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Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill

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Abstract Somatic embryogenesis was induced from juvenile explants of *Eucalyptus globulus* Labill. Mature zygotic embryos, isolated cotyledons, hypocotyls, leaves and stems were cultivated at 24°C in darkness on Murashige and Skoog medium supplemented with 3% (w/v) sucrose and different growth regulator combinations. Callus was formed at the surface of the explant in all tested media containing sucrose but not in those containing mannitol. Calli were transferred to the same medium without growth regulators (MSWH) after 25 days. Somatic embryogenesis was observed in callus derived from cotyledon explants and from entire mature zygotic embryos in the presence of 3–15 mg l^{-1} α-naphthalene acetic acid (NAA) alone or in the presence of 1 mg l^{-1} NAA combined with 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid. Best embryogenic rates were obtained in the presence of 3–5 mg/l NAA, as approximately 30% of callus formed on these media produced somatic embryos. Exposure, for >1 week, to the highest NAA concentrations tested (15 mg l^{-1}) failed to induce somatic embryogenesis. Addition of 500 mg l^{-1} casein hydrolysate and 500 mg l^{-1} glutamine to the induction medium increased the number of abnormal somatic embryos. Conversion of somatic embryos to plantlets (21%) was obtained when they were transferred to medium free of growth-regulators.

Keywords *Eucalyptus* · *Eucalyptus globulus* · Somatic embryogenesis · Embryogenic callus

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Abbreviations *6-BAP*: 6-Benzylaminopurine · *2,4-D*: 2,4-Dichlorophenoxyacetic acid · *Glu*: Glutamine · *MS*: Murashige and Skoog · *MSWH*: MS medium without growth regulators · *NAA*: α-Naphthalene acetic acid

Introduction

The genus *Eucalyptus* comprises approximately 700 species and varieties (Watt et al. 1999) although only 1% of them are used for industrial purposes. Although exact figures for the total plantation area are difficult to obtain, there is an estimated 10 million ha of *Eucalyptus* plantations (Kellison 2001) and the *Eucalyptus* kraft pulp industry is based largely on two species, namely *E. globulus* and *E. grandis* hybrids. The natural genetic diversity within and between *Eucalyptus* species is enormous and can be further enhanced by interspecific hybridisation making it an attractive genus for breeding (Eldridge et al. 1993). Besides the production of pulpwood, *Eucalyptus* is also used for timber, veneer, firewood, shelter, ornamentals and essential oil production (Watt et al. 1999). *E. globulus* is nowadays grown worldwide (e.g. Australia, South America, South Africa, Portugal, Spain, USA) due to its versatility, fast growth and fibre characteristics. *E. globulus* was introduced to Portugal 150 years ago and nowadays it represents the third forest species in Portugal, covering approximately 672,149 ha of forest (Direcção Geral Florestas 2001).

Propagation of this species has been carried out mainly from seed. However, *E. globulus* is an outcrossing species and a high level of heterozygosity is found in seeds. Therefore, clonal propagation offers the possibility to capture both additive and non-additive variation created by conventional breeding as well as improving uniformity for cost-effective mechanical harvesting. However, *E. globulus* has a very irregular adventitious rooting behaviour (5–64%) (Marques et al. 1999) that hampers vegetative propagation of some desired genotypes. Somatic embryogenesis has the advantage that

Table 1 Embryogenic potential of *Eucalyptus globulus* explants. *E. globulus* callus production, rhizogenesis and embryogenic potential obtained on Murashige and Skoog medium without growth regulators (MSWH) (after 8 weeks) for different explant sources (leaf, stem, cotyledon, hypocotyl and zygotic embryos) grown for the first 25 days on different induction media (E1–E15). All media had the basal MS composition supplemented with different growth regulators (E1–E15 media) and different carbon sources. Non-destructive visual rating of callus production and rhizogenesis based on quantity produce: + poor, ++ good, +++ best. *6-BAP* 6-Benzylaminopurine; *2,4-D* 2,4-dichlorophenoxyacetic acid, *NAA* α-naphthalene acetic acid, *NT* not tested

Treatment	Additives	Explant response	Leaf	Stem	Cotyledon	Hypocotyl	Zygotic embryos
E1	1 mg l^{-1} 2,4-D+0.01 mg l^{-1} 6-BAP (30 g l^{-1} sucrose)	Callus production	+	+	+	+	NT
		Rhizogenesis	+	+	++	++	
		Somatic embryo formation	No	No	No	No	
E2	2 mg l^{-1} 2,4-D+0.5 mg l^{-1} 6-BAP (30 g l^{-1} sucrose)	Callus production	++	+	+	+	NT
		Rhizogenesis	+	+	++	++	
		Somatic embryo formation	No	No	No	No	
E3	1 mg l^{-1} 2,4-D+2 mg l^{-1} zeatin (30 g l^{-1} sucrose)	Callus production	++	+	++	++	NT
		Rhizogenesis	+	+	++	++	
		Somatic embryo formation	No	No	No	No	
E4	0.5 mg l^{-1} Dicamba (30 g l^{-1} sucrose)	Callus production	+++	++	+++	+++	NT
		Rhizogenesis	+	+	+	+	
		Somatic embryo formation	No	No	No	No	
E5	1 mg l^{-1} 2,4-D (30 g l^{-1} sucrose)	Callus production	NT	NT	++	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E6	1 mg l^{-1} 2,4-D+1 mg l^{-1} NAA (30 g l^{-1} sucrose)	Callus production	NT	NT	++	NT	+
		Rhizogenesis			++		++
		Somatic embryo formation			Yes		Yes
E7	0.5 mg l^{-1} Dicamba+1 mg l^{-1} zeatin (30 g l^{-1} sucrose)	Callus production	NT	NT	+	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E8	1 mg l^{-1} 2,4-D (60 g l^{-1} sucrose)	Callus production	NT	NT	+	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E9	1 mg l^{-1} 2,4-D (90 g l^{-1} sucrose)	Callus production	NT	NT	+	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E10	1 mg l^{-1} 2,4-D (36.44 g l^{-1} mannitol)	Callus production	NT	NT	0	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E11	0.5 mg l^{-1} Dicamba (36.44 g l^{-1} mannitol)	Callus production	NT	NT	0	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E12	3 mg l^{-1} NAA (30 g l^{-1} sucrose)	Callus production	NT	NT	++	NT	++
		Rhizogenesis			++		++
		Somatic embryo formation			Yes		Yes
E13	5 mg l^{-1} NAA (30 g l^{-1} sucrose)	Callus production	NT	NT	++	NT	+++
		Rhizogenesis			+++		+++
		Somatic embryo formation			Yes		Yes
E14	5 mg l^{-1} NAA+500 mg l^{-1} casein hydrolysate+500 mg l^{-1} Glu (30 g l^{-1} sucrose)	Callus production	NT	NT	++	NT	++
		Rhizogenesis			+++		+++
		Somatic embryo formation			Yes		Yes
E15	15 mg l^{-1} NAA (30 g l^{-1} sucrose)	Callus production	NT	NT	NT	NT	++
		Rhizogenesis					+++
		Somatic embryo formation					Yes/No[a]

[a] Somatic embryogenesis was only obtained in calli that were grown for 1 week on induction medium: *Yes* presence of somatic embryos, *No* absence of somatic embryos

both a root and a shoot meristem are present simultaneously in somatic embryos. Furthermore, somatic embryogenesis largely simplifies the conservation methods by using a limited space for a large number of genotypes while they are being field tested. In fact, as in other forest species, genotype×environment interactions are particularly important in eucalypt species (Zobel 1993).

Somatic embryogenesis in the *Eucalyptus* genus has been described for *E. citriodora* (Muralidharan and Mascarenhas 1987, 1995; Muralidharan et al. 1989), *E. dunnii* (Termignoni et al. 1996; Watt et al. 1999), *E. grandis* (Watt et al. 1991, 1999) and for *E. nitens* (Bandyopadhyay et al. 1999; Bandyopadhyay and Hamill 2000). However, *E. globulus* has previously been

Table 2 Average number of roots and somatic embryos formed in *E. globulus* callus. Average number of roots and somatic embryos formed on MSWH medium in *E. globulus* callus produced in the cotyledon region of zygotic embryos that grew on E12, E13, E14 and E15 induction media

Treatment	Induction period (days)	No. of explants tested	Responsive explants (%)	Roots formed per explant (range)	Embryogenic callus	Total embryos indirectly formed in all explants	Abnormal embryos (%)
E12	25	20	30	0–9	Yes	13	61
E13	25	14	28.5	2–11	Yes	19	63
E14	25	14	21.4	0–17	Yes	9	100
E15	8	7	14.2	0–14	Yes	9	90
E15	15	7	–	0–12	No	–	–
E15	25	7	–	0–15	No	–	–

reported as being extremely recalcitrant to regeneration through somatic embryogenesis, and in vitro plant regeneration was only obtained through organogenesis (Serrano et al. 1996; Bandyopadhyay et al. 1999). Recently, Bandyopadhyay et al. (1999) reported the appearance of embryogenic structures in *E. globulus* from seedling explants. Also Nugent et al. (2001) reported somatic embryogenesis from cotyledons and hypocotyls but with no plantlet development. We herewith describe, for the first time, a reproducible protocol for somatic embryogenesis in *E. globulus* from mature zygotic embryos.

Materials and methods

Plant material and sterilisation

Half-sib seeds of *Eucalyptus globulus* ssp. *globulus* Labill (Stora-Enso Celbi, Leirosa, Portugal) were imbibed and surface-sterilised by immersion in 50% absolute ethanol (v/v) for 15 min and rinsed in three changes of sterile distilled water (15–20 min per wash), then with 0.1% (w/v) Benlate (Rhône-Poulenc) and finally extensively rinsed in sterile distilled water and left to imbibe for 16 h. Germination was carried out aseptically on Murashige and Skoog (1962) medium (MS) supplemented with 2% sucrose (w/v) and 0.3% gelrite. All media were autoclaved at 121°C for 15 min. Cultures were maintained at 24±1 C either in darkness or under a 16-h photoperiod at a photon flux of 98±2 μmol m^{-2} s^{-1} at plant level. All compounds used in this work were purchased from Duchefa (Haarlem, Netherlands). Data refer to three independent experiments with $n>7$ each.

Induction of somatic embryogenesis

Cultures were initiated from cotyledons and hypocotyls of 3-day-old seedlings, and from leaves and stems of 2-month-old plants. Explants ($n>40$) were grown on MS medium supplemented with 0.3% (w/v) gelrite and the pH adjusted to 5.8. Different carbon sources (sucrose and mannitol) and combinations of growth regulators were tested (E1–E15 media; Table 1). Callus induction always took place in darkness at 24±1 C. Explants were transferred 25 days after explant inoculation to MS medium without growth regulators (MSWH). Cultures were sub-cultured onto fresh medium every 4 weeks and maintained for >1 year.

When entire mature zygotic embryos were used, the seed coat was removed and they were transferred to MS medium supplemented with 3% (w/v) sucrose, and with different α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations (E6, E12, E13, E14 and E15; Table 1) to induce somatic embryogenesis. After 25 days on callus induction media, explants from E12 ($n=20$), E13 ($n=14$) and E14 ($n=14$) media were transferred to MSWH. Explants growing on E15 medium (15 mg l^{-1} NAA) were divided into three groups ($n=7$) that were transferred to MSWH medium 8, 15 and 25 days after callus induction, respectively (Table 2).

Development of somatic embryos

After being transferred to dim light for 2 weeks on MSWH, somatic embryos were kept under a 16-h photoperiod (98 μmol m^{-2} s^{-1}) at 24±1°C. Cotyledon-derived somatic embryos were isolated and transferred to fresh MSWH medium and incubated under the conditions described above for somatic embryo development.

Results

Callus proliferation was observed in all tested media containing sucrose, while mannitol (36.44 g l^{-1}) did not promote callus formation (Table 1) although explants remained green. Increasing sucrose levels (E8 and E9 media) decreased callus formation (Table 1) and increased phenolic production (data not shown).

Somatic embryogenesis induction only occurred in the presence of NAA, either alone or in combination with 2,4-D (Tables 1, 2). Embryogenic calli emerged mainly from cotyledons of entire zygotic embryos in the presence of NAA (Fig. 1A).

Germination of entire mature zygotic embryos was observed in all media tested. Two weeks after inoculation, the germination process stopped and callus production was initiated mainly in cotyledons. Embryogenic calli were compact, white-brownish and were composed mostly of small isodiametric cells. At this stage, globular structures could already be observed in the callus formed from cotyledons (Fig. 1A, B).

Somatic embryos formed indirectly from embryogenic calli (Fig. 1A), but occasionally direct somatic embryogenesis also occurred from the upper surface of hypocotyls (Fig. 1C). Somatic embryogenesis was observed for all concentrations of NAA tested (3–15 mg l^{-1}), but best results were obtained with the lower NAA concentrations. On the other hand, exposure for >1 week to the highest concentration of NAA (15 mg l^{-1}) inhibited embryogenic capacity.

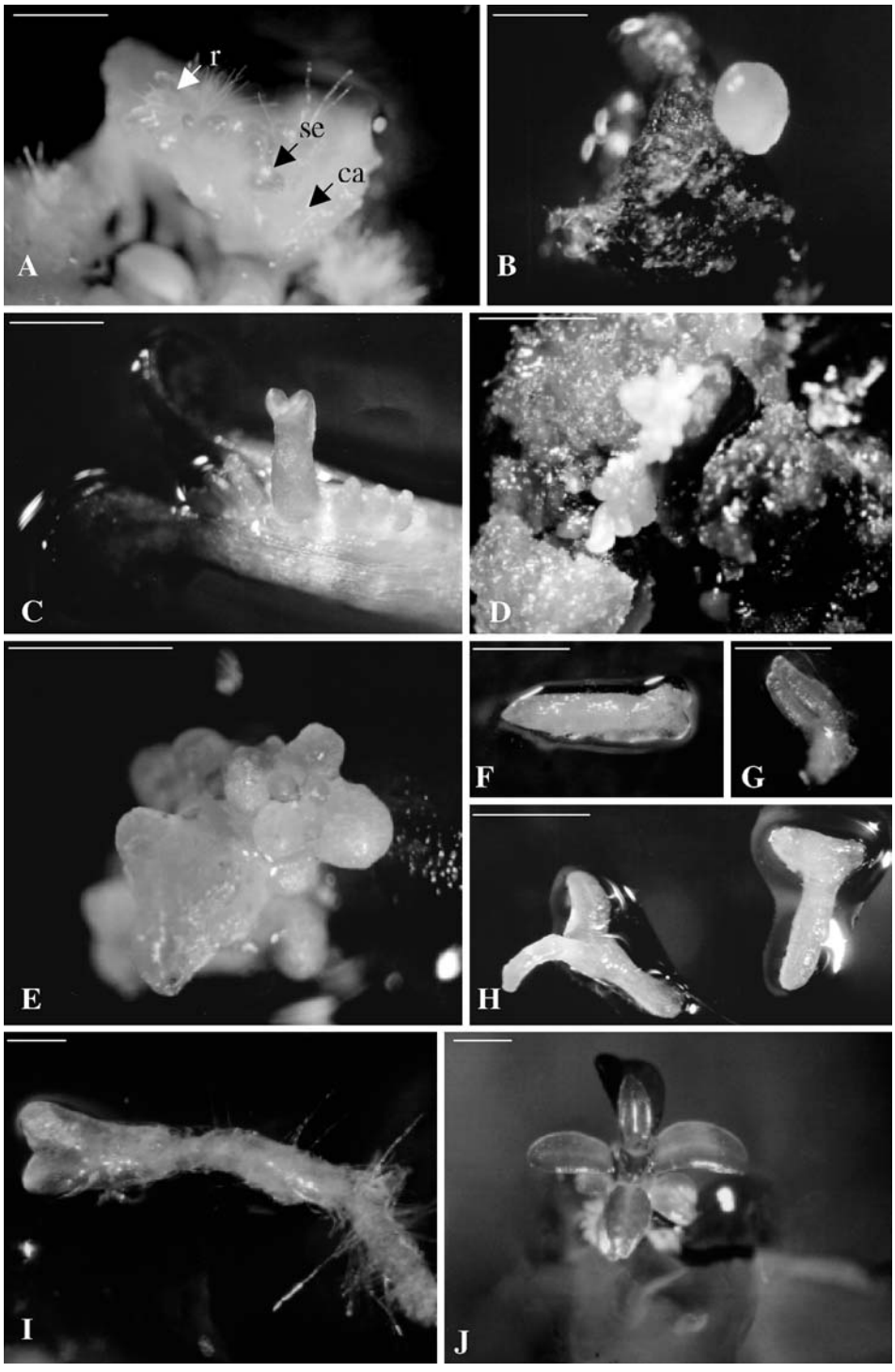

Fig. 1A–J Somatic embryogenesis and plant regeneration in *Eucalyptus globulus.* **A** Embryogenic callus (*ca*) with somatic embryos (*se*) and abundant roots (*r*) produced on a cotyledon of a zygotic embryo explant (*bar*=1 mm). **B** Globular somatic embryo (*bar*=1 mm). **C** Direct somatic embryogenesis from the upper surface of a hypocotyl of a zygotic embryo explant (*bar*=1 mm). **D** Cluster of somatic embryos surrounded by tissue with accumulated phenolic compounds (*bar*=1 mm). **E** Cluster of somatic embryos (*bar*=1 mm). **F** Torpedo stage somatic embryo (*bar*=1 mm). **G** Cotyledon-stage somatic embryo (*bar*=1 mm). **H** Cotyledon-stage somatic embryo 6 weeks after culture initiation (*bar*=1 mm). **I** Conversion of a somatic embryo on Murashige and Skoog medium without growth regulators (MSWH) (*bar*=1 mm). **J** Plantlet from somatic embryo conversion on MSWH, 10 weeks after induction (*bar*=1 mm)

Embryogenic calli, with globular structures (Fig. 1B), were transferred to MSWH. Two weeks after transfer to this medium, callus browning was intense but it was possible to detect yellowish clusters of embryos (Fig. 1D) at different stages of development (Fig. 1E). However, due to the abundant root formation (Fig. 1A), somatic embryos were not always evident in the embryogenic callus. Some of these embryos (after transfer to light conditions) developed a greenish colour and, rarely, some red pigmentation (anthocyanins) was observed. A few days later it was possible to isolate complete torpedo (Fig. 1F) and cotyledon-phase somatic embryos (Fig. 1G, 1H) that subsequently converted to plants (Fig. 1I, J). Conversion of somatic embryos to plantlets (21%) was obtained and acclimation is underway.

The addition of organic nitrogen supplements (500 mg l^{-1} casein hydrolysate and 500 mg l^{-1} glutamine) stimulated root formation and callus growth but a

higher proportion of abnormal somatic embryos was observed (Table 2).

Discussion

The results reported here showed that regeneration through somatic embryogenesis was obtained in *E. globulus* juvenile explants grown on MS medium supplemented with NAA. The use of NAA to induce somatic embryogenesis has already been reported for other *Eucalyptus* species such as *E. citriodora* (Muralidharan and Mascarenhas 1987; Muralidharan et al. 1989) and *E. dunnii* (Termignoni et al. 1996).

Treatments with 2,4-D (1.0–2.0 mg l^{-1}), either alone or in combination with the cytokinin 6-benzylaminopurine (6-BAP) (0.01–0.5 mg l^{-1}) or zeatin (2 mg l^{-1}), failed to induce somatic embryos. Dicamba (0.5 mg l^{-1}) induced a highly friable callus but only root regeneration was observed. The lack of embryo formation on calli induced by Dicamba has previously been described for this species (Trindade 1996).

Regeneration through somatic embryogenesis has been described only for a few species of *Eucalyptus* [E. *citriodora* (Muralidharan and Mascarenhas 1987; Muralidharan et al. 1989), *E. dunnii* (Termignoni et al. 1996) and *E. grandis* (Watt et al. 1991)]. More recently somatic embryogenesis was also reported for *E. nitens* (Bandyopadhyay et al. 1999; Bandyopadhyay and Hamill 2000) and *E. globulus* (Bandyopadhyay et al. 1999; Nugent et al. 2001) but plantlet regeneration was not achieved. One of the main reported problems for establishing embryogenic cultures in *Eucalyptus* species is phenolic accumulation (Nugent et al. 2001). In fact, phenolic accumulation was also evident in the embryogenic callus of *E. globulus* after transfer to MSWH. Darkness was reported to decrease phenolic accumulation in *E. citriodora* (Muralidharan et al. 1989); however, light is also an important signal for somatic embryogenesis since *E. dunnii* responded positively to light with somatic embryogenesis occurring under a 16-h photoperiod of approximately 40 µmol m^{-2} s^{-1} (Termignoni et al. 1996).

The accumulation of phenolic compounds is probably one of the causes involved in the low induction frequencies observed in this work. Nevertheless, the induction frequencies that were obtained in this study are higher than those reported previously for the same species (Bandyopadhyay et al. 1999; Nugent et al. 2001) and highly reproducible for the open-pollination family used. The extrapolation of this methodology to other families and genotypes is underway.

Although casein hydrolysate and glutamine were reported to improve somatic embryogenesis in *E. citriodora* (Muralidharan et al. 1989), in our experiments addition of casein hydrolysate (500 mg l^{-1}) and glutamine (500 mg l^{-1}) did not improve somatic embryo production and a higher number of abnormal somatic embryos were observed. Somatic embryo development in *E. globulus* was asynchronous and all phases up to the cotyledon phase could be observed simultaneously in the same embryogenic callus, as previously described for *E. citriodora* (Muralidharan et al. 1989). In fact, asynchrony and the high frequency of abnormal embryos still hampers the industrial application of this regeneration process for plant production and further studies have to be carried out to overcome these problems.

The data reported demonstrated for the first time the regeneration of somatic embryos from juvenile explants of *E. globulus*. The production of somatic embryos in *E. globulus* opens up a new way to overcome rooting difficulties in traditional cloning techniques, as somatic embryos are bipolar structures carrying both a root and shoot meristem.

Effective somatic embryogenesis techniques offer the possibility to mass multiply material that has been genetically improved by breeding and preserve a large number of genotypes in a confined space while they are being field tested (breeding-cloning strategy). Although somatic embryogenesis in eucalypt species is not yet ready to be used commercially, the fact that the process is amenable to automation can mean that it will eventually become cheaper than other clonal propagation techniques in use. In fact, vegetative propagation has an important role in progeny testing since the use of clonal replicates allows the estimation of additive and non-additive genetic variance. Furthermore, the correct ranking of individuals within families is largely improved and therefore the cumulative genetic gain obtained during each cycle of breeding increased (Mullin and Park 1992).

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