

## *In vitro* multiplication of *Eucalyptus hybrid* via temporary immersion bioreactor: culture media and cytokinin effects

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**Abstract:** This study aimed to assess the influence of culture medium and cytokinin type on *in vitro* multiplication of an *Eucalyptus grandis* x *E. urophylla* hybrid clone via temporary immersion bioreactor (TIB<sup>®</sup>). JADS, modified MS at a (NO<sub>3</sub><sup>-</sup>):(NH<sub>4</sub><sup>+</sup>) ratio, and WPM were used as liquid media; and BAP, KIN, and TDZ were the cytokinins used in the study. According to the results, explants under the influence of modified MS presented better results for all traits. BAP was the most suitable plant growth regulator for axillary bud shoot proliferation. By the leaf histological analysis, most of the shoots grown in the presence of BAP either exhibited normal morphology or only a few hyperhydricity symptoms. All these results were obtained at 19 days after *in vitro* cultivation, a shorter period when compared with other works in the literature, showing the protocol efficiency in the multiplication of this hybrid clone using TIB<sup>®</sup>.

**Key words:** 6-benzilaminopurine, hyperhydricity, plant biotechnology, *in vitro* propagation.

### INTRODUCTION

Bioreactor technology is a mechanized system used in plant tissue culture to improve the large-scale micropropagation in laboratories and biofactories. Therefore, bioreactor comes as an alternative technological tool to the conventional micropropagation process aimed at optimizing the *in vitro* cultivation by the automation of the process (Maciel et al. 2016, Mariateresa et al. 2014, Oliveira et al. 2011b). These mechanized systems can help achieve a higher plant biomass gain and reduce the time needed for propagation (Máximo et al. 2015). Such gains are the result of the greater contact between explant and culture medium. This fact enables a higher nutrient absorption by the explant and a greater *in vitro* environment aeration, which renews the gaseous atmosphere inside culture vessels (Mariateresa et al. 2014, Zobayed et al. 2001).

The *in vitro* cell and tissue development depend on the interaction of internal (genotype, age, physiological conditions) and external factors (media composition, growth regulators, temperature, luminosity) (Pinto et al. 2008). According to previous studies, the culture media, such as MS (Murashige and Skoog 1962), JADS (Correia et al. 1995), and WPM (Lloyd and McCown 1981) are the most commonly used in *Eucalyptus spp.* culture (Brondani et al. 2012). Nevertheless, new studies must be carried out to determine which formulation

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is more adequate for each species or hybrid.

Besides determining the culture medium composition, the evaluation on which growth regulator is the most efficient for the *in vitro* multiplication of each species is fundamental. This is because regulators are associated to plant morphogenetic responses (Garcia et al. 2011). At the *in vitro* multiplication stage, cytokinins are essential growth regulators for the apical dominance rupture and induction of axillary bud proliferation. Furthermore, the cytokinins type strongly influences the success of *in vitro* propagation of many species, including *Eucalyptus* sp. (Brondani et al. 2012).

This study aimed to assess the efficiency of culture media and cytokinin types in the cultivation of a commercial *Eucalyptus grandis* x *E. urophylla* hybrid as an effort to establish a specific protocol for such procedure by using a temporary immersion bioreactor (TIB<sup>®</sup>).

## MATERIAL AND METHODS

### Plant material

Shoots from an *E. grandis* x *E. urophylla* hybrid matrix were excised and sterilized with paraformaldehyde for 40 minutes. Shoot tips were isolated and inoculated onto MS medium (Murashige and Skoog 1962) supplemented with 20 g L<sup>-1</sup> sucrose and Phytigel<sup>®</sup> (SIGMA) gelling agent at 5.8 pH. Explants were kept in darkness for five days and transferred to MS medium supplemented with 5.7 µM of indol-3-acetic acid (IAA) and 0.14 µM of 6-benzilaminopurine (BAP). Afterward, the plant material was maintained for 40 days in growth room, with 16 h photoperiod, with 40 µmol m<sup>-2</sup> s<sup>-1</sup> irradiance, at a temperature of 26 ± 2 °C.

### Temporary immersion bioreactor (TIB<sup>®</sup>)

A temporary immersion bioreactor (TIB<sup>®</sup>) coupled to a pneumatic chassis MFE – 1001 (Fitoclone, Viçosa, MG, Brazil) was used in all experiments. The temporary immersion cycle was controlled by an electronic system (timer) set as follows: transfer of medium between vessels every 2 h; contact between explant and medium was kept for 10 s; and finally, air was injected for *in vitro* atmosphere renewal every 1 h. Air injection was performed using 0.20-µm-pore filters to promote sterilization. All vessels were chemically sterilized with a 0.036% (m/v) sodium hypochlorite solution during 12 h. The media had their pH adjusted to 5.8. The bioreactor was kept in a growth room at 26 ± 2 °C, with 16 h photoperiod, and 40 µmol m<sup>-2</sup> s<sup>-1</sup> irradiance, provided by white fluorescent lamps, which were controlled by the electronic system.

### Liquid medium employed in the TIB<sup>®</sup>

The stem of *in vitro* shoots, each one containing two nodal segments, were used as explants. The explants were inoculated into bioreactor vessels containing 400 mL of the following liquid media: MS medium (Murashige and Skoog 1962) modified on nitrate: ammonium ratio 3:1 (NO<sub>3</sub><sup>-</sup>):(NH<sub>4</sub><sup>+</sup>) (for more information, see Máximo et al. 2015), JADS (Correia et al. 1995), and WPM (Lloyd and McCown 1981). All media were supplied with 20 g L<sup>-1</sup> sucrose. Inoculation was conducted in a horizontal laminar flow chamber, and the explants were maintained according to the cultivation conditions previously established for TIB<sup>®</sup> section.

At 19 days after inoculation, the following traits were assessed: number of shoots, fresh weight, length of the highest shoot, and number of leaves of the highest shoot. A completely randomized design was used, with 30 repetitions, each one represented by two nodal segments.

### *In vitro* multiplication by cytokinin supplementations

After the selection of the adequate medium in the previous experiment, the effect of different cytokinins during *in vitro* multiplication was assessed. Explants were the same as mentioned above: stem of *in vitro* shoots. These explants were inoculated into bioreactor vessels containing 400 mL of modified MS medium 3:1 (NO<sub>3</sub><sup>-</sup>):(NH<sub>4</sub><sup>+</sup>) added of 5.7 µM of IAA and 20 g L<sup>-1</sup> sucrose. Treatments with cytokinin consisted of applying 0.14 µM of either 6-benzilaminopurine (BAP), kinetin (KIN), or thidiazuron (TDZ) to the culture medium. A treatment with no cytokinin application was used as a control. Inoculation was conducted in a horizontal laminar flow chamber, and explants were maintained according to the cultivation conditions established in the TIB<sup>®</sup> section.

At 19 days after inoculation, the evaluated traits were the same as the prior experiment, but with 21 repetitions (two nodal segments each) per treatment.

### Histological analysis

Shoots cultivated in TIB® containing modified MS 3:1 (NO<sub>3</sub><sup>-</sup>):(NH<sub>4</sub><sup>+</sup>) supplemented or not with cytokinins BAP, KIN, and TDZ at 0.14 µM had their leaves cut from the third node to undergo light microscopy. The leaves from the third node of each explant were used in the experiment as a standard. Furthermore, these leaves were apparently more physiologically active and completely expanded.

All samples were fixed in FAA 70 (Johansen 1940) and stored in 70 % ethanol (v/v). Each sample was dehydrated for 1 h with the 80, 90, and 100 % ethanol concentration series – the last one was applied twice. Samples were infiltrated by a 1:1 epoxy resin solution (Historesin® Leica) added with ethanol for 24 h and transferred to a 100 % concentration-epoxy-resin solution for 48 h. The infiltrated material was blocked in 15:1 resin+polymer by water-soluble cylindrical capsules and maintained in an oven (HL-2000 Hybrilinker) at 37 °C, for approximately 72 h. A semiautomatic microtome (Easypath EP-31-20091) was used to make 3.0 µm-thick transversal cuts, which were fixed in glass slides. These materials were stained with safrablau (0.1% safranin + 1% astra blue) (Kraus and Arduin 1997) and finally evaluated under light microscopy (Zeis, Axio Scope). The UTHSCSA Image Tool was used to analyze the adaxial epidermis, leaf and mesophyll thicknesses, and palisade and spongy parenchyma thicknesses.

A completely randomized design was used, using three leaves from each shoot. For slide assembly, each leaf yielded ten transversal cuts, being five cuts per slide. Three visual fields were selected from each cut to measure the variables.

### Statistical data analysis

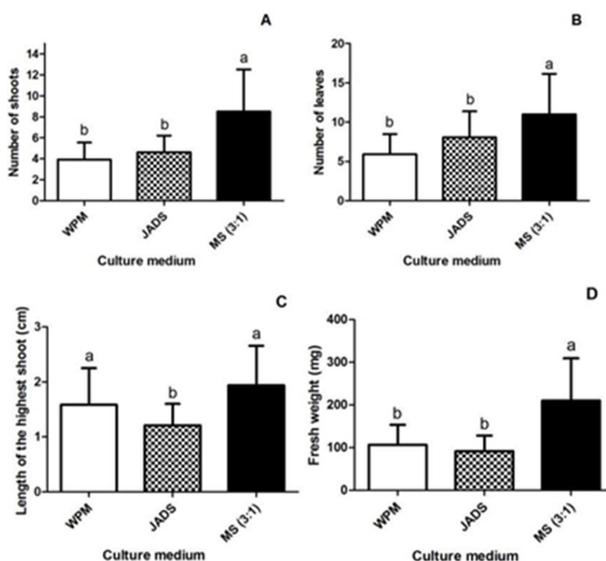
All data were submitted to the analysis of variance (ANOVA) through the Sisvar software (Ferreira 2014) for statistical analysis. Mean values were compared by the Tukey’s test at 5% significance.

## RESULTS AND DISCUSSION

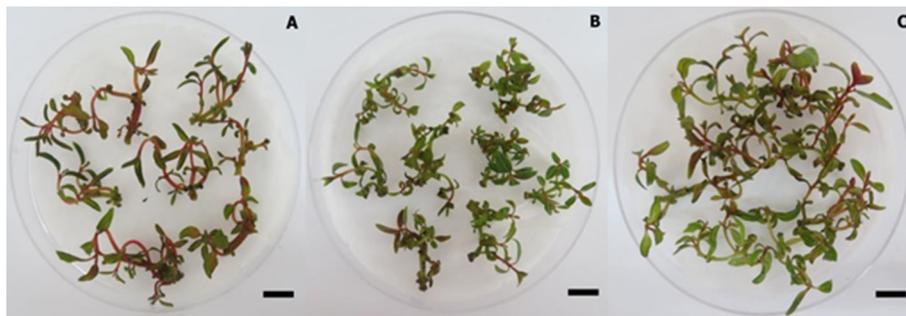
Little information about using temporary immersion bioreactors is available for *Eucalyptus* spp. *in vitro* multiplication, especially on the use of different culture media (Oliveira et al. 2011a). Most of the discussion of this study is mainly based on results found in semisolid medium cultivation. A statistical difference was observed for all studied traits by the Tukey’s test (p<0.05). The shoots taken from the modified MS medium presented the best results. Conversely, no significant effect was observed in shoot growth when using the JADS medium (Figure 1).

Explants cultivated in the modified MS medium had a mean of 8.5 shoots per explant, 11 leaves in the highest shoot, 1.94 cm also in the highest shoot, and a total fresh weight of 210.28 mg. These data are consistent with those found in the literature, confirming that MS provides promising results for cultivation, regeneration, and multiplication of *Eucalyptus* spp. when compared with JADS and/or WPM (Oliveira et al. 2011a,). Other works have demonstrated that modifications on nitrate and ammonium ratio in the MS medium can also provide enhanced results during *in vitro* multiplication of *E. grandis* x *E. urophylla* clones (Máximo et al. 2015, Oliveira et al. 2011b).

Oliveira et al. (2011a) reported about 8 shoots per



**Figure 1.** Effect of culture media WPM, JADS, and modified MS 3:1 (NO<sub>3</sub><sup>-</sup>):(NH<sub>4</sub><sup>+</sup>) on the development of explants grown on TIB® for 19 days. Graphics show the mean of A) number of shoots; B) number of leaves from the highest shoot; C) length of the highest shoot; and D) fresh weight of shoots.



**Figure 2.** Visual aspect of shoots cultivated for 19 days in TIB<sup>®</sup> using the media A) WPM; B) JADS; C) modified MS 3:1 ( $\text{NO}_3^-$ ):( $\text{NH}_4^+$ ). All petri dishes above contain eight explants each. Bar = 10 mm.

explant and a fresh weight of about 300 mg after cultivating shoot tips of an *E. grandis* x *E. urophylla* clone in RITA<sup>®</sup> bioreactors. Although these results are similar to those found in the present work, the cultivation period in RITA<sup>®</sup> was 28 days, while the cultivation period in TIB<sup>®</sup> was 19 days. In general, RITA<sup>®</sup> vessels are smaller than TIB<sup>®</sup>, and thus bigger vessels are expected to have more headspace. Considering the same aeration time in both bioreactors, TIB<sup>®</sup> might have accumulated less toxic gases (like ethylene) in the *in vitro* environment, and thus had less influence on plant growth, explaining the similar performance in both TIB<sup>®</sup> and RITA<sup>®</sup> explants growth, but with a shorter period in TIB<sup>®</sup>.

Shoots cultivated under influence of the modified MS medium morphologically presented greater strength, greener leaves, and greater uniformity. Conversely, explants cultivated on JADS were smaller and less homogenous. Regarding shoots grown on WPM, some morphological alterations were noted, such as twisted reddish stems and a smaller number of leaves (Figure 2). Works using clone shoots from this hybrid cultivated on WPM also presented reddish stems and callus formation on leaves, while those cultivated on MS showed better morphological traits (Oliveira et al. 2011a).

Reportedly, MS is a saline medium, rich in nutrient and nitrogen, while WPM presents low nitrogen levels (Pinto et al., 2008). Furthermore, adequate ( $\text{NO}_3^-$ ):( $\text{NH}_4^+$ ) ratios could also stimulate plant morphogenesis (Ramage and Williams 2002), suggesting the results found for the two media were different due to the less nitrogen availability in WPM. Therefore, this fact indicates that the 3:1 ( $\text{NO}_3^-$ ):( $\text{NH}_4^+$ ) ratio applied in MS was the most appropriate for shoots of the *E. grandis* x *E. urophylla* hybrid clone.

When the nitrate level is higher than the ammonium level, the nitrate reductase (NR) activity may increase as a response of the enzyme to its substrate (Lea et al. 2006). The result of the enzyme activity is the greater nitrate absorption and shoot growth. In addition, under aerobic soil conditions, nitrate is the main source of nitrogen absorbed by plants. This ion acts as a signaling molecule regulating the expression of genes related to root development and leaf expansion, consequently adjusting plant growth according to its availability (Ho and Tsay 2010). Thus, such resemblance between soil condition and TIB<sup>®</sup> may have occurred when nitrate availability was higher in modified MS medium.

As the proportion between nitrate and ammonium is very similar in the WPM medium composition, such ammonium availability might be impairing a properly nitrate absorption. Ammonium absorption is more energetically economical than nitrate owing to its reduced form; however, higher ammonium levels in tissues may be toxic (Britto and Kronzucker 2002, Ho and Tsay 2010). Although plant toxicity responses are little understood, external environment acidification, acid/base balance disruption, and energy loss caused by excessive export of ammonium may negatively affect plant growth and subsequently its survival (Ho and Tsay 2010). This could explain, for example, the twisted reddish stems observed in shoots cultivated in WPM.

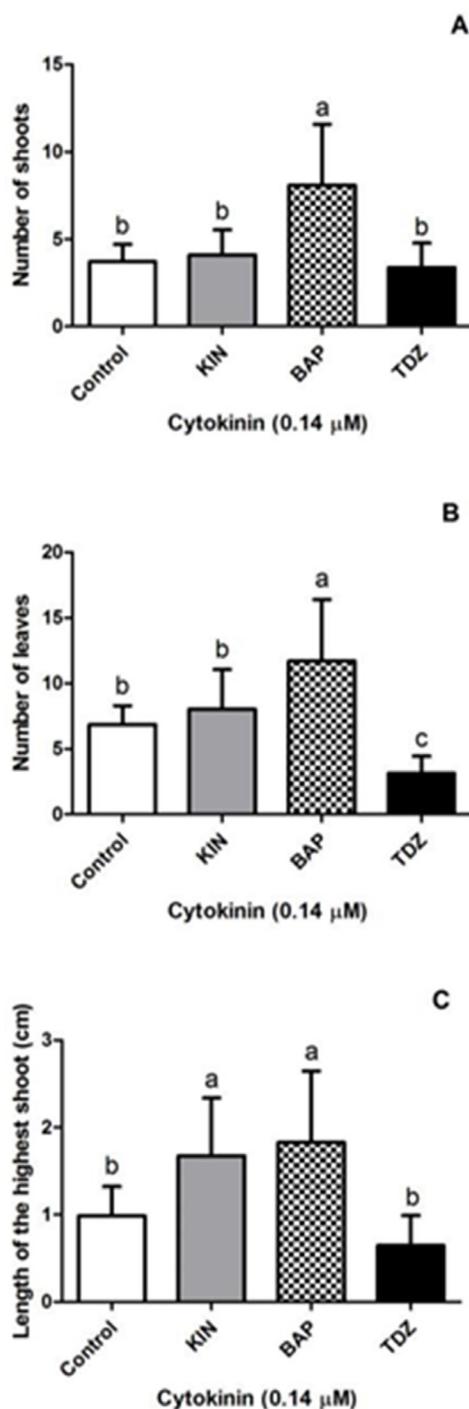
Works using JADS as culture medium to cultivate *E. grandis* have demonstrated positive results during the *in vitro* multiplication stage (Correia et al. 1995), even though the composition of the medium is formulated with significantly reduced salt concentrations, specifically potassium ions when compared with MS. These results show that genotype is dependent on the culture medium, emphasizing that a few changes in the medium composition may be considered as essential to elucidate shoot phenotype and explain differences in efficiency of culture media on plant growth.

All traits, except for fresh weight, were statistically influenced by the cytokinin type used in the cultivation on TIB® (Figure 3). Mean number of shoots (8.1 shoots) and mean number of leaves (11.71 leaves) from explants grown in media containing BAP were the best results among cytokinin treatments. Although shoots cultivated under BAP influence showed better results for the traits analyzed, they presented no significant difference when compared with those cultivated under KIN influence regarding the length of the highest shoot. BAP is directly related to cell division and adventitious shoot development (Andrade et al. 2006), while KIN not only influences cell division but also increases the number of microtubule cells (Romanov et al. 2000), which might promote elongation over new shoot production.

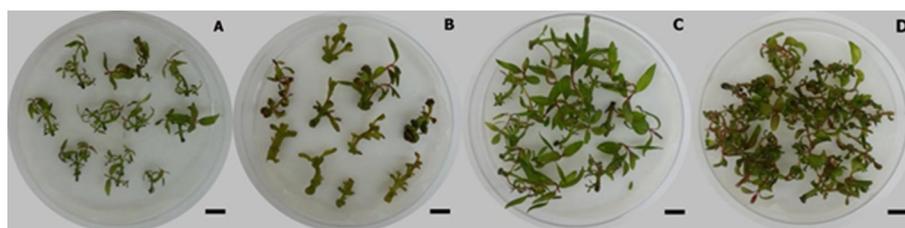
Shoots grown in medium containing BAP not only were outstanding and numerous but also presented greener leaves, sturdier stems, and no apparent hyperhydricity aspect. Conversely, explants under the influence of KIN presented a smaller number of shoots, thinner and more translucent stems, and apparent hyperhydric leaves (Figure 4C). The choice of the cytokinin type during the *in vitro* cultivation has been considered as a crucial factor that might affect the developmental stage and quality of shoots, once cytokinins may act on the photosynthetic apparatus function and interfere in the leaf chlorophyll content (Dobrąnszki and Mendler-Drienyovszki 2014).

In another research, shoots from *E. grandis* x *E. urophylla* hybrids cultivated in a RITA® bioreactor at low BAP concentrations did not present hyperhydricity symptoms (Oliveira et al. 2011a). Hyperhydric plants show sturdier, thicker, more wrinkled, more translucent, more rigid stems and leaves (Kevers et al. 2004, Picoli et al. 2001), as well as node shortening, and an apparent water accumulation (Vasconcelos et al. 2012). Shoots cultivated in medium containing TDZ displayed abnormal formation, small size, and less developed vitrified leaves, besides callus formation at the stem base of shoots and on the leaf surface (Figure 4B). *In vitro* works with *Aloe polyphylla* have also demonstrated that the use of TDZ led to callus formation, abnormal morphology leaf, and vitrified small shoots (Ivanova and Van Staden 2011).

A statistical difference ( $p < 0.05$ ) was also observed for leaf histological analysis of shoots cultivated under cytokinins influence. Shoots grown in the presence of BAP had higher means of leaf, mesophyll, and palisade thicknesses, besides displaying a spongier parenchyma. Nevertheless, both TDZ and KIN showed greater adaxial epidermis thickness (Table 1).



**Figure 3.** Influence of the cytokinins BAP, KIN, and TDZ (all at 0.14 μM) on the development of shoots cultivated on TIB®. Graphics show the mean of A) number of shoots; B) number of leaves from the highest shoot; and C) length of the highest shoot for *E. grandis* x *E. urophylla* explants. Control contains no cytokinin in the modified MS medium 3:1 (NO<sub>3</sub><sup>-</sup>):(NH<sub>4</sub><sup>+</sup>).



**Figure 4.** Morphological aspect of shoots cultivated on TIB<sup>®</sup> under the influence of different cytokinin in the modified MS medium (NO<sub>3</sub><sup>-</sup>:(NH<sub>4</sub><sup>+</sup>) for 19 days. Shoots grown in the absence of cytokinin (control) (A) and in the presence of TDZ (B) exhibited small number of shoots; however, callus formation occurred only in (B). Explants cultivated on medium containing KIN (C) were more elongated, whereas explants grown in the presence of BAP (D) presented greater number of shoots. All petri dishes above contain ten explants. Bar = 10 mm.

**Table 1.** Mean of leaf thickness (LT), mesophyll thickness (MT), adaxial epidermis (AE), palisade parenchyma (PP), spongy parenchyma (SP), and spongy and palisade parenchyma ratio (SOR) during TIB<sup>®</sup> cultivation of shoots under the influence of different cytokinin types. All variables were measured in micrometers (μm)

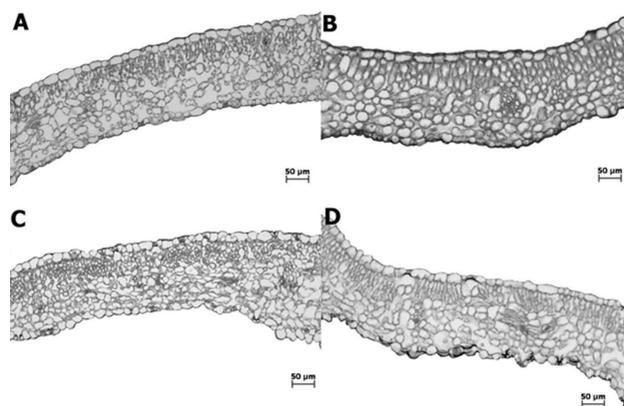
Cytokinin	LT	MT	AE	PP	SP	SOR
Control	182.23 b	149.27 b	17.39 bc	54.12 b	99.44 ab	1.84
TDZ	184.20 b	142.03 b	20.06 ab	52.69 b	93.94 b	1.78
KIN	182.91 b	143.38 b	20.28 a	51.71 b	95.09 b	1.84
BAP	214.97 a	181.28 a	17.18 c	73.23 a	114.55 a	1.56

\*Similar letters in the columns do not statistically differ by the Tukey's test at 5% significance (P < 0.05).

Usually, the palisade tissue of vitrified leaves is either absent or greatly reduced, while the mesophyll has a spongy and vacuolated aspect, as well as a great number of intercellular space (Vasconcelos et al. 2012). In hyperhydric compact leaves, a poorly differentiated palisade cell layer is commonly present, while vitrified lacunae leaves present a decreased differentiation of the palisade tissue, with both intercellular space and spongy mesophyll enlargement (Picoli et al. 2001). Such information confirms the results found in the present study since the shoots from the BAP treatment presented relatively normal leaves, while the leaves from the other cytokinin treatments exhibited a higher quantity of intercellular space (ratio between the thickness of palisade and spongy parenchyma), which evidences the profile of lacunous hyperhydric leaves (Figure 5). Other studies have reported this disorder and adverse results in the multiplication of some species cultivated with TDZ (Bosela and Michler 2008, Sunagawa et al. 2007), confirming that this plant growth regulator was not appropriate for the *Eucalyptus* hybrid evaluated in our study.

## CONCLUSIONS

The modified MS medium with 3:1 (NO<sub>3</sub><sup>-</sup>):(NH<sub>4</sub><sup>+</sup>) ratio containing BAP at 0.14 μM is efficient for *in vitro* multiplication of *E. grandis* x *E. urophylla* hybrid clone using TIB<sup>®</sup>. Most shoots exhibited hyperhydricity symptom; however, the shoots under BAP influence presented lower evidence of this abnormality. Therefore, the protocol developed in this study can provide a greater number of vigorous shoots to be used in subsequent micropropagation steps.



**Figure 5.** Mesophyll aspects of shoots cultivated under the influence of different types of cytokinin on TIB<sup>®</sup>. Above, a leaf transverse section from the shoots cultivated in A) absence of cytokinin (Control), and in the presence of B) BAP, C) KIN, and D) TDZ for 19 days.

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