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MICROPROPAGATION OF *EUCALYPTUS SALIGNA* SM. FROM COTYLEDONARY NODES

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Abstract

Eucalyptus saligna is an important woody plant used to lumber and cellulose. The aim of this research was to establish a protocol for micropropagation of this species from cotyledonary nodes. Plantlets with 16 days old were used as a donor explants. The induction of cotyledonary nodes consisted of two parts: a dark culture followed by a light culture. Basal medium was MS added with 30g.L^{-1} sucrose, 10% coconut water and solidified with 7g.L^{-1} agar. For the dark culture the media were supplemented with 3.6µM NAA (Naftalenoacetic acid) and 4.4 µM BAP (6-Benzylaminopurine) and for the light culture the media were supplemented with 2.7µM NAA and 1.1 µM BAP. The period for dark and light culture was 20 days. Shoots were multiplied on MS medium, 30 g.L^{-1} sucrose supplemented with 1.1 µM BAP. Shoots were elongated on MS medium free of plant growth regulators. Shoots were rooting on half-strength MS salts. Acclimatization was performed in a hydroponics floating system. Moreover, the shoot multiplication in liquid medium with different $CaCl_2$ levels was carried out under agitation. Organogenesis of cotyledonary nodes was characterized by simultaneous occurrence of shoot and callus. Shoots presented hyperhydricity under liquid medium, however, the $CaCl_2$ reduces the hyperhydricity in liquid medium; nevertheless, it had been not effective in eliminating hyperhydricity due to toxicity of chlorine. The hydroponics acclimatization results in 90% plant survival. An efficient protocol for micropropagation of *E. saligna* was suitable established and can be used for clonal propagation or genetic transformation.

Key words: Hyperhydricity; Nitrogen source; Eucalypt; Phenolic oxidation; Browning explant; Callogenesis.

Introduction

The species *Eucalyptus saligna* (Myrtaceae) is originated from Australia, it generally occurs in the coastal area and in the valleys of the mountainous chains close to the coast of New South Wales, and to the south of Queensland. *E. saligna* is tipic of humid and hot climate, this species presents high potential of adaptation and growth (e.g. mainly different soil types) and its lumber is very used to produce wooden cross ties (e.g. tramway sleepers), crosspieces, sawed wood and pulp to paper industry. Nevertheless, this species is susceptible to cold and frost, whereas the largest restriction to *E. saligna* culture is in the regions where cold and frosts occur. Frost is too severe that eucalypt trees show since burned tip until dead of the aerial part (Selle & Vuaden, 2008). In regions where the incidence of severe frosts affects the establishment of eucalypts forests, the adaptation to low temperatures is one of the most important characteristics that can be introduced via genetic transformation or hybridization (Brondani *et al*., 2011; Brondani *et al*., 2012).

Some advances for genetic transformation were already reached (Shinwari *et al*., 1998), a protocol for genetic transformation via *Agrobacterium tumefaciens* was developed using leaf explants, nevertheless a number extremely low of transformed plants was regenerated, being one plant per each 200 explants, 0.5% efficiency (Dibax *et al*., 2010). Some attempts using shoot tips as explants for transformation via *Agrobacterium tumefaciens* were already performed (Lopes da Silva *et al*., 2011). Moreover, some efforts to elucidate others factors involved with the increase of transient expression were already performed, as the influence of pre-culture and the medium type for *Agrobacterium* resuspension. This study had demonstrated which the MS/2 medium and the pre-culture of one day increase the efficiency of the transient expression of the gene *uidA* in leaf explants of *E. saligna* (Lopes da Silva *et al*., 2013a). Nevertheless, the main difficulty is the low rate of transformed plant regeneration.

Among the eucalypt species, *E. saligna* represents one of the most easy to cultivate *In vitro* as compared to other eucalypt species. There are few reports of *In vitro* propagation for *E. saligna*, however these studies include: a protocol for *In vitro* establishment of nodal segments collected in field from seedlings or adult plants (Le Roux & Van Staden, 1991) and a protocol using leaf and cotyledonary explants via indirect organogenesis (Dibax *et al*., 2010). However, when an explant is transformed its time of regeneration is larger than when this same explant is not transformed. Eucalypt as the most of woody plants presents high browning rate and probably this represents the main difficult for *In vitro* regeneration of explants. Several causes can be associated to the high browning rates; nevertheless, one of them, can be related the thydiazuron (TDZ) presence (which some protocols use), whereas the senescence induction in explants cultivated with TDZ is caused by its action in the ethylene production (Carvalho *et al*., 2011). The ethylene is associated to high browning percentage in explants cultured *In vitro*.

Therefore, the aim of this study was to establish a protocol for *In vitro* regeneration of *E. saligna* from cotyledonary nodes.

Materials and Methods

Disinfection and *In vitro* germination of seeds: The seeds of *Eucalyptus saligna* Sm. used in this research were provided by EMBRAPA – Forests (Colombo, Brazil), these seeds were a mixture of the following progenies: BR00-519 (27%), BR00-523 (13%), BR01-197 (12%), BR00-534 (12%), BR01-201 (12%), BR00-530 (12%) and BR00-539 (12%). The seeds were disinfected with ethanol 70% during 3 min., and after in NaOCl 6% (with 5 drops Tween-20® per 100 mL) for 30 min., and after washed three times with distilled and autoclavated water (Lopes da Silva *et al*., 2010). Seeds were sowed on half strength MS medium (Murashige & Skoog, 1962) supplemented with 20 $g.L^{-1}$ sucrose and solidified with 7 $g.L^{-1}$ agar. The pH was adjusted to 5.8 before autoclaving. These explants were cultivated in flasks.

Organogenesis of cotyledonary nodes: Seedlings (16 days old) were the cotyledonary node donors, which were excised and inoculated in the following treatments: M1 – MS (Murashige & Skoog, 1962); M2 – MS with 10% coconut water (Sigma®); M3 – MS with half strength nitrogen (13.3 mM NH_4NO_3 and 9.4 mM KNO_3) and M4 - MS with half strength nitrogen (13.3 mM NH_4NO_3 and 9.4 mM KNO_3) with 10% coconut water (Sigma®). All these media were supplemented with 3.6 µM 1-Naphthaleneacetic acid (NAA) and 4.4 µM 6-Benzylaminopurine (BAP), 30 $g.L^{-1}$ sucrose and 7 $g.L^{-1}$ agar. The pH was adjusted to 5.8. All cultures were kept in dark during 20 days. After the dark culture, the explants were evaluated with regard the oxidation (%), survival (%), callogenesis (%), cotyledonary nodes with shoots (%) and shoot number per cotyledonary node. After the evaluation, the explants were transferred to same treatments described above, nevertheless with modifications in level of plant growth regulators, being 2.7 µM NAA and 1.1 µM BAP, these cultures were kept for 20 days under light conditions. After 20 days in the light, the explants were evaluated again with regard to the same characteristics used to evaluate the explants in dark. These explants were cultivated in flasks.

Evaluation of the residual effect of the media used for organogenesis of cotyledonary nodes in *In vitro* multiplication: In order to evaluate the residual effect of the media used for organogenesis of cotyledonary nodes (M1, M2, M3 and M4), these explants were cultured on the multiplication medium (i.e. MS modified with 13.3 mM NH_4NO_3 and 9.4 mM KNO_3 (50% of standard level of this medium) and supplemented with 20$g.L^{-1}$ sucrose, 1.11 µM BAP and solidified with 7$g.L^{-1}$ agar) (Dibax *et al*., 2010); and these plants were not mixed in other treatments. The browning (%), survival (%), callogenesis (%), cotyledonary nodes with shoots (%) and shoot number per cotyledonary node were evaluated after 20 days of culture. These explants were cultivated in flasks.

***In vitro* multiplication in Petri dishes:** Shoots obtained from cotyledonary nodes according the process described above cultivated on medium M2 were used as explants. The experiment was carried out in Petri dishes with 9 cm diameter, 2 cm height and 100 mL volume. The medium volume used was 20mL. The basal medium was MS, 30 $g.L^{-1}$ sucrose and solidified with 7 $g.L^{-1}$ agar. The experiment was organized in a three-way ANOVA (2 x 2 x 3). The factor A consisted of two explant type (isolated shoot and multiple shoots), the factor B consisted of two subcultures (28 and 56 days) and the factor C consisted of three BAP levels (0, 1.11 and 2.22 µM). It was used 5 explants per dish. The shoot number per explant was evaluated after 28 and 56 days of culture. The proliferation rate was estimated by the shoot number obtained at 56 days of culture divided by the shoot number obtained at 28 days of culture. The means of proliferation rate was compared independent of three-way ANOVA.

Effects of amount of MS medium nitrogen for *In vitro* multiplication: Shoots (5mm) obtained from cotyledonary nodes according the process described above cultivated on medium M2 were used as explants. These shoots were cultivated in flasks. The treatments consisted of three different levels of KNO_3 and NH_4NO_3 of MS medium: (1) 5.15 mM NH_4NO_3 and 4.7 mM KNO_3 (25% N compared to standard of MS medium), (2) 13.3mM NH_4NO_3 and 9.4 mM KNO_3 (50% N compared to standard of MS medium) and 20.6 mM NH_4NO_3 and 18.16mM KNO_3 (100% N, level standard of MS medium). The basal medium was MS supplemented with 1.11 µM BAP, 30 $g.L^{-1}$ sucrose and solidified with 7 $g.L^{-1}$ agar. The shoot number per explant, fresh and dry mass were evaluated after 28 days of culture

Effect of $CaCl_2$ to control the hyperhydricity in liquid medium: Shoots (5 mm height) were cultured in liquid MS medium, 30 $g.L^{-1}$ sucrose, 1.11 µM BAP. The treatments were: 0 (Level standard of MS medium (i.e. 440 $mg.L^{-1}$ $CaCl_2.2H_2O$)), 440, 880 and 1320 $mg.L^{-1}$ $CaCl_2$. These explants were shaking at 80 rpm. The shoot number per explant, hyperhydricity (%), browning (%), fresh mass (mg) and dry mass (mg) were evaluated after 21 days of culture.

***In vitro* elongation and *In vitro* rooting:** The new shoots originated from multiplication medium were elongated on MS medium supplemented with 30 $g.L^{-1}$ sucrose, 2.5 $g.L^{-1}$ activated charcoal, free of plant growth regulators and solidified with 7 $g.L^{-1}$ agar (Vetec™) (Dibax *et al*., 2010). The height of the aerial part (cm) was determined after 20 days of *In vitro* culture. Elongated shoots (with approximately 1.5 cm height) were transferred to the rooting medium, which consisted of half strength MS medium (Murashige & Skoog, 1962) supplemented with 30 $g.L^{-1}$ sucrose, 2.5 $g.L^{-1}$ activated charcoal and solidified with 7 $g.L^{-1}$ agar (Vetec™). The height of the aerial part (cm), fresh mass of plant, roots and aerial part (g), root number, root total length (cm) and rooting percentage were evaluated after 50 days of culture.

Acclimatization: In order to acclimatize the rooted plants, it was used a pre-acclimatization which consisted of the lid removal of the culture flasks. Ten mL of distilled autoclaved water were added into the flasks due to evapotranspiration of plants and culture medium. After 72 hours of the flask opening, the plants were cultivated in thick sand in an expanded polyethylene tray floating on a nutritive solution consisting of modified liquid MS medium (salts and FeEDTA with one-half and one-quarter of the original concentration, respectively, and without organic constituents) inside a plastic tray. Trays containing the hydroponic culture were placed in a growth room for five days and then transferred to a greenhouse. Hydroponic culture lasted 14 days, then the plants were transferred to Plantmax™ HT substrate.

Culture conditions and statistical analysis: All media had the pH adjusted to 5.8 and were autoclaved at 1.1 kg/cm^2 and 121ºC for 20 min. The cultures were kept at 25 ± 2ºC under white fluorescent light (30 µM m^{-2} s^{-1}) with a 16 h photoperiod. All treatments consisted of five replicates with five explants. The data was submitted in a homogeneity analysis for the Bartlett´s test and, followed by analysis of variance (ANOVA) followed by Duncan´s test, both at the levels of $p \leq 0.05$. Variables from counting were transformed to $\sqrt{x+0.5}$ and variables from percentage were transformed to $\arcsin\sqrt{x/100}$. All the statistical analyses were performed using the software GENES (Cruz, 2001). The culture flasks had 6.5 cm diameter, 8.5 cm height, 250 mL volume and were sealed with rigid polypropylene caps (The culture medium volume used was 30 mL per flask).

Results and Discussion

Organogenesis of cotyledonary nodes: After 20 days of culture in dark, the M3 and M4 medium favored the shoot number per cotyledonary nodes during this first phase, and after 20 days of culture in light the best results for this variable was obtained in M2 and M4 (Table 1). The presence of coconut water in M2 and M4 due to its unique chemical composition of sugars, vitamins, minerals, amino acids and phytohormones (Yong *et al*., 2009), can have influenced larger shoot number per explant. On the other hand, the M3 medium presented higher shoot number just in first phase in dark; and when they were cultivated in light presented a low shoot number (Table 1), probably due to absence of coconut water.

The calli percentage was low, varying from 12 to 36% in dark phase and from 33.3 to 61% in light phase (Table 1). Nevertheless, the percentage of cotyledonary nodes with shoots was high, from 96 to 100% in dark phase and from 86 to 96% in light phase (Table 1). Organogenesis of cotyledonary nodes was characterized by simultaneous occurrence of shoot and callus. It is possible that callus and shoots can have different origins in different tissues, what can be caused by the presence of pieces of cotyledons and hypocotyls, which remained after the excision of the cotyledonary nodes. Similar results were found in nodal explants of fox grape cv. Bordô (*Vitis labrusca* L.) cultivated on MS supplemented with 1-5 µM BAP, where it induced simultaneous formation of shoot and callus (Carvalho *et al*., 2013). However, the cotyledonary nodes demonstrated an excellent ability to regenerate and can be explored for genetic transformation. On the other hand, leaf explants of *E. saligna* presented a low ability to regenerate (from 5 to 30% shoots) and this can be associated with high browning rate (from 70 to 80%), this protocol consist of two phases: calli induction and bud regeneration, the calli induction use 1-2 µM TDZ and 0.1 µM NAA and bud regeneration uses 0.67 µM NAA and 1.11 BAP (Dibax *et al*., 2010).

There were not statistical differences in all treatments for survival percentage; nevertheless, it varied from 88.8 to 96% in dark and from 91 to 96% in light (Table 1). The browning was the cause of death explants. The browning percentage of the explants was not influenced during the first phase in dark and the percentages were low (Table 1). The culture in dark is used to inhibit the calli browning, whereas the light induces the release of phenolic compounds (Mayer, 1987). However, when they were cultivated in light (second phase), the browning percentage had increased (Table 1). The M2, M3 and M4 presented lower browning percentage than M1; demonstrating that the N reduction of MS medium (50%) and the presence of coconut water decreases browning percentage. It is possible that the decrease of browning percentage in M2 and M4 media does not be only by the reduction of nitrogen level (50%), but by the salt reduction as observed in explants of the hybrid *E. benthamii x E.dunii* cultivated on MS medium, wherein the browning symptoms did not occur on explants cultivated on half strength MS medium (½ MS) (Brondani *et al*., 2011). The effect of coconut water in the decrease of browning symptoms is already known, as observed in *E. nitens* and *E. globulus*, wherein the addition of coconut water (5-10% v/v) showed a beneficial effect on appearance and growth of calli cultivated on MS medium supplemented with 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP compared to medium without coconut water (Spokevicius *et al*., 2005).

Evaluation of the residual effect of the media used for organogenesis of cotyledonary nodes on *In vitro* multiplication: The media used for organogenesis of cotyledonary nodes did not present residual effects on *In vitro* multiplication for the browning (%), survival (%), calli (%) and shoots (%) (Table 2). Nevertheless, there is a residual effect for the shoot number per explant as observed in M4 medium (Table 2). Probably the decrease of nitrogen level and the absence of coconut water had influenced this characteristic; however, the reduction of nitrogen level, but with the presence of coconut water did not affect the reduction of the shoot number per explant as observed in explants from M2 medium transferred to multiplication medium. Therefore, we do not recommend the M4 medium to organogenesis of cotyledonary medium.

Table 1. Organogenesis of cotyledonary nodes of *Eucalyptus saligna* Sm. in two phases (a dark culture of 20 days followed by a light culture of 20 days). The media were: M1–MS (Murashige & Skoog, 1962); M2–MS with 10% coconut water (Sigma®); M3–MS with half strength nitrogen (13.3 mM NH_4NO_3 and 9.4 mM KNO_3) and M4-MS with half strength nitrogen (13.3 mM NH_4NO_3 and 9.4 mM KNO_3) with 10% coconut water (Sigma®). All media were supplemented with 30 g.L^{-1} sucrose and solidified 7 g.L^{-1} agar (Vetec®). These explants were cultivated in flasks.

Luminosity conditions	Culture media	Plant growth regulators	Browning (%)	Survival (%)	Calli (%)	Nodes with shoots (%)	Shoots per cotyledonary node
Dark 20 days	M1	3.6 µM NAA + 4.4 µM BAP	12 a[1]	88.8 a	20 a	100 a	1.1 b
	M2		4 a	96 a	36 a	96 a	0.8 b
	M3		4 a	96 a	12 a	100 a	1.9 a
	M4		12 a	87 a	20 a	100 a	1.6 a
	CV (%)		57.7	15.2	45.7	2.0	0.3
Light 20 days	M1	2.7 µM NAA + 1.1 µM BAP	79.2 a	96 a	33.3 b	96 a	3.3 b
	M2		52.2 b	96 a	61 a	86 a	4.5 a
	M3		52.2 b	96 a	44 ab	92 a	3.4 b
	M4		52.2 b	91 a	41 b	86 a	4.8 a
	CV (%)		22.9	17.2	26.1	15.4	9.3

[1] Means within a column followed by the same letter for each parameter are not different at p<0.05 by Duncan's test

Table 2. Evaluation of the residual effect of the media (M1, M2, M3 and M4) used for organogenesis of cotyledonary nodes in *In vitro* multiplication of *Eucalyptus saligna* after 20 days. Media: M1–MS (Murashige & Skoog, 1962); M2–MS with 10% coconut water (Sigma®); M3–MS with half strength nitrogen (13.3 mM NH_4NO_3 and 9.4 mM KNO_3) and M4–MS with half strength nitrogen (13.3 mM NH_4NO_3 and 9.4 mM KNO_3) with 10% coconut water (Sigma®). All media were supplemented with 30 g.L^{-1} sucrose and solidified 7 g.L^{-1} agar (Vetec®). These explants were cultivated in flasks.

Culture media	Browning (%)	Survival (%)	Calli (%)	Shoot (%)	Shoot number per explant
M1	4 a[1]	96 a	12 a	96 a	17.3 a
M2	4 a	96 a	16 a	88 a	21.3 a
M3	12 a	92 a	8 a	92 a	18.5 a
M4	8 a	92 a	0 a	100 a	11.8 b
CV (%)	13.7	4.5	22.3	33.5	18.6

[1]Means within a column followed by the same letter for each parameter are not different at p<0.05 by Duncan's test.

In some cases the residual effect of the medium exert a significant influence on the explants during the others subcultures (same medium) or new media (others culture phases), as demonstrated in *Lavandula angustifolia* cultivated on MS medium supplemented with 1320 mg.L^{-1} $CaCl_2.2H_2O$, wherein the shoot-tip necrosis had increased (approximately 20%) in the second subculture, suggesting that the calcium uptake is limited (Machado *et al*., 2014). In tobacco, a residual effect was promoted by the addition of cefotaxime to the regeneration medium; resulting in rooting inhibition this shoots after the transferring to rooting medium (Nauerby *et al*., 1997). The residual effect of the culture medium can influence even in the acclimatization, as observed in *Lavandula angustifolia* cultivated on elongation and rooting media constituted with different levels and sources of GA_3 (gibberellic acid), plants obtained from culture medium supplemented with 0.25 mg.L^{-1} GA_3 contained in 7.8 mL.L^{-1} fermented extract of *Fusarium moniliforme* presented larger development in the height of the aerial part, fresh weight of the aerial part, leaf number, dry weight of roots and lateral shoots (Lopes da Silva *et al*., 2013b).

***In vitro* multiplication in Petri dishes:** Shoot multiplication in Petri dishes was not efficient. A low shoot number per explant was obtained. The evaluated characteristics for the multiplication of isolated shoots or multiple shoots were significant for the three factors studied; nevertheless, there was interaction only for subculture time and BAP levels. The BAP was indispensable to enhance the shoot number. The largest multiplication rates were reached with the presence of 1.11 µM BAP in culture medium, being 3.4 shoots per isolated shoots and 3.1 shoots per multiple shoots, comparing to 1.0 and 1.7 shoots per multiple shoots and isolated shoots in medium free of BAP, respectively (Table 3). The shoot separation enhances the explant number and the multiplication using isolated shoots is the same obtained using the multiple shoots (Table 3). The shoot multiplication using Petri dishes is not recommended due to high browning percentage, 100% of the explants presented browning symptoms and this probably resulted in low multiplication rates, it were observed much parts of tissues with browning symptoms, what it becomes necessary to excise this necrosed parts before to subculture these explants to new fresh medium. The *In vitro* multiplication in culture flasks was more efficient than the Petri dishes, whereas the highest multiplication rate in culture flasks was 21.3 shoots per explants (20 days of culture) (Table 2) comparing to the largest multiplication rate obtained in Petri dishes, which was 12.8 shoots per explant in the second subculture (56 days of culture) (Table 3).

Several factors can be involved to induce browning in explants cultivated in Petri dishes. We can consider the great exposition area to light incidence, whereas Petri dishes has not a rigid polypropylene lid to avoid the direct light incidence as the culture flasks has. The phenolic oxidation is a great problem for tissue culture, mainly because none tissue lacks phenolic compounds and high concentrations can be found in actively growing cells (Ozyigit *et al*., 2007). Moreover, these phenolic compounds (i.e. molecules responsible to explant browning) are secondary metabolites in plant tissue and their biosynthesis is greatly influenced by light (Crippen & Morrison, 1986; Mayer, 1987).

Table 3. Shoot number per explant and proliferation rate of isolated shoots and multiple shoots obtained from cotyledonary nodes of *Eucalyptus saligna* cultivated on different BAP levels on Petri dishes after 28 and 56 days.

Explant type	BAP (µM)	Shoot number per explant		Proliferation rate
		28 days	56 days	
Isolated shoot	0.00	1.8 bB1	3.1 cA	1.7 b
	1.11	3.4 aB	11.5 aA	3.4 a
	2.22	3.3 aB	10.5 bA	3.2 a
CV (%)		9.3		14.6
Multiple shoots	0.00	3.3 bA	3.3 cA	1.0 c
	1.11	4.1 aB	12.8 aA	3.1 a
	2.22	4.1 aB	11.1 bA	2.7 b
CV (%)		9.3		14.6

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's test. The proliferation rate was estimated by the shoot number obtained at 56 days of culture divided by the shoot number obtained at 28 days of culture

Another consideration to regard the use of Petri dishes to favor browning in eucalypt explants is the small volume (small headspace) and the sealing these dishes (i.e. a polyvinylchloride film or parafilm is used to seal the Petri dishes). This consideration was raised due to the hypothesis of the accumulation of ethylene; it is possible that wounded tissues and/or tissues exposed to light incidence can produce ethylene. The culture flasks (250 mL total volume) had a difference of 150 mL more than Petri dishes (100 mL total volume); of course, we must discount the volume occupied by the culture medium too, 30 mL and 20 mL for culture flasks and Petri dishes, respectively. Moreover, the culture flasks were not sealed with polyvinylchloride film presenting larger gas exchange than Petri dishes. The short or none gas exchange associated to a minor container volume and larger exposure to light can enhances the ethylene accumulation inside the Petri dish resulting in a high browning in tissues with detrimental consequences which can induce death explants. Similar results were found in *Eucalyptus camaldulensis*, which was observed the favourable effect of a half-litre glass vial with a greater headspace in the development of the explants (Arezki *et al*., 2001). This headspace effect has also been mentioned for micropropagated *Prunus* rootstock cultivated in different container types (Demeester *et al*., 1995). Reductions in explant size in a reduced confined atmosphere, has been attributed to the effect of accumulated ethylene (Kevers *et al*., 1992).

Of course there are very reports about the success of different *In vitro* morphogenesis of eucalypt explants using Petri dishes, nevertheless, the subcultures were carried out in a very short time, varying from 1 to 2 weeks (Hervé *et al*., 2001). Nevertheless, these frequent manipulations of the explants (pealing) increase the contamination risk of cultures. Some these cultures were carried out in dark (Arezki *et al*., 1998; Arezki *et al*., 2001; Trindade & Pais, 2003; Pinto *et al*., 2008; Dibax *et al*., 2010) and others in light (Arruda *et al*., 2000; Nugent *et al*., 2001; Prakash & Gurumurthi, 2009; Prakash & Gurumurthi, 2010). Nevertheless, it is possible that the *In vitro* multiplication these explants from cotyledonary nodes can be made in Petri dishes cultivated in dark and the subcultures performed more frequent (one or two weeks).

Effects of amount of MS medium nitrogen for *In vitro* multiplication. Different nitrogen levels in the MS medium did not influence the shoot number per explant; however, the shoot number per explant had varied from 10.3 to 14.8 among the treatments (Table 4). There were statistical differences for fresh and dry mass among the treatments, being the best result obtained in nitrogen standard level of MS medium (i.e. 20.6 mM NH_4NO_3 and 18.16 mM KNO_3). However, in a study for *In vitro* morphogenesis of *Eucalyptus saligna* was used the level of 13.3 mM NH_4NO_3 and 9.4 mM KNO_3 in several media (i.e. represents the half strength for nitrogen standard of MS medium), such as shoot multiplication medium, leaf callogenesis medium, medium for bud regeneration from calli and shoot elongation medium (Dibax *et al*., 2010). Nevertheless, in this prior study the nitrogen levels were not evaluated. Our data suggests the nitrogen standard level of MS medium can be used with better results for *In vitro* culture of *E. saligna*, whereas *E. saligna* already was one of the most cultured eucalypt species in Brazil, and was substituted by *E. grandis* due to its great necessity of nutrient consumption; therefore, the culture of *E. saligna* is most expensive due to high cost for fertilization. Although, some plant habits is similar *In vivo* and *In vitro* as proposed by the high demand of nutrients of *E. saligna*.

The half strength of nitrogen of MS medium is common used to avoid or to reduce the explant hyperhydricity as demonstrated in *Eucalyptus tereticornis*, which was observed high vigor, growth and reduced hyperhydricity when cultured on MS medium with 50% of NH_4NO_3 and 36.6% of KNO_3 (Sharma & Ramamurthy, 2000). Other example was observed in the cultivation of *Eucalyptus grandis* on MS medium with 50% concentrations of KNO_3 and NH_4NO_3 in temporary immersion system, the average production was 230 plants per flask (500 mL) in six weeks, at a frequency of a three minutes immersion every 12 hours (Castro & Gonzáles, 2002). Nevertheless, in our study we do not observe any hyperhydricity symptom for *E. saligna* cultivated in solid media.

Table 4. Amount of the nitrogen contained in the MS medium for *In vitro* multiplication of *Eucalyptus saligna* after 28 days. These explants were cultivated in flasks.

Nitrogen (%)	NH_4NO_3 (mM)	KNO_3 (mM)	Shoot number per explant	Fresh mass (mg)	Dry mass (mg)
25	5.15	4.7	10.3 a[1]	140.9 b	81.0 b
50	13.3	9.4	11.8 a	154.8 b	89.6 b
100	20.6	18.16	14.8 a	324.2 a	150.8 a
	CV(%)		11.4	15.5	16.6

[1] Means within a column followed by the same letter for each parameter are not different at p<0.05 by Duncan's test

Table 5. Effects of $CaCl_2$ in *In vitro* multiplication of *Eucalyptus saligna* cultivated in liquid medium under shaking (80 rpm) after 21 days. These explants were cultivated in flasks.

$CaCl_2$ ($mg.L^{-1}$)	Shoot number per explant	Hyperhydricity (%)	Browning (%)	Fresh mass (mg)	Dry mass (mg)
0[1]	3.8 a[3]	72 b	4 a	121.1 a	18.2 a
440[2]	6.1 a	75 b	10 a	127.8 a	19.0 a
880[2]	5.9 a	46 a	8 a	123.2 a	16.8 a
1320[2]	4.3 a	56 a	48 b	99.3 b	13.7 b
CV(%)	14.4	39.8	46.1	26.1	19.6

[1]Level standard of MS medium (i.e. 440 $mg.L^{-1}$); [2]Level supplemented to the level standard of MS medium; [3]Means within a column followed by the same letter for each parameter are not different at p<0.05 by Duncan's test

Effect of $CaCl_2$ to control the hyperhydricity in liquid medium: The levels of 880 and 1320 $mg.L^{-1}$ decreased the hyperhydricity occurrence, 46 and 56% hyperhydricity, respectively (Table 5). Similar results were observed in *Lavandula angustifolia* which the hyperhydricity percentage was reduced in shoots cultivated with the concentration of 1320 $mg.L^{-1}$ $CaCl_2.2H_2O$ (Machado *et al*., 2014). The $CaCl_2$ levels did not influence the shoot number per explant. On the other hand, the level of 1320 $mg.L^{-1}$ $CaCl_2$ presented detrimental effects on fresh and dry mass. These results are associated to stress promoted by chlorine as demonstrated in these studies (Ulisses, 2000; Sajid & Aftab, 2012; Brondani *et al*., 2013). Moreover, the concentration of 1320 $mg.L^{-1}$ $CaCl_2$ had increased the browning oxidation, demonstrating chloride toxicity in high levels. Chloride had a high oxidant power, and in high levels presents great toxicity. However, it is possible to use others calcium sources, which have no chlorine. The $CaCl_2$ reduces the hyperhydricity in *E. saligna* cultivated in liquid medium under agitation; nevertheless, it had been not effective in eliminating hyperhydricity due to toxicity of chlorine.

Calcium is an integral component in the cell wall and plays an important role in maintaining membrane integrity (Marschner, 1995; Machado *et al*., 2010), also it can modify the permeability of plant membranes (Taiz & Zeiger, 2006). Calcium is associated to processes such as membrane structure and function, ions uptake, reactions with growth regulators and enzymatic activation (via calmodulin) (Malavolta *et al*., 1997). The structural function of calcium is characterized by the use in the synthesis of new cell walls, particularly the middle lamellae that separate newly divided cells (Taiz & Zeiger, 2006). Calcium is an element relatively immobile in most plant species. The *In vitro* environment (i.e. it presents high relative humidity, approximately 95 to 100%) limits the calcium uptake due to low transpiration of the explants cultivated in flasks. The absence or deficiency of calcium is one the causes of hyperhydricity as demonstrated in several studies. In *Oncidium leucochilum*, the hyperhydricity was induced in a medium free of calcium (Lopes da Silva *et al*., 2014). In studies on calcium deficiency in tissue cultures, it was shown that an increase in calcium in the medium reduced hyperhydricity and tip necrosis (Sha *et al*., 1985).

There are no reports on the hyperhydricity occurrence in *E. saligna* cultivated on solid medium. However, in liquid medium *E. saligna* show hyperhydricity symptoms. Shoots of *E. saligna* cultivated in multiplication and elongation liquid media under stationary culture after 21 days demonstrated 100% hyperhydricity (data not shown). Various factors can be involved with the hyperhydricity occurrence; however, the most common is the liquid culture (Scheidt *et al*., 2011). The culture in liquid media is an alternative to reduce cost due to eliminate the use of agar, whereas the agar is the component more expansive used in culture media. Moreover, the liquid culture media are used in bioreactors for plant tissue culture automation. Other advantages in the liquid culture media utilization is the *In vitro* culture under stationary culture (i.e. *In vitro* culture using liquid medium without agitation), which also aid reduced production costs because the electricity that is required to move shakers is not necessary (Lopes da Silva *et al*., 2012). Nevertheless, the most eucalypt species as the *E. saligna* present hyperhydricity. In this context, strategies to overcome this problem must be developed. One of them; can be the culture in high calcium levels. In this case, new sources of calcium must be tested.

***In vitro* elongation, *In vitro* rooting and acclimatization:** The *In vitro* elongation of the shoots presented relative variation on the height of the shoots, varying from 0.3 to 1.6 cm after 20 days of culture. Several the shoot multiples during *In vitro* elongation showed rooting. Similar results were found in another

study with *E. saligna*, which elongated shoots also rooting (Dibax *et al*., 2010). The absence of homogeneity in shoot elongation can be due to BAP residual effect from prior multiplication medium (Silva *et al*., 2008). Other cause can be due to the genetic characteristics of the explants (Brondani *et al*., 2009; Dutra *et al*., 2009). The rooting percentage obtained was 85%, it was obtained approximately two roots per shoot. Shoots reached a mean of 3.0 cm height of the aerial part and fresh mass of 0.14 g (being 0.11 g roots and 0.03 g aerial part). The shoot length is often correlated with the rooting percentage (Lopes da Silva *et al*., 2006; Bisognin *et al*., 2008), of course, others factors are associated too, as the presence of plant growth regulators, the ionic strength the salts and genotype. There was formation of lateral shoots in these rooted plants, suggesting a largest period of *In vitro* elongation. None callus was observed in rooted plants. The acclimatization performed by hydroponics resulted in 90% survival, demonstrating the efficiency of this protocol to *In vitro* propagation this species.

E. saligna is more sensitive than others species acclimatized using hydroponics as *Dyckia agudensis* (Lopes da Silva *et al*., 2007) and taro (*Colocasia esculenta* spp.) (Nhut *et al*., 2004). Therefore, a pre-acclimatization (uncapped culture flasks with addition of water by three days before hydroponics) is necessary to promote a suitable acclimatization using hydroponics (Data not shown).

Conclusions

This is the first report of induction of organogenesis from cotyledonary nodes in *E. saligna*. The induction of cotyledonary nodes consisted of two parts: a dark culture followed by a light culture. Basal medium was MS added with 30 $g.L^{-1}$ sucrose, 10% coconut water and solidified with 7 $g.L^{-1}$ agar. For the dark culture the media were supplemented with 3.6 μM NAA and 4.4 μM BAP and for the light culture the media were supplemented with 2.7μM NAA and 1.1 μM BAP. The period for dark and light culture was 20 days. Shoots were multiplied on MS medium, 30 $g.L^{-1}$ sucrose supplemented with 1.1 μM BAP. Shoots were elongated on MS medium free of plant growth regulators. Shoots were rooting on half-strength MS salts. Acclimatization was performed in a hydroponics floating system.

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