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Micropropagation of an *Eucalyptus* hybrid (*Eucalyptus benthamii* x *Eucalyptus dunnii*)

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ABSTRACT. This study was designed to micropropagate *E. benthamii* x *E. dunnii*, by testing chlorine concentrations for explant asepsis, the optimal concentrations of benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) for bud proliferation, and the ratio between BAP and gibberellic acid (GA_3) in two nutrient media for shoot elongation. Nodal segments from H12, H19 and H20 clones were disinfected with 0.5, 1.0, 1.5 and 2.0% (v v^{-1}) of chlorine. Explants were grown on ½MS medium supplemented with BAP (0, 0.25, 0.50, 0.75 and 1.00 mg L^{-1}) and NAA (0, 0.025, 0.050, 0.075 and 0.100 mg L^{-1}) for bud production. They were elongated on MS and ½MS media supplemented with BAP (0, 0.05 and 0.10 mg L^{-1}) and GA_3 (0, 0.1, 0.2 and 0.3 mg L^{-1}). The 0.50 mg L^{-1} BAP and 0.050 mg L^{-1} NAA combination was optimal for bud proliferation for H12 and H20. GA_3 concentrations of 0.10 and 0.20 mg L^{-1} combined with 0.10 mg L^{-1} BAP on ½MS resulted in the longest shoots, for H12 and H20, respectively. Regardless of clone, the rooting rate was low, with an average of 12.0% and 14.4% of plants having roots for *in vitro* and *ex vitro* conditions, respectively.

Keywords: *in vitro* establishment, culture medium, cloning, BAP, NAA, GA_3.

RESUMO. Micropropagação de um híbrido de *Eucalyptus* (*Eucalyptus benthamii* x *Eucalyptus dunnii*). Objetivou-se micropropagar *E. benthamii* x *E. dunnii*, testando concentrações de cloro para a assepsia de explantes, a concentração ótima de benzilaminopurina (BAP) e ácido naftalenoacético (ANA) para a proliferação de gemas e a relação entre BAP e ácido giberélico (GA_3) em dois meios de cultura para o alongamento de brotações. Segmentos nodais dos genótipos H12, H19 e H20 foram desinfetados com 0,5; 1,0; 1,5 e 2,0% (v v^{-1}) de cloro. Os explantes foram multiplicados em meio ½MS suplementado com BAP (0; 0,25; 0,50; 0,75 e 1,00 mg L^{-1}) e ANA (0; 0,025; 0,050; 0,075 e 0,100 mg L^{-1}) para produção de gemas, e alongados nos meios MS e ½MS suplementados com BAP (0; 0,05 e 0,10 mg L^{-1}) e GA_3 (0; 0,1; 0,2 e 0,3 mg L^{-1}). A combinação de 0,50 mg L^{-1} de BAP e 0,050 mg L^{-1} de ANA proporcionou melhor proliferação de gemas para os genótipos H12 e H20. As concentrações de 0,10 e 0,20 mg L^{-1} de GA_3 combinadas com 0,10 mg L^{-1} de BAP em ½MS, promoveram os melhores resultados no alongamento, para os clones H12 e H20, respectivamente. O enraizamento foi baixo, com média de 12,0% para condições *in vitro* e 14,4% *ex vitro*.

Palavras-chave: estabelecimento *in vitro*, meio de cultura, clonagem, BAP, ANA, GA_3.

Introduction

Economically important *Eucalyptus* species or varieties adapted to severe climate conditions are limited, but *E. benthamii* and *E. dunnii* stand out among these species, offering the possibility of climate adaptation and quality wood production. In regions where severe frosts compromise the establishment and growth of *Eucalyptus*, resistance to cold conditions is a crucial feature that may be introduced by interbreeding (BORÉM, 2007).

The natural hybridization between *Eucalyptus* species is common (BUTCHER et al., 2005). Recently, it was shown that the occurrence of spontaneous hybrid formation between *E. benthamii* and *E. dunnii* at Embrapa Forests in Colombo, Paraná State, might present frost-tolerant varieties. *E. benthamii* and *E. dunnii* hybrids are an alternative for future forest plantations, particularly due to frost tolerance and low temperature resistance (HIGA et al., 2000; JOVANOVIC et al., 2000). However, there are limitations regarding seed production,

mainly because of the high price and low availability of hybrid seeds. Moreover, hybrid clones have low rooting rates when propagated by macropropagation techniques, such as mini-cutting (BRONDANI et al., 2008; BRONDANI et al., 2010a and b). Therefore, before the commercial use of these hybrids can be completed, studies are required to identify methods for obtaining seeds or plants from those hybrids.

One method for plant production is *in vitro* plant propagation (KOMATSU et al., 2011; MERKLE; NAIRN, 2005; NEHRA et al., 2005; VILLA et al., 2006; WATT et al., 2003), with micropropagation as the most widespread technique, with applications in wood production (BORÉM, 2007). According to Joshi et al. (2003), this technique is linked to genetic improvement programs that aim to maximize or maintain genetic material. Borém (2007) suggests that micropropagation from organ segments, with direct bud induction (direct organogenesis) or pre-existent buds stimulus through apical dominance suspension, is the most recommended method for plant propagation and results in lower genetic variation than other methods.

Several studies with *in vitro* cultivation of *Eucalyptus* species have been performed with a variety of modifications in the MS (MURASHIGE; SKOOG, 1962) nutrient medium, being the most widely used (AREZKI et al., 2000, 2001; BARRUETO CID et al., 1999; BENNETT et al., 1994; BUNN, 2005; BUNN et al., 2005; GLOCKE et al., 2006a and b; JOSHI et al., 2003; NUGENT et al., 2001; SHARMA; RAMAMURTHY, 2000). There is a diversity of mechanisms that control the processes of adventitious rooting (BALTIERRA et al., 2004; CORRÊA et al., 2005; FOGAÇA; FETT-NETO, 2005; TRINDADE; PAIS, 1997). However, little is known about clonal propagation of subtropical *Eucalyptus*, which may be used for forest plantations in colder climates.

Due to the absence of a protocol for *E. benthamii* x *E. dunnii* hybrids using *in vitro* cultivation, the present study was designed to: (i) to determinate the best chlorine concentration for the asepsis of nodal segments; (ii) to verify the best benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) ratio for bud proliferation and (iii) to verify the best BAP and gibberellic acid (GA_3) ratio and culture medium conditions for shoot elongation. A protocol was developed for optimal rooting and survival of explants *ex vitro*.

Material and methods

Plant material

The material used to obtain the mini-cuttings came from 1-year-old plants, located in Guarapuava, Paraná State, Brazil. The three best *E. benthamii* x *E. dunnii* hybrids (H12, H19 and H20) were propagated by conventional cutting techniques. Cuttings 15 cm long and 90 days old were planted in gutters containing sand and grown in a hydroponic system. After 20 days, the first pruning was performed at 5 cm high from the base of each rooted cutting. Plants received daily nutrient solution by dripping at a flow of 5 L m^{-2} (BRONDANI et al., 2009, 2010a).

Explant sampling and preparation

One week before shoots were removed for propagation, the mini-stumps were sprayed with Kumulus DF® fungicide at 3 g L^{-1} (p v^{-1}). Shoots from the 13th collection were transported in polyethylene bags with 1% of ascorbic acid solution (p v^{-1}). In the laboratory, leaves were removed and the vegetative material was washed with deionized water, for removal of dust and particles.

Culture medium preparation and *in vitro* conditions

The pH value was adjusted to 5.8 using HCl (0.1 M) and NaOH (0.1 M) and 250 mg L^{-1} PVP 40, BAP and NAA were added to the culture medium. After this, 6 g L^{-1} agar was added and the culture medium was autoclaved at 121°C (≈ 1.0 kgf cm^{-2}) during 20 minutes. After autoclaving, GA_3 was added to the culture medium into a laminar flow chamber using a disposable filter Millex-GP®, with a 0.22 μm sterile membrane. Explants were cultivated in an incubation room, at 25°C (± 2°C), 16-hour photoperiod and 40 μmol m^{-2} s^{-1} of luminosity.

Plant establishment

Nodal segments from the medium portion of shoots, with one pair of axillary buds, with leaves removed and a mean size of 1.5 cm were immersed in 70% solution of alcohol (v/v) during 15 seconds, washed in deionized and autoclaved water, and then submitted to 0.5, 1.0, 1.5 and 2.0% (v v^{-1}) chlorine (NaOCl) with Tween 20® (0.05% v v^{-1}) during 10 minutes. At the end, nodal segments were washed three times in deionized and autoclaved water, and inoculated in 7 x 3 cm test tubes containing 10 mL of MS medium, supplemented with 30 g L^{-1} sucrose (Merck®).

After 21 days, fungal and bacterial contamination and oxidation were evaluated. The number of leaves and shoots and total shoot length were quantified from the established explants. The experiment was performed in a randomized block design (3 x 4), with three clones (H12, H19 and H20), four chloride concentrations (0.5, 1.0, 1.5 and 2.0%) and five replications. Each replication was composed of five explants.

Shoot multiplication

Shoots with three to five buds were inoculated in 10 x 2 cm test tubes with 10 mL of ½MS medium supplemented with 0.25, 0.50, 0.75 and 1.0 mg L^{-1} BAP (Merck®) and 0.025, 0.050, 0.075 and 0.100 mg L^{-1} NAA (Merck®). Sucrose at 15 g L^{-1} was added to the medium. After 30 days, explants were transferred to a new culture medium maintaining the same treatments.

The number of developed buds per explant was quantified 60 days after the inoculation. The experiment was performed in a randomized block design (2 x 5 x 5), testing two clones (H12 and H20), five concentrations of BAP combined with five concentrations of NAA, and five replications. Each replication was composed of five explants.

Shoot elongation

Explants with 15 to 20 buds were grown in 7 x 6 cm recipients during 21 days in 30 mL of ½MS medium, which were supplemented with 250 mg L^{-1} PVP 40 (Merck®), 15 g L^{-1} sucrose, 6 g L^{-1} agar (Merck®) and pH adjusted to 5.8. After this, explants were inoculated in the same size recipients containing 30 mL of MS medium with 30 g L^{-1} sucrose or ½MS with 15 g L^{-1} sucrose, both supplemented with 0.05 and 0.10 mg L^{-1} BAP and 0.1, 0.2 and 0.3 mg L^{-1} GA_3 (Merck®). The culture medium was supplemented with 0.5 mg L^{-1} NAA.

The number of elongated shoots and the length of shoot mean per explant were quantified 30 days after inoculation. The experiment was performed in a randomized block design (2 x 2 x 3 x 4), testing two clones (H12 and H20), two culture media (MS and ½MS), three concentrations of BAP combined with four concentrations of GA_3 and five replications. Each replication was composed of five explants.

In vitro rooting

Elongated shoots of H12 and H20 clones with 1.5 cm long and one pair of buds were inoculated in 10 x 2 cm recipients with 10 mL of ½MS medium with 15 g L^{-1} sucrose. 0.1 g L^{-1} calcium pantotenate (Merck®), 0.1 mg L^{-1} Biotin (Merck®), 0.1 g L^{-1} Mio innosytol (Merck®) and 2 mg L^{-1} IBA (indole-3-butyric acid) (Merck®) were added to the culture medium (LE ROUX; VAN STADEN, 1991). Rooting percentage was defined as the percentage of plants with roots and was quantified after 30 days.

Ex vitro rooting

Shoots elongated *in vitro* of H12 and H20 clones with 1.5 cm long and one pair of buds were treated with 1,000 mg L^{-1} IBA (alcohol : water, 1:1, v v^{-1}) and transferred to potting compost (55 cm^3). The substratum was a mixture of carbonized rice bark and vermiculite medium (1:1, v v^{-1}), incorporated with 4 kg m^{-3} Simple Superphosphate® (20% P_2O_5 and 14% S) and 1.5 kg m^{-3} FTEBR12® (9% Zn, 3% Fe, 2% Mn, 0.1% Mo, 1.8% B and 0.8% Cu).

Shoots remained inside the greenhouse for 40 days, with relative air humidity (RH ≥ 80%) and air temperature (T < 30°C) automatically controlled. Afterwards, shoots were transferred to a shade house (50% of light reduction) for acclimatization for 22 days. The micro aspersion system was controlled by timers and consisted of 12 micro aspersion nibs with 2.0 kg cm^{-2} of pressure, and flow of 144 L $hour^{-1}$ (2.4 L $minute^{-1}$), which were turned on during two minutes every two hours. After that, shoots were transferred to outdoor conditions during 28 days, for plant hardening and growth. The aspersion system worked with net water pressure (2.0 kg cm^{-2}). The system consisted of 12 micro aspersion nibs of 97 L $hour^{-1}$, which were turned on three times a day during 20 minutes.

After 90 days, the rooting percentage was quantified. The experiment was performed in a randomized block design (2 x 2), testing two clones (H12 and H20), with presence and absence of IBA. To that end, six replications composed by five explants were used.

Statistical analysis

Data were analyzed using the Lilliefors test ($p < 0.05$), and then, with analysis of variance (ANOVA) ($p < 0.01$ and $p < 0.05$). Qualitative data were compared by Tukey test ($p < 0.05$) and quantitative data by polynomial regression ($p < 0.01$ and $p < 0.05$).

Results and discussion

Establishment

There was no significant interaction between the clone and the concentration of chlorine on plant asepsis. However, there was a significant effect among clones regarding the percentage of plants with bacterial contamination ($p < 0.01$), oxidation ($p < 0.05$) and plants that were established ($p < 0.01$). H19 had 9% of the plants with bacterial contamination, much greater than H12 and H20, which had 1%, or the absence of bacterial contamination, respectively. The H20 clone did not exhibit oxidation and statistically differed from H19, which had 6% of oxidized explants. The H12 clone did not differ statistically from the others, presenting on average 3% of oxidation (Table 1).

Table 1. Average values of bacterial contamination, oxidized and established explants of *E. benthamii* x *E. dunnii*, 21 days after inoculation.

Clone	Bacterial Contamination [a]	Oxidized Explants [a]	Established Explants [a]
	---------- (%)----------		
H12	1.0 B ± 4.47	3.0 AB ± 7.33	45.0 B ± 14.01
H19	9.0 A ± 12.10	6.0 A ± 9.40	46.0 B ± 7.31
H20	0.0 B ± 0.00	0.0 B ± 0.00	66.0 A ± 11.62

In the columns, the mean values followed by the same letter do not significantly differ by Tukey test ($p < 0.05$). [a]data presented as: mean ± standard deviation.

H12 and H19 did not differ regarding the success of explant establishment, with 45 and 46% of the explants surviving after final acclimation, respectively. H20 had the greatest percentage of established explants, with 66% of the plants surviving (Table 1). From established explants, the number of leaves, number of shoots and mean shoot length did not statistically vary among clones, with a mean value of approximately 5 leaves per explant and 2 shoots of about 0.7 cm in length per explant.

Shoot Multiplication

Despite of its satisfactory establishment, the H19 clone proved to be highly recalcitrant in the following stages, with a high oxidation index during bud proliferation, which limited its *in vitro* cultivation under tested conditions.

The concentration of NAA and BAP significantly affected the number of bud induction for H12 and H20 clones ($p < 0.01$; Figure 1). The greatest number of buds for H12 occurred with 0.51 mg L^{-1} BAP combined with 0.057 mg L^{-1} NAA. Under these concentrations, there were 8.8 buds per explant (Figure 1a). For H20, 0.45 mg L^{-1} BAP and 0.040 mg L^{-1} NAA resulted in the most buds, with about 13 buds per explant after 60 days of inoculation (Figure 1b).

Concentrations above 0.75 mg L^{-1} of BAP did not result in increased bud proliferation. Furthermore, under concentrations of 1 mg L^{-1} BAP combined with 0.1 mg L^{-1} NAA, vitrification, explant oxidation and mortality were verified.

Shoot elongation

The medium and growth regulator concentrations significantly affected the number of shoots per explant (Figure 2). In ½MS medium, H12 had the greatest number of elongated shoots with medium supplemented with 0.11 mg L^{-1} BAP and 0.13 mg L^{-1} GA_3 with 10.6 shoots per explant (Figure 2a). H20 had the most shoots under nutrient conditions of 0.08 mg L^{-1} BAP with 0.22 mg L^{-1} GA_3, with 5.82 elongated shoots per explant (Figure 2b).

Despite the fact that the estimation of BAP critical point for H12 was mathematically greater than the highest concentration tested on ½MS medium (Figure 2a), it can be noticed that the concentration of 0.1 mg L^{-1} BAP positively influenced the number of shoots, regardless of clone. Nevertheless, on MS medium, the H12 clone had, on average, 2.58 elongated shoots per explant under 0.07 mg L^{-1} BAP and 0.11 mg L^{-1} GA_3 (Figure 2c). The H20 clone, under 0.06 mg L^{-1} BAP and 0.18 mg L^{-1} GA_3 had 2.91 shoots (Figure 2d). When grown on MS medium, the clones had 3 elongated shoots per explant with 0.1 mg L^{-1} BAP concentration.

The clones had distinct behaviors with regard to GA_3. Regardless of medium, the concentration of 0.1 mg L^{-1} and 0.2 mg L^{-1} GA_3 produced the most shoots for H12 and H20, respectively. Furthermore, H12 had approximately twice the number of shoots compared to H20, when it grew on ½MS medium (Figures 2a and b).

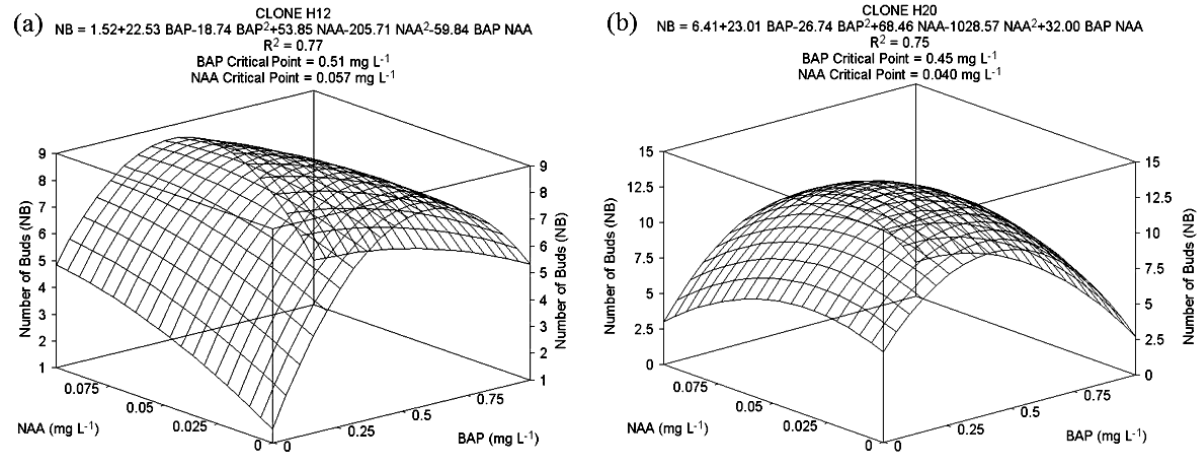

Figure 1. Average values for number of buds (NB) per explant of *E. benthamii* x *E. dunnii* under NAA and BAP treatments, 60 days after inoculation. (a): H12 clone and (b): H20 clone.

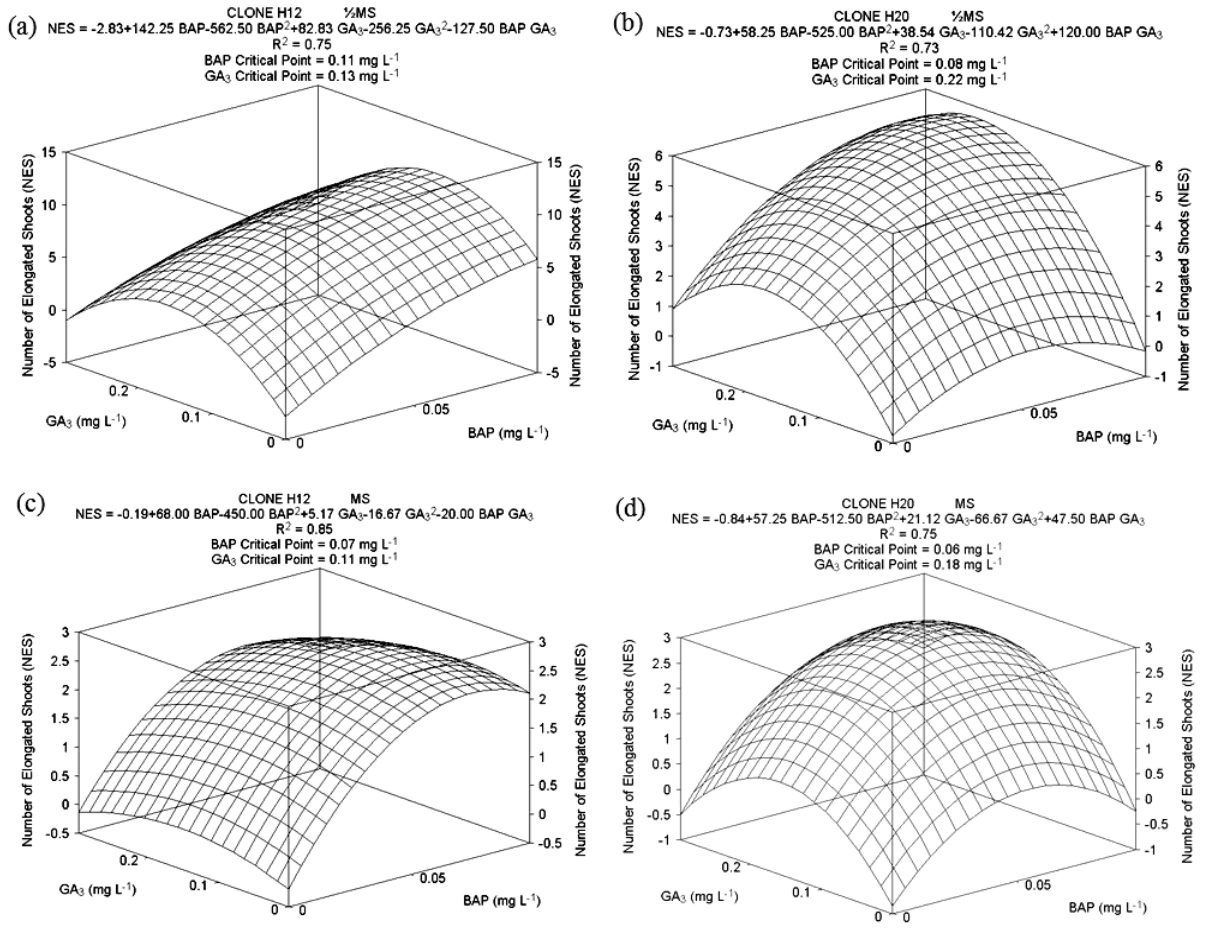

Figure 2. Average values for number of elongated shoots (NES) per explant of *E. benthamii x E. dunnii* under GA_3 and BAP treatments, 30 days after inoculation. (a): H12 clone and (b): H20 clone on ½MS medium, (c): H12 clone and (d): H20 clone on MS medium.

When it was grown on MS medium, H12 had a reduced percentage of shoots per explant (75% lower) compared to plants from ½MS medium. Similar results were found for the H20 clone, which had a 50% reduction in the number of shoots per explant when grown on MS medium. Furthermore, explants cultivated on MS medium showed oxidation symptoms, which did not occur when the culture medium had a 50% reduction in its saline composition (½MS).

H12 had the longest shoot length with medium supplemented with 0.07 mg L^{-1} BAP and 0.22 mg L^{-1} GA_3, with shoots on average of 1.52 cm in length when grown on ½MS (Figure 3a). H20 had the longest shoot length, at 1.5 cm, with medium supplemented with 0.08 mg L^{-1} BAP and 0.21 mg L^{-1} GA_3 (Figure 3b).

When full-strength MS was used, H12 clones were 1.13 cm long with medium containing 0.08 mg L^{-1} BAP and 0.16 mg L^{-1} GA_3 (Figure 3c). For H20, the longest shoots were, on average, 1.43 cm long and occurred with medium containing 0.12 mg L^{-1} BAP and 0.30 mg L^{-1} GA_3 (Figure 3d).

In general, the longest shoots were found with medium supplemented with 0.10 mg L^{-1} BAP and 0.20 mg L^{-1} GA_3, regardless of clone and MS concentration. Exceptions occurred for H20 cultivated on full-strength MS medium, where the GA_3 concentration that provided the longest shoots was at 0.30 mg L^{-1}.

Rooting

Both H12 and H20 had a rooting percentage of 12% of plants with roots *in vitro* (data not shown), 30 days after inoculation on ½MS medium. For *ex vitro* rooting, genetic materials did not differ in relation to IBA treatment, regarding survival after greenhouse (41.0%), after shade house (22.2%) and rooting under outdoor conditions (14.4%) (Table 2).

The rooted material was vigorous and had excellent aboveground development (Figure 2d). Although significant differences were not found, it was observed that the H12 clone tended to have the highest mean values (Table 2).

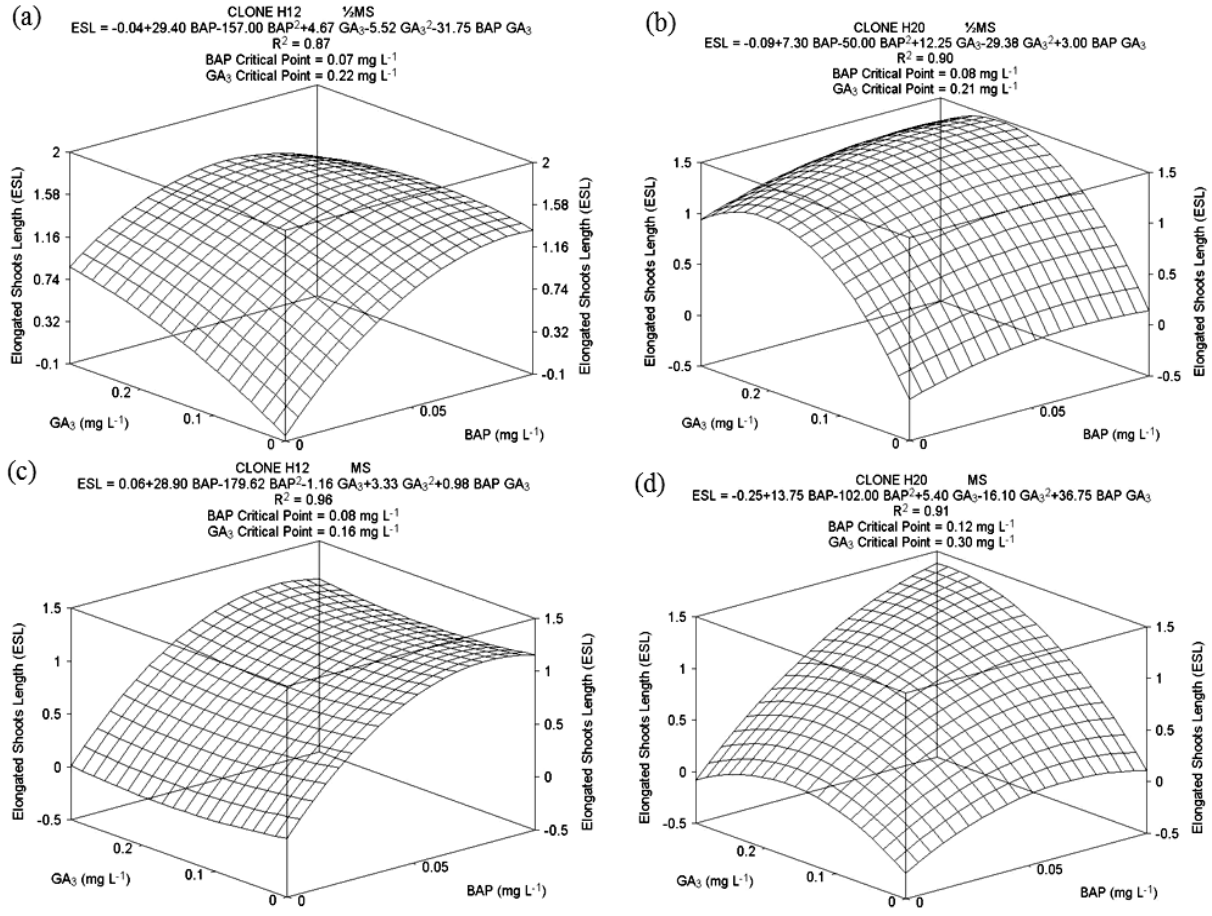

Figure 3. Average values for elongated shoot length (ESL) per explant of *E. benthamii* x *E. dunnii* under GA_3 and BAP treatments, 30 days after inoculation. (a): H12 clone and (b): H20 clone on ½MS medium, (c): H12 clone and (d): H20 clone on MS medium.

Table 2. Average values of *E. benthamii* x *E. dunnii* shoots survival after leaving greenhouse (GHS), shade house (SHS) and rooting under outdoor conditions (ROC), 90 days after planting.

Clone	IBA (mg L^{-1})	GHS [a]	SHS [a]	ROC [a]
		---------- (%)----------		
H12	0 (control)	46.7 ± 08.17	25.0 ± 12.25	16.7 ± 07.53
	1,000	43.3 ± 18.05	25.0 ± 21.68	18.3 ± 19.66
	Average	45.0	25.0	17.5
H20	0 (control)	38.7 ± 11.89	20.5 ± 26.69	12.6 ± 31.38
	1,000	35.3 ± 09.56	18.2 ± 23.56	10.2 ± 27.88
	Average	37.0	19.4	114
	General Average	41.0	22.2	14.4

[a]data presented as: mean ± standard deviation.

Regardless of the clone, there were a high percentage of plants with fungal contamination (41.33%, on average). The identification of contaminant sources (e.g. fungal or bacterial) during establishment becomes essential. Further studies on fungal and bacterial contamination are required, utilizing methods that reduce contamination with products application to source plants before shoots are collected, collaborating to the maximization of hybrid micropropagation processes.

The percentage of established explants were similar for our results compared to those found by Joshi et al. (2003) for 30-year-old *E. tereticornis* x *E. grandis* trees, both at approximately 50% survival after acclimation. Here, 50% of plants survived when treated with 30% concentration (v v^{-1}) of $NaOCl_2$ for 20 minutes. A similar treatment was performed by Bennett et al. (1994), with sodium hypochlorite at 1% (v v^{-1}) during 20 minutes for asepsis of 4- to 5-year-old nodal segments of *E. globulus* trees.

George et al. (2008) emphasize that the physiological condition of stock plants, from which the explants are obtained, has a high influence on the later behavior of cultures. This fact may have influenced the clone response, because the clonal mini-garden management system provided homogeneity in the phytosanitary conditions. Due to the non-significant effect among tested sodium hypochlorite concentrations, 0.5% chlorite concentration is recommended for the asepsis of explants of hybrid material when it comes from a clonal mini-garden cultivated in sand.

Eucalyptus micropropagation responses are variable according to concentration of plant growth

regulators and depend on work conditions. Gomes and Canhoto (2003) verified that 0.9 μM BAP ($\approx$ 0.20 mg L^{-1}) combined with 0.05 μM NAA ($\approx$ 0.01 mg L^{-1}) on ½MS medium was the optimum concentration for *E. nitens* bud proliferation. Despite the low rate of bud proliferation (2.25 per explant), their results were promising because of the high recalcitrance of this species. Joshi et al. (2003) observed that *E. tereticornis* x *E. grandis* produced 20 to 25 buds per explant after 150 days on MS medium supplemented with 1 mg L^{-1} BAP and 1 mg L^{-1} NAA.

Similar results were obtained by Bisht et al. (1999) for *E. tereticornis* x *E. camaldulensis*, where the highest multiplication, regardless of clone, occurred after 120 days on MS medium supplemented with 1 mg L^{-1} BAP and 1 mg L^{-1} NAA. For *E. tereticornis*, Sharma and Ramamurthy (2000) obtained the greatest number of buds (20 to 30 per explant) on MS medium supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA.

George et al. (2008) emphasize that excess cytokinin on the culture medium may be toxic to the explant, causing severe problems in the following stages. Bisht et al. (1999) verified for *E. tereticornis* x *E. camaldulensis* that the highest BAP concentrations (over 1 mg L^{-1}) reduced the number of buds per explant, regardless of the studied clone. Bennett et al. (1994) recorded that BAP concentrations above 2.5 µM ($\approx$ 0.56 mg L^{-1}) on MS medium reduced the average number of buds per explant of *E. globulus*, which was similar to the results found in the present study, where concentrations over 0.5 mg L^{-1} BAP reduced the number of buds per explant (Figure 1). This fact supports that concentrations above 0.5 mg L^{-1} BAP are not recommended during the bud multiplication stage for the studied hybrid.

In general, H12 and H20 clones produced similar number of buds in relation to regulator concentrations. Furthermore, the combination of 0.50 mg L^{-1} BAP with 0.05 mg L^{-1} NAA was the most promising, and similar to the best values estimated by mathematical models, which increased the number of buds per explant (Figure 4a).

Several reports used different cytokinin and gibberellin combinations to induce shoot elongation, mainly for genetic materials of difficult *in vitro* propagation. Joshi et al. (2003) observed multiplication followed by shoot elongation of 30-year-old *E. tereticornis* x *E. grandis* trees. The mean length of elongated shoots varied from 2.5 to 3 cm in length during 30 days when grown on MS medium supplemented with 1 mg L^{-1} BAP and 0.04 mg L^{-1} GA_3.

Similar effects were verified by Bisht et al. (1999) for *E. tereticornis* x *E. camaldulensis*, who reported multiplication followed by elongation after 120 days on MS medium supplemented with BAP and NAA. After 180 days on MS medium, all BAP and NAA treatments had elongation, producing shoots of 2.0 cm to 3.5 cm in length. Barrueto Cid et al. (1999) elongated buds regenerated from cotyledon leaves on SP medium (modified MS) supplemented with 1.0 µM BAP ($\approx$ 0.24 mg L^{-1}), 0.5 µM NAA ($\approx$ 0.09 mg L^{-1}) and 2.0 µM GA_3 ($\approx$ 0.7 mg L^{-1}) and, after 20 to 30 days of growth, produced vigorous shoots with mean length of 1.5 cm.

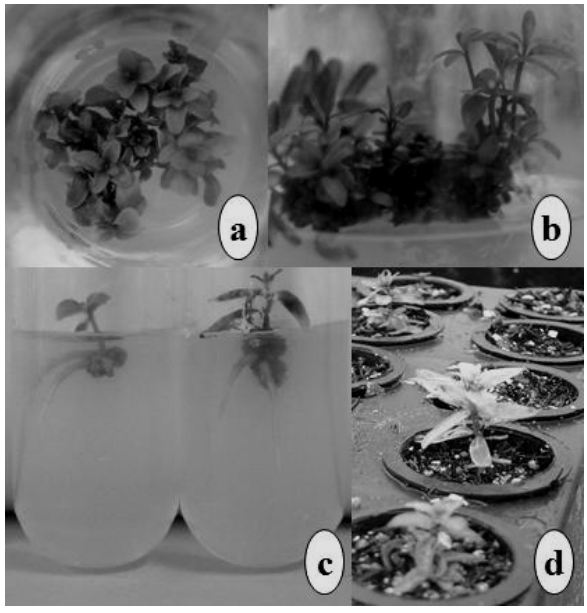

Figure 4. *E. benthamii* x *E. dunnii* micropropagation. (a) bud proliferation, (b) shoot elongation, (c) *in vitro* rooting, (d) *ex vitro* rooting.

In general, explants cultivated on ½MS supplemented with 0.10 mg L^{-1} BAP and 0.10 mg L^{-1} GA_3 for the H12 clone and 0.10 mg L^{-1} BAP and 0.20 mg L^{-1} GA_3 for the H20 clone had either the best standard development of number of elongated shoots and shoots length, then shoots presented a good aspect and vigor, with mean size around 1.5 cm in length (Figure 4b), 30 days after inoculation. The thinnest and the most fragile elongated shoots were produced in culture medium supplemented with GA_3 and without BAP; thus, its application is not recommended in the genetic material studied.

H12 and H20 clones had similar rooting responses, in both *in vitro* and *ex vitro* conditions. Joshi et al. (2003) verified the highest rooting rates on ½MS culture medium for *in vitro* conditions. However, the maximum rooting (75% of rooting) occurred with 1 mg L^{-1} IBA, as concentrations above

1 mg L^{-1} IBA promoted callus induction. Bisht et al. (1999) had the same response where the maximum rooting was verified only after adding 1 mg L^{-1} IBA to MS medium for *in vitro* conditions. It is relevant to emphasize for the studied hybrid material, that 2 mg L^{-1} IBA concentration induced callus formation followed by rooting (Figure 4c), an effect similar to that verified by Joshi et al. (2003) and Bisht et al. (1999). Lower IBA concentrations might increase rooting rates without callus induction (Figure 4d).

It was verified that the genotype had low rooting rates, even when cultivated *in vitro*, similar to the rates observed for *ex vitro* conditions. These results show that *E. benthamii* x *E. dunnii* hybrid material can be directly rooted *ex vitro*, thus maximizing micropropagation process tested. Despite low rooting rates through micropropagation, the results are promising due to rooting recalcitrance had by the hybrid at macropropagation (BRONDANI et al., 2008, 2010a e b). Further studies regarding this type of research will be complementary and necessary to develop a more efficient micropropagation protocol for the genotypes.

Conclusion

For nodal segments sterility, the use of 0.5% chlorine (NaOCl) is recommended.

The combination of 0.50 mg L^{-1} BAP and 0.05 mg L^{-1} NAA on ½MS basic medium provided the greatest number of buds for H12 and H20 clones. The concentrations of 0.10 mg L^{-1} GA_3 for the H12 clone and 0.20 mg L^{-1} GA_3 for the H20 clone, both combined with 0.10 mg L^{-1} BAP on ½MS basic medium, produced the longest shoots.

The GA_3 effect on shoot elongation depended on the basic composition of culture medium, where the ½MS provided the best results for the characteristics analyzed. Both H12 and H20 clones had low rooting rates, with an average of 12% of plants having roots for *in vitro* conditions and only 14.4% of plants having roots for *ex vitro* conditions, regardless of clone.

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References

AREZKI, O.; BOXUS, P.; KEVERS, C.; GASPAR, T. Hormonal control of proliferation in meristematic agglomerates of *Eucalyptus camaldulensis* Dehn. **In Vitro Cellular and Developmental Biology – Plant**, v. 36, n. 5, p. 398-401, 2000.

AREZKI, O.; BOXUS, P.; KEVERS, C.; GASPAR, T. Changes in peroxidase activity, and level of phenolic compounds during light-induced plantlet regeneration from *Eucalyptus camaldulensis* Dehn. nodes *in vitro*. **Plant Growth Regulation**, v. 33, n. 3, p. 215-219, 2001.

BALTIERRA, X. C.; MONTENEGRO, G.; GARCÍA, E. Ontogeny of *in vitro* rooting processes in *Eucalyptus globulus*. **In Vitro Cellular and Developmental Biology – Plant**, v. 40, n. 5, p. 499-503, 2004.

BARRUETO CID, L. P.; MACHADO, A. C. M. G.; CARVALHEIRA, S. B. R. C.; BRASILEIRO, A. C. M. Plant regeneration from seedling explants of *Eucalyptus grandis* x *E. urophylla*. **Plant Cell, Tissue and Organ Culture**, v. 56, n. 1, p. 17-23, 1999.

BENNETT, I. J.; McCOMB, J. A.; TONKIN, C. M.; McDAVID, D. A. J. Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. **Annals of Botany**, v. 74, n. 1, p. 53-58, 1994.

BISHT, P.; SHARMA, V. K.; JOSHI, I.; KAPOOR, M. L. Micropropagation of newly produced F_1 hybrid of *Eucalyptus* (*E. tereticornis* Sm. x *E. camaldulensis* Dehn. Southern Form). **Silvae Genetica**, v. 48, n. 2, p. 104-108, 1999.

BORÉM, A. **Biotecnologia florestal**. Viçosa: Suprema, 2007.

BRONDANI, G. E.; WENDLING, I.; ARAUJO, M. A.; PIRES, P. P. Ácido indolbutírico em gel para o enraizamento de miniestacas de *Eucalyptus benthamii* Maiden & Cambage x *Eucalyptus dunnii* Maiden. **Scientia Agraria**, v. 9, n. 2, p. 153-158, 2008.

BRONDANI, G. E.; DUTRA, L. F.; GROSSI, F. G.; WENDLING, I.; HORNIG, J. Estabelecimento, multiplicação e alongamento *in vitro* de *Eucalyptus benthamii* Maiden & Cambage x *Eucalyptus dunnii* Maiden. **Revista Árvore**, v. 33, n. 1, p. 11-19, 2009.

BRONDANI, G. E.; GROSSI, F.; WENDLING, I.; DUTRA, L. F.; ARAUJO, M. A. Aplicação de IBA para o enraizamento de miniestacas de *Eucalyptus benthamii* Maiden & Cambage x *Eucalyptus dunnii* Maiden. **Acta Scientiarum. Agronomy**, v. 32, n. 4, p. 667-674, 2010a.

BRONDANI, G. E.; WENDLING, I.; GROSSI, F.; DUTRA, L. F.; ARAUJO, M. A. Miniestaquia de *Eucalyptus benthamii* × *Eucalyptus dunnii*: (II) sobrevivência e enraizamento de miniestacas em função das coletas e estações do ano. **Ciência Florestal**, v. 20, n. 3, p. 453-465, 2010b.

BUNN, E. Development of *in vitro* methods for *ex situ* conservation of *Eucalyptus impensa*, an endangered mallee from southwest Western Australia. **Plant Cell, Tissue and Organ Culture**, v. 83, n. 1, p. 97-102, 2005.

BUNN, E.; SENARATNA, T.; SIVASITHAMPARAM, K.; DIXON, K. W. *In vitro* propagation of *Eucalyptus phylacis* L. Johnson and K. Hill., a critically endangered relict from Western Australia. **In Vitro Cellular and Developmental Biology – Plant**, v. 41, n. 6, p. 812-815, 2005.

BUTCHER, P. A.; SKINNER, A. K.; GARDINER, C. A. Increased inbreeding and inter-species gene flow in remnant populations of the rare *Eucalyptus benthamii*. **Conservation Genetics**, v. 6, n. 2, p. 213-226, 2005.

CORRÊA, L. R.; PAIM, D. C.; SCHWAMBACH, J.; FETT-NETO, A. G. Carbohydrates as regulatory factors on the rooting of *Eucalyptus saligna* Smith and *Eucalyptus globulus* Labill. **Plant Growth Regulation**, v. 45, n. 1, p. 63-73, 2005.

FOGAÇA, C. M.; FETT-NETO, A. G. Role of auxin and its modulators in the adventitious rooting of *Eucalyptus species* differing in recalcitrance. **Plant Growth Regulation**, v. 45, n. 1, p. 1-10, 2005.

GEORGE, E. F.; HALL, M. A.; KLERK, G. J. **Plant propagation by tissue culture**. 3rd ed. The Netherlands: Springer, 2008. v. 1.

GLOCKE, P.; COLLINS, G.; SEDGLEY, M. 6-benzylamino purine stimulates *in vitro* shoot organogenesis in *Eucalyptus erythronema*, *E. stricklandii* and their interspecific hybrids. **Scientia Horticulturae**, v. 109, n. 4, p. 339-344, 2006a.

GLOCKE, P.; DELAPORTE, K.; COLLINS, G.; SEDGLEY, M. Micropropagation of juvenile tissue of *Eucalyptus erythronema* x *Eucalyptus stricklandii* cv. 'Urrbrae Gem'. **In Vitro Cellular and Developmental Biology – Plant**, v. 42, n. 2, p. 139-143, 2006b.

GOMES, F.; CANHOTO, J. M. Micropropagation of *Eucalyptus nitens* Maiden (Shining Gum). **In Vitro Cellular and Developmental Biology – Plant**, v. 39, n. 3, p. 316-321, 2003.

HIGA, R. C. V.; HIGA, A. R.; TREVISAN, R.; SOUZA, M. V. R. Resistência e resiliência a geadas em *Eucalyptus dunnii* Maiden plantados em Campo Tenente, PR. **Boletim de Pesquisa Florestal**, n. 40, p. 67-76, 2000.

JOSHI, I.; BISHT, P.; SHARMA, V. K.; UNIYAL, D. P. *In vitro* clonal propagation of mature *Eucalyptus* F_1 hybrid (*Eucalyptus tereticornis* Sm. x *E. grandis* Hill ex. Maiden). **Silvae Genetica**, v. 52, n. 3-4, p. 110-113, 2003.

JOVANOVIC, T.; ARNOLD, R.; BOOTH, T. Determining the climatic suitability of *Eucalyptus dunnii* for plantations in Australia, China and Central and South America. **New Forests**, v. 19, n. 3, p. 215-226, 2000.

KOMATSU, Y. H.; BATAGIN-PIOTTO, K. D.; BRONDANI, G. E.; GONÇALVES, A. N.; ALMEIDA, M. *In vitro* morphogenic response of leaf sheath of *Phyllostachys bambusoides*. **Journal of Forestry Research**, v. 22, n. 2, p. 209-215, 2011.

LE ROUX, J. J.; VAN STADEN, J. Micropropagation of *Eucalyptus* species. **Journal of Hortscience**, v. 26, n. 5, p. 199-200, 1991.

MERKLE, S. A.; NAIRN, C. J. Hardwood tree biotechnology. **In Vitro Cellular and Developmental Biology – Plant**, v. 41, n. 5, p. 602-619, 2005.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, v. 15, n. 3, p. 473-497, 1962.

NEHRA, N. S.; BECWAR, M. R.; ROTTMANN, W. H.; PEARSON, L.; CHOWDHURY, K.; CHANG, S.; WILDE, H. D.; KODRZYCKI, R. J.; ZHANG, C.; GAUSE, K. C.; PARKS, D. W.; HINCHEE, M. A. Forest biotechnology: innovative methods, emerging opportunities. **In Vitro Cellular and Developmental Biology – Plant**, v. 41, n. 6, p. 701-717, 2005.

NUGENT, G.; CHANDLER, S. F.; WHITEMAN, P.; STEVENSON, T. W. Adventitious bud induction in *Eucalyptus globulus* Labill. **In Vitro Cellular and Developmental Biology – Plant**, v. 37, n. 3, p. 388-391, 2001.

SHARMA, S. K.; RAMAMURTHY, V. Micropropagation of 4-year-old elite *Eucalyptus tereticornis* trees. **Plant Cell Reports**, v. 19, n. 5, p. 511-518, 2000.

TRINDADE, H.; PAIS, M. S. *In vitro* studies on *Eucalyptus globulus* rooting ability. **In Vitro Cellular and Developmental Biology – Plant**, v. 33, n. 1, p. 1-5, 1997.

VILLA, F.; PASQUAL, M.; PIO, L. A. S.; ASSIS, F. A. Multiplicação *in vitro* de porta-enxerto de videira em variações do meio MS. **Acta Scientiarum. Agronomy**, v. 28, n. 3, p. 345-349, 2006.

WATT, M. P.; BERJAK, P.; MAKHATHINI, A.; BLAKEWAY, F. *In vitro* field collection techniques for *Eucalyptus* micropropagation. **Plant Cell, Tissue and Organ Culture**, v. 75, n. 3, p. 233-240, 2003.

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