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A N I N T E R N A T I O N A L J O U R N A L

Plant Regeneration from Cotyledonary Explants of *Eucalyptus camaldulensis* Dehn and Histological Study of Organogenesis *in Vitro*

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ABSTRACT

The present work aimed at regenerating plants of Eucalyptus camaldulensis *from the cotyledonary explants and describing the anatomy of the tissues during callogenesis and organogenesis processes, in order to determine the origin of the buds. The cotyledonary leaves of* E. camaldulensis *were cultured in Murashige and Skoog (MS), WPM and JADS media supplemented with 2.7 µM NAA and 4.44 µM BAP. The best results for bud regeneration were obtained on MS and WPM media (57.5 and 55% of calluses formed buds, respectively). Shoot elongation and rooting (80%) were obtained on MS/2 medium (with half-strength salt concentration) with 0.2% activated charcoal. Acclimatization was performed in the growth chamber for 48 h and then the plants were transferred to a soil:vermiculite mixture and cultured in a greenhouse. Histological studies revealed that the callogenesis initiated in palisade parenchyma cells and that the adventitious buds were formed from the calluses, indicating indirect organogenesis.*

Key words: Adventitious buds; Callogenesis; Organogenesis; Tissue culture; Anatomical study

INTRODUCTION

Eucalyptus is a genus of Myrtaceae family, composed of shrubs and forest trees of large size. Native to Australia, it was successfully introduced in many regions of the world for commercial plantations. Nowadays, the eucalypts occur in all tropical and subtropical regions. In Brazil, it is considered as the most important forestry genus, covering more than four millions of hectare (FAO, 2001). As the demand for the wood and sub-products is increasing, many studies of genetic improvement have been developed to maximize the production of the species and to attend the exigencies of the forestry market (FAO, 2001). The application of conventional methods of genetic improvement to forest trees, although efficient, is relatively slow, due to the time needed to complete each generation of crossings and to obtain the progenies. The introduction of exogenous genes into progenies and market clones of eucalypts through genetic transformation offers an excellent opportunity to accelerate the process of obtention of genotypes with interesting characteristics (Barrueto-Cid et al., 1999). The establishment and understanding of the regenerating process are essential for the success of this process. Studies on plant regeneration through organogenesis have already been reported for *E. camaldulensis* (Muralidharan and

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Mascarenhas, 1987; Mullins et al., 1997; Ho et al., 1998; Dibax et al., 2005; Quisen, 2007), *E. tereticornis* (Subbaiah and Minocha, 1990; Parthiban et al., 1999), *E. grandis* (Warrag et al., 1991; Lainé and David 1994; Hajari et al., 2006), *E. urophylla* (Tibok et al., 1995), *E. globulus* (Serrano et al. 1996), *E. gunnii* (Hervé et al., 2001) and *E. grandis* x *E. urophylla* (Gonzales et al., 2002; Tournier et al., 2003; Alves et al., 2004). However, these mainly describe the protocol of plant regeneration and do not refer to the anatomical process. The purposes of this study were to describe a procedure of adventitious bud regeneration from the cotyledonary leaves, the subsequent development of rooted shoots and to elucidate the origin of adventitious buds by histological techniques.

MATERIAL AND METHODS

Plant material

Seeds of *Eucalyptus camaldulens*is, collected in Açailândia, MA, Brazil, were provided by the Instituto de Pesquisas Florestais (IPEF, Piracicaba, SP, Brazil).

In vitro culture conditions and media

Seeds were surface-sterilized by immersion in 70% (v/v) ethanol for two min and then in a 6% (v/v) sodium hypochlorite solution supplemented with Tween® (5 drops in 100 mL) for 20 min. They were subsequently rinsed three times in sterile distilled water and sown in Petri dishes on MS/2 medium, composed of half-strength MS (Murashige and Skoog, 1962) mineral salts, MS vitamins and organic compounds, 20 $g.L^{-1}$ sucrose and 7 $g.L^{-1}$ agar (Vetec®). All the cultures were maintained in a growth chamber under cold white fluorescent light, with a photon flux density of approximately 30 $\mu mol.m^{-2}.s^{-1}$, 16/8 light/dark regime and 25 ± 2°C. The cultures were performed in Petri dishes (10 cm diameter and 2 cm height), containing 25 mL of culture medium and sealed with PVC film, or in glass flasks (6 cm diameter and 9 height) containing 40 mL of culture medium each and sealed with rigid polypropylene caps. All media had the pH adjusted to 5.8 and were autoclaved for 20 min at 120°C.

For the plant regeneration from the cotyledonary leaves, three treatments were compared: MS, WPM (Lloyd and McCown, 1981) and JADS (Correia, 1993). All media contained 2.70 µM NAA (naphthaleneacetic acid) and 4.44 µM BAP (benzylaminopurine), 20 $g.L^{-1}$ sucrose and 6 $g.L^{-1}$ agar (Vetec®). The growth regulators and their concentrations were chosen according to the results of Dibax et al. (2005). Fifteen days after sowing, the plantlets were used as sources of explants. The cotyledonary leaves were excised at the petiole base and cultured with their adaxial face in contact with the culture medium. The cultures were kept in darkness in the growth chamber for 60 days, with a subculture after 30 days. At the end of this period, the percentage of explants forming calluses, their colour, the percentage of explants that regenerated the buds from the callus, the number of buds per explant and percentage of oxidised explants were recorded. Experimental design was totally randomized, with four replicates per treatment and 10 explants per flask. The values used for statistical analysis were the means obtained for each treatment. The treatment effects were analysed by ANOVA and means were compared by Duncan's multiple range test ($\alpha = 0.05$).

Micropropagation of regenerated buds

After the induction of bud, one clone was selected for its *in vitro* performance and multiplied on MS medium with thiamine 1 $mg.L^{-1}$ instead of 0.1 $mg.L^{-1}$, supplemented with 0.5 µM NAA and 2.64 µM BAP, 30 $g.L^{-1}$ sucrose and 7 $g.L^{-1}$ agar (Vetec®). The buds were subcultured for three periods of 20 days in the same medium (Fig. 1). For elongation and rooting, regenerants were isolated and transferred to the flasks containing MS/2 medium with 30 $g.L^{-1}$ sucrose, 7 $g.L^{-1}$ agar (Vetec®) and 0.2% (w/v) activated charcoal for two 30-day-periods. The acclimatization of rooted microcuttings was performed by the opening of flasks in the growth chamber for 48 h and then they were transferred to a soil:vermiculite mixture (1:1) and cultured in a greenhouse with intermittent mist (5s/15min) during 20 days.

Histological techniques

Fifteen days after the sowing, the plantlets were utilized as the source of cotyledonary explants as described above. The explants were cultured on MS medium containing 20 $g.L^{-1}$ sucrose and 6 $g.L^{-1}$ agar (Vetec®). Two treatments were compared: (1) MS medium without growth regulators (control) and (2) MS medium supplemented with 2.7 µM NAA and 4.44 µM BAP. The cultures were kept in darkness during the first 30 days, at a

temperature of 27 ± 2°C. After this period, the explants were transferred to the same medium and kept under light. Five samples were collected at day 0, 15, 30, 45 and 60 after the explant inoculation. The samples were fixed in formaldehyde/acetic acid/alcohol 70% (v/v) under vacuum for 24h, in accordance with Johansen (1940) protocol. After that the material was transferred into 70% (v/v) ethanol and dehydrated in an ethanol series. The samples were then embedded in glycol methacrylate (GMA) following the procedure described by Feder and O'Brien (1968) and the manufacturer's instructions (JB4 - PolyScience®). After the inclusion, longitudinal sections were made with a rotary microtome with steel razor type C, giving a thickness of 7 µm. The cuttings were disposed in slides and stained with 0.05% (v/v) toluidine blue for 10 min (O'Brien et al., 1964). Then the slides were washed under tap water for approximately 5 min, dried and mounted in synthetic resin (Entelan®). The pictures were made with digital camera under microscope (Olympus®). The scales were projected in the same optical conditions.

RESULTS AND DISCUSSION

Medium effect on organogenesis induction

The initial morphogenetic response was the explant swelling up after 7-10 days of *in vitro* culture, as reported by Azmi et al. (1997) for *Eucalyptus globulus* and Dibax et al. (2005) for *E. camaldulensis*. Callus formation and bud regeneration were located at the periphery of protuberances near the petiole basis (Fig. 2). The best results of bud formation were observed on MS and WPM media (Table 1 and Fig. 3 and Fig. 4). Ho et al. (1998) and Diallo and Duhoux (1984) also achieved the bud regeneration from cotyledonary leaves of *E. camaldulensis* on MS media but they used other combinations of NAA and BAP: 16.2 µM NAA and 4.44 µM BAP or 5.4 µM NAA and 2.22 µM BAP. For the other variables, the statistical analysis showed no significant differences among the three media, but the negative effect of callus oxidation on the bud formation was observed. The morphological appearance of regenerated tissues was different in the three treatments: the JADS medium induced the formation of hyperhydric dark green buds of small size (Fig. 5), while in the MS medium, the buds formed were bigger than those obtained on WPM medium (Fig. 3 and Fig. 4). Symptoms of hyperhydricity are known to be correlated with the concentration of ingredients of the culture medium, with the type and concentration of the gelling agent, with high concentrations of growth regulators, low light intensity, genotype and high humidity (Daguin and Letouzé, 1986; Gribble et al., 1996; Chakrabarty et al., 2006). In the present case, only mineral formulation differed among the three culture media. For *in vitro* culture of *Eucalyptus* spp, the required salt formulation can differ among the species. According to George (1993) and Radice (2004), the MS medium is the most appropriate and widely used in dicotyledonous morphogenic processes due to the high NO^{3-} and NH^{4+} concentration and proportion between these nitrogen forms. Rodrigues and Vendrame (2003) reported that eucalypt micropropagation efficiency was highly influenced by the culture medium ionic balance. According to these authors, studies based on the nutritional characteristics between different species are crucial to establish the media formulation for *in vitro* culture of *Eucalyptus*. The JADS culture medium (Correia, 1993) was formulated with significant reductions in the mineral salts concentration, mainly potassium ions, in comparison to MS medium, and showed positive results for *E. grandis* micropropagation. Overall, the statistical data presented here, along with the visual observations, indicated the superiority of both MS and WPM media for callus induction and bud regeneration of a *E. camaldulensis* genotype despite the low total ion concentration of WPM medium.

Table 1 - Effect of MS, WPM and JADS culture media supplemented with 2.70 µM NAA and 4.44 µM BAP on direct organogenesis from cotyledonary leaf explants of *Eucalyptus camaldulensis*, after 60 days of culture.

Mineral medium	Callogenesis (%)	Bud formation from callus (%)	Bud number per callus	Bud oxidation (%)
MS	37,5 a	57,5 a	4,8 a	5,0 a
WPM	42,5 a	55,0 a	4,2 a	2,5 a
JADS	55,0 a	35,0 b	3,5 a	10,0 a
VC %	27,8	29,4	35,6	42,3

Means followed by the same letter in a column do not differ (P < 0,05) Duncan's multiple range test. VC: variation coefficient

Rooting and acclimatization

Microcuttings (Fig. 6) root emission rate of 80% was observed after 10-15 days on the culture medium and 2 to 4 cm plants were obtained after 30 days on the same medium. Ho et al. (1998) also achieved the successful rooting of *E. camaldulensis* microcuttings on half-strength MS culture media. Similar results were reported by Ito et al. (1996) for *E. botryoides*, *E. camaldulensis*, *E. deglupta* and *E. grandis* microcuttings, using B5 (Gamborg et al., 1968) medium with half-strength salt concentration in the absence of growth regulators. Different results were obtained by Barrueto Cid et al. (1999). The bud elongation from cotyledonary leaves of *E. grandis* x *E. urophylla* was stimulated when the SP medium containing BAP, NAA and GA_3 was used and after an interval between 20 and 30 days, 1.5 cm length plants showed spontaneous root induction. Furthermore, Mullins et al. (1997) reported that the buds resulting from leaf explants of *E. camaldulensis* showed occasionally spontaneous elongation and rooting when cultured on WPM medium containing 2 µM BAP and 2.5 µM NAA. A comparison between elongation and rooting results obtained for *E. camaldulensis* in the present work and others reported in the literature indicated that the culture medium formulation should be chosen according to the studied species. Acclimatization method described in this work was efficient and provided a survival rate of 87% (Fig. 7). Bennett and McComb (1982) reported a 50% rate for *E. marginata* acclimatization using a peat and sand (3:1) sterile mixture. In their case, the initial acclimatization was done in a growth chamber and the humidity was gradually reduced until the fourth week, and finally the plants were transferred to a greenhouse. Similar strategy was used by Tibok et al. (1995) who acclimatized *E. urophylla* plants under growth chamber conditions and progressive flask opening during a period of 14 days, and then the plants were cultured under greenhouse. Tournier et al. (2003) used a sand and fertilizer mixture for *E. grandis x E. urophylla* plant acclimatization under greenhouse conditions that resulted in a 95% survival rate. This practice confirmed the recommendations proposed by Grattapaglia and Machado (1998). According to these authors, pre-acclimatization or gradual plant exposition to external environment could contribute to the future survival of the plant under greenhouse conditions.

Figure 1 - 7 - Plant regeneration of *Eucalyptus camaldulensis* from cotyledonary explants. Bud multiplication on MS medium supplemented with 1.3 µM BAP subcultured for three periods of 20 days on the same medium (1). Indirect in vitro organogenesis in cotyledonary explants of *E. camaldulensis* on MS medium supplemented with 2.70 µM NAA and 4.44 µM BAP after 30 days of culture (2). Differences of organogenic response of cotyledonary explants of *E. camaldulensis* cultured on different culture media containing 2.7 µM NAA and 4.4 µM BAP after 60 days of culture. (3) – MS (Murashige and Skoog, 1962), (4) - WPM (Lloyd and McCown, 1981), (5) - JADS (Correia, 1993). Bud elongation on MS/2 medium with 0.2% activated charcoal after a 30 day-period of growth (6). Plant acclimatization on soil:vermiculite (1:1) mixture under greenhouse after 20 days (7). (Bars = 2.0 mm (1), 2.5 mm (2), 1.0 cm (3-5), 1.5 cm (6), 2.5 cm (7)).

Histological studies

The transversal section of the cotyledonary leaves cultured on the plant growth regulator-free medium showed uniserial epidermis with thin walls and stomata on both faces.

The mesophyll was dorsiventral with approximately three layers of palisade parenchyma. The vascular bundles were surrounded by a sheath with parenchyma cells (Fig. 8). The first divisions occurred in the palisade parenchyma cells after 15 d of culture on a medium containing 2.7 µM NAA and 4.44 µM BAP (Figs. 9–10). After 30 d on this medium, the callogenesis was evident in the portions of peripherical parenchymatic tissue in subepidermic position where the callus formation was starting (Figs. 11 to 13). In some samples, the cellular proliferation was intense and broke the layers of the epidermis of the adaxial face (Fig. 11). Caulinar meristems were formed in the parenchyma (Figs. 11 to 13).

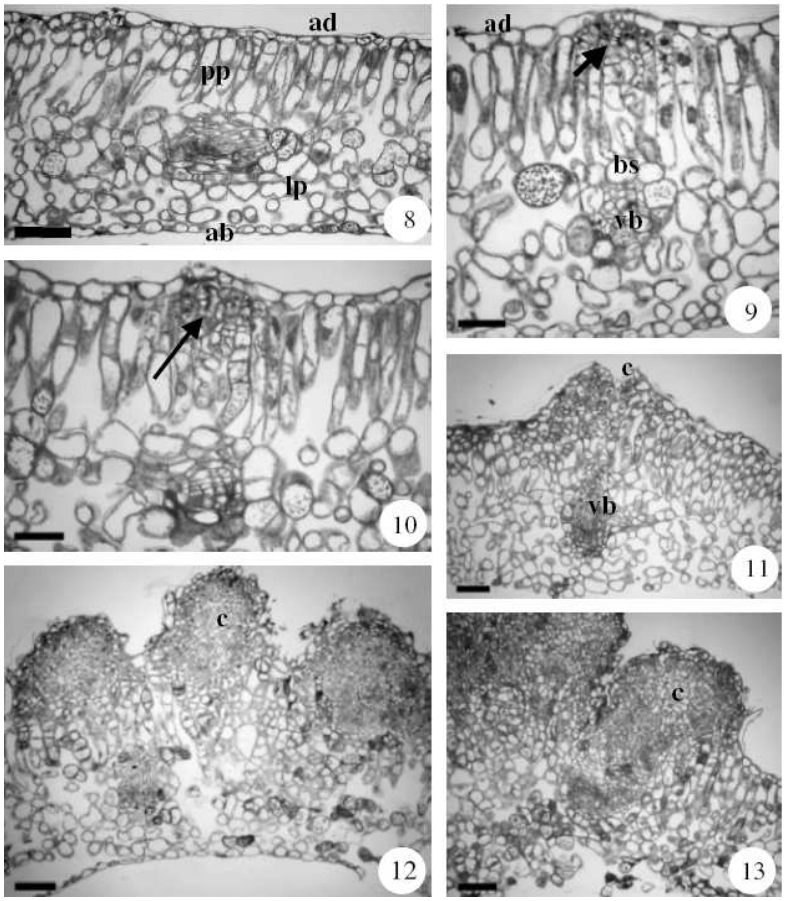

Figure 8 - 13 - Cotyledonary leaves of *Eucalyptus camaldulensis* cultured *in vitro*. Transversal sections. Explant on the first day of culture, showing dorsiventral mesophyl after 15 days of culture (8-9). Note the cell divisions in the palisade parenchyma (arrow) 30 days of culture (10). Callus originated by cell proliferation in the parenchyma. Observe the rupture of the epidermis (11). Presence of several calluses constituted by parenchyma (12-13). (ad) adaxial face, (ab) abaxial face, (pp) palisade parenchyma, (lp) lacunar parenchyma, (bs) vascular bundle sheath, (c) callus, (vb) vascular bundle. (Bars = 100 µm (8 - 13)).

This confirmed the results obtained by Alves et al. (2004) that indicated the adventitious origin of the buds formed in the leaf explants of the hybrid *E. grandis* x *E. urophylla*. In this case, the buds were formed on calli developed from the mesophyll of the abaxial face of the leaf after 35 d on culture medium containing 4.44 µM BAP. The knowledge of the exact local where cell divisions start before callus formation is important for the studies of genetic transformation, as they indicate the

superficial origin of adventitious bud induction (Hervé et al., 2001). The cotyledons of *Racosperma mangium* formed adventitious buds when cultured in MS medium containing 4-CPPU and 2,4-D. Their anatomical study revealed the most intense differentiation near the epidermis too (Takemori et al., 2000). Similar results were described by Stipp et al. (2001) who obtained adventitious buds in the cotyledons of *Cucurbita melo* L. var. *inodorus* cultured on MS medium supplemented with 4.44 µM BAP.

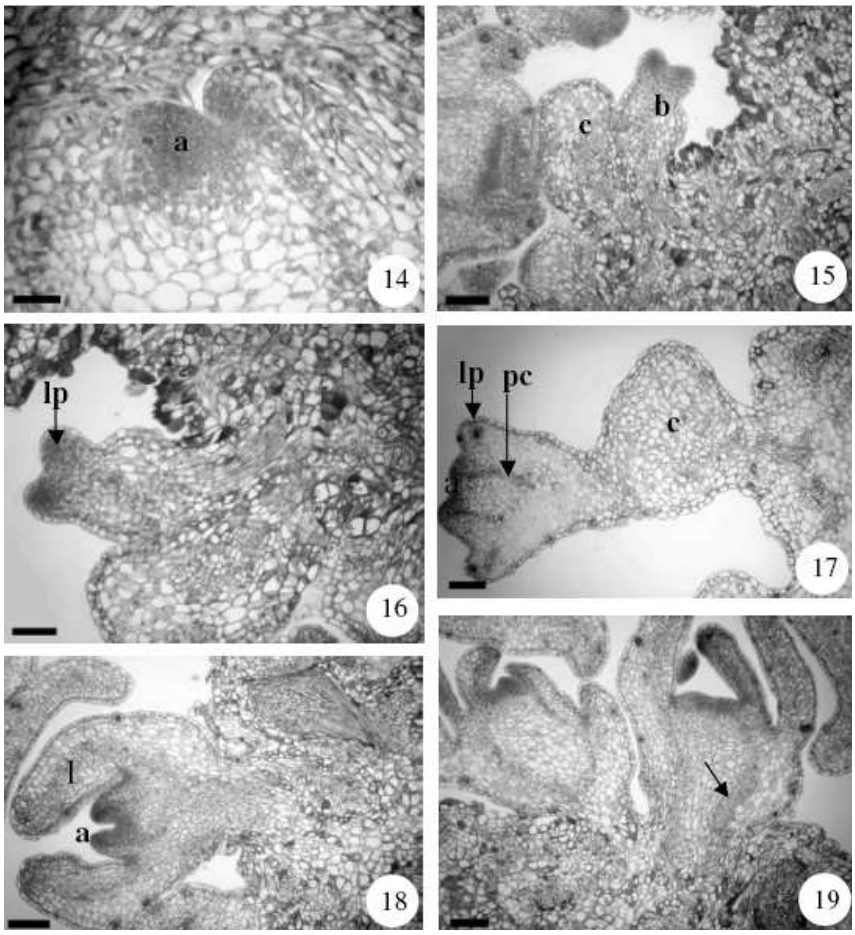

Figure 14 - 19 - Organogenesis in cotyledonary leaves of *Eucalyptus camaldulensis* after 45 days of culture (14 – 16). Formation of the caulinar apical meristem (14). Callus and bud showing the leaf primordia in the bud after 60 days of culture (15-16). Bud showing apical meristem, leaf primordia and procambium (17). Bud showing the caulinar apical meristem with young leaves (18). Caulinar buds in advanced stage of development showing the procambium (arrow) (19). (a) caulinar apical meristem, (c) callus, (b) bud, (pc) procambium, (lp) leaf primordium, (l) young leaf. (Bars = 50 µm (14); 100 µm (15 –19)).

In this case too, many meristematic regions were observed near the epidermis. After 45 and 60 d in the presence of NAA and BAP, the calli were highly developed and showed concentric groups of vascular cells and adventitious buds with visible caulinar meristems (Figs. 14-19). In the histological studies of leaf, node and internode explants of *E. gunnii* Hook, cultured in the presence of Picloram (0.04 µM) and BAP (2.25 µM), the same results were observed (Hervé et al. 2001). In this study with *E. camaldulensis*, the callus cells appeared to have a restricted number of divisions before differentiation was triggered and the yield of shoot regeneration was limited by

the size of the callus. Similar results have been observed on *E. urophylla* organogenic callus (Tibok et al., 1995).

In conclusion, the cotyledonary leaves cultured in the conditions described here originated adventitious buds and acclimatizated plants were obtained. Histological study showed that the calluses were formed by the dedifferentiation of palisade parenchyma cells and caulinar meristems appeared in this tissue, indicating that the regeneration in *E. camaldulensis* occurred through the indirect organogenesis

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RESUMO

Este trabalho teve como objetivo a obtenção de plantas de *Eucalyptus camaldulensis* a partir de folhas cotiledonares e o estudo da anatomia dos tecidos durante a calogênese e organogênese para determinar a origem das gemas. Folhas cotiledonares foram cultivadas em meios de cultura MS, WPM e JADS suplementados com 2,7 µM de ANA e 4,44 µM de BAP. Os melhores resultados para a regeneração de gemas foram obtidos com os meios MS e WPM. Para o alongamento e enraizamento, o meio de cultura MS/2 contendo 0,2% de carvão ativado apresentou-se eficiente para ambas as etapas. A aclimatização foi realizada mediante a abertura dos frascos na sala de crescimento por 48 horas, seguido da transferência para casa-de-vegetação com nebulização intermitente. Estudos histológicos foram conduzidos e revelaram que a calogênese teve início nas células do parênquima paliçádico e que as gemas adventícias formaram-se a partir dos calos, indicando a organogênese indireta.

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