

Callogenesis in Stem Explants of *Eucalyptus grandis* (Hill ex Maiden) Showing Resilience in Oxide Calluses

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ABSTRACT

Callogenesis was achieved using growth regulators at different concentrations to obtain the best characteristics regarding the calluses texture aspect with the main objective to produce friable calluses from stem explants of *Eucalyptus grandis*. Stem segments of approximately 1 cm were excised from 30-day seedlings. The treatments were prepared based on the MS medium added with TDZ alone or combined with AIA; 2,4-D; ANA. Each treatment consisted of five repetitions with five explants per plate. After 210 days of *in vitro* culture, the calluses were evaluated visually and by manipulation for texture. The explants that did not produce calluses were those exposed to treatments with 2,4-D 50.0 μM ; TDZ 3.0 μM and in absence of a growth regulator (control). The best protocols for maintenance of friable calluses of stem explants of *Eucalyptus grandis* under the conditions performed in this work were those using only TDZ.

Keywords: stem explants, protocols, friable calluses.

Calogênese em Explantes Caulinares de *Eucalyptus grandis* (Hill ex Maiden) Mostrando Resiliência em Calos Oxidados

RESUMO

A calogênese foi efetuada utilizando-se reguladores de crescimento em diferentes concentrações para obtenção das melhores características em relação ao aspecto da textura dos calos, com o objetivo principal de produzir calos friáveis de explantes caulinares de *Eucalyptus grandis*. Segmentos caulinares de aproximadamente um centímetro foram excisados de mudas de 30 dias. Os tratamentos foram elaborados tendo como base o meio MS adicionado de TDZ isolado ou combinado com AIA, 2,4-D, ANA. Cada tratamento constou de cinco repetições, com cinco explantes por placa. Os calos foram avaliados visualmente e por manipulação quanto à textura, após 210 dias de cultivo *in vitro*. Os explantes que não produziram calos foram aqueles expostos a tratamentos com 50,0 μM de 2,4-D e 3,0 μM de TDZ, e em ausência de regulador de crescimento (controle). Os melhores protocolos para manutenção de calos friáveis de explantes caulinares de *Eucalyptus grandis*, nas condições realizadas neste trabalho, foram os que utilizaram apenas TDZ.

Palavras-chave: explantes caulinares, protocolos, calos friáveis.

1. INTRODUCTION

Plant biotechnology has arisen with the purpose of trying to solve agricultural problems under a new optics, using in vitro methodology when necessary, in vegetable production, in terms of quantity and quality (Borgatto & Hayashi, 2002). In this context, the genetic potential of plants can be explored by a wide range of in vitro techniques.

Callogenesis, an indirect process usually used for plant production, is among these techniques. In this work, callogenesis was used for posterior cell suspension culture. However, most studies on vegetable tissue culture address the development of protocols for the regeneration of whole plants from cells, calluses and protoplast (Chen et al., 2000).

Aiming to develop a vanilla regeneration protocol (*Vanilla planifolia* Andr), Janarthanam & Seshadri (2008) experimented with two types of explants, leaf and nodal segment, for calluses induction. They found that the best results were recorded in leaf explants, with maximum production obtained when the culture medium was supplemented with 2,4-D 4.52 mM and BAP 2.22 mM, and that the explants cultured in the absence of these growth regulators did not form calluses in any of the explant types used.

However, as highlighted by Smith & Krikorian (1990), there is a possibility that, at the time of callus induction, the plant endogenous hormone synthesis system be activated, resulting in the increase of these hormones levels, allowing cells proliferation and differentiation, even without exogenous implementation with growth regulators.

Growth regulators, synthetic substances similar to the hormones found in plants, have been of great use in vegetable tissues culture, especially for the production of callus and cells used in several biochemical studies, including woody plants.

According to Roberts (1988), many, – if not all physiological processes seem to be ruled by two or more hormone classes (auxin, ethylene, gibberellin, cytokinin) simultaneously, or in any sequential order. The most important classes of vegetable hormones in culturing vegetable tissues are auxins and cytokinins. These hormones are the substances that control the growth and development of vegetable cells cultured in vitro (Kamínek, 1992). When evaluating

the effects of different types of explants, growth regulators, and regeneration on callus induction in garlic (*Allium sativum* L.), Luciani et al. (2006) obtained the best response in meristem explants with dichlorophenoxyacetic acid (2,4-D), which increased the production and quality of calluses.

1-phenyl-3-(1,2,3-thiadiazole-5-yl) urea (TDZ) is a substance similar to cytokinins, and it is considered the most active substance for tissue culture of woody plants (Huetteman & Preece, 1993).

Nevertheless, Venturieri & Venturieri (2004) concluded that TDZ did not produce the expected effects in the induction of calluses for somatic organogenesis or embryogenesis of the cocoa hybrid (*Theobroma cacao*) _ *Theobromagrandiflorum* × *Theobromaobovatum*.

In this work, callus production was achieved using growth regulators at different concentrations for obtaining the best characteristics regarding callus texture, aiming the posterior cell suspension culture of *Eucalyptus grandis*.

The general objective of this work was to produce friable calluses from stem explants of *Eucalyptus grandis*.

2. MATERIAL AND METHODS

2.1. Seed disinfection

Eucalyptus grandis seeds were obtained from the 'Instituto de Pesquisa e Estudos Florestais' (IPEF). They were disinfested by immersion in 100 mL 1% hypochlorite solution containing two Tween 20 drops for 20 minutes under stirring. Then, they were washed five times with deionized water in autoclave for five minutes.

2.2. Seed germination

The disinfested seeds were put in glass flasks (with capacity for 250 mL) containing 25 mL of nutritive medium (MS) (Murashige & Skoog, 1962), supplemented with 0.3% phytigel and 3% sucrose. The flasks containing the culture medium were autoclaved at 121 °C for 20 minutes and exposed to UV radiation for 20 minutes; the medium pH was adjusted to 5.8 before phytigel addition.

Approximately 5 to 10 seeds were inoculated in each flask. The cultures were kept in growth room at 25°C with photoperiod of 16 hours and luminous intensity of 30 mmol m⁻².s⁻¹ during 30 days for germination.

2.3. Callus induction

Stem segments of 1 cm were excised from 30-day seedlings after in vitro germination of the seeds. The explants were inoculated in MS medium containing different concentrations and combinations of cytokinin TDZ and auxins AIA, ANA and 2,4-D; aiming callus induction.

The treatments were prepared based on the MS medium added with TDZ alone or combined with AIA; 2,4-D; ANA (Table 1). In order to minimize possible contaminations, Benomyl (100 mg/L) and Rifampicin (150 mg/L) were added to all treatments after culture medium autoclaving. The explants were inoculated in 90 mm-diameter and 15 mm-height glass Petri dishes containing 40 mL of solid nutritive medium in BOD in darkness regimen at 25°C for 210 days, being sub-cultured every 30 days. Each treatment consisted of five repetitions (five plates per treatment) with five explants per plate.

2.3.1. Treatments

The treatments containing different concentrations of the cytokinin TDZ used alone or in combination with auxins AIA, 2,4-D and ANA, and different concentrations of the auxin 2,4-D used alone, for obtaining calluses in explants from stem segments in *Eucalyptus grandis* are presented in Table 1.

2.4. Evaluation

The calluses were evaluated for texture after 210 days of in vitro culture. The following evaluation criteria were used: I (friable callus - cells loosely attached); II (compact callus - cells strongly attached); III semi-friable callus (callus with intermediate characteristics between friable and compact, i.e., presenting both compact and friable portions).

3. RESULTS AND DISCUSSION

No antioxidant was used throughout the production and maintenance the calluses (210 days). The non-addition of antioxidant to the culture

Table 1. Treatments with different concentrations of growth regulators for callus induction in stem segments of *Eucalyptus grandis*.

Treatments	Auxins (µM)	Cytokinins (µM)
T 01	AIA 15.0	TDZ 2.0
T 02	AIA 30.0	TDZ 2.0
T 03	2,4-D 25.0	TDZ 2.0
T 04	2,4-D 50.0	TDZ 2.0
T 05	ANA 10.0	TDZ 2.0
T 06	ANA 20.0	TDZ 2.0
T 07	-	TDZ 2.0
T 08	-	TDZ 1.0
T 09	-	TDZ 1.5
T 10	-	TDZ 2.5
T 11	-	TDZ 3.0
T 12	ANA 0.11	TDZ 4.4
T 13	ANA 0.7	TDZ 0.7
T 14	ANA 2.69	TDZ 2.27
T 15	2,4-D 4.53	-
T 16	2,4-D 9.06	-
T 17	2,4-D 18.12	-
T 18	2,4-D 0.23	-
T 19	2,4-D 0.45	-
T 20	2,4-D 0.68	-
T 21	-	-

medium did not hamper the initial production of calluses, but their maintenance. Nevertheless, it was possible to verify that some calluses of dark color and compact texture recovered the aspect with the subcultures, regarding color and texture (Figure 1). Callogenesis did not occur in only three of the 21 (twenty-one) treatments (Table 2).

3.1. Texture

The calluses treated with AIA at two different concentrations (15 and 30 μM) and the same TDZ concentration (2 μM) did not present change in texture, both treatments (T 01 and T 02) provided calluses of semi-friable texture (III) (Figures 2 and 3). Nevertheless, callus induction in leaf explant of Greek sage (*Salvia fruticosa* Mill.) was completely inhibited in the absence of AIA and TDZ, and the largest callus was obtained with the combination of AIA 3 μM and TDZ 6.9 μM (KARAM et al., 2003).

In treatments T 03 and T 04 (Table 1), combination of 2,4-D in concentrations of 25 and 50 μM with the same TDZ concentration (2.0 μM), the first combination (25 μM 2,4-D with TDZ 2.0 μM) presented calluses with texture III and when a combination with a higher concentration of 2,4-D (50 μM) was used, there was no callus formation (Figure 4). In the case of calluses obtained from stem explants of Moss-rose (*Portulaca grandiflora* Hook), Rossi-Hassani & Zryd (1995) obtained friable

calluses with kinetin (5 μM) combined with 2,4-D (3 μM).

Therefore, the very high concentration (50 μM) in stem explants of *Eucalyptus grandis* may have been

Table 2. Results of treatments of callus texture after 210 days.

Treatments	Texture
T 01	III
T 02	III
T 03	III
T 04	NF
T 05	I
T 06	III
T 07	I
T 08	I
T 09	II
T 10	I
T 11	NF
T 12	II
T 13	III
T 14	III
T 15	II
T 16	III
T 17	III
T 18	II
T 19	II
T 20	II
T 21	NF

I= friable callus; II= compact callus; III = semi-friable callus; NF = no callus formation.

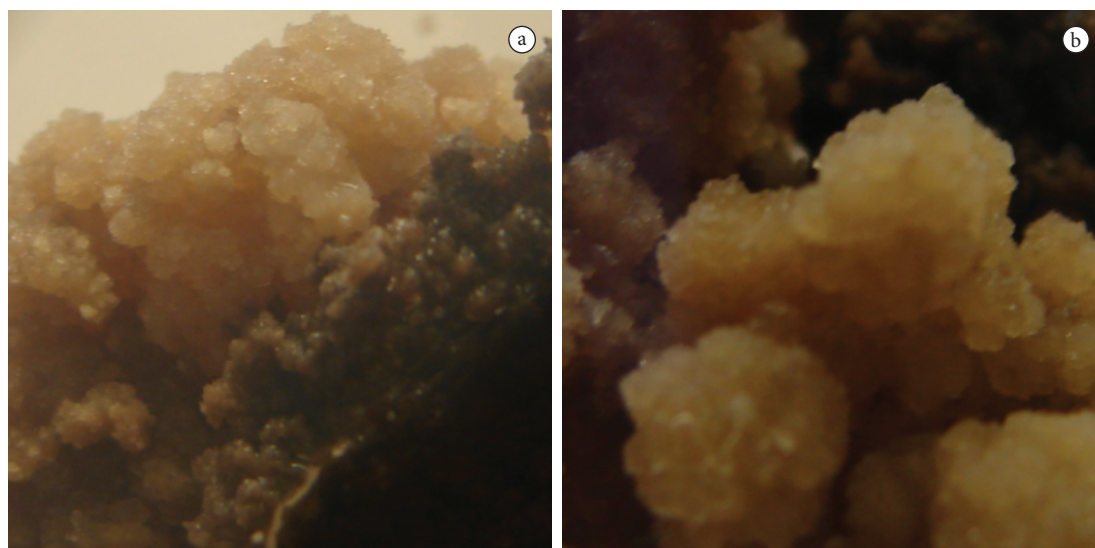


Figure 1. a) Side view of treatment T 05, callus formed in MS medium containing ANA 10 μM and TDZ 2.0 μM , 210 days after in vitro culture; b) Upper view of the same callus showing its much darkened base.

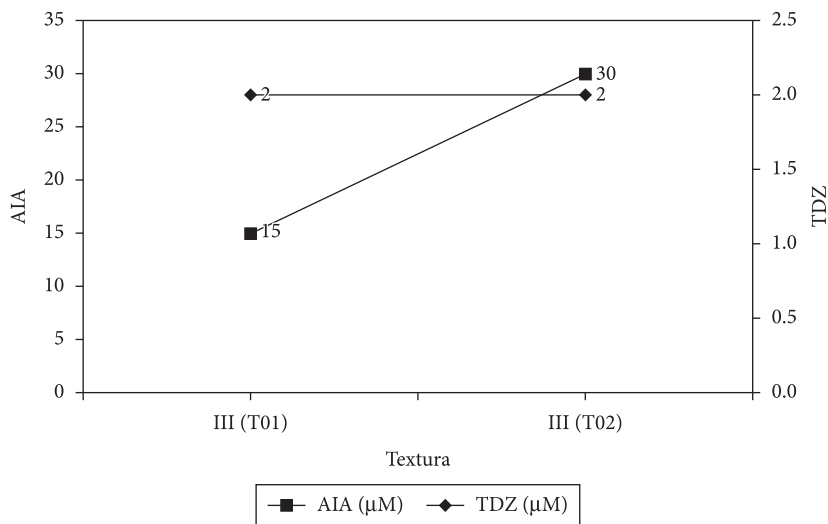


Figure 2. Relation of regulators AIA and TDZ combination in callus texture. Note: III – semifriable callus.

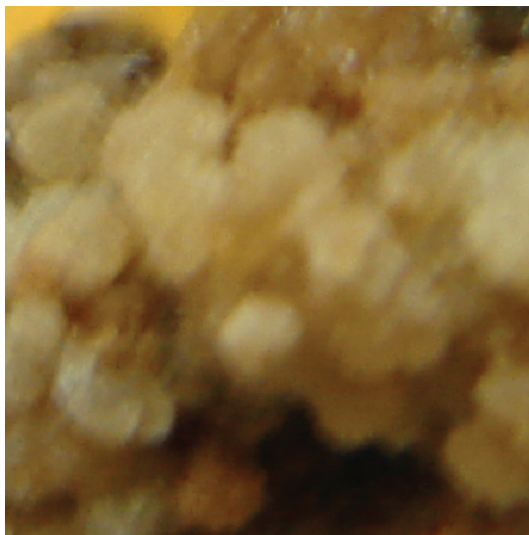


Figure 3. Semi-friable texture callus (treatment T 02).

toxic, preventing formation of calluses. Possibly, the TDZ concentration was not enough to minimize the toxic effect of the extremely high concentration (2,4-D) in treatment T 04. In leaf explants of long pepper *Piper hispidinervum* C.DC.), the medium composed of 2,4-D 5.0 mg.L⁻¹ (22.65 µM) and BAP 10,2 mg.L⁻¹ (45.28 µM) offered the best formation of friable calluses and these results were possibly due to the high concentration of cytokinin used in combination with 2,4-D, which may have annulled the 2,4-D toxic effect (COSTA et al., 2008).

In nodal segments and roots of *Pfaffiatuberosa* (Spreng.) Hiken, higher proliferation of friable

calluses was observed when the nodal segments were treated with BAP 1 mM and 2,4-D 10 mM (FLORES et al., 2006). Häggman et al.(1999), experimenting with *Pinus sylvestris* L., concluded that the best results of callus induction occurred with the use of 2,4-D at the concentrations of 13.6 µM or 9.1 µM combined with BAP at the concentration of 2.2 µM. In dicotyledonous; 2,4-D was also successful (Lee et al., 2001).

With the combination of growth regulators ANA and TDZ, it was possible to observe the three textures in the calluses formed from stem segment explants of *Eucalyptus grandis* after 210 days (Figure 5). Treatments T 06, T 13 and T 14 presented calluses with texture III. The calluses treated with an ANA concentration much lower than the TDZ (T 12) presented compact texture (II). The calluses in T 05 presented texture I, where the explants were exposed to ANA 10 µM combined with TDZ 2 µM. The same reaction was observed in callus derived from okra (*Abelmoschus esculents* L.Moench.) hypocotyl explants inoculated in MS medium supplemented with ANA 2.0 mg.L⁻¹ (10.74 µM) combined with TDZ 0.5 mg.L⁻¹ (2.27 µM); they presented friable texture (ANISUZZAMAN et al., 2010).

In an experiment carried out with leaf explants from hybrids of *Eucalyptus urophylla* x *Eucalyptus grandis* inoculated in TDZ and ANA, respectively, at the following concentrations (0.5 + 0.5; 0.5 + 0.1; 0.05 + 0.01; 1.0 + 0.1 mg.L⁻¹) which corresponds to

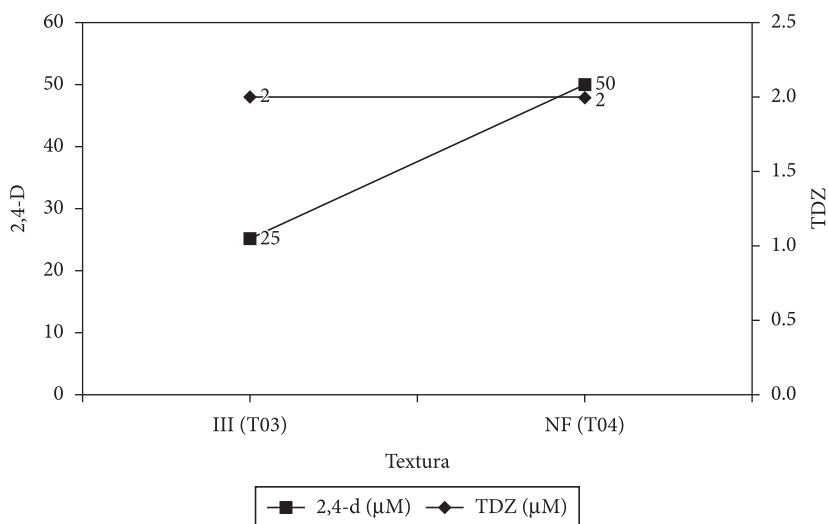


Figure 4. Relation of regulators 2,4-D and TDZ combination and concentration in callus texture. Note: III – semifriable callus; NF – no callus formation.

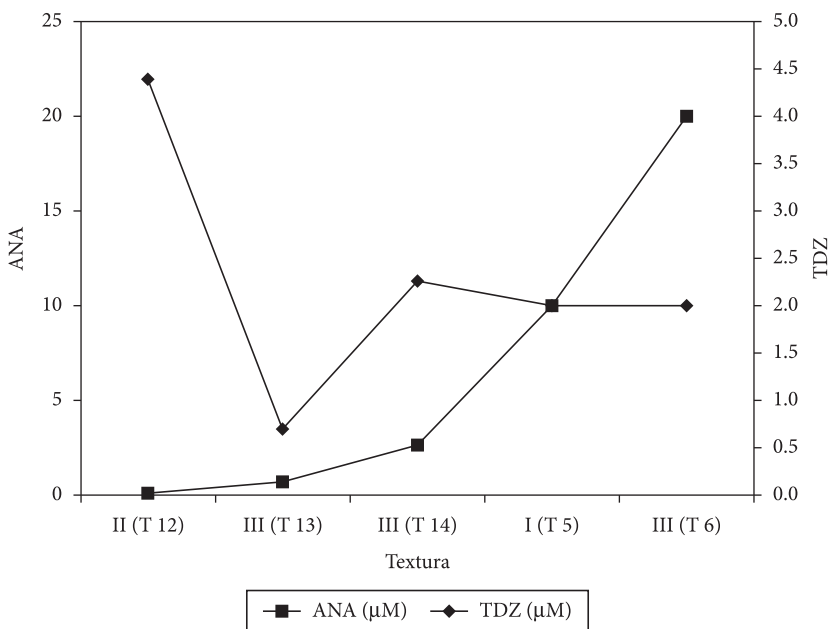


Figure 5. Relation of regulators ANA and TDZ combination and concentration in callus texture. Note: I- friable callus; II- compact callus; III- semi-friable callus.

(2.27 + 2.69; 2.27 + 0.54; 0.23 + 0.054; 4.54 + 0.54 μM) resulted, mostly, in semi-friable calluses, while under the same conditions (explant and regulators concentration) in hybrid of *Eucalyptus grandis* x *Eucalyptus urophylla* clones, the calluses presented compact texture (Alves et al., 2004). TDZ and ANA concentrations of 0.5 + 0.5 mg.L⁻¹ (2.27 + 2.69 μM) respectively used in leaf explants of *Eucalyptus urophylla* x *Eucalyptus grandis* hybrid were the same

used in this work with stem explants of *Eucalyptus grandis* in treatment T 14. In addition to treatment T 14, the same texture occurred in treatments T 06 (ANA 20.0 μM with TDZ 2.0 μM) and T 13 (ANA 0.7 μM with TDZ 0.7 μM). In this case, this shows greater influence of the regulators (combination and concentrations) rather than the explant type. Thus, the influence of regulators ANA and TDZ on the semi-friable texture of stem explants of *Eucalyptus*

grandis occurred with balanced concentrations (T 13) or when the ratio ANA/TDZ was much higher, which occurred in treatment T 06.

The ANA combination is also very used with other cytokinins, among not only at callus production, but also texture; e.g., with leaf explants of mahogany (*Swieteniamacrophylla* King), Rocha & Quoirin (2004) observed that the highest amount of calluses was obtained in the presence of cytokinin and auxin combination: BA (4.4 µM) with ANA (0.11 µM), and BA (8.9 µM) with ANA (0.11 µM or 0.54 µM).

There two highlights for the TDZ treatments: treatments T 09 (Figure 6) and T 11 (Figure 7), the first resulted in compact texture and the second did

not form calluses. Treatments T 07, T 08 and T 10 (Figure 8), which also used only TDZ, resulted in friable texture. Cotyledon explants and hypocotyls of 15-20-day seedlings of *Eucalyptus grandis* x *Eucalyptus urophylla* hybrid were inoculated in SP medium (composed of 1/2 mineral salts and ¼ FeEDTA from MS medium) supplemented with TDZ 2.0 µM for callus induction and it was possible to observe after 30 days of incubation in darkness that, in addition to 95 to 100% of callus formation in the explants, all the calluses formed presented compact texture and homogeneity, despite the different genotypes of seedlings used as explant sources (BARRUETO CID et al., 1999). As in treatment T 07 (TDZ 2.0 µM), which presented friable texture, the same concentration and growth regulator used by Barrueto Cid et al. (1999), and which obtained compact texture; it is possible that the friability reached with T 07 treatment in *Eucalyptus grandis* was due to the long culture period (210 days with 7 subcultures). TDZ concentrations higher than 1 µM may stimulate callus formation in explants of woody nature (Huetteman & Preece, 1993). However, the TDZ 3.0 µM concentration of treatment T 11 in stem segment explants of *Eucalyptus grandis* may have caused some phototoxic effect, preventing the formation of calluses. In hybrid *Eucalyptus grandis* x *Eucalyptus urophylla* clones, the TDZ concentration of 4.5 µM favored callus formation (Lima & Gonçalves, 1998).



Figure 6. Compact texture callus (treatment T 09).

The high cytokinin activity and positive response of woody species to TDZ has placed this regulator

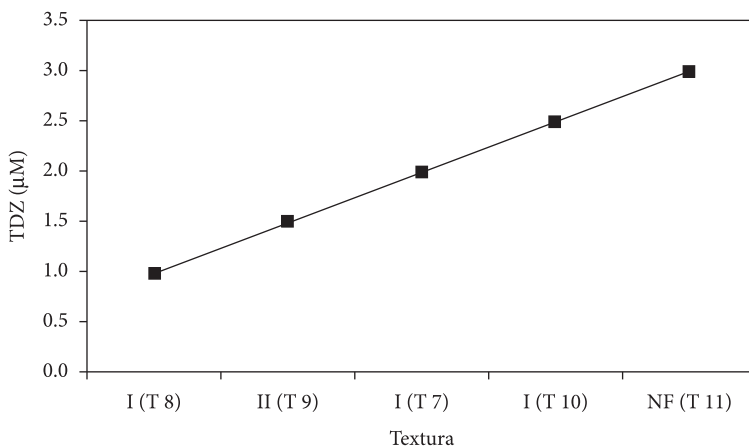


Figure 7. Relation of regulator TDZ concentrations in callus texture. Note: I- friable callus; II- compact callus; NF- no callus formation.

among the most active cytokinins in the handling of many woody species (Huetteman & Preece, 1993). In some species, TDZ stimulates cell division influencing the formation of calluses that depends on cytokinin (Lu, 1993). Nevertheless, Morales et al. (1999) concluded that apple tree internodes (*Malus* spp.) are excellent explants in calluses induction, and BAP was greater than TDZ in this fruit tree callogenesis.

In an experiment carried out by Roy et al. (2008), *Gynemasylyvestris* R.Br. calluses presented friable texture at 25 days receiving only 2,4-D (4.53-9.06 μM). Differently from what occurred in treatments (T 15 and T 16) of calluses formed with stem explants of *Eucalyptus grandis*, which used the same concentrations and presented compact and semi-friable texture, respectively (Table 2): T 15 (4.53 μM)

and T 16 (9.06 μM); and with lower concentrations of the treatments T 18 (0.23 μM), T 19 (0.45 μM) and T 20 (0.68 μM), which also obtained compact texture.

Semi-friable texture was obtained in treatment T 17 (18.12 μM) and treatment T 16 (Figure 9). In lychee tree (*Litchi chinensis* Sonn.), a 2,4-D high concentration (8 mg.L^{-1}) (36.24 μM) inhibited callus formation (Mesquita, 1999). Callogenesis inhibition also occurred in intercotyledonal explants of paricá *Schizolobium parahyba* var. *amazonicum* (Huber ex Ducke) Barneby inoculated in MS medium supplemented with 2,4-D 6 mg.L^{-1} (27.18 μM) (Reis et al., 2007).

The largest amount of calluses in stem explants of *Stevia rebaudiana* Bert occurred when they were inoculated in MS medium with 2,4-D 3.0 mg/L (13.59 μM). However, the medium with 50 mg/L (226.5 μM) of the same growth regulator provided a smaller amount of calluses (Uddin et al., 2006).

The explants that did not produce calluses were those exposed to treatments with 2,4-D 50.0 μM ; TDZ 3.0 μM , and in absence of growth regulator (control).

Calluses presenting friable texture were produced in MS medium supplemented only with TDZ or a combination of ANA 10.0 μM and TDZ 2.0 μM .

Auxins AI, 2,4-D and ANA contributed, in general, to semi-friable texture.

The best protocols for the maintenance of friable calluses from stem explants of *Eucalyptus grandis*



Figure 8. Friable texture callus (treatment T 10).

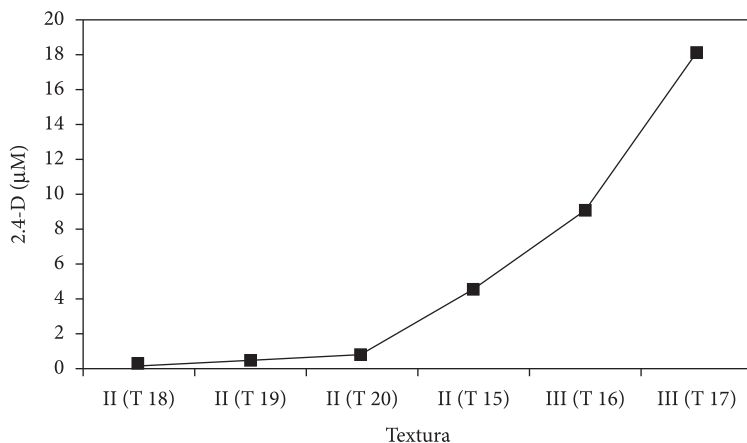


Figure 9. Relation of regulator 2,4-D concentrations in callus texture.

under the conditions performed in this work were those using only TDZ.

4. CONCLUSIONS

TDZ was confirmed as the regulator that provides the highest stability in friability after 7 subcultures of stem explants of *E. grandis*. However, the ANA and TDZ combination produced resilience in calluses with very advanced oxidation.

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