SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN *Eucalyptus globulus* LABILL.

Cristian Gómez, Darcy Ríos y Manuel Sánchez-Olate

SUMMARY

Induction of somatic embryos of Eucalyptus globulus *from seeds, hypocotyls, cotyledons and micropropagated leaves was studied. The material with the highest proliferation rate was transferred to differentiation treatments. The best treatments were determined based on growth, oxidation and embryogenic development stages. Finally, the somatic cotyledonary embryos were transferred to germination conditions, to induce conversion to plantlets. In the induction, >60% callogenesis was obtained when using mature seeds and leaves and during proliferation; the highest calli multiplication was achieved with culture medium B5 + 2.0mg·l^{-1} 2,4-D + 0.5mg·l^{-1} BAP (I3) when employing seeds, cotyledons and hypocotyls. During differentiation on B5 + 0.1mg·l^{-1} ANA + 0.1mg·l^{-1} BAP (D3), mature cotyledonary states were obtained that produced leaves, hypocotyls and secondary roots after being transferred to germination conditions. In conclusion, a lesser cell differentiation and ontogenic age of calli coming from seeds, allowed the achivement of high callogenesis, less oxidation and differentiation of cotyledonary stages capable of germinating to obtain plantlets, in contrast with the other explants.*

Introduction

Somatic embryogenesis is a tool of high potential in the improvement of productivity since it allows, among other applications, the propagation of selected genotypes at a large scale, the production of artificial seeds and the cryopreservation of material, as well as providing a method to regenerate genetically modified plants (Vicient and Martínez, 1998; Von Arnold, 2008).

Early reports of embryogenic induction in *Eucalyptus globulus* were published by Muralidharan and Mascarenhas (1987) and Muralidharan *et al.* (1989). Starting from zygotic embryos of *Eucalyptus citriodora* they managed to induce somatic embryos in B5 (Gamborg, mineral solution; Gamborg *et al*., 1968) medium supplemented with glutamine, hydrolyzed casein and 5mg·l^{-1} naftalenacetic acid (NAA), achieving embryo germination into plantlets. Further studies of Watt *et al.* (1991) in *E. grandis* indicated the possibility of obtaining somatic embryos from leaves of micropropagated seedlings, but at low rates (30%) and with asynchrony problems during germination. Major *et al.* (1997) in a preliminary study of embryogenic induction in *E. grandis*, described the formation of three types of embryogenic *calli* when employing mature zygotic embryos and hypocotyls, as well as the control of oxidation problems of the *callus* with the use of phitagel (Sigma®) as a gelifying agent.

In *E. dunnii* (Termignoni *et al*., 1996) and in *E. nitens* (Ruaud *et al*., 1997) a lower somatic embryo formation rate and *callus* oxidation occurred during the proliferation stage. Nevertheless, the authors indicated that induction of somatic embryos was possible starting from hypocotyls, cotyledons, leaves and epicotyls, observing the formation of globular stages without progression to more advanced stages. Trindade and Pais (1999, 2003), working with seeds and adult *E. globulus* material, described the formation of globular stages representing somatic embryos and nodules that later evolved to plantlets. Nugent *et al.* (2001) described the formation of all the development stages, together with the formation of abnormal somatic embryos.

The formation of somatic embryos was also reported in studies of indirect callogenesis of *E. nitens*, although it had a low frequency and was conditioned by growth regulators (Bandyopadhyay *et al*., 1999).

On the other hand, Bandyopadhyay and Hamill (2000) in a comparative study of zygotic and somatic embryos, described the internal morphology and cell organization of somatic embryos, which was similar to the one observed in zygotic embryos. In both embryo types, the cotyledons were formed by highly compact cells with a great number of vacuoles and dense cytoplasm. In the meristematic zones of each embryo the cells were small and presented prominent nuclei and nucleoli.

Pinto *et al*. (2002), using mature *E. globulus* zygotic embryos, achieved induction and finally the conversion of somatic embryos to seedlings with 21% success. These authors established that the addition of nitrogenated supplements like hydrolyzed casein and glutamine favor the embryogenic induction; however, they stimulated root and abnormal somatic embryo formation. This formation of abnormal embryos, already described previously by Nugent *et al.* (2001) reached 61-100% in treatments that induced embryo formation.

Likewise, Prakash and Gurumurthi (2005) described a high frequency (54%) of embryo formation from mature zygotic embryos in *E. tereticornis* when transferring the induced cells to MS medium (Mu-

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RESUMEN

Se estudió la inducción y diferenciación de embriones somáticos de Eucalyptus globulus *desde semillas, hipocótilos, cotiledones y hojas micropropagadas, determinando los mejores tratamientos en cuanto a crecimiento, oxidación y desarrollo de estados embriogénicos. Finalmente, los embriones somáticos cotiledonares se transfirieron a condiciones de germinación para inducir la conversión a plántulas. En la inducción se obtuvieron porcentajes de callogénesis >60% al utilizar semillas maduras y hojas, y durante la proliferación, la mayor multiplicación de callos se logró con medio de cultivo B5 + 2,0mg·l^{-1} 2,4-D + 0,5mg·l^{-1} BAP (I3) al utilizar semillas, cotiledones e hipocótilos. Durante la diferenciación, en el medio de cultivo B5 + 0,1mg·l^{-1} ANA + 0,1mg·l^{-1} BAP (D3) se obtuvieron estados cotiledonares maduros, que al ser transferidos a condiciones de germinación, desarrollan hojas, hipocótilos y raíces secundarias. Se concluye que la menor diferenciación celular y edad ontogénica de callos procedentes de semillas permite obtener alta callogénesis, menor oxidación y diferenciación de estados cotiledonares capaces de germinar y obtener plántulas, a diferencia de los otros explantes.*

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RESUMO

Estudou-se a indução e diferenciação de embriões somáticos de Eucalyptus globulus *desde sementes, hipocótilos, cotilédones e folhas micropropagadas, determinando os melhores tratamentos quanto a crescimento, oxidação e desenvolvimento de estados embriogênicos. Finalmente, os embriões somáticos cotiledonares se transferiram a condições de germinação para induzir a conversão em plântulas. Na indução foram obtidas porcentagens de calogênese >60% ao utilizar sementes maduras e folhas, e durante a proliferação, a maior multiplicação de calos se conseguiu com meio de cultivo B5 + 2,0mg·l^{-1} 2,4-D + 0,5mg·l^{-1} BAP (I3) ao utilizar sementes, cotilédones e hipocótilos. Durante a diferenciação, no meio de cultivo B5 + 0,1mg·l^{-1} ANA + 0,1mg·l^{-1} BAP (D3) se obtiveram estados cotiledonares maduros, que ao ser transferidos a condições de germinação, desenvolvem folhas, hipocótilos e raízes secundarias. Conclui-se que a menor diferenciação celular e idade ontogenética de calos procedentes de sementes permite obter alta calogênese, menor oxidação e diferenciação de estados cotiledonares capazes de germinar e obter plântulas, a diferença dos outros explantes.*

rashige and Skoog, 1962) containing 2.22µM 6-benzylaminopurine (BAP). The germination was accomplished in MS basal medium obtaining low mortality when transplanting the seedlings to containers.

Finally, in a comparative study through flow cytometry, between somatic and zygotic embryos with respect to their genetic material content, Pinto *et al*. (2004) did not find significant differences between the two, which could indicate that somatic embryogenesis is a technique that maintains ploidy.

In general, in all previous studies embryogenic induction was achieved from mature somatic embryos or material derived from them, and by leaves of plants propagated *in vitro*. The *Eucalyptus* genus is difficult to study because of its low embryogenic potential, the high oxidation rates of phenolic compounds; the asynchrony in the induction and maturation processes, and the difficult regeneration of plants. The scarce existing literature doesn't establish clearly if somatic embryo formation is associated to a certain type of explants with embryogenic competence. For other woody species such as *Quercus* (Manzanera, 1992) o *Castanea* spp. (Vieitez *et al*., 1990; Merckle *et al*., 1991) it has been determined which explants are more appropriated.

Considering the importance of somatic embryogenesis as a biotechnological technique for the propagation of selected genotypes for the forestry programs of genetic improvement and the fact that, in Chile, no studies have been carried out in this species, it was established as a general objective of this research, to induce the formation and differentiation of somatic embryos of *E. globulus* Labill using mature seeds, cotyledons, hypocotyls and leaves of micropropagated plants.

Materials and Methods

Plant material

Mature *Eucalyptus globulus* seeds from the region of Inzunsa, Colcura Valley, province of Arauco, Chile (37º8'S; 73º9'W) were used. Seeds were disinfected with 70% ethanol (v/v) during 1min, followed by a commercial sodium hypochlorite 50% (v/v) solution (Clorox ®), plus one drop of Quix ® surphactant during 20min and, finally, three washings in sterile distilled water during 1, 2 and 3min each. Four types of explants were tested.

In half of the seeds, the seed coat was removed, and mature seeds (Se) were used as the first type of explant.

Germination of zygotic embryos

The other half of the seeds were cultivated in MS mineral medium (Murashige and Skoog, 1962) with the macronutrients diluted to 25%, without growth regulators, 3% sucrose and 0.7% agar (Merck), during 21 days in darkness at 24 ±1ºC. The second and third type of initial explants were the cotyledons (Co) and a 1cm portion of hypocotyls (Hy), excised after germination.

The other portion of germinated seeds, was transferred to 25 ±1ºC with a 16h photoperiod with cold light (Philips Tube TLD 36W/54), under a photon flux of 80µmol·m^{-2}·s^{-1} until two pairs of leaves were formed and reached an average of 5cm in length. The roots were then eliminated and the stem was inoculated in culture containers 9.5cm tall and 6cm in diameter, containing 20ml of proliferation medium (MS + 1mg·l^{-1} BAP + 0.01mg·l^{-1} NAA). After 50 days, the leaves (Le) formed were excised, and they constituted the fourth and last type of initial explants. All the mediums employed were sterilized in an

autoclave at 120ºC (1atm) for 20min.

Essay establishment

The four types of explants were inoculated on glass Petri dishes 9cm in diameter with 20ml of culture medium for each induction treatment, sealed with parafilm and maintained in continuous darkness at 25 ±1°C for 90 days.

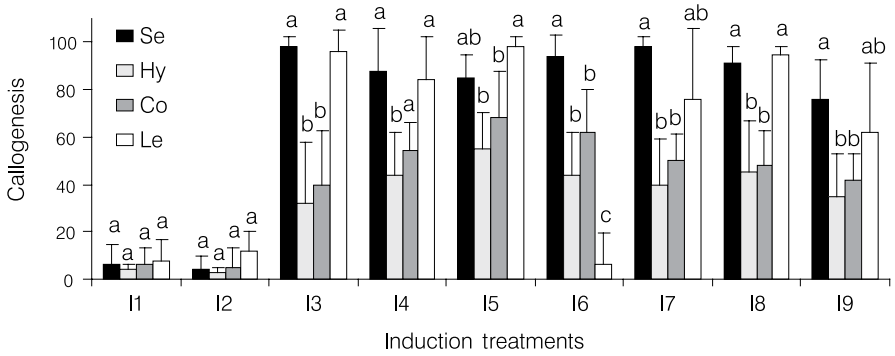

Figure 1. Callogenic responses (%) for the different type of induction mediums and explant types. Se: mature seed, Hy: hypocotyl, Co: cotyledon, Le: leaves of micropropagated shoots. Over the bars the standard deviation is indicated. In each treatment, different letters indicate significant differences between the type of explant in each treatment. (α=0.05).

Induction

For embryogenic *callus* induction, nine treatments were used (media I1-I9) which were constituted by MS and B5 media. In some variations, 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), hydrolyzed casein and glutamine supplementation were tested, in the following combinations:

I1: B5 without regulators (Control 1)

I2: MS without regulators (Control 2)

I3: B5 + 2.0mg·l^{-1} 2,4-D + 0.5mg·l^{-1} BAP

I4: MS + 2.0mg·l^{-1} 2,4-D + 0.5mg·l^{-1} BAP

I5: MS + 1.0mg·l^{-1} 2,4-D + 1.0mg·l^{-1} NAA

I6: MS + 3.0mg·l^{-1} NAA

I7: MS + 5.0mg·l^{-1} NAA

I8: MS + 5.0mg·l^{-1} NAA + 0.5mg·l^{-1} hydrolyzed casein + 0.5mg·l^{-1} glutamine

I9: MS + 10.0mg·l^{-1} NAA

The percentage of explants that formed *callus* was evaluated at this stage (callogenesis).

Proliferation

The *calli* formed following the different treatments were transferred to proliferation media, constituted by B5 and MS mineral salts, during 30 days. These cultures were maintained in the dark at 25 ±1ºC with the objective of increasing the amount of callogenic material to be used in the following stages.

Morphological, cytological and histological study

At the end of the embryogenic induction period, a macro-morphological analysis was carried out, which included aspects such as texture, color and *callus* friability; as well as a micro-morphological analysis that consisted in disaggregation smears of small portions of the *callus* (2-3mm). The samples selected were stained with lacto-acetic orcein (2% w/v), following the methodology described by Álvarez (1996). Observation with an optical microscope allowed to determine the existence of embryogenic and non-embryogenic cells from the callogenic material formed in the different treatments and types of explants.

The histological study was performed in the *calli* obtained from treatments that presented a globular stage (GS) development. *Calli* portions of 5-6mm were fixed during 7 days in formaldehyde, 70% alcohol and glacial acetic acid (FAA), dehydrated through a series of ascending grade alcohols, and embedded in paraffin. Thin 15µm sections were obtained using a rotation microtome Microm HM135 and stained with aqueous safranine 1% w/v and Fast Green 0.05% w/v (Merck).

In addition, scanning electron microscopy was carried out, using samples taken from *calli* that presented embryogenic characteristics, which were gradually dehydrated for 4 days, vacuum dried and covered with a gold-palladium layer in a sputtering camera, for 2min. The observation in the scanning electron microscope allowed to corroborate the initial development the proembryos as well as their frequency and asynchrony.

Differentiation

With the morphological and histological analysis, the best types of explants could be selected, as well as the best culture medium and growth regulator combinations. In this manner, pieces of *calli* 0.5cm in diameter were selected, which were deposited in Petri dishes with 20ml of the following differentiation treatments:

D1: B5 without regulators (Control)

D2: B5 + 0.05mg·l^{-1} NAA + 0.05mg·l^{-1} BAP

D3: B5 + 0.1mg·l^{-1} NAA + 0.1mg·l^{-1} BAP

D4: B5 + 0.5mg·l^{-1} NAA + 0.5mg·l^{-1} BAP

D5: B5 + 1.0mg·l^{-1} NAA + 1.0mg·l^{-1} BAP

D6: B5 + 1.0mg·l^{-1} NAA

D7: B5 + 1.0mg·l^{-1} BAP

The cultures were maintained in the dark at 25 ±1°C for 45 days. The variables analyzed in this stage were: average *callus* diameter, oxidation level of the *callus* (0: 0-25% oxidation, 1: 25-50% oxidation, 2: 50-75% oxidation, and 3: 75-100% oxidation), number of globular states (GS) per *callus* mass, and macro-morphological aspects.

Germination

Those embryos that reached the mature cotyledonary stage were subcultured in B5 medium supplemented with 30g·l^{-1} sucrose, without growth regulators under light conditions, for root emergence and foliage development.

Statistical analysis

In the induction phase, the experimental unit was a Petri dish with 10 explants. Ten, replicates were used per treatment. For the differentiation phase, the experimental unit was a Petri dish with 6 *calli* replicated 6 times per treatment. The data had a normal distribution and a homogeneous variance; therefore, an ANDEVA parametric and the Tuckey test were applied, with a 5% significance level for multiple comparisons, using Statistica 6.0 software. In case the suppositions were not met, the non-parametric Kruskal-Wallis test and the Dunn test were applied with a significance level of 5% for multiple comparisons using the software Infostat.

Results

Induction

When seeds were used as explants, a callogenic induction response >76% was obtained in all treatments. In the control, *calli* formation ranged from 4 to 6% (Figure 1) due mainly to germination. The best treatment with respect to callogenesis percentage was I5 (MS supplemented with 1.0mg·l^{-1} 2,4-D and 1.0mg·l^{-1} NAA). The *callus* originated mainly from the cotyledons and the seminal root, and in some treatments (I3, I4, I7 and I8) *calli* with

root hair formation were observed. These were organogenic associated responses. In qualitative terms the *calli* formed in treatment I3 (B5 + 2.0mg·l^{-1} 2,4-D + 0.5mg·l^{-1} BAP), I4 (MS + 2.0mg·l^{-1} 2,4-D + 0.5mg·l^{-1} BAP) and I5 (MS + 1.0mg·l^{-1} 2,4-D + 1.0mg·l^{-1} NAA) had the best aspect quality, being yellow and friable. In addition, the *callus* mass was greater than in he other treatments. On treatments I6 (MS + 3.0mg·l^{-1} NAA) and I9 (MS + 10.0 mg·l^{-1} NAA) *calli* were less developed and were not so friable. The presence of a notorious root development, indicated their organogenic rather than their callogenic potential.

The hypocotyls were the type of explants with the lowest values of callogenesis in all treatments except in I6 (MS + 3.0mg·l^{-1} NAA). In the control (I1 and I2) the values varied from 3 to 4%, while the highest values (55%) were obtained in treatments I3, I4 and I5. Treatments I7, I8 and I9 generated smaller *calli* that had a rhizogenic response. Although numerically the values were low, the quality of the *calli* was good, because they presented a reduced oxidation rate, a yellow color with small brown zones and a friable aspect. It was noticed that the *callus* was formed mainly from the cut zone and from the explant interior, apparently from the parenquimatous cells that surround the vascular bundle.

In the case of the cotyledons, controls (I1 and I2) presented values of 5 to 6%, while the highest value was obtained in treatment I5 (MS + 1.0mg·l^{-1} 2,4-D + 1.0mg·l^{-1} NAA) with 68% callogenesis. In general for all the treatments, quality was lower than hypocotyl-derived *calli* regarding size and oxidation grade. Treatments I3 and I4 generated more translucent and friable *calli* than I7, I8 and I9.

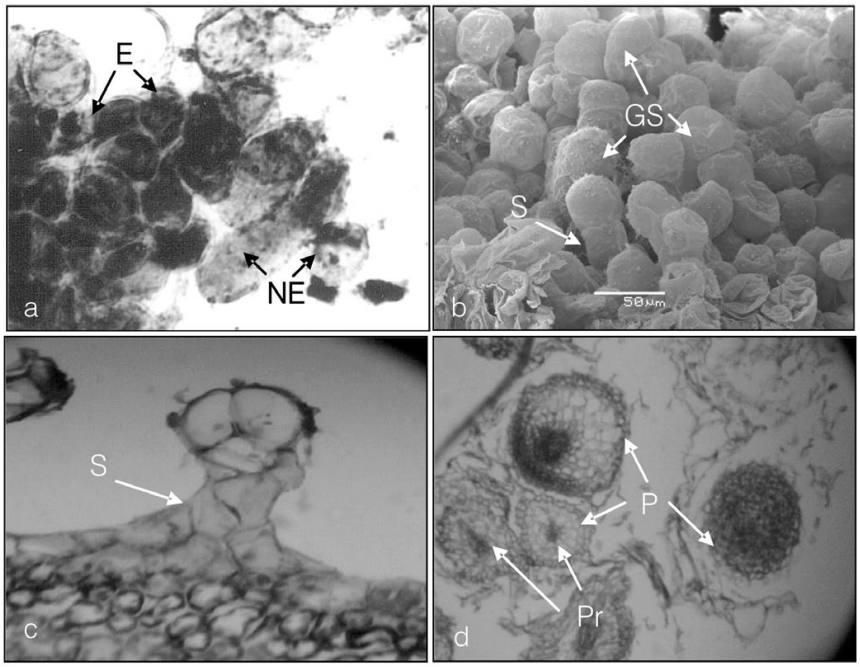

Figure 2. Morphologic and histologic aspects of induction and embryogenic differentiation. a: embryogenic cells (E) and non-embryogenic (NE) obtained in the induction phase from cotyledons, treatment I3 (x400); b: scanning electron microscopy of the embryogenic *callus* in the proliferation stage, obtained from a mature seed in treatment I5 (MS + 1.0mg·l^{-1} 2,4-D + 1.0mg·l^{-1} NAA) (GS: globular state, S: suspensor, bar= 50µm); c: longitudinal section of a GS in differentiation phase, treatment D7 (B5 + 1.0mg·l^{-1} BAP) (x200); d: transverse section of a GS showing internal differentiation, P: protoderm, Pr: procambium (x100).

When using leaves, in the control (I1 and I2) values of 8 to 12% callogenesis were obtained, which surpass those obtained with the other explants. The best treatment was I5 (90%) and the lowest was I6 (6%). No organogenic response was observed except in treatment I7, where root formation was noticed. In spite of the fact that the percentages callogenesis were high in seeds, *callus* formation was slower.

Treatments combining 2,4-D 2mg·l^{-1} and BAP 0,5mg·l^{-1} (I3, I4), or 1mg·l^{-1} 2,4-D and NAA (I5) produced friable *calli* that barely oxidized, forming from the leaf surface. In the other treatments in the presence of NAA (I6 to I9) *callus* formation was incomplete, because only the leaf borders reacted and formed a *callus* mass with a compact aspect and a high oxidation rate.

The cytologic studies indicated the presence of embryogenic cells mainly when using cotyledons and mature seeds as explants. These cells were characterized mainly for presenting a strong coloration and being of smaller size than non-embryogenic cells (Figure 2a.).

On the other hand, at the end of the induction period electron microscopy evidenced the presence of a great number of proembryos in the *callus* surface, which were formed by a small suspensor and embryogenic pole that was composed by no more than two cells.

Proliferation

During the proliferation phase, the *calli* obtained from seeds, hypocotyls and cotyledons kept their color and friable characteristics, only a small increase in oxidation was observed. In contrast the material coming from the leaves was compacted and presented an increased oxidation, leading to a high percentage of necrosis.

Due to the large proliferation obtained with the material from B5 medium supplemented with 2,4-D and BAP, at 2 and 0,5mg·l^{-1}, respectively, (I3) and the high necrosis percentage in *calli* obtained from leaves, only the *calli* proceeding from mature seeds, hypocotyls and cotyledons from treatment I3 were used for further experiments.

Differentiation

In general, all types of explants responded to almost all the treatments except in D2 and D3, where total necrosis of *calli* coming from hypocotyls and cotyledons was produced. Also the *calli* coming from seeds in D5 did not respond well and, therefore, these will not be presented on the graphics.

Regarding *callus* growth, it was greater in controls that in the other treatments; also, no significant differences were observed between the type of explants in each differentiation treatment, except in D4 (Figure 3a).

The *calli* proceeding from seeds grew more than those from other origins, in all the treatments except in D5, where they underwent necrosis. The lower growth of the *callus* masses was obtained on those originated from cotyledons in the treatments D1 (Control), D4 and D6. *Callus* growth was an important factor to consider during the development of somatic embryogenesis, because a greater *callus* mass means more cells that can be induced to develop somatic embryos. This is a factor to consider when it is desired to massify and clone selected genotypes (Figure 3a). With regard to oxidation in this phase, the control presented a lower level than all the treatments. Treatment D4 presented the highest levels obtained in the three *callus* types. In general, the material proceeding from hypocotyls and cotyledons presented higher levels of oxidation than the *callus* proceeding from seeds (Figure 3b).

Finally, regarding the number of globular states (GS) per *callus* mass, a higher amount was observed in treatments with

growth regulators, in comparison with controls. The *calli* proceeding from seeds presented the highest number of GS with treatment D7, where the highest values (10 GS) per 8mm *callus* mass were obtained, although there were no significant differences with the other types of *callus* (Figure 3c).

The histological studies indicated differentiation of the pro-embryos at the end of the induction phase, consisting in an increase of mitotic division at suspensor level and the embryogenic pole; at the end of this phase took place the development of a spherical multicellular *callus* mass covered by a unilayered protoderm. In the interior, a series of parenquimatous type cells and in the center a vascular procambium were observed (Figures 2c, d).

Even though the GS were observed in all the differentiation and proliferation treatments, and mainly when using *calli* from mature seeds in B5 medium supplemented with 1.0mg·l^{-1} BAP (D7), the differentiation of the other somatic embryos stages was also possible in treatment D3 (B5 + 0.1mg·l^{-1} BAP + 0.1mg·l^{-1} NAA). In this treatment, heart stages were differentiated, characterized by the loss of the globular form and the appearance of the cotyledonary primordials (Figure 4d). The torpedo stage is a more advanced stage, characterized by an elongated form in which mature vascular tissue differentiation begins, of oval form that extends throughout the entire embryo. This stage was associated to globular stages forming small embryogenic masses (Figure 4e). Some advanced torpedo stages presented two small protuberances in the superior zone that corresponded to the future cotyledons and radicular hairs of the inferior zone (Figures 4f, g). Finally, at day 45 in germination conditions, the presence of 2 to 3 pairs of leaves, a developed hypocotyl and the differentiation of a radicular and a shoot pole could be seen (Figure 4h).

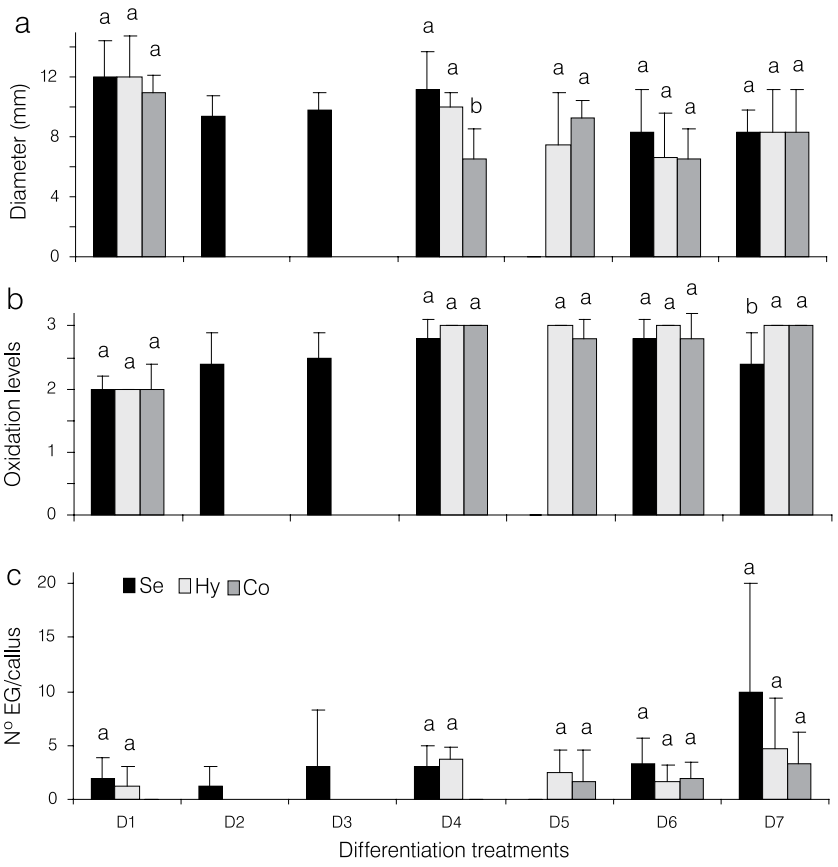

Figure 3. Responses in the distinct differentiation treatments. a: average *callus* diameter, b: *callus* oxidation levels, c: average number of GS per *callus*. Over the bars the standard deviation is indicated. In each treatment, different letters indicate significant differences between the type of explant in each treatment. (α=0.05). Se: mature seed, Hy: hypocotyl, Co: cotyledon.

Germination

At the end of the differentiation period of 45 days, the germination of cotyledonary stages was observed and presented radicular emergence of at least 2 to 4 times longer than cotyledonary leaves (Figure 4i). When transferred to a base medium without regulators and photoperiod conditions, in some embryos (10 day) the development of secondary globular states was observed; in other cases, the growth and development of a root (Figures 4j, k). At day 20 it was already evident the development of small leaves upon the cotyledons and the presence of a high number of root hairs (Figures 4l, m). Finally, at day 45 under germination conditions, the presence of 2 to 3 pairs of leaves, a developed hypocotyl and secondary roots were observed.

Discussion

Specialized studies have establish that along with the manifestation of *callus* formation, it is possible to find caulogenic and ryzogenic responses. These responses depend on the type of explants and types and concentrations of growth regulators used, and are due to the presence of cells and tissue with different morphogenic capacities (Ahuja, 1993; Rodríguez *et al*., 2005).

The analysis of the responses obtained corroborate the fact that small doses of NAA induce an organogenic effect on the explants, mainly the formation of sprouts. In other studies in *Eucalyptus* (Trindade *et al*., 1990; Tapia and González, 2000), NAA has been used to favor adventitious sprout proliferation, which has been confirmed in the present study. On the other hand Pinto *et al*. (2002) established that the exposure to high concentrations of NAA for more than a week, inhibited somatic embryo formation.

On what concerns growth regulators, the best somatic embryo inducer has been considered to be 2,4-D **(**Margara, 1988; Pierik, 1990; Manzanera, 1992**).** In *Eucalyptus*, Termignoni *et al*. (1996) accomplished the formation of somatic embryos in *E. dunni* with the application of 2,4-D in combination with NAA. But some reports (Muralidharan and Mascarenhas, 1987; Muralidharan *et al*., 1989; Ruaud *et al*., 1997; Trindade and Pais, 1999) managed somatic embryo induction using NAA alone or in combination with cytokinine. It is pertinent to say that high concentrations of 2,4-D (>10mg·l^{-1}) usually are very noxious for somatic embryogenesis because they induce the formation of abnormal embryos or they decrease drastically the embryogenic potential of the *callus*. In general, for somatic embryogenesis of *Eucalyptus,* the combination of NAA with BAP produces better results at shorter induction periods, while 2,4-D in combination with BAP favors it in longer induction periods.

In general, the *calli* obtained from mature seeds, hypocotyls and cotyledons, following treatments I3, I4 and I5 were much more friable, of irregular aspect and lighter color, than those obtained from all the other treatments, including when using leaves. These characteristics, which have been attributed to embryogenic type *calli* (Watt *et al*., 1991; Rodríguez *et al*., 2005), were corroborated in this study by micro-morphological analysis that evidenced the presence of *calli* with embryogenic cells (Figure 2a).

The embryogenic cells presented the characteristics of

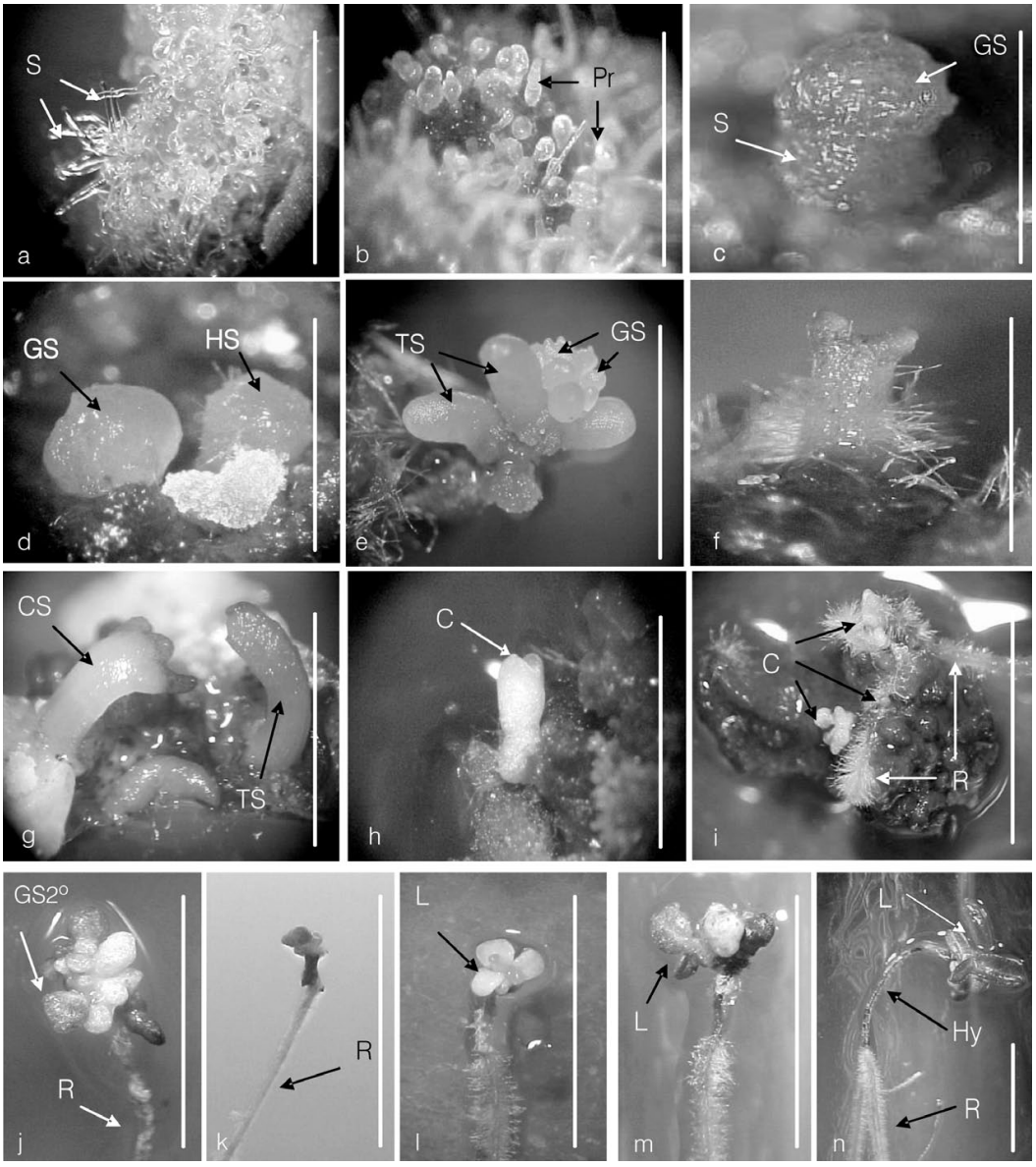

Figure 4. Macromorphological aspects of the induction sequence, differentiation and germination of somatic embryos. a and b: initial induction phases (S: suspensor, Pr: proembryos), c: globular stage (GS, S: suspensor), d: globular stage (GS) and heart stage (HS), e: mass proliferation of globular stages (GS) and torpedo stage (TS), f: early cotyledonary state with formation of root hairs, g: early cotyledonary state (CS) and torpedo state (TS), h: mature cotyledonary state (C: cotyledons), i: cotyledonary embryo germination (C: cotyledon, R: root), j and k: somatic embryos at 10 days of germination (R: root, GS2º: formation of secondary globular stages from the main embryo), l and m: leaf differentiation in somatic embryo germinated after 20 days (L: leaves), n: somatic embryo after 35 days in B5 medium with the development of 5 leaves (L), hypocotyls (Hy) and secondary roots (R). Vertical bars in a-i = 1mm; in j-n = 1cm).

cells in constant division: small size, dense cytoplasm and well stained, large nuclei and nucleoli, small vacuoles and a great amount of starch granules (Figure 2a). In *E. grandis* (Watt *et al*., 1991) and in *E. nitens* (Bandyopadhyay and Hamill, 2000) this cell type has been described, as individual cells or in small clusters from which the somatic embryos develop. In general, it is concurred that direct somatic embryogenesis is produced from highly competent cells, which immediately form somatic embryos following cell division. On the other hand, the indirect somatic embryogenesis is produced from cells that differentiate and later on divide and develop somatic embryos.

In the case of the species of the *Eucalyptus* genus, there are no investigations in somatic embryogenesis that may clarify whether the origin of embryos is multicelullar or unicellular.

During proliferation, the oxidation of phenolic compounds was the main factor that influenced *callus* growth and development. This has been described in the majority of the existing reports in callogenesis and somatic embryogenesis of *Eucalyptus* (Watt *et al*., 1991; Termignoni *et al*., 1996; Ruaud *et al*., 1997; Trindade and Pais 1999; Nugent *et al.,* 2001; Pinto *et al*., 2002). However, no specific references to the type of explants with a larger occurrence of oxidation is presented.

The phenolic oxidations can, on occasions, constitute a serious problem in the establishment and survival of meristem, apex and/or *callus*, This phenomena, although being reported in a wide range of plants, is more acute in woody species, representing a difficulty for the establishment of *in vitro* cultures (Ahuja, 1993; Jiménez, 1998). During the differentiation phase, the formation of GS was observed on the surface as in the interior of the *callus* mass, in the same way as it has been described in previous studies (Gómez *et al*., 2006).

In embryogenic induction in *E. nitens* Ruaud *et al.* (1997) also described the formation of protoderm and parenquimatous tissue, but did not observe vascular tissue development. On the contrary, Hervé *et al.* (2001) in *E. gunni* organogenesis, and Trindade and Pais (2003) in *E. globulus*, described the development of new sprouts starting from nodular structures with internal differentiation of vascular strands, but despite of the GS described in this study, these structures maintain a strong connection with the callogenic tissue.

The globular and torpedo stages to cotyledonary are coincident, in their morphology, to the results described by Nugent *et al*. (2001) and Pinto *et al*. (2002) in *E. globulus*. However, the cotyledonary embryos differ from those described in *E. nitens* by Bandyopadhyay and Hamill (2000), who established that the somatic embryos found presented an epidermis layer cover similar to the one found in zygotic embryos.

The germination results demonstrate the capacity of somatic embryos to germinate without entering into a dormancy period like zygotic embryos, although afterwards a more advanced development of the root is observed, in comparison to the shoot system. This is the first report of somatic embryogenesis

in *E. globulus* that describes all the developments stages of the somatic embryo. Also described here are the morpho-anatomical processes involved from the embryogenic cells to the conversion to plantlets.

Conclusions

When using mature seeds as an initial explant, high callogenic induction percentage was obtained in B5 mineral medium supplemented with distinct concentrations of 2,4-D and BAP. A combination of NAA ($0.1 mg \cdot l^{-1}$) and BAP ($0.1 mg \cdot l^{-1}$) allowed to achieve the differentiation of the induced embryos to the mature cotyledonary stage, from which the germination and conversion to plantlets was possible.

Most of the phenolic oxidation and the difficulty in obtaining embryonic stages after the globular stage, in the other type of explants (cotyledons, hypocotyls and leaves), can be explained by the higher tissue differentiation and ontogenic age of this material and consequently its own physiology.

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