest concentrations the balance between auxin and cytokine was not adequate for the formation of shoots, since in addition to the exogenous source, it is highly likely that the explants had sufficient endogenous levels of auxin to balance the exogenous cytokine, favoring tumescence. However, the elevated cytokine levels promoted shoot production.

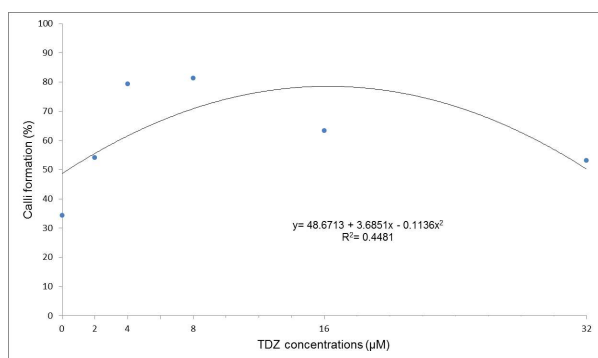


Figure 4 – Average percentage of *Eugenia involucrata* DC. nodal segments forming calli as a function of Thidiazuron (TDZ) concentration (0, 2, 4, 8, 16, or 32 μM), regardless of the absence or presence of α -Naphthaleneacetic acid (NAA; at 0.5 μM) after 60 days of *in vitro* culture on $\frac{1}{2}$ MS nutritive medium. Santa Maria, RS, UFSM, 2010.

Figura 4 – Porcentagem média de formação calogênica em segmentos nodais de *Eugenia involucrata* DC. em função das concentrações 0, 2, 4, 8, 16 ou 32 μM de Thidiazuron (TDZ), independentemente da ausência ou presença (a 0,5 μM) de Ácido α -Naftalenoacético (ANA), após 60 dias de cultivo *in vitro* em meio nutritivo MS, cuja concentração de sais foi reduzida à metade ($\frac{1}{2}$ MS). Santa Maria, RS, UFSM, 2010.

Similar observations were made by Faisal et al. (2005) in *Rauvolfia tetraphylla*, in which there was an increase in the number of shoots produced by nodal segments due to the supplementation of the nutritive medium with TDZ. However, the intermediate concentrations were more propitious to multiplication than the highest concentration tested, in contrast with the results of the present study with *E. involucrata*. However, the highest concentration tested on *R. tetraphylla* did not correspond to even one third of the concentration that best promoted *E. involucrata* multiplication.

The largest number of shoots regenerated with TDZ in *Tectona grandis* were observed at a concentration of 8.8 μM (Fermino Júnior et al., 2014). TDZ was used in the *in vitro* multiplication of *T. grandis* shoots (Akram and Aftab, 2008), and was the most efficient in the protocol proposed by Kozgar and Shahzad (2012), followed by CIN and BAP. TDZ also promoted the highest multiplication rates for *Cassia angustifolia* nodal segments, both alone and in combination with NAA or Indolacetic Acid (IAA) (Siddique and Anis, 2007). Similarly, the positive effect of TDZ was evident on

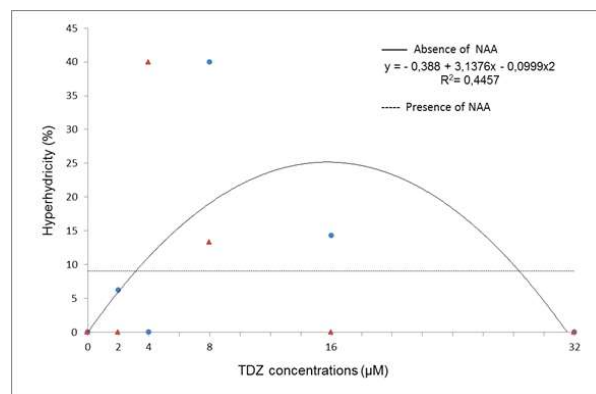


Figure 5 – Average percentage of hyperhydricity in nodal segments of *Eugenia involucrata* DC. as a function of the interaction between the absence or presence of α -Naphthaleneacetic acid (NAA; at 0.5 μM) and Thidiazuron (TDZ) at 0, 2, 4, 8, 16, or 32 μM after 60 days of *in vitro* culture on $\frac{1}{2}$ MS nutritive medium. Santa Maria, RS, UFSM, 2010.

Figura 5 – Porcentagem de hiperhidricidade em segmentos nodais de *Eugenia involucrata* DC. em função da interação entre a ausência ou presença (a 0,5 μM) de Ácido α -Naftalenoacético (ANA) e concentrações 0, 2, 4, 8, 16 ou 32 μM de (Thidiazuron) TDZ, após 60 dias de cultivo *in vitro*, em meio nutritivo MS, cuja concentração de sais foi reduzida à metade ($\frac{1}{2}$ MS). Santa Maria, RS, UFSM, 2010.

the multiplication of *Balanites aegyptiaca* (Siddique and Anis, 2009). In this case, the highest TDZ concentration tested was 13.6 μM , well below the optimal concentration of 32 μM for *E. involucrata*, according to the results of the present study.

With respect to the presence of adventitious buds, there was a synergism between NAA and TDZ. Similar to what we observed for *E. involucrata*, an increase in the TDZ concentration in the nutritive medium induced bud formation in *Pinus pinea* (Cortizo et al., 2009). In *Ricinus communis*, bud induction was evident when TDZ was used at a concentration of 2.5 mg L^{-1} (Sousa, 2013).

Fewer leaves were produced in the absence of the phytohormones as well as in the presence of TDZ alone, either at 16 or 32 μM . At the tested concentrations, the combined use of NAA and TDZ was not effective in promoting the production of *E. involucrata* leaves. In the *in vitro* culture of *Caesalpinia pyramidalis*, the largest number of leaves was also observed when no phytohormones were added to the nutritive medium. However, the smallest averages were observed when higher TDZ concentrations were used (Silva et al., 2013). Likewise, in *Thymus vulgaris*, the largest number of leaves was produced at an NAA concentration of 2.7 μM (Rubin et al., 2007).

The number of leaves plays an important role during *in vitro* multiplication, since it is at the insertion between the stem and leaf that buds are formed, thus potentially producing new shoots and increasing the production of new seedlings (Costa et al., 2010). Additionally, plants with a larger number of leaves have better chances of survival in nature, as leaves are responsible for utilizing sunlight to fix organic matter through photosynthesis (Moreira et al., 2006).

Callus formation was higher in the presence of phytohormones. The supplementation of the nutritive medium with these compounds often results in callus induction in *E. involucrata* (Golle, 2010) and other species, such as *Morinda citrifolia* (Baque et al., 2010), *Hyptis leucocephala* (Pereira et al., 2012), *Litchi chinensis* (Ma et al., 2009), and *Passiûora cincinnata* (Silva et al., 2009; Pinto et al., 2010).

Besides phytohormones, the type of explant affects callus formation, as observed with *E. involucrata* in another study, in which leaf discs showed a higher percentage of callogenesis (Golle, 2010) compared with

apical and nodal segments (Golle and Reiniger, 2013). Nevertheless, in the present work, in which we aimed to evaluate the multiplication of *E. involucrata* explants, callus formation was not desired.

In general, hyperhydricity did not occur as is typical; the low development of the explants led to the vitreous aspects of the leaves produced and they easily broke upon touching. Hyperhydricity was observed at intermediate TDZ concentrations. However, as the concentration increased, this phenomenon no longer appeared in the cultures. The observed hyperhydricity may have been caused by factors other than the presence of TDZ in the culture medium, such as stress conditions (Saher et al., 2004); the high humidity characteristic of *in vitro* cultures; nutritional conditions, such as mineral and carbohydrate levels; and low luminosity (Majada et al., 2000). Nevertheless, it must be noted that high levels of phytohormones are the most frequent cause of plant physiological disorders, such as hyperhydricity (Majada et al., 2000).

Hyperhydricity induced by cytokines may be more difficult to reverse, particularly in cases where the phytohormone is required for proliferation (Palma et al., 2011). The presence of TDZ caused low shoot regeneration and high hyperhydricity in *Aloe polyphylla*, and this effect seemed to be species-specific (Ivanova and Staden, 2011). In *Gypsophila paniculata*, the highest percentage of hyperhydricity (49.5%) occurred in the absence of NAA (Quoirin et al., 2008).

The use of certain cytokines, especially TDZ, may cause hyperhydricity (Barrueto, 2000). Among the cytokines whose effects on multiplication were tested, TDZ induced the highest rates of hyperhydricity in *Pyrus pyrifolia* plants cultivated *in vitro* (Kadota and Niimi, 2003). Bosela (2009) observed that this phenomenon appeared in several *Populus* hybrids when the nutritive medium was supplemented with antibiotics, which induced a significantly higher rate of hyperhydricity when combined with TDZ.

5. CONCLUSION

The highest number of shoots per explant and the average number of adventitious buds is observed with the combined use of 0.5 μM NAA and 32 μM TDZ.

The lowest percentage of callus formation occurs in the absence of phytohormones. However, the presence of calli does not affect plant development *in vitro*.

No hyperhydricity is observed at TDZ concentrations associated with the highest averages of shoot and bud formation.

6. ACKNOWLEDGEMENTS

We thank CNPq for the fellowships awarded.

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